Mycologia, 104(3), 2012, pp. 682–697. DOI: 10.3852/11-088

Three new species of *Aspergillus* section *Flavi* isolated from almonds and maize in Portugal

Célia Soares

Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus Gualtar, 4710-057, Braga, Portugal

Paula Rodrigues

Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus Gualtar, 4710-057, Braga, Portugal, and CIMO-Escola Superior Agrária de Bragança, Campus Santa Apolónia, 5301-855 Bragança, Portugal

Stephen W. Peterson

Bacterial Foodborne Pathogens and Mycology Research Unit, National Center for Agricultural Utilization Research, US Department of Agriculture 1815 North University Street Peoria, Illinois 61604

Nelson Lima

Armando Venâncio[1]

Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus Gualtar, 4710-057, Braga, Portugal

Abstract: Three new aflatoxin-producing species belonging to *Aspergillus* section *Flavi* are described. They are *Aspergillus mottae*, *A. sergii* and *A. transmontanensis*. These species were isolated from Portuguese almonds and maize. An investigation examined morphology, extrolite production and DNA sequence data to characterize these isolates and describe the new species. Phylogenetic analysis showed that *A. transmontanensis* and *A. sergii* form a clade with *A. parasiticus* whereas *A. mottae* shares a most recent common ancestor with the combined *A. flavus* and *A. parasiticus* clade.

Key words: aflatoxins, *Aspergillus chungii*, *A. mottae*, *A. sergii*, *A. transmontanensis*, cyclopiazonic acid, multilocus sequence analysis

Introduction

Aspergillus section *Flavi* includes several economically important species that are closely related and morphologically similar. The section includes species that are important spoilage agents of food and producers of toxic metabolites (mycotoxins) and also species that are useful in the food industry and in biotechnological applications (Horn et al. 2009, Samson and Varga 2009).

Aflatoxins are highly toxic secondary metabolites and aflatoxin B1 (AFB1) is regarded as the most potent naturally occurring carcinogen (Bennet and Klich 2003). *A. flavus* also can produce cyclopiazonic acid (CPA), an indole tetramic acid (Luk et al. 1977), and the co-occurrence of these two mycotoxins can result in additive effects on consumers and may increase the toxigenic potential of *A. flavus* (Bamba and Sumbali 2005). There is continuing concern about the effects of mycotoxins in foods and feeds due to species from *Aspergillus* section *Flavi*. Some of the species within the section, such as *A. flavus*, *A. parasiticus* and *A. nomius*, can cause serious damage in stored commodities and produce aflatoxins (Godet and Munaut 2010). Recently described species that are less important economically or are rarely isolated, such as *A. pseudotamarii* (Ito et al. 2001), *A. bombycis* (Peterson et al. 2001), *A. parvisclerotigenus* (Frisvad et al. 2005), *A. minisclerotigenes* and *A. arachidicola* (Pildain et al. 2008), also have the ability to produce mycotoxins.

Research carried out on the fungi causing food spoilage and on the mycotoxins they produce requires accurate identification of the microorganisms (Pitt and Hocking 2009). Samson and Varga (2009) argue that no single method is flawless in recognizing species and recommend a polyphasic approach, where morphological, physiological, extrolite and DNA sequence data are considered together.

Classical identification of *Aspergillus* section *Flavi* strains is performed by examining several macroscopic (e.g. colony color and cultural characteristics) and microscopic (e.g. spore size and shape) phenotypic characteristics observed from cultures grown on different media (Rodrigues et al. 2009, Godet and Munaut 2010, Varga et al. 2011). Conventional methods used in extrolite analyses involve culturing the isolate in suitable inducing media, extracting compounds with organic solvents and monitoring their presence usually by chromatographic techniques (Lin et al. 1998).

Nucleic acid sequence information is a useful tool for species description. Molecular phylogenetics can be used to determine the limits of a species through the concordance of several genes (Baum and Shaw 1995, Samson and Varga 2009, Taylor et al. 2000). The information obtained from a single locus

Submitted 11 Mar 2011; accepted for publication 26 Oct 2011.
[1] Corresponding author. E-mail: avenan@deb.uminho.pt

generally cannot resolve the phylogeny with a satisfactory level of confidence nor can it solve questions of species identification (Lutzoni et al. 2004, Godet and Munaut 2010), but concordance analysis of multilocus DNA data provides a reliable method for species identification (Taylor et al. 2000, Dettman et al. 2006, Peterson 2008).

Molecular genetic analyses are increasingly providing evidence of sexual reproduction in ascomycetous fungi previously thought to be strictly asexual; asexuality is thought to be a common feature of fungal evolution because approximately 20% of fungi (mostly ascomycetes) lack the morphology of sexual reproduction and reproduce clonally (LoBuglio and Taylor 2002). Among some of those clonally reproducing species successful heterothallic matings have been demonstrated between strains possessing either *MAT1-1* or *MAT1-2* mating-type genes (Horn et al. 2009). Nucleotide comparisons among *MAT* genes revealed that these genes appear to be evolving faster than other sequences in the genome (Turgeon 1998). Single nucleotide polymorphisms (SNPs) occurring in these genes seem to cause high amino acid variations between species but low within-species variation (Ramirez-Prado et al. 2008).

In this study 22 strains belonging to *Aspergillus* section *Flavi* were investigated. These strains were isolated from Portuguese almonds and maize, and a polyphasic taxonomic approach was used for the characterization and identification of the isolates. Phylogenetic placement of the isolates was determined with multilocus DNA sequence analysis and the phenotypic analysis including macro- and micromorphology, aflatoxins and CPA production, and growth at different temperatures was used to describe these isolates.

MATERIALS AND METHODS

Fungal isolates.—Twenty-two fungi were isolated from almonds or maize grown in four Portuguese regions; 14 were isolated from almonds provided by producers and processors from Trás-os-Montes and Algarve and eight were isolated from maize samples provided by the National Association of Maize and Sorghum Producers (ANPROMIS) in the regions of Ribatejo and Alentejo. All isolates are deposited in the Micoteca da Universidade do Minho (MUM) culture collection, Braga, Portugal. Type strains of the three proposed new species also are deposited at CBS, with the accession numbers CBS 130015 (*Aspergillus transmontanensis* MUM 10.214), CBS 130016 (*Aspergillus mottae* MUM 10.231) and CBS 130017 (*Aspergillus sergii* MUM 10.219). For comparison purposes additional strains were obtained from the Agricultural Research Service Culture Collection (NRRL), Peoria, Illinois (TABLE I).

Morphology and mycotoxin production.—Morphological characterization was performed according to Klich (2002). A loopful of spores was suspended in 0.2% agar and used for three-point inoculations on plates of Czapek yeast autolysate agar (CYA), Czapek agar with 20% sucrose (CY20S) and malt extract agar (MEA); all cultures were grown 7 d in the dark at 25 C. Colony appearance and diameters were recorded. Cultures also were grown on *Aspergillus flavus* and *parasiticus* agar (AFPA; Pitt et al. 1983) to confirm group identification by colony reverse color. Microscopic examination was performed with a Leica DMR microscope with bright field, phase contrast and DIC optics. A Leica EC3 camera was mounted on the microscope for photomicrography. Microscope mounts were made in cotton blue from colonies grown on MEA with exception of the species *A. mottae* where microscope mounts were made from colonies grown on G25N (Pitt and Hocking 2009) at 42 C. A drop of alcohol was added for removing excess conidia. SEM micrography was performed with NanoSEM-FEI Nova 200 (FEG/SEM); EDAX-Pegasus X4M (EDS/EBSD) equipment. The conidia and conidiophores for SEM were obtained from CYA colonies of *A. sergii* and *A. transmontanensis* and a CY20S colony of *A. mottae*. The samples were prepared by rubbing a standard SEM aluminum stub across a growing colony and coating it with gold.

Extrolites.—Aflatoxin and cyclopiazonic acid (CPA) production of the isolates was analyzed by HPLC-FL and HPLC-UV respectively, as described in Rodrigues et al. (2009). Briefly, all strains were tested for aflatoxin production in aflatoxin-inducing yeast extract sucrose agar medium (YES). Strains were inoculated on 6 cm diam plates and incubated at 25–27 C for 7 d in the dark. Aflatoxins were extracted by removing three agar plugs from one colony and placing them into a 4 mL vial with1 mL methanol. After 60 min the methanol extract was filtered through a 0.45 μm sieve, evaporated and dissolved in 1 mL mobile phase. Aflatoxins detection limits were 1 ng/mL for each AFB and AFG. For cyclopiazonic acid analysis all strains were inoculated on 6 cm diam plates with CYA, incubated at 25 C for 14 d in the dark and extracted as described for aflatoxin analysis. CPA detection limit was 25 ng/mL.

DNA extraction and amplification.—A loopful of spores was suspended in 0.2% agar and used for inoculation of MEA slants in 15 mL polypropylene tubes. After 7 d growth in the dark at 25 C a 1% solution of thimerosal was added in sufficient quantity to cover the cultures and left 24 h to kill the cultures. A portion of biological material was transferred from the dead 7 d old culture into a 1.5 mL microcentrifuge tube containing CTAB buffer and 0.5 mm diam glass beads (Sigma, St Louis, Missouri) and vortexed 5 min at maximum speed. Polysaccharides and proteins were precipitated by adding chloroform and mixing by inversion and were separated by centrifugation at maximum speed for 10 min in a microcentrifuge. Cleaned supernatant was transferred to a new tube and an equal volume of isopropanol was added. This solution was gently mixed by inversion and centrifuged at maximum speed for 2 min to collect precipitated nucleic acids. The DNA pellet was washed with 70% ethanol, centrifuged at maximum speed for 2 min and air dried after gently discarding the

TABLE I. *Aspergillus* type strains and *Aspergillus* isolates from maize and almonds used in this study

Strain	Species	Source	Substrate	Country
CBS 117610	*A. arachidicola* Pildain, Frisvad & Samson		*Arachis glabrata* leaf	Argentina
CBS 117611	*A. arachidicola*		*Arachis glabrata* leaf	Argentina
CBS 117612	*A. arachidicola*		*Arachis glabrata* leaf	Argentina
CBS 117615	*A. arachidicola*		*Arachis glabrata* leaf	Argentina
NRRL 517	*A. avenaceus* G. Sm.	George Smith, London School of Hygiene and Tropical Medicine	Seed peas	England
NRRL 26010	*A. bombycis* S.W. Peterson, Yoko Ito, B.W. Horn & T. Goto	Tetsuhisa Goto, National Food Research Institute Tskuba, Japan	Silk worm excrement	Japan
NRRL 25528	*A. caelatus* B.W. Horn	Bruce W. Horn, Natlional Peanut Lab., Dawson, GA	Peanut field soil	USA
NRRL 4868	*A. chungii* Y.K. Shih		Air	Central China
NRRL 13603	*A. coremiiformis* Bartoli& Maggi	International Mycological Institute, Egham, England	Soil	Ivory Coast
NRRL 506^T	*A. effuses* Tirab.			
CBS 110.55^T	*A. fasciculatus* Bat. & H. Maia		Air	Brazil
NRRL 1957	*A. flavus* Link	Chemical Warfare Service	Cellophane diaphragm of an optical mask	
NRRL 28986	*A. flavus*	David Geiser, Penn St. Univ	Peanut field soil	Australia
NRRL 28987	*A. flavus*	David Geiser, Penn St. Univ	Peanut field soil	Australia
NRRL 28992	*A. flavus*	David Geiser, Penn St. Univ	Peanut field soil	Australia
MUM 10.206	*A. flavus*		Almond	Portugal
MUM 10.232	*A. flavus*		Maize	Portugal
NRRL 4818^T	*A. flavus* var. *columnaris* Raper& Fennell		Butter	Japan
NRRL 3751^T	*A. kambarensis* Sugiy.		Soil	Japan
NRRL 3648	*A. lanosus Kamal & Bhargava*			
NRRL 28998	*A. minisclerotigenes* Vaamonde, Frisvad & Samson	David Geiser, Penn. St. Univ.	Peanut field soil	Australia
NRRL 29000	*A. minisclerotigenes*	David Geiser, Penn. St. Univ.	Peanut field soil	Australia
NRRL 29002	*A. minisclerotigenes*	David Geiser, Penn. St. Univ.	Peanut field soil	Australia
MUM 10.226	*A. minisclerotigenes*		Maize	Portugal
MUM 10.228	*A. minisclerotigenes*		Maize	Portugal
MUM 10.229	*A. minisclerotigenes*		Maize	Portugal
MUM 10.230	*A. minisclerotigenes*		Maize	Portugal
MUM 10.227	*A. minisclerotigenes*		Maize	Portugal
MUM 10.203	*A. minisclerotigenes*		Almond	Portugal
MUM 10.231^T	*A. mottae*		Maize	Portugal
MUM 10.233	*A. mottae*		Maize	Portugal
NRRL 13137	*A. nomius* Kurtzman, B.W. Horn & Hesselt.	A.F. Schindler, Food and Drug Administration, Washington D.C.	Wheat	USA

TABLE I. Continued

Strain	Species	Source	Substrate	Country
LEMI 250/CBS 126849 [T]	*A. novoparasiticus* Gonçalves, Stchigel, Cano, Colombo & Guarro		Sputum, leukemic patient	Brazil
LEMI 267	*A. novoparasiticus*		Sputum, leukemic patient	Brazil
LEMI 149IOP/CBS 126850	*A. novoparasiticus*		Hospital air	Brazil
NRRL 447[T]	*A. orzyae* (Ahlb.) E. Cohn		Unknown source	Japan
NRRL 502	*A. parasiticus* Speare	Speare, Honolulu	Insect, Mealybug on sugar cane	USA
MUM 10.225	*A. parasiticus*		Almond	Portugal
MUM 10.212	*A. parasiticus*		Almond	Portugal
MUM 10.224	*A. parasiticus*		Almond	Portugal
MUM 10.215	*A. parasiticus*		Almond	Portugal
CBS 121.62[T]	*A. parvisclerotigenus* (Mich. Saito & Tsuruta) Frisvad & Samson		*Arachishy pogaea*	Nigeria
NRRL 25517	*A. pseudotamarii* Yoko Ito, S.W. Peterson, Wicklow & T. Goto	Tetsuhisa Goto, National Food Research Institute Tskuba, Japan	Field soil collected in tea fields	Japan
MUM 10.219[T]	*A. sergii*		Almond	Portugal
MUM 10.208	*A. sergii*		Almond	Portugal
MUM 10.214[T]	*A. transmontanensis*		Almond	Portugal
MUM 10.205	*A. transmontanensis*		Almond	Portugal
MUM 10.223	*A. transmontanensis*		Almond	Portugal
MUM 10.211	*A. transmontanensis*		Almond	Portugal
MUM 10.221	*A. transmontanensis*		Almond	Portugal
MUM 10.222	*A. transmontanensis*		Almond	Portugal
NRRL 20818	*A. tamarii* Kita	Quartermaster Corps Culture Collection	Activated carbon	
CBS 822.72	*A. toxicarius* Murak.		*Arachishy pogaea*	Uganda
NRRL 4181	*Petromyces alliaceus* Malloch& Cain	D.I. Fennell, University of Wisconsin, Madison, Wisconsin	Soil	Australia

TABLE II. Details of the primers used and target zone

Primer pair	Target zone	Sequences	Reference
Bt2a-Bt2b	Beta tubulin gene	f 5′GGTAACCAAATCGGTGCTGCTTTC3′ r 5′ACCCTCAGTGTAGTGACCCTTGGC3′	Glass and Donaldson (1995)
Cf1-Cf4	Calmodulin gene	f 5′GCCGACTCTTTGACYGARGAR3′ r 5′TTTYTGCATCATRAGYTGGAC3′	Peterson (2008)
I5-D2r	ITS and partial 1su-rDNA	f 5′GGAAGTAAAAGTCGTAACAAGG3′ r 5′TTGGTCCGTGTTTCAAGACG3′	White et al. (1990), Peterson (2008)
Mf-Mr	*Mcm7* gene	f 5′ACIMGIGTITCVGAYGTHAARCC3′ r 5′GAYTTDGCIACICCIGGRTCWCCCAT3′	Schmidt et al. (2009)
M1f-M1r	Mating type gene	f 5′ATTGCCCATTTGGCCTTGAA3′ r 5′TTGATGACCATGCCACCAGA3′	Ramirez-Prado et al. (2008)
M2f-M2r	Mating type gene	f 5′GCATTCATCCTTTATCGTCAGC3′ r 5′GCTTCTTTTCGGATGGCTTGCG3′	Ramirez-Prado et al. (2008)
5F-7R	RNA polymerase	f 5′GAYGAYMGWGATCAYTTYGG3′ r 5′GAYTGRTTRTGRTCRGGGAAVGG3′	Liu et al. (1999)
Tsr1-Tsr2	*Tsr1* gene	f 5′CCACGCTCATTCAAATCTTCT3′ r 5′CCGGTAGTTCGACCACTCGCATA3′	Schmidt et al. (2009)

f = forward; *r* = reverse.

supernatant. DNA was dissolved in 100 µL of sterile deionized water and diluted 1 : 100 for amplifications.

Beta tubulin (*BT2*), calmodulin (*CF*), ITS and partial 1su-rDNA (*ID*) and RNA polymerase (*RPB2*) were amplified under conditions described by Peterson (2008). The amplification process consisted of a predenaturation step at 94 C/2 min, followed by 35 cycles of denaturation at 96 C for 30 s, annealing at 51 C for 1 min and extension at 72 C for 1 min, plus a final extension at 72 C for 5 min. Pre-rRNA processing protein (*Tsr1*), minichromosome maintenance complex component 7 (*Mcm7*) and mating-type genes (*MAT1-1* and *MAT1-2*) were amplified under the same conditions, except the annealing temperature for *Tsr1*was 53 C, while for *Mcm7* and *MAT1-1/MAT1-2* the annealing temperature was 56 C and a 40 cycle amplification program. The set of primers used is provided (TABLE II). PCR products were purified with ExoSAP-IT® (USB, Affymetrix Inc) following the supplier's protocol. Purified PCR products were used as a sequencing template. DNA sequencing was performed with dye terminator technology (3.1) and an ABI 3730 sequencer, both from Applied Biosystems (Foster City, California). PCR products were sequenced in both directions.

Data analysis.—Sequencing errors were detected and corrected with Sequencher (Gene Codes Corp, Ann Arbor, Michigan). DNA sequences were aligned for phylogenetic analysis with Clustal X (Larkin et al. 2007). After alignment the leader elements were trimmed with a text editor. PAUP* 4.0b10 (Swofford 2003) was used to conduct parsimony analysis and to generate phylogenetic trees for single gene alignments as well as on combined alignment. Bootstrapping (bs) was performed in PAUP* with maximum parsimony criterion and TBR branch swapping for 1000 replicates. MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003) was used to calculate Bayesian posterior probabilities (pp) of branches. Mostly default settings were used. *Mcm7*, *Tsr1*and *RPB2* datasets included only protein coding, whereas *MAT1-1/MAT1-2*, *BT2* and *CF* loci included protein-coding and intronic regions and accordingly were partitioned into intron and exon regions. Markov chain Monte Carlo (MCMC) analysis was conducted for up 5×10^6 generations until the chains converged. Exclusionary principle of Baum and Shaw (1995) and genealogical concordance phylogenetic species recognition concepts (Taylor et al. 2000) were used for concordance analysis. Congruence was based on strongly supported branches with bootstrap values and posterior probability above 90% and 0.90 respectively.

RESULTS

Molecular analysis.—The *BT2* dataset contained 535 characters (239 exon positions, 296 intron positions). The *CF* sequence set contained 756 aligned sequence positions (379 exon positions, 377 intron positions). The *Mcm7* sequence set contained 616 protein-coding positions. The *RPB2* aligned sequence set contained 1001 protein-coding positions. The *Tsr1* aligned sequence set contained 788 protein-coding positions. ITS sequences were determined for the species and are deposited in GenBank as JF412767–JF412787. Although not used in this phylogenetic study they are useful for identification purposes using ITS sequences.

BT2 and *CF* sequences from our isolates were aligned with homologous section *Flavi* sequences obtained from GenBank (TABLE III) and used to generate the maximum parsimony tree (FIG. 1). A branch with > 95% bootstrap support connects several isolates and putative species including the ex-type isolate of *A. flavus*. Other isolates in that clade include the ex-type isolates of *A. orzyae*, *A. fascicula-*

TABLE III. Accession numbers of the deposited sequences at GenBank

Code	ID	Mating type	MCM7	Beta-tubulin	Calmodulin	RNA-Polymerase	TSR1	Species
MUM 10.206	JF412783	HM803049	HM803079	HM803091	HM803019	HM802977	HM802992	*A. flavus*
MUM 10.232	JF412782	HM803048	HM803080	HM803096	HM803018	HM802975	HM802990	*A. flavus*
MUM 10.226	JF412776	HM802956	HM803061	HM803088	HM803016	HM802971	HM802993	*A.minisclerotigenes*
MUM 10.227	JF412777	HM803043	HM803068	HM803094	HM803032	HM802970	HM802995	*A.minisclerotigenes*
MUM 10.228	JF412778	HM803038	HM803062	HM803098	HM803025	HM802976	HM803000	*A.minisclerotigenes*
MUM 10.229	JF412779	HM803052	HM803078	HM803095	HM803031	HM802989	HM802998	*A.minisclerotigenes*
MUM 10.230	JF412780	HM803045	HM803063	HM803097	HM803014	HM802987	HM802994	*A.minisclerotigenes*
MUM 10.203	JF412781	HM802963	HM803067	HM803083	HM803026	HM802973	HM802999	*A.minisclerotigenes*
MUM 10.225	JF412784	HM803057	HM803074	HM803085	HM803027	HM802984	HM803001	*A. parasiticus*
MUM 10.212	JF412785	HM803036	HM803069	HM803092	HM803012	HM802969	HM803011	*A. parasiticus*
MUM 10.224	JF412786	HM803058	HM803075	HM803100	HM803033	HM802986	HM803007	*A. parasiticus*
MUM 10.215	JF412787	HM803037	HM803073	HM803081	HM803022	HM802974	HM803008	*A. parasiticus*
MUM 10.208	JF412770	HM802957	HM803076	HM803099	HM803017	HM802978	HM803004	*A. sergii*
MUM 10.219^{T}	JF412769	HM802967	HM803071	HM803082	HM803029	HM802985	HM803005	*A. sergii*
MUM 10.205	JF412771	HM803035	HM803070	HM803087	HM803021	HM802979	HM803002	*A. transmontanensis*
MUM 10.211	JF412772	HM803054	HM803060	HM803102	HM803023	HM802968	HM803003	*A. transmontanensis*
MUM 10.221	JF446612	HM803056	HM803072	HM803093	HM803028	HM802972	HM802996	*A. transmontanensis*
MUM 10.222	JF412773	HM803047	HM803064	HM803089	HM803030	HM802981	HM803009	*A. transmontanensis*
MUM 10.214^{T}	JF412774	HM803050	HM803065	HM803101	HM803020	HM802980	HM802997	*A. transmontanensis*
MUM 10.223	JF446613	HM802958	HM803077	HM803084	HM803024	HM802983	HM803010	*A. transmontanensis*
MUM 10.233	JF412768	HM803040	HM803066	HM803090	HM803013	HM802982	HM802991	*A. mottae*
MUM 10.231^{T}	JF412767	HM803042	HM803059	HM803086	HM803015	HM802988	HM803006	*A. mottae*
CBS 117610	EF409241			EF203158	EF202049			*A. arachidicola*
CBS 117611				EF203160	EF202052			*A. arachidicola*
CBS 117612				EF203159	EF202051			*A. arachidicola*
CBS 117615				EF203161	EF202050			*A. arachidicola*
NRRL 4868	JN185451			JN185450				*A. chungii*
NRRL 506	AF459735			JN185446	JN185447			*A. effuses*
CBS 110.55	FJ491463			EF203135	EF202056			*A. fasciculatus*
NRRL 1957	AF027863			AF255064	AF255041			*A. flavus*
NRRL 4818	EF661563			EF661489	EF661512			*A. flavus* var. *columnaris*
NRRL 3751	EF661554			EF661488	EF661511			*A. kambarensis*
NRRL 447	EF661560			EF661483	EF661506			*A. oryzae*
NRRL 502	AY373859			AY373859	AF255040			*A. parasiticus*
CBS 822.72	FJ491470			EF203163	EF202046			*A. toxicarius*

CBS = Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands.
MUM = Micoteca da Univerdade do Minho.
NRRL = Agricultural Research Service Culture Collection, Peoria, Illinois.

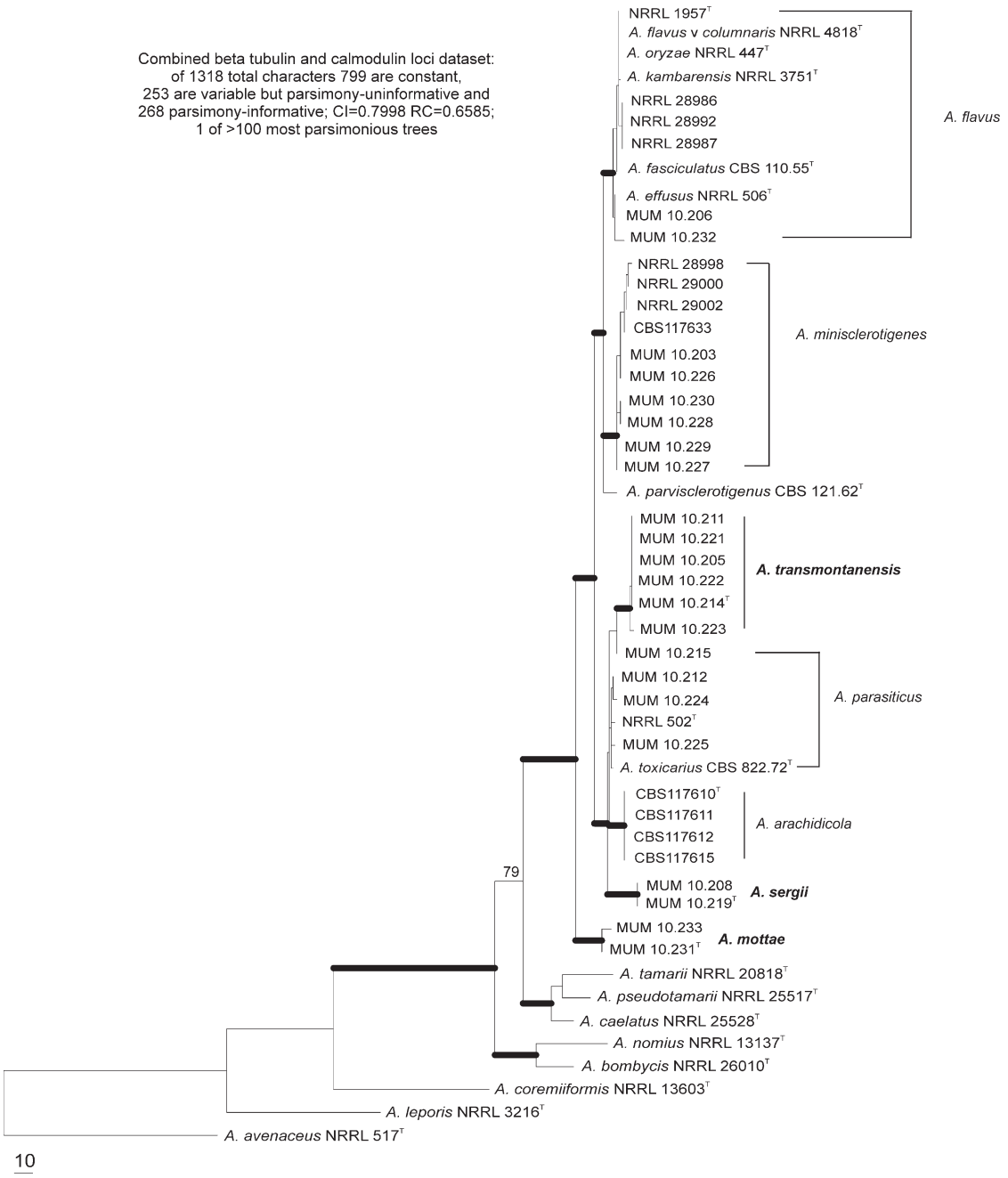

FIG. 1. Phylogenetic tree calculated from combined *BT2* and *CF* data. Double bold branches are supported with > 90% bootstrap value and > 0.90 Bayesian posterior probability. A number of named species appear synonymous with *A. flavus* because of their presence on the same strongly supported branch. *A. toxicarius* appears to be synonymous with *A. parasiticus* for the same reason. *A. transmontanensis*, *A. sergii* and *A. mottae* have distinct strongly supported positions in the tree.

tus, *A. kambarensis*, *A. effusus* and *A. flavus* var. *columnaris* (FIG. 1). There is no evidence from *BT2* and *CF* sequences that these latter species are distinct from *A. flavus*. *A. minisclerotigenes* and *A. parvisclerotigenus* occur on distinct and statistically supported sibling branches showing that they are species distinct from *A. flavus*. *A. parasiticus* isolates occur on a strongly supported branch that includes the ex-type isolate of *A. toxicarius*. These data provide no evidence that *A. toxicarius* is a distinct species. We also sequenced *BT2* and ITS regions from the ex-type isolate of *A. chungii* (NRRL 4868) but were unable to get a readable sequence from the calmodulin locus. BLAST comparisons of the *A. chungii* sequences showed that they are identical to the homologous loci from an ex-type isolate of *A. parasiticus* (NRRL 502)

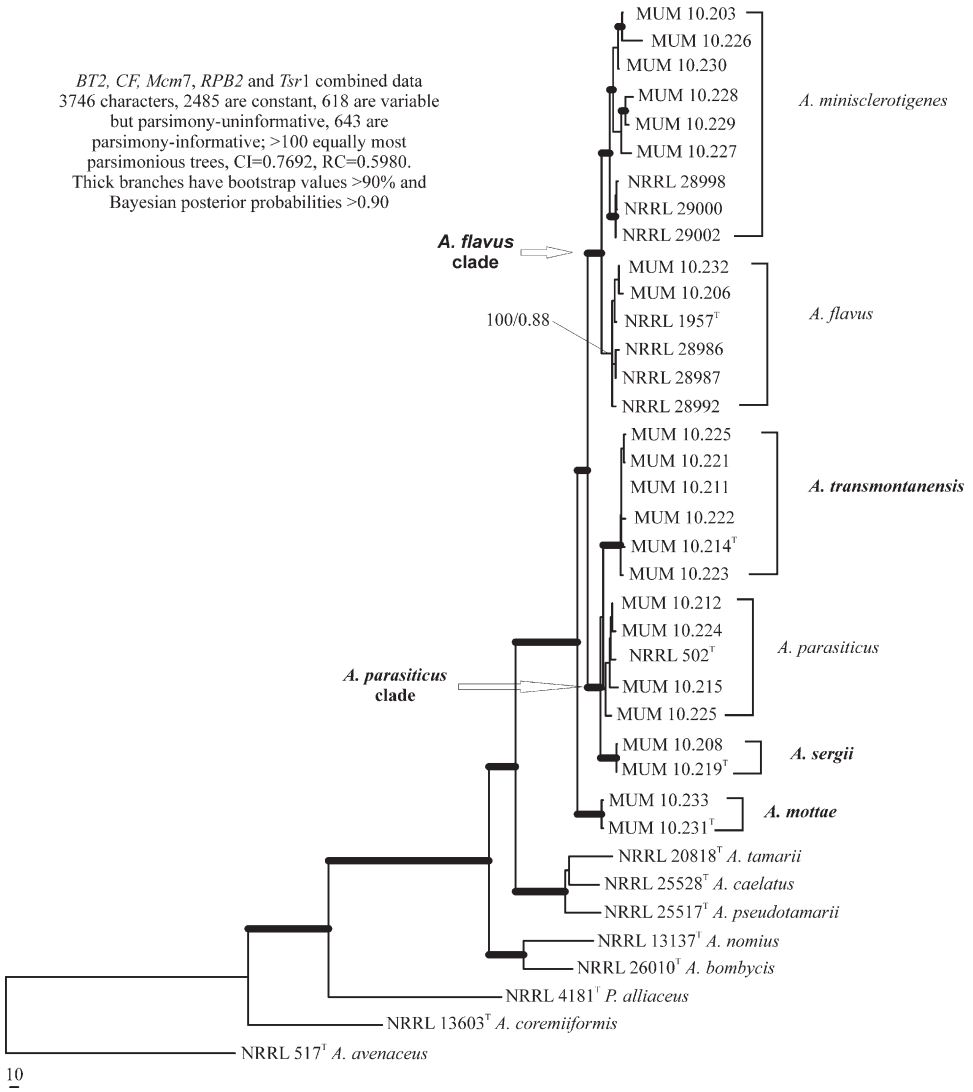

FIG. 2. Phylogenetic tree calculated from combined *BT2*, *CF*, *Mcm7*, *RPB2* and *TSR1* data. Double bold branches are supported with > 90% bootstrap value and > 0.90 Bayesian posterior probability. *A. mottae* has a strongly supported position as sharing a most recent common ancestor with either *A. flavus* or *A. parasiticus* and can serve as an outgroup for determining the polarity of character changesin both of these clades.

(SUPPLEMENTARY FIG. 1, for *BT2* sequence). Gonçalves et al. (2011) provided unpublished *BT2* sequences for their recently described species *A. novoparasiticus* (isolates LEMI 250/CBS 126849^T, LEMI249IPO/CBS 126850 and LEMI267) for comparisons with our newly proposed species. BLAST comparisons of the *A. novoparasiticus* sequences showed that they are similar to *A. parasiticus* but are different from the three new species reported in this work. This difference is also supported by phylogenetic analysis for *BT2* sequence (SUPPLEMENTARY FIG. 1).

The new species along with *A. flavus*, *A. parasiticus*, *A. parvisclerotigenus*, *A. arachidicola* and *A. minisclerotigenes* form a monophyletic group in section *Flavi* that is composed of (i) an *A. flavus* clade containing *A. flavus*, *A. parvisclerotigenus and A. minisclerotigenes*, (ii) an *A. parasiticus* clade containing *A. parasiticus*, *A. arachidicola*, *A. transmontanensis* and *A. sergii* and (iii) *A. mottae* as a sibling to the clade containing both *A. parasiticus* and *A. flavus* (FIG. 1).

In the broad *A. flavus* clade *A. minisclerotigenes* isolates form a strongly supported clade (100% bs/1.00 pp) with a subclade containing three isolates from Australia (FIG. 2, TABLE I). Some of the individual locus trees (SUPPLEMENTARY FIGS. 1–5) have strongly supported subclades that include both Australian and Portuguese isolates of *A. minisclerotigenes*. *A. flavus* isolates examined (except type strain NRRL 1957) also originated from either Portugal or Australia. As with the *A. minisclerotigenes* isolates,

Species	Strains	MAT1-1 amino acid sequence
A. mottae	MUM 10.231	PEQLEELLKYLQDAKSQENTQSSYPKENLQSCLEFKADKNNGSTTPASANPRSSASRGKRTSDAKRRPLNSFIAFRSYYSVMFP-DLTQKAKSGILRFLWQNDPFKA
A. mottae	MUM 10.233	.....
A. flavus	MUM 10.206	.....F.....N.....V.....A.....
A. flavus	MUM 10.232	.....F.....N.....V.....A.....
A. flavus	NRRL 1957	.....F.....N.....V.....A.....
A. flavus	NRRL_28987	.....F.....N.....V.....A.....
A. minisclerotigenes	MUM 10.227	.....N.....A.....
A. minisclerotigenes	MUM 10.228	.....N.....A.....
A. minisclerotigenes	MUM 10.229	.....N.....A.....
A. minisclerotigenes	MUM 10.230	.....N.....A.....
A. minisclerotigenes	NRRL 29002	....K.....N.....A.....
A. parasiticus	MUM 10.212	.....N.....A.....I.....
A. parasiticus	MUM 10.215	.....N.....A.....I.....
A. parasiticus	MUM 10.224	.....N.....A.....I.....
A. parasiticus	MUM 10.225	.....N.....A.....I.....
A. transmontanensis	MUM 10.205	.....N....S.....A.....
A. transmontanensis	MUM 10.211	.....N....S.....A.....
A. transmontanensis	MUM 10.214	.....N....S.....A.....
A. transmontanensis	MUM 10.221	.....N....S.....A.....
A. transmontanensis	MUM 10.222	.....N....S.....A.....
A. caelatus	NRRL 25528	.....A.G.P.P...N..I.....H.....S...T.....A.....
A. pseudotamarii	NRRL 25517	.....A.G.P.....N.....H.....S...T.....A.....
A. tamarii	NRRL 20818	.....A.G.P.....N....P...S.....H...I.....S...T.....V.....
A. nomius	NRRL 13137	.....V.A...S.....N.KF.....SH.....S.T...V.....A.....

FIG. 3. Amino acid sequence alignment of a 317-bp fragment of *MAT1-1* amplification. Regions with dots indicate identical amino acids residues; regions with amino acids are not conserved.

three *A. flavus* isolates originally from Australia formed a subclade at some loci while at other loci they occurred in strongly supported subclades intermixed with Portuguese isolates. *A. flavus* and *A. minisclerotigenes* are strongly supported as sibling species (FIG. 2).

In the broad *A. parasiticus* clade *A. parasiticus* isolates had variable sequences at each of the five loci and the isolates form a clade at each locus (SUPPLEMENTARY FIGS. 1–5). There was strong statistical support for the *A. parasiticus* clade in four of the five loci examined. *Aspergillus transmontanensis* isolates were isogenic at the *BT2* locus and variable at the other loci. In four of the five loci statistical support for this clade was strong. While the isolates were sequence variable, no consistent subclades were present in the data. *Aspergillus sergii* isolates were isogenic at four of the five loci and formed a distinct strongly statistically supported clade sister to *A. parasiticus* (FIG. 2).

Aspergillus mottae isolates were isogenic at three of five loci and in each case formed a distinct clade with greater than 85% bs support at four of the five loci (SUPPLEMENTARY FIGS. 1–5). *A. mottae* is basal in the tree relative to the *A. flavus* and *A. parasiticus* clades with very strong statistical support (FIG. 2).

Two of the 22 Portuguese isolates were identified as *A. flavus*, six as *A. minisclerotigenes* and four as *A. parasiticus*. Examination of morphological characters combined with the analysis of aflatoxin and CPA production and molecular data revealed that 10 of the *Aspergillus* section *Flavi* strains isolated did not match described species in section *Flavi*. Therefore we propose three new species, *A. mottae*, *A. sergii* and *A. transmontanensis* (FIGS. 1, 2), to accommodate these Portuguese isolates.

Type strains and all Portuguese isolates were screened to determine mating type, either *MAT1-1* or *MAT1-2*, and these genes subsequently were sequenced. The resulting PCR showed that all strains are heterothallic with 24 strains assigned to *MAT1-1* and 13 strains to*MAT1-2*. The *MAT1-1* sequence set contained 375 aligned sequence positions (317 exon positions, 58 intron positions). The *MAT1-2* aligned sequence set contained 238 sequence positions (182 exon positions, 56 intron positions). In silico translation of the DNA sequences showed amino acid differences in some cases (FIGS. 3, 4). In the case of *MAT1-1* all species showed intraspecific single-nucleotide polymorphisms (SNPs). In the alignment (FIG. 3) *A. mottae* was used as the reference sequence because it is ancestral to the other new species and to

Species	Strains	Mating type 1-2 protein sequence
A. minisclerotigenes	MUM 10.226	AYPDFTNNEISIILGKQWKAESEEVKMQFRNMAEELKKKHAEDHPDYHYTPRKP
A. minisclerotigenes	MUM 10.203	.....
A. flavus	NRRL 28986	.....K.....
A. parasiticus	NRRL 502	.....
A. transmontanensis	MUM 10.223	.....
A. sergii	MUM 10.208	.....
A. sergii	MUM 10.219	.....
A. bombycis	NRRL 26010	.....D.....
A. lanosus	NRRL 3648	.....M.....A.V...S.....R.....Y.....
P. alliaceus	NRRL 20602	.....M.....A.V...S.....R.....---
P. alliaceus	NRRL 4181	.....M.....A.V...S.....R.....
A. alliaceus	NRRL 5108	...N.....M.....A.V...S.....R.....
A. avenaceus	NRRL 517	...E.....S.T..I.V...G..D.....

FIG. 4. Amino acid sequence alignment of a 238-bp fragment of *MAT1-2* amplification.

TABLE IV. Production of aspergillic acid (AA); aflatoxins B_1 (AFB1), B_2 (AFB2), G_1 (AFG1) and G_2 (AFG2); and cyclopiazonic acid (CPA) of *Aspergillus* section *Flavi* isolates using different media (AFPA, YES and CYA)

Code	Region	AA AFPA	AFG2 YES	AFG1 YES	AFB2 YES	AFB1 YES	CPA CYA	Species
MUM 10.206	Tras–os–Montes	+	−	−	+	+	+	*A. flavus*
MUM 10.232	Alentejo	+	+	+	+	+	+	*A. flavus*
MUM 10.226	Alentejo	+	+	+	+	+	+	*A.minisclerotigenes*
MUM 10.227	Alentejo	+	+	+	+	+	+	*A.minisclerotigenes*
MUM 10.228	Alentejo	+	−	−	+	+	+	*A.minisclerotigenes*
MUM 10.229	Alentejo	+	+	+	+	+	+	*A.minisclerotigenes*
MUM 10.230	Alentejo	+	+	+	+	+	+	*A.minisclerotigenes*
MUM 10.203	Tras-os-Montes	+	−/+	+	−/+	+	+	*A.minisclerotigenes*
MUM 10.225	Tras-os-Montes	+	+	+	+	+	−	*A. parasiticus*
MUM 10.212	Algarve	+	−	−/+	+	+	−	*A. parasiticus*
MUM 10.224	Algarve	+	−	−	+	+	−	*A. parasiticus*
MUM 10.215	Algarve	−	+	+	+	+	−	*A. parasiticus*
MUM 10.208	Tras-os-Montes	+	+	+	+	+	+	*Aspergillus sergii*
MUM 10.219	Algarve	+	+	+	+	+	+	*Aspergillus sergii*
MUM 10.205	Tras-os-Montes	+	+	+	+	+	−	*Aspergillus transmontanensis*
MUM 10.211	Tras-os-Montes	−	+	+	+	+	−	*Aspergillus transmontanensis*
MUM 10.221	Tras-os-Montes	+	+	+	+	+	−	*Aspergillus transmontanensis*
MUM 10.222	Tras-os-Montes	+	+	+	+	+	−	*Aspergillus transmontanensis*
MUM 10.214	Algarve	+	+	+	+	+	−	*Aspergillus transmontanensis*
MUM 10.223	Tras-os-Montes	+	+	+	−/+	+	−	*Aspergillus transmontanensis*
MUM 10.233	Alentejo	+	+	+	+	+	−	*Aspergillus mottae*
MUM 10.231	Ribatejo	+	+	+	+	+	+	*Aspergillus mottae*

− not detected; −/+ very weak signal; + detected.

A. flavus and *A. parasiticus*. *A. flavus* isolates had four synapomorphic amino acid differences from *A. mottae*. *A. parasiticus*, *A. transmontanensis* and *A. minisclerotigenes* had one or more synapomorphic amino acid differences from *A. mottae* and other species. *A. minisclerotigenes* NRRL 29002 from Australia had a single apomorphic difference from all other isolates of the species. *Aspergillus tamarii*, *A. caelatus and A. pseudotamarii* each represented by the type isolate shared seven synapomorphic differences from the *A. flavus/A. parasiticus* clade isolates and also had several apomorphic differences from each other. Similarly *A. nomius* differed from each of the preceding clades about equally. The two isolates of *A. sergii* were both *MAT1-2*.

The *MAT1-2* amino acid sequences (FIG. 4) were quite similar, with *A. sergii*, *A. parasiticus*, *A. minisclerotigenes* and *A. transmontanensis* having identical amino acid sequences (FIG. 4). Our only *A. flavus MAT1-2* sequence and our only *A. bombycis MAT1-2* sequence differed from the above and each other at one amino acid site. *A. lanosus*, *A. alliaceus* and *A. avenaceus* display 5–7 amino acid differences from the sequences of the other species.

Mycotoxin production.—CPA was produced by 11 of the 22 Portuguese isolates examined (TABLE IV). *A. flavus* isolates MUM 10.232 and MUM 10.206 showed very different mycotoxin profiles. The almond isolate (MUM 10.206) was a weak AFB producer but produced CPA as expected for this species. The maize isolate (MUM 10.232) produced AFBs and AFGs in high quantities and CPA only very weakly. Five of the six strains identified as *A. minisclerotigenes* produced AFBs, AFGs and CPA as described by Pildain et al. (2008), while one isolate (MUM 10.228) did not produce AFGs. The maize isolates were strong producers of aflatoxins, whereas the almond strain (MUM 10.203) was a weak producer. *A. sergii* isolates produced AFGs, AFBs and CPA. Isolate MUM 10.208 showed strong aflatoxin production ability, but isolate MUM 10.219 produced aflatoxins at lower levels. *A. mottae* isolates produced both B and G aflatoxins and one of the two isolates produced CPA. Aspergillic acid is produced by most of the tested strains (20/22), not being produced by one isolate of *A. parasiticus* and one of *A. transmontanensis*.

A. parasiticus isolates (MUM 10.212; 10.215; 10.224 and 10.225) did not produce CPA, as expected, but in contrast to Frisvad et al. (2006), Pitt and Hocking (2009) MUM 10.212 is a weak producer of AFGs and AFGs were not detected in MUM 10.224 (TABLE IV). All isolates of the newly described species *A. transmontanensis* produced AFBs and AFGs but not CPA. However HPLC chromatograms of AFs and CPA

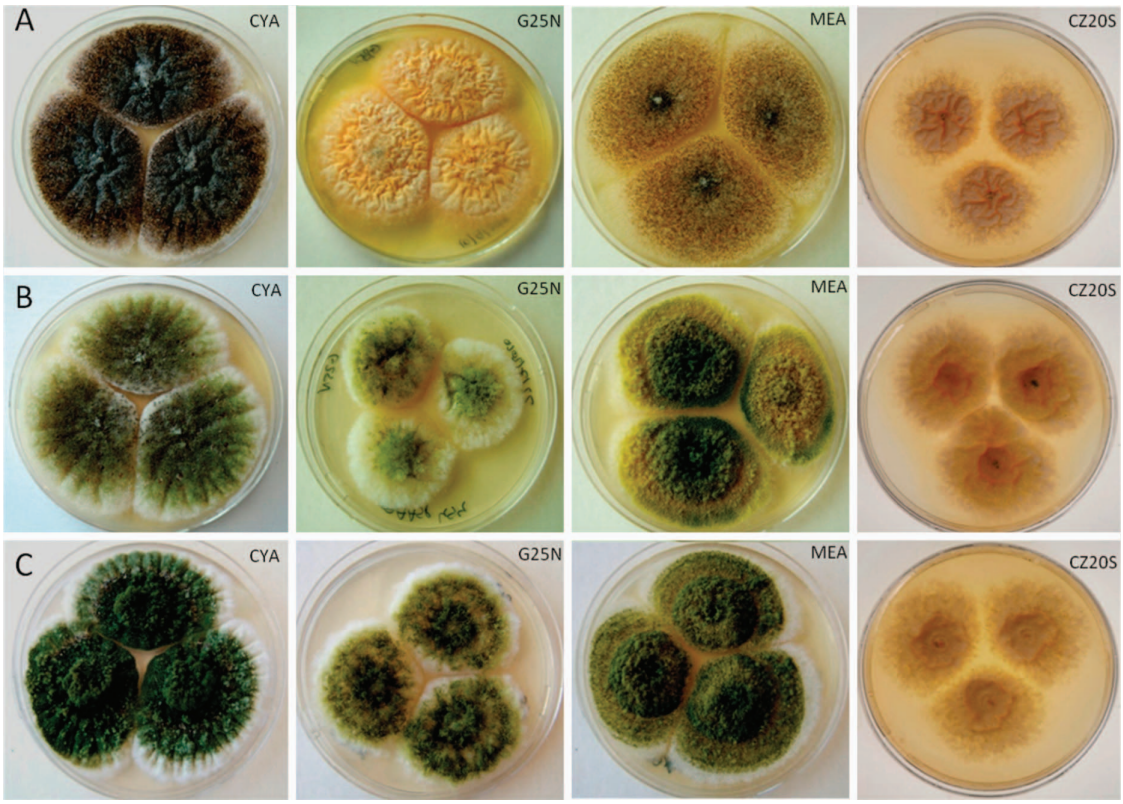

FIG. 5. A. *Aspergillus mottae* MUM 10. 231. B. *Aspergillus sergii* MUM 10.219. C. *Aspergillus transmontanensis* MUM 10.214.Colonies grown on CYA, G25N, MEA and CZ20S at 25 C for 7 d.

for *A. transmontanensis* were distinctive from those of *A. parasiticus*. A. *transmontanensis* isolates all produced higher amounts of AFGs than AFBs. Diener and Davis (1966) reported that to be a characteristic of all A. *parasiticus* isolates when grown at 25 C but, in our study under similar test conditions a high AFG/AFB ratio was detected only for A. *transmontanensis* while A. *parasiticus* isolates produced equivalent amounts of both toxins.

TAXONOMY

Aspergillus mottae C. Soares, S.W. Peterson et A. Venâncio sp. nov. FIGS. 5A; 6a, b, c
MycoBank MB561841

A simili *Aspergillo flavor* capitulis luteo-flavis sparsissimis, sed 42 C augmentatis, sclerotiis parvis luteis copiosis et exudatione aflatoxini B et G et acidi cyclopiazonici distinguendus.

Holotype. MUM-H 10.231. Preserved dried colonies of MUM 10.231 deposited in the herbarium, Braga, Portugal.

Etymology. The epithet pays tribute to Prof Dr Manuel Mota who has been a mentor for many generations of Portuguese biotechnologists and is currently head of the Biological Engineering Centre of Minho University. He has been a great supporter of the fungal culture collection Micoteca da Universidade do Minho (MUM).

Colonies on CYA attained > 70 mm diam in 7 d at 25 C, 50–54 mm diam at 37 C and 20–40 mm diam at 42 C; colonies on MEA attained > 70 mm diam at 25 C, 50–53 mm diam at 37 C and 20–40 mm diam at 42 C; colonies on G25N attained 40 mm diam at 25 C, 30 mm diam at 37 C and 17–20 mm diam at 42 C; colonies on CZ20S attained 35–40 mm diam at 25 C, > 70 mm diam at 37 C; and 17–20 mm diam at 42 C; no growth occurred at 5 C. Colony surface plane, mycelia white, yellow-green conidia heads scarce at 25 C, numerous dark brown small sclerotia, 249–371 µm diam, covering the plate on CYA and MEA, sclerotia yellow and fewer on G25N and CZ20S media (FIG. 5A), conidial heads more plentiful with growth at 42 C. Conidial heads normally biseriate but uniseriate heads also occur. Vesicles globose to subglobose 36–43 µm diam; metulae 9.0–11.8 × 3.6–5.4 µm; phialides 5.8–8.2 × 2.8–4.8 µm; stipes hyaline, smooth; conidia globose to subglobose, smooth to finely rough, 3.3–4.3 µm diam (FIG. 6a, b, c).

Mycotoxin production. aspergillic acid, aflatoxins B1, B2, G1, G2.

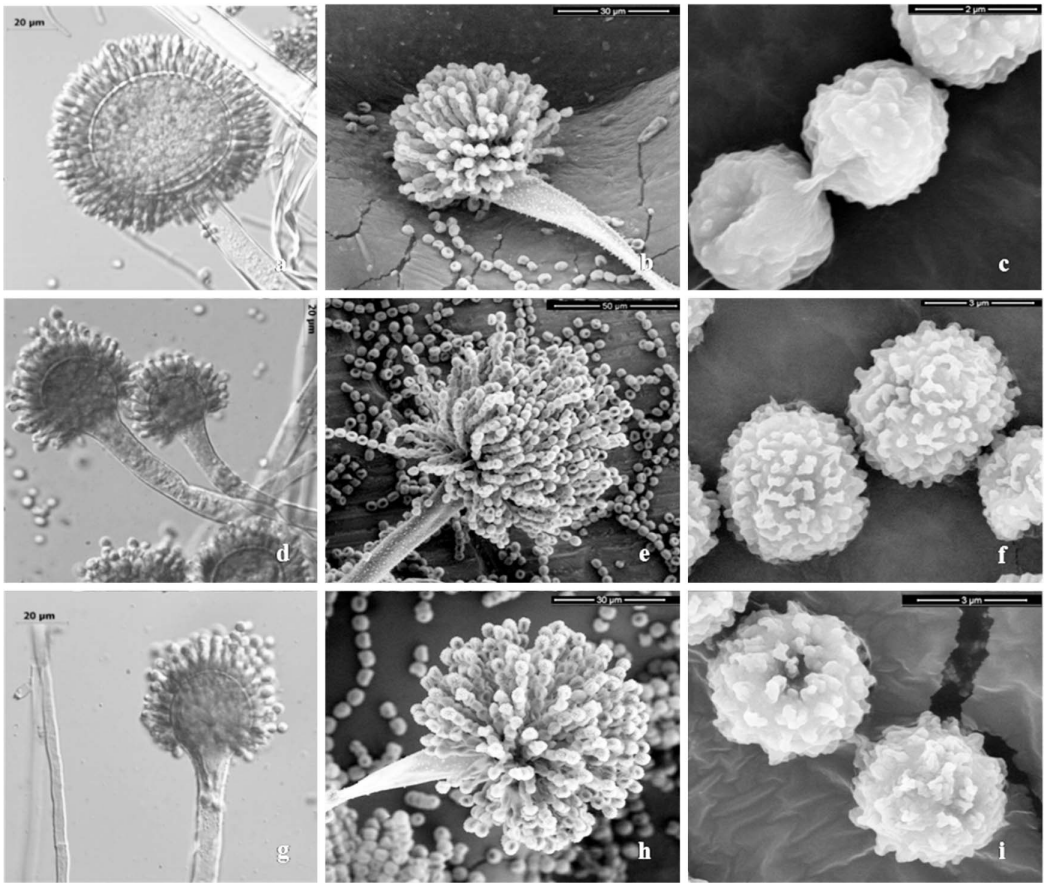

FIG. 6. *Aspergillus mottae* MUM 10.231, conidiophores (a, b) and conidia (c). *Aspergillus sergii* MUM 10.219, conidiophores (d, e) and conidia (f). *Aspergillus transmontanensis* MUM 10.214, conidiophores (g, h) and conidia (i).

On a phenotypic basis *A. mottae* resembles *A. flavus*, *A. nomius*, *A. bombycis*, *A. arachidicola* and *A. minisclerotigenes* in having yellow-green biseriate conidial heads. *A. arachidicola* and *A. bombycis* are not known to produce sclerotia, whereas *A. mottae* produces numerous small dark sclerotia, such as *A. minisclerotigenes*, *A. parvisclerotigenus* and some strains of *A. flavus*. *A. flavus* isolates are variable for sclerotium production, producing dark sclerotia when present, and *A. flavus* vesicles are up to 85 µm diam, whereas the vesicles of *A. mottae* are 36–43 µm diam. Conidia of *A. mottae* appear smooth to finely rough, similar to those of *A. flavus*. A notable distinction of *A. flavus* and *A. mottae* is that *A. flavus* produces B aflatoxins while *A. mottae* produces both B and G aflatoxins. The species also can be distinguished on the basis of beta tubulin or calmodulin DNA sequences.

Isolates examined.—MUM 10.231 (CBS 130016), PORTUGAL. RIBATEJO, Riachos place, from maize seed, Apr 2009, *Célia Soares 09MAsp200*; ex-type culture. MUM 10.233, PORTUGAL. RIBATEJO, Riachos place, from maize seed, Apr 2009, *Célia Soares 08MAsp571*.

Habitat. In kernels of *Zea mays* L.

Distribution. Known only from Portugal.

Aspergillus sergii P. Rodrigues, S.W. Peterson, A. Venâncio et N. Lima sp. nov. FIGS. 5B; 6d, e, f). MycoBank MB561842

A simili *Aspergillo parasítico* coloniis pallidioribus luteoviridibus et exudatione acidi cyclopiazonici differt; aflatoxina B et G etiam producit.

Holotype. MUM-H 10.219. Dried colonies of MUM 10.219 deposited in the herbarium, Braga, Portugal.

Etymology. The epithet pays tribute to Prof Dr Sérgio Machado dos Santos who was rector of Minho University 1985–1998 when the Micoteca da Universidade do Minho (MUM) was established. He has been a great supporter of this fungal culture collection.

Colonies after 7 d growth on CYA attained 55 mm diam at 25 C, 60 mm diam at 37 C and 15–25 mm diam at 42 C; colonies on MEA attain 55 mm diam at 25 C, 55 mm diam at 37 C and 15–25 mm diam at 42 C, colonies on G25N attained 37 mm diam at 25 C, 40 mm at 37 C and 10–20 mm at 42 C; colonies on CZ20S attained 40 mm diam at 25 C, > 70 mm diam

at 37 C and 15–25 mm diam at 42 C; no growth at 5 C. Colony surface is plane, velvety and dense; conidial heads in a uniform, dense layer but sparse in the areas of sclerotium production showing a color between those of *A. flavus* and *A. parasiticus* (FIG. 5B); sclerotia brown, type L, 513–551 μm diam. Conidial heads uniseriate; vesicles globose, 26–36 μm diam; phialides 5.5–6.8 × 2.5–3.1 μm; stipes hyaline, smooth; conidia globose to subglobose, rough, greenish, 3.3–4.3 μm diam (FIG. 6d, e, f).

Mycotoxin production. aspergillic acid, aflatoxins B1, B2, G1, G2 and CPA.

Aspergillus sergii most closely resembles *A. parasiticus* because of the rough conidia and the production of predominantly uniseriate conidial heads. The two species differ in colony color, which is a lighter green in *A. sergii*, and on phialide and conidial sizes. *A. sergii* phialides are 5.5–6.7 × 2.5–3.1 μm, while *A. parasiticus* phialides are 7–10 × 2.5–5; conidia of *A. sergii* are 3.3–4.2 μm diam and roughened, while those of *A. parasiticus* are 4–6 μm diam and roughened to echinulate. *A. sergii* also differs from *A. parasiticus* by CPA production. The morphological species concept for *A. sergii* is based on two isolates and is necessarily subject to revision as additional isolates are found and studied. DNA sequences from the beta tubulin or calmodulin locus also distinguish this species.

Isolates examined.—MUM 10.219 (CBS 130017), PORTUGAL. TRÁS-OS-MONTES, (processor plant), from in-shell almond originating Faro, 22 May 2009, *Paula Rodrigues 09AAsp494*; ex-type culture. MUM 10.208, PORTUGAL. TRÁS-OS-MONTES, Moncorvo (almond orchard), in-shell fruit at the time of harvest, 12 Sep 2008, *Paula Rodrigues 08AAsp183*. MUM 10.248, PORTUGAL. TRÁS-OS-MONTES, Alfândega da Fé (processor plant), from shelled almond originating from Faro, 22 May 2009, *Paula Rodrigues 09AAsp305*. MUM 10.251, PORTUGAL. TRÁS-OS-MONTES, Alfândega da Fé (processor plant), from shell of almonds originating from Faro, 22 May 2009, *Paula Rodrigues 09AAsp488*.

Habitat. Fruits of *Prunus dulcis* (Miller) D.A. Webb.

Distribution. Known only from Portugal.

Aspergillus transmontanensis P. Rodrigues, S.W. Peterson, N. Lima et A. Venâncio sp. nov. FIGS. 5C, 6g, h, i MycoBank MB561843

A simili *Aspergillo parasítico* sclerotiis brunneis, copiosis, 450–610 μm diam differt.

Holotype. Preserved as dried colonies and deposited in the herbarium MUM-H 10.214

Etymology. The epithet refers to the Portuguese region Trás-os-Montes where this fungus was collected and where Prof Dr Sérgio Machado dos Santos and Prof Dr Manuel Mota grew up as ''transmontanos''.

Colonies grown 7 d on CYA attained 55–57 mm diam at 25 C, 55–57 mm diam at 37 C and 10–20 mm diam at 42 C; colonies on MEA attained 55–57 mm diam at 25 C, 55–57 mm diam at 37 C and 10–20 mm diam at 42 C; colonies on G25N attained 45 mm diam at 25 C, 40 mm diam at 37 C and 10–20 mm diam at 42 C; colonies on CZ20S attained 45–47 mm diam at 25 C, 55–60 mm diam at 37 C and 10–20 mm diam at 42 C; no growth at 5 C. Colony surface on CYA is dense and velutinous; conidial heads form a uniform, dense layer but are more sparse in the areas of sclerotium production and are dark yellow-green (FIG. 5C). Colonies on MEA and G25N are similar to growth on CYA with conidia heads more dense and floccose on MEA and more velutinous on G25N (FIG. 5C). Sclerotia brown, type L, 458–609 μm diam; conidial heads mostly uniseriate, vesicles globose to subglobose 27.5–38.2 μm diam; metulae (when present) 7.1–12.3 × 3.0–4.6 μm; phialides 6.8–8.4× 3.0–4.8 μm; stipes hyaline smooth; conidia globose to subglobose, rough, greenish 4.1–5.1 μm diameter (FIG. 6G, H, I).

Mycotoxin production. aflatoxins B1, B2, G1, G2.

Aspergillus transmontanensis is similar to *A. parasiticus* in colony growth and microscopic characters, with conidia, metulae, phialides, vesicles and conidiophores having overlapping sizes. While *A. transmontanensis* has primarily biseriate conidial heads, *A. parasiticus* usually has primarily uniseriate conidial heads. However *A. toxicarius*, which was shown to be a synonym of *A. parasiticus* by Pildain et al (2008), was noted by Christensen (1981) to be predominantly biseriate. *A. parasiticus* sclerotia are 160–530 μm diam, while *A. transmontanensis* produces larger (450–610 μm diam) brown sclerotia in abundance.

Isolates examined. MUM 10.214 (CBS 130015), PORTUGAL. TRÁS-OS-MONTES, Alfândega da Fé (processor plant), from shelled almond originating from Faro, 20 Mar 2009, *Paula Rodrigues 09AAsp260*; ex-type culture.MUM 10.205, PORTUGAL. TRÁS-OS-MONTES, Alfândega da Fé (processor plant), from shelled almond originating from Moncorvo, 29 Feb 2008, *Paula Rodrigues 08AAsp67*. MUM 10.211, PORTUGAL. TRÁS-OS-MONTES, Alfândega da Fé (processor plant), from shelled almond originating from Moncorvo, 20 Mar 2009, *Paula Rodrigues 09AAsp146*. MUM 10.221, PORTUGAL. TRÁS-OS-MONTES, Alfândega da Fé (processor plant), from shelled almond originating from Moncorvo, 20 Mar 2009, *Paula Rodrigues 09AAsp152*. MUM 10.222, PORTUGAL. TRÁS-OS-MONTES, Alfândega da Fé (processor plant), from in-shell almond originating from Faro, 20 Mar 2009, *Paula Rodrigues 09AAsp201*. MUM 10.223, PORTUGAL. TRÁS-OS-MONTES, Alfândega da Fé (processor plant), from shelled almond originating from Moncorvo, 22 May 2009, *Paula Rodrigues 09AAsp298*. MUM 10.243, PORTUGAL. TRÁS-OS-MONTES, Alfândega da Fé (processor plant), from shelled almond originating from Moncorvo, 20 Mar 2009, *Paula Rodrigues 09AAsp153*. MUM

10.244, PORTUGAL. TRÁS-OS-MONTES, Alfândega da Fé (processor plant), from in-shell almond originating from Faro, 20 Mar 2009, *Paula Rodrigues 09AAsp233*. MUM 10.245, PORTUGAL. TRÁS-OS-MONTES, Alfândega da Fé (processor plant), from in-shell almond originating from Faro, 20 Mar 2009, *Paula Rodrigues 09AAsp239*. MUM 10.246, PORTUGAL. TRÁS-OS-MONTES, Alfândega da Fé (processor plant), from in-shell almond originating from Faro, 20 Mar 2009, *Paula Rodrigues 09AAsp261*. MUM 10.247, PORTUGAL. TRÁS-OS-MONTES, Alfândega da Fé (processor plant), from shelled almond originating from Moncorvo, 22 May 2009, *Paula Rodrigues 09AAsp299*. MUM 10.250, PORTUGAL. TRÁS-OS-MONTES, Alfândega da Fé (processor plant), from in-shell almond originating from Faro, 22 May 2009, *Paula Rodrigues 09AAsp487*.

Habitat. Fruits of *Prunus dulcis*.

Distribution. Known only from Portugal.

DISCUSSION

The genealogical concordance phylogenetic species concept is based on recognizing the boundaries of species by concordance of the tree diagrams from different unlinked loci and the intraspecific variation of the isolates of each species. *Aspergillus transmontanensis* fulfills these requirements to be recognized as a species. We have insufficient isolates of *A. sergii* and *A. mottae* to apply the concordance species concept, but each is on a unique branch that is statistically distinct from other accepted species.

Like others (e.g. Varga et al 2009) we consider several species used in the phylogenetic study to be synonyms. Ex-type isolates of *A. orzyae*, *A. fasciculatus*, *A. kambarensis*, *A. effusus* and *A. flavus* var. *columnaris* were treated as synonyms of *A. flavus*; ex-type isolates of *A. toxicarius* and of *A. chungii* (NRRL 4868) were not considered distinct from *A. parasiticus* (NRRL 502). Comparisons of the newly described species *A. novoparasiticus* with our newly proposed species demonstrate that *A. novoparasiticus* is not a synonym of any of the three new species (*A. transmontanensis*, *A. sergii* and *A. mottae*) being described.

Aspergillus mottae and *A. sergii* are separated from *A. parasiticus* and *A. minisclerotigenes* by relatively minor phenotypic differences, and the two isolates of each species available to us leave some doubts about how well these distinctions will separate the species once additional isolates of each are known, but the DNA sequence distinctions have high statistical support.

The acquisition of additional isolates of *A. sergii* and *A. mottae* will enable concordance analysis and testing of our taxonomic hypothesis. In the combined data tree (FIG. 2) and in some individual loci the *A. minisclerotigenes* isolates from Australia are strongly supported as a distinct clade, but at other loci Australian and Portuguese isolates reside in a single clade that is contradictory to the combined data tree. It is interesting that an *A. minisclerotigenes* isolate from Australia differs at one amino acid site from all other isolates of the species in the *MAT1-1* mating-type gene. These isolates appear to be components of a single gene pool and are regarded here as conspecific. By the results presented here we extend the known range of *A. minisclerotigenes* to Europe in addition to South America and Australia (Pildain et al. 2008).

A. transmontanensis, *A. sergii* and *A. mottae* are well supported by phylogenetic analysis of the multilocus combined data. Their branches are fully congruent because the same group of isolates always occurs as a terminal group at each locus and there is strong statistical support for that grouping, either by bootstrap analysis or Bayesian posterior probability analysis (Peterson 2008). *Aspergillus mottae* shares a most recent common ancestor with both the *A. flavus* and *A. parasiticus* clades. For this reason study of *A. mottae* might be useful in analyzing the evolution of characters and toxins in the *A. flavus* and *A. parasiticus* clades (FIG. 2). *A. transmontanensis* and *A. sergii* share a most recent common ancestor with *A. parasiticus*. Donner et al. (2009) reported the existence of a strain (S_{BG}), isolated from maize from Nigeria, that produces high AFGs and AFBs and numerous small sclerotia. These are some of the characteristics of the new proposed species *A. mottae*, but we have not had access to S_{BG} isolates. Molecular phylogenetics suggest that *A. transmontanensis* and *A. sergii* represent species distinct from both *A. flavus* and *A. parasiticus*.

Nucleotide sequences comparison of the mating-type gene reveals that there are larger variations between species than within species; most isolates in this study showed species-specific SNPs. In some cases the SNPs were insufficient to change the amino acid residues. This is the case for all *MAT1-1* isolates of *A. parasiticus*, which had three SNPs but without amino acid replacement (FIG. 3). The small changes in the nucleotide sequence of the *MAT* gene might indicate the normal variation within species. Bigger changes however might indicate the loss of the *MAT* gene function (Turgeon 1998).

The mycotoxin profile of strains belonging to *Aspergillus* section *Flavi* has been routinely used for identification purposes (Rodrigues et al. 2009). *A. parasiticus* strains are usually more consistent than *A. flavus*, being relatively uniform in their toxin production, strongly producing aflatoxins AFBs and AFGs but not CPA. Non-aflatoxigenic strains are unusual (Vaamonde et al. 2003). However two isolates

identified as *A. parasiticus*(MUM 10.212 and MUM 10.224) showed that one does not produce detectable amounts of AFG while the other has a weak production but these isolates appear in an internal branch in the main clade of *A. parasiticus* with bootstrap value of 100% and 1.00 of Bayesian probability and share all morphological characteristics of this species.

A. flavus populations have been found to be extremely diverse in toxigenicity (Giorni et al. 2007) and that can be seen with the two *A. flavus* isolates in our study. While the isolate MUM 10.206 produces AFBs and CPA, as it is most commonly accepted, the isolate MUM 10.232 shows high production of AFGs and AFBs and moderate production of CPA, as well as large quantities of sclerotia under 400 µm diam. Strains of *A. flavus* with these characteristics already have been isolated from agricultural soils in Thailand (Saito et al. 1986) and from maize soils in Nigeria (Donner et al. 2009), but it is possible that some of these strains are not *A. flavus*.

A. minisclerotigenes strains have been described as AFGs, AFBs and CPA producers (Pildain et al. 2008). However one of the isolates identified as belonging to this species does not produce AFGs. The Portuguese *A. minisclerotigenes* isolates form a different branch from the Australian ones with 100% bootstrap values and 1.00 posterior probability but share the morphological characteristics of this species.

ACKNOWLEDGMENTS

The authors appreciate the Latin translation of the diagnoses by Dr Walter Gams. The mention of firm names or trade products does not imply that they are endorsed or recommended by the U. S. Department of Agriculture over other firms or similar products not mentioned. USDA is an equal opportunity provider and employer. Célia Soares and Paula Rodrigues were supported by grants from Fundação para a Ciência e Tecnologia (references SFRH/BD/37264/2007 and SFRH/PROTEC/49555/2009 respectively).

LITERATURE CITED

Bamba R, Sumbali G. 2005. Co-occurrence of aflatoxin B1 and cyclopiazonic acid in sour lime (*Citrus aurantifolia* Swingle) during post-harvest pathogenesis by *Aspergillus flavus*. Mycopathologia 159:407–411, doi:10.1007/s11046-004-8401-x

Baum DA, Shaw KL. 1995. Genealogical perspectives on the species problem. In: Hoch PC, Stephenson AG, eds. Experimental and molecular approaches to plant biosystematics. St Louis: Missouri Botanical Garden. p 289–303.

Bennett JW, Klich M. 2003. Mycotoxins. Clin Microbiol Rev 16:497–516, doi:10.1128/CMR.16.3.497-516.2003

Christensen M. 1981. A synoptic key and evaluation of species in the *Aspergillus flavus* group. Mycologia 73:1056–1084, doi:10.2307/3759676

Dettman JR, Jacobson DJ, Taylor JW. 2006. Multilocus sequence data reveal extensive phylogenetic species diversity within the *Neurosporadiscreta* complex. Mycologia 98:436–446, doi:10.3852/mycologia.98.3.436

Diener UL, Davis ND. 1966. Aflatoxin production by isolates of *Aspergillus flavus*. Phytopathology 56:1390–1393.

Donner M, Atehnkeng J, Sikora RA, Bandyopadhyay R, Cotty PJ. 2009. Distribution of *Aspergillus* section *Flavi* in soils of maize fields in three agroecological zones of Nigeria. Soil Biol Biochem 41:37–44, doi:10.1016/j.soilbio.2008.09.013

Frisvad JC, Skouboe P, Samson RA. 2005. Taxonomic comparison of three different groups of aflatoxin producers and a new efficient producer of aflatoxin B1, sterigmatocystin and 3-o-methylsterigmatocystin, *Aspergillus rambellii* sp. nov. Syst Appl Microbiol 28:442–453, doi:10.1016/j.syapm.2005.02.012

———, Thrane U, Samson RA, Pitt JI. 2006. Important mycotoxins and the fungi which produce them. In: Hocking AD, Pitt JI, Samson RA, Thrane U, eds. Advances in food mycology. New York: Springer Science. p 3–31.

Giorni P, Magan N, Pietri A, Bertuzzi T, Battilani P. 2007. Studies on *Aspergillus* section *Flavi* isolated from maize in northern Italy. Int J Food Microbiol 113:330–338, doi:10.1016/j.ijfoodmicro.2006.09.007

Glass NL, Donaldson G. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microbiol 61:1323–1330.

Godet M, Munaut F. 2010. Molecular strategy for identification in *Aspergillus* section *Flavi*. FEMS Microbiol Lett 304:157–168, doi:10.1111/j.1574-6968.2009.01890.x

Gonçalves SS, Stchigel AM, Cano JF, Godoy-Martinez PC, Colombo AL, Guarro J. 2011. *Aspergillus novoparasiticus*: a new clinical species of the section *Flavi*. Med Mycol 50:152–160, doi:10.3109/13693786.2011.593564

Horn BW, Moore GG, Carbone I. 2009. Sexual reproduction in *Aspergillus flavus*. Mycologia 101:423–429, doi:10.3852/09-011

Huelsenbeck JP, Ronquist F. 2001. MrBayes: Bayesian inference of phylogeny. Bioinformatics 17:754–755, doi:10.1093/bioinformatics/17.8.754

Ito Y, Peterson SW, Wicklow DT, Goto T. 2001. *Aspergillus pseudotamarii*, a new aflatoxin producing species in *Aspergillus* section *Flavi*. Mycol Res 105:233–239, doi:10.1017/S0953756200003385

Klich MA. 2002. Identification of common *Aspergillus* species. Utrecht, the Netherlands: Centraalbureau voor Schimmelcultures. 116 p.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. 2007. Clustal W and Clustal X 2.0. Bioinformatics 23:2947–2948, doi:10.1093/bioinformatics/btm404

Lin L, Jun Z, Ping W, Yuesong W, Jiping C. 1998. Thin-layer chromatography of mycotoxins and comparison with other chromatographic methods. J Chromatograph A 815:3–20, doi:10.1016/S0021-9673(98)00204-0

Liu Y, Whelen S, Hall BD. 1999. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. Mol Biol Evol 16:1799–1808.

LoBuglio KF, Taylor JW. 2002. Recombination and genetic differentiation in the mycorrhizal fungus *Cenococcum geophilum* Fr. Mycologia 94:772–780, doi:10.2307/3761692

Luk KC, Kobbe B, Towsend JM. 1977. Production of cyclopiazonic acid by *Aspergillus flavus* Link. Appl Environ Microbiol 33:211–212.

Lutzoni F, Kauff F, Cox CJ, Mclaughlin D, Celio G, Dentinger B, Padamsee M, Hibbett D, James TY, Baloch E, Grube M, Reeb V, Hofstetter V, Schoch C, Arnold E, Miadlikowska J, Spatafora J, Johnson D, Hambleton S, Crockett M, Shoemaker R, Sung G, Lücking R, Lumbsch T, O'donnell K, Binder M, Diederich P, Ertz D, Gueidan C, Hansen K, Harris RC, Hosaka K, Lim Y, Matheny B, Nishida H, Pfister D, Rogers J, Rossman A, Schmitt I, Sipman H, Stone J, Sugiyama J, Yahr R, Vilgalys R. 2004. Assembling the fungal tree of life: progress, classification and evolution of subcellular traits. Am J Bot 91:1446–1480, doi:10.3732/ajb.91.10.1446

Peterson SW. 2008. Phylogenetic analysis of *Aspergillus* species using DNA sequences from four loci. Mycologia 100:205–226, doi:10.3852/mycologia.100.2.205

———, Ito Y, Horn BW, Goto T. 2001. *Aspergillusbombycis*, a new aflatoxigenic species and genetic variation in its sibling species, *A. nomius*. Mycologia 93:689–703, doi:10.2307/3761823

Pildain MB, Frisvad JC, Vaamonde G, Cabral D, Varga J, Samson RA. 2008. Two novel aflatoxin-producing *Aspergillus* species from Argentinean peanuts. Int J Syst Evol Microbiol 58:725–735, doi:10.1099/ijs.0.65123-0

Pitt JI, Hocking AD. 2009. Fungi and food spoilage. 3rd ed. New York: Springer. 519 p.

———, ———, Glenn DR. 1983. An improved medium for the detection of *Aspergillus flavus* and *A. parasiticus*. J Appl Bacteriol 54:109–114, doi:10.1111/j.1365-2672.1983.tb01307.x

Ramirez-Prado JH, Moore GG, Horn BW, Carbone I. 2008. Characterization and population analysis of the mating-type genes in *Aspergillus flavus* and *Aspergillus parasiticus*. Fungal Genet Biol 45:1292–1299, doi:10.1016/j.fgb.2008.06.007

Rodrigues P, Venâncio A, Kozakiewicz Z, Lima N. 2009. A polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* section *Flavi*. Int J Food Microbiol 129:187–193, doi:10.1016/j.ijfoodmicro.2008.11.023

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574, doi:10.1093/bioinformatics/btg180

Saito M, Tsuruta O, Siriacha P, Kawasugi S, Manabe M, Buangsuwan D. 1986. Distribution and aflatoxin productivity of the atypical strains of *Aspergillus flavus* isolated from soils in Thailand. Proc Japan Assoc Mycotoxicol 24:41–46.

Samson RA, Varga J. 2009. What is a species in *Aspergillus*? Med Mycol 47(Suppl 1):S13–S20, doi:10.1080/13693780802354011

Schmitt I, Crespo A, Divakar PK, Fankhauser JD, Herman-Sackett E, Kalb K, Nelsen MP, Nelson NA, Rivas-Plata E, Shimp AD, Widhelm T, Lumbsch HT. 2009. New primers for promising single copy genes in fungal phylogenetics and systematics. Persoonia 23:35–40, doi:10.3767/003158509X470602

Swofford DL. 2003. PAUP* 4.0: phylogenetic analysis using parsimony (*and other methods). Sunderland, Massachusetts: Sinauer Associates.

Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett DS, Fisher MC. 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genet Biol 31:21–32, doi:10.1006/fgbi.2000.1228

Turgeon BG. 1998. Application of mating type gene technology to problems in fungal biology. Annu Rev Phytopathol 36:115–37, doi:10.1146/annurev.phyto.36.1.115

Vaamonde G, Patriarca A, Pinto VF, Comeria R, Degrossi C. 2003. Variability of aflatoxin and cyclopiazonic acid production by *Aspergillus* section *Flavi* from different substrates in Argentina. Int J Food Microbiol 88:79–84, doi:10.1016/S0168-1605(03)00101-6

Varga J, Frisvad JC, Samson RA. 2009. A reappraisal of fungi producing aflatoxins. World Mycotoxin J 2:263–277, doi:10.3920/WMJ2008.1094

———, ———, ———. 2011. Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi*. Stud Mycol 69:58–80.

White TJ, Bruns T, Lee S, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR protocols: a guide to methods and application. New York: Academic Press. p 315–324.