Aspergillus pseudotamarii, a new aflatoxin producing species in *Aspergillus* section *Flavi*

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A recent report of an aflatoxin producing isolate of *Aspergillus tamarii* prompted a taxonomic re-examination of aflatoxigenic and non-aflatoxigenic isolates identified as *A. tamarii* as well as the closely related *A. caelatus*. Representatives of each species, including atypical isolates, were compared morphologically, for mycotoxin production, and for divergence in ITS, 28S, β-tubulin and calmodulin gene sequences. Because of genetic, morphological, and mycotoxin differences, the aflatoxin producing isolates of *A. tamarii* are given species rank as *Aspergillus pseudotamarii* sp. nov.

INTRODUCTION

Aflatoxin is a potent natural carcinogen (WHO 1979), and it is produced by certain species in *Aspergillus* section *Flavi* (Samson *et al*. 1995). *Aspergillus flavus*, *A. parasiticus* and *A. nomius* are widely known and widely distributed aflatoxin producing fungi (Samson *et al*. 1995) that cause damage to crops and commodities. Two aflatoxin producing isolates that are clearly not isolates of the known aflatoxigenic species and that resemble *A. tamarii* were recently reported (Goto *et al*. 1997). These strains were morphologically characterized as members of the bronze series of *A. tamarii* (Goto, Wicklow & Ito 1996). This was unexpected because no other isolates of *A. tamarii* are known aflatoxin producers. Peterson *et al*. (in press) studied intraspecific variation of the ITS, 5.8S rDNA and 28S rDNA from 42 isolates of *A. caelatus* and 158 isolates of *A. tamarii* that had geographic origins in 6 continents (collected between 1914 and 1997). They found that *A. tamarii* and *A. caelatus*, as well as the aflatoxigenic *A. tamarii* isolates have distinct sequences in this locus. Ito, Peterson & Goto (1999) screened *A. flavus* group soil isolates collected from the region of Japan where one of the aflatoxigenic *A. tamarii* isolates originated, but no additional aflatoxigenic isolates were found. Due to the lack of additional isolates, taxonomic disposition of the aflatoxin producing strains of *A. tamarii* was reexamined using the two known aflatoxigenic isolates.

We compared the morphological, physiological and genetic characteristics of aflatoxigenic and non-aflatoxigenic species in this part of section *Flavi*, using *ex type* cultures as well as new isolates from acidified tea-field soils in Japan, in order to determine whether the aflatoxigenic *A. tamarii* isolates are members of *A. tamarii*.

MATERIALS AND METHODS

Isolates

The isolates used in this study are permanently preserved in the Agricultural Research Service Culture Collection (NRRL), Peoria, Illinois, USA and are listed in Table 1.

Methods for morphological study

Isolates were inoculated on Czapek Dox agar plates (Cz), composed of 35 g of Difco Czapek Dox Broth and 17 g of Difco Bacto Agar in one litre of distilled water, and cultured at 25 °C in the dark to compare their micro-morphological characters (Raper & Fennell 1965). Also, plates were incubated in the dark at 5, 25, 37, 42 and 45 ° and colony size was measured at 7 d. The cryo observation technique for scanning electron microscopy (FE-SEMS S-4200, Hitachi) was used to observe fungal cultures and to make some micro-measurements. One week old cultures were frozen in liquid nitrogen and observed at 3000 to 5000 × magnification without coating.

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Table 1. *Aspergillus* isolates used in this study and their origins.

A. parasiticus
NRRL 502. *Ex-type*. Isolated from mealy bug on sugar cane, Hawaii, 1913.
A. flavus
NRRL 1957. *Ex-type*, isolated from cellophane, South Pacific, 1944
A. nomius
NRRL 13137. *Ex-type*. Isolated from wheat, USA, 1965.
NRRL 25393. Isolated from tea field soil, Okinawa, Japan, 1994.
A. pseudotamarii
NRRL 25517. *Ex-type*. Isolated from tea field soil, Miyazaki, Japan, 1993.
NRRL 443. Sent from Argentina, 1923 by Da Fonseca.
A. tamarii
NRRL 20818. *Ex-lectotype*. Isolated from activated charcoal, 1913.
NRRL 25565. Isolated from tea field soil, Kochi, Japan, 1995.
NRRL 26594. Isolated from tea field soil, Fukuoka, Japan, 1993.
A. caelatus
NRRL 25528. Isolated from peanut field soil, Georgia, USA, 1996.
NRRL 25566. Isolated from tea field soil, Kochi, Japan, 1995.
NRRL 25576. Isolated from tea field soil, Shizuoka, Japan, 1996.
NRRL 26592. Isolated from tea field soil, Saga, Japan, 1993.
Aspergillus leporis
NRRL 3216. *Ex-type*. Isolated from jack-rabbit dung, USA, 1996.

Mycotoxin production

Aflatoxin productivity was tested using glucose yeast extract (GY) medium, as reported by Wongurai, Goto & Manabe (1990), followed by TLC analysis. Isolates were inoculated in 10 ml of GY liquid medium in a test tube (18 × 180 mm with cotton plug) and incubated at 27 ° for 7 days in the dark. Aflatoxins were extracted with chloroform and the chloroform extracts were dried under a stream of nitrogen gas. The residue was dissolved in toluene-acetonitrile (98:2, v/v) and spotted on a TLC plate (Merck No. 5721). The plate was developed with toluene-ethyl acetate-88% formic acid (6:3:1, v/v/v) or chloroform-acetone (9:1, v/v) in a glass tank. Aflatoxins were detected as blue and green spots under long wave length (365 nm) uv light.

Cyclopiazonic acid productivity was examined by growing the isolates in 10 ml of modified Czapek–Dox medium in a text tube (18 × 180 mm with cotton plug) in the dark for 10 days at 30 ° (Goto *et al*., 1987). The samples for cyclopiazonic acid analysis were prepared using a mini test tube method, and then analysed by HPLC with monitoring by uv absorption at 284 nm (Goto *et al*. 1987)

Kojic acid production of the isolates was determined by growing the fungi in 10 ml of glucose-peptone medium in 18 × 130 mm test tube incubated for 2 wk at 30 ° in the dark. The culture medium was diluted 20 times using mobile phase and injected into an HPLC column. Kojic acid was detected by absorbance of 270 nm (Manabe *et al*. 1984).

DNA sequence

The DNA analysis procedures were those of Peterson *et al*. (in press). Cultures were grown 36–48 h and harvested by filtration over cheesecloth. Harvested cells (*ca* 0.2 g) were suspended in 3 ml of breaking buffer (composed of 100 mM Tris, 50 mM EDTA and 1% sarcosyl, pH 8.0) and 1.5 g of glass beads (0.5 mm diam.) was added. Cell walls were broken by vortexing for 30–45 s. Phenol-chloroform (1:1, w/v) was added to the tube (3 ml) and an emulsion was formed by gentle rocking. Aqueous and organic phases were separated by low speed centrifugation. Nucleic acids were precipitated from the aqueous phase by addition of 1.3 volumes of ethanol. Nucleic acids were dissolved in TE/10 (1 mM Tris, 0.1 mM EDTA, pH 8.0) and further purified by adsorption to a silica matrix (Geneclean, Bio101) according to the manufacturer's instructions. DNA was eluted from the matrix in TE/10 and stored frozen (−20 °) until used for PCR amplification.

A DNA fragment of about 1200 nucleotide length that includes the internal transcribed spacer regions (ITS1, ITS2), the 5.8S rDNA, and about 600 bases from the 5′ end of the 28S rDNA was amplified from the genomic DNA using PCR (Peterson *et al*. in press). The primers ITS1 (White *et al*. 1990) and D2R (Peterson, 1993) were used and amplification was accomplished during 30 thermal cycles (96 °–30 s; 51 °–30 s; 72 °–120 s). A short (about 450 nt) fragment of the 5′ portion of β-tubulin was also amplified using the protocol and primers of Geiser, Frisvad & Taylor (1998b). This β-tubulin fragment contains introns 3, 4 and 5 and exons 4, 5 and 6 (based on the complete *A. flavus* sequence, GenBank M38265). A segment of the calmodulin gene was amplified using the primers and reaction conditions described by Fiebelman *et al*. (1998). This segment contains introns 2, 3 and 4 and exons 3, 4 and 5 (based on the complete *A. oryzae* sequence GenBank D44468). The amplified DNA fragments were purified by adsorption to a silica matrix (Geneclean), eluted into TE/10, and stored frozen (−20 °) until used in sequencing reactions. DNA sequences were determined using Applied Biosystems (ABI) DyeDeoxy sequencing kits (fluorescent labelling, Taq polymerase) and the ABI 373 or 377 DNA sequencer. Both strands of each fragment were sequenced.

Sequences were aligned using ClustalW (ver. 1.74, Thompson, Higgins & Gibson 1994) and alignments were further refined by visual inspection. Aligned sequence sets were analysed by maximum parsimony using the heuristic

search option in PAUP* (ver. 4.0 β4a, Swofford, 1998), with maximum parsimony criterion, treating characters as unordered and equal weight, and gaps were treated as missing data. In tree building, the most distant branches were added first, and branch lengths of zero were kept as polytomies. The 'MulTrees' option was in effect. Bootstrap values were determined using heuristic searches with 1000 replications. The partition homogeneity test in PAUP* was applied to the data sets, excluding uninformative characters, and using 10000 replicate samples.

RESULTS

DNA sequence analysis

The aligned rDNA data set composed of the contiguous region from the 3′ terminal bases of the 18S rDNA through ITS1, the 5.8S rDNA, ITS2 and part of the 28S rDNA (the rDNA data) contained 1169 aligned nucleotide positions. After eliminating 19 characters because of uncertain alignment, 25 of the remaining characters were parsimony-informative. Six of the 25 informative characters separated *A. tamarii*, *A. caelatus*, *A. pseudotamarii* and *A. flavus* isolates. Heuristic search of the data produced 19 equally parsimonious trees of 69 steps. The consensus tree, with bootstrap values above 70% on the tree, is shown in Fig. 1. The four species are not resolved by the rDNA data. The four *A. caelatus* isolates do not come out as a clade, but each has 'equal weight' with the others and with the basal branch of the other three species. Consequently, β-tubulin and calmodulin sequence data were added to resolve the relationships of the species *A. caelatus*, *A. tamarii* and aflatoxigenic *A. tamarii*.

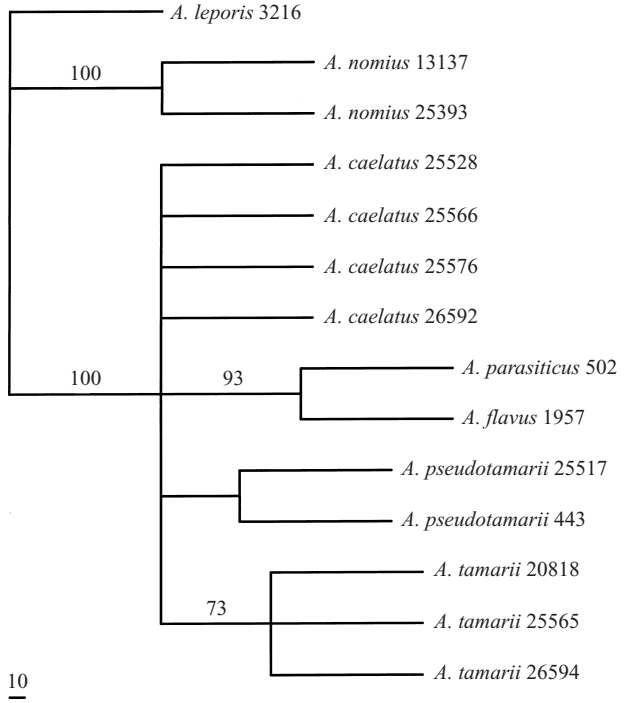


Fig. 1. Consensus tree diagram of *Aspergillus* section *Flavi* based on the ITS1, 5.8S rDNA, ITS2 and 28S rDNA (partial) data set, using *A. leporis* as the outgroup species. Heuristic search found 19 equally parsimonious trees. Numbers over nodes are bootstrap values based on heuristic search with 1000 replicate samples. The consistency index (CI) was 0.9565, homoplasy index (HI) was 0.0435, the retension index (RI) was 0.9333 and the rescaled consistency index (RC) was 0.8928. Genbank numbers: AF027860, AF027862, AF027863, AF027864, AF004931, AF004929, AF004930 and AF104443. Isolates of each species had identical sequences and Genbank numbers are not provided for some isolates. The scale bar at the bottom refers to branch length in terms of the number of steps in the tree construction.

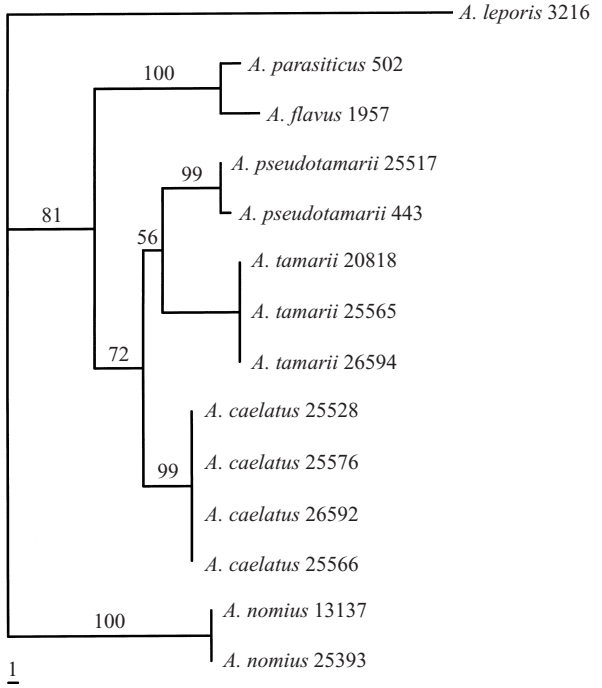


Fig. 2. Phylogenetic tree based on partial β-tubulin gene sequences, using *A. leporis* as the outgroup. A single most parsimonious tree was generated using PAUP* with the heuristic search option. Tree statistics were CI = 0.9098, HI = 0.0902, RI = 0.8991, and RC = 0.8180. Numeric values over tree nodes are the bootstrap values based on a heuristic search of the data and 1000 bootstrap samples. Genbank numbers AF2555062–AF255075. The scale bar at the bottom refers to branch length in terms of the number of steps in the tree construction.

The segment of β-tubulin examined contained 454 aligned positions, 27 positions were eliminated because of length differences or poor alignment, and 59 parsimony-informative sites remained in the data set. In the protein coding region, eight variable nucleotide positions are found in the third positions of codons and these codons translate to the same amino acid. All other variable positions were in the introns. Heuristic search of these data, using maximum parsimony, resulted in a single most probable tree of 122 steps, shown in Fig. 2, with bootstrap values placed on the branches.

The amplified calmodulin fragment contained 597 aligned nucleotide positions, with 13 positions eliminated because of uncertain alignment. The remaining positions included 75 variable but uninformative sites and 73 parsimony informative sites. Thirty-three variable nucleotide positions were found in the protein coding regions, but none of the substitutions caused differences in the translated amino acid. Other variable sites were distributed among the three intron regions. The

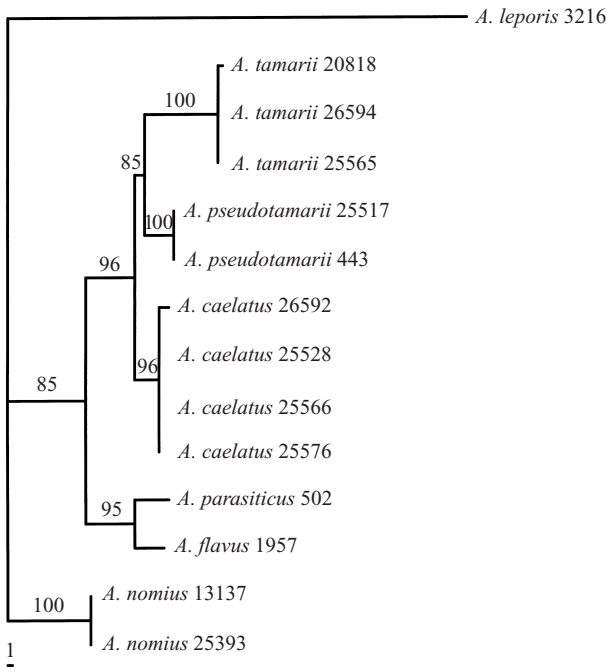


Fig. 3. Phylogenetic tree based on calmodulin gene sequences, using *Aspergillus leporis* as the outgroup. A single most parsimonious tree was generated in the heuristic search, and bootstrap values (based on 1000 bootstrap samples) are placed on the tree nodes. Tree statistics were CI = 0.8901, HI = 0.1099, RI = 0.8712, and RC = 0.7754. Genbank numbers AF255030–AF255043. The scale bar at the bottom refers to branch length in terms of the number of steps in the tree construction.

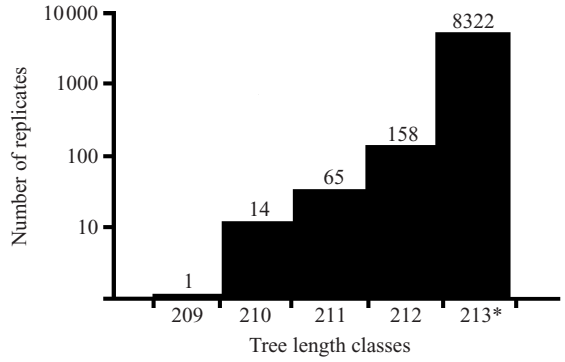


Fig. 4. Tree length distribution histogram obtained in the partition homogeneity test (PAUP*) partitioning β-tubulin and calmodulin sequences. The tree length with an asterisk is the value found before resampling. Because no trees were longer than the single most parsimonious tree, the *P*-value for this test is $P = 1-(0/10000) = 1$, and the two data sets are compatible.

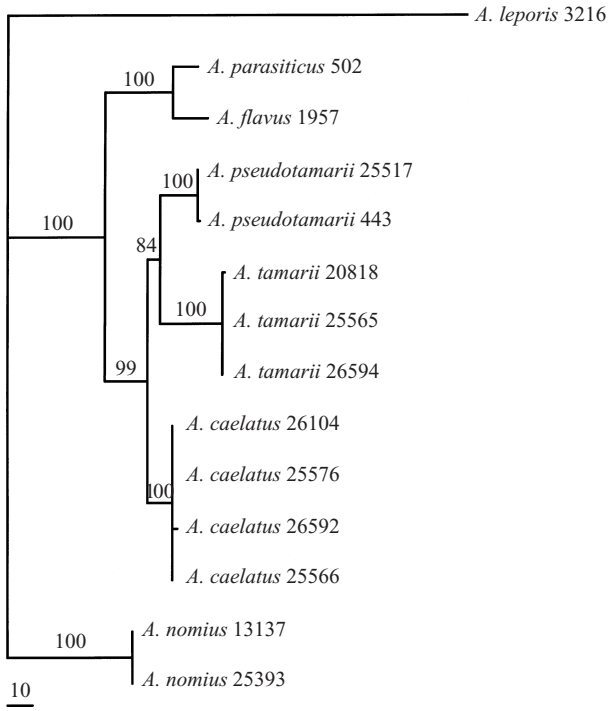


Fig. 5. Phylogenetic tree obtained by heuristic search of the combined rDNA, β-tubulin and calmodulin sequences. The numbers above tree nodes are bootstrap values based on 1000 bootstrap samples. The tree statistics were CI = 0.9075; (HI) = 0.0925; (RI) = 0.8886, and (RC) = 0.8064. The scale bar at the bottom refers to branch length in terms of the number of steps in the tree construction.

Table 2. Colony diameter (mm) of *Aspergillus* species after 7 d growth on Czapek's agar at various temperatures.

	Growth temperature (°C)			
	45	42	37	25
A. parasiticus (NRRL 502)	—	17	72	57
A. flavus (NRRL 1957)	2	38	69	54
A. flavus (NRRL 25394)	1	39	69	57
A. nomius (NRRL 13137)	—	7	54	67
A. nomius (NRRL 25393)	—	13	49	54
A. pseudotamarii (NRRL 25517)	—	—	33	72
A. pseudotamarii (NRRL 443)	—	—	2	72
A. tamarii (NRRL 20818)	—	5	54	66
A. tamarii (NRRL 25565)		1	43	62
A. tamarii (NRRL 26594)	—	2	64	78
A. caelatus (NRRL 25528)	—	—	46	60
A. caelatus (NRRL 25566)	—	—	32	66
A. caelatus (NRRL 25576)	—	—	45	58
A. caelatus (NRRL 26592)	—	—	37	62

data were subjected to heuristic search, which resulted in a single most parsimonious tree of 191 steps, shown in Fig. 3 with bootstrap values on the branches.

The rDNA, β-tubulin and calmodulin data were combined into a single data set and their combinability was tested using the partition homogeneity test (PHT). The combined data set contained 2215 total characters and 165 parsimony informative sites. The PHT was run in all pairwise combinations, with uninformative characters excluded, and 10000 replications. The resulting tree length distribution for the β-tubulin-calmodulin comparison is shown in Fig. 4, and the *P*-value is $1-(0/10000) = 1$. The comparisons of β-tubulin-rDNA and rDNA-calmodulin also gave *P*-values of 1. This *P*-value is insufficient to exclude combining the data sets so further analysis was performed on the combined rDNA, *b*-tubulin and calmodulin data set. The combined data set was subjected to an heuristic search that found a single most parsimonious tree of 400 steps. The single most parsimonious tree with bootstrap values placed on the branches is shown in Fig. 5.

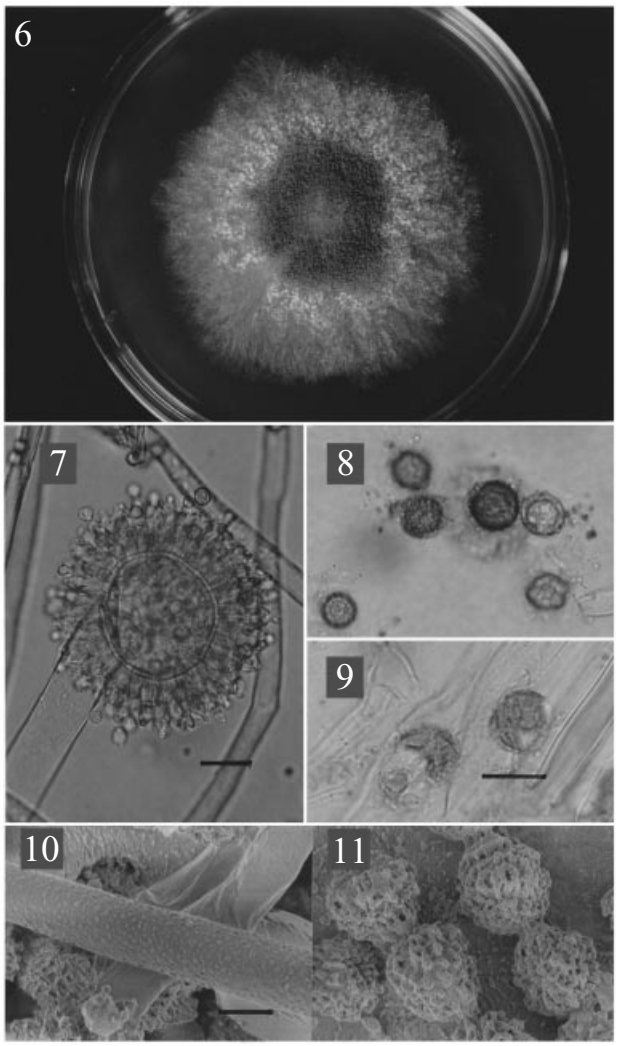
Growth of the species at different temperatures and their mycotoxin production profiles are presented in Tables 2 and 3, respectively.

Table 3. Ability of species in *Aspergillus* section *Flavi*, to produce aflatoxins (AF) B and G, cyclopiazonic acid (CPA) and kojic acid (KA).

	AFB_1	AFB_2	AFG_1	AFG_2	CPA	KA
A. parasiticus	+	+	+	+	−	+
A. flavus	+*1	+*1	−	−	+*1	+
A. pseudotamarii	+	+	−	−	+	+
A. nomius	+	+	+	+	−	+
A. tamarii	−	−	−	−	+	+
A. caelatus	−	−	−	−	−	+

*1: some isolates do not produce these mycotoxins.

Data compiled from this study, as well as Goto *et al.* (1987, 1996, 1997), Horn *et al.* (1996), Ito *et al.* (1999) and Manabe *et al.* (1987).



Figs 6–11. *Aspergillus pseudotamarii*. **Fig. 6.** Colony on Czapek's agar plate, grown at 25 °, 7 days in the dark. **Fig. 7.** Light micrograph of conidial heads on Czapek's agar plate at 25 °, 7 days in the dark. **Fig. 8.** Double walled conidia seen in the light microscope. **Fig. 9.** Conidia with the outer wall separating from the inner wall. **Fig. 10.** Very finely roughened stipe, SEM. **Fig. 11.** SEM view of coarsely roughen conidia.

Aspergillus pseudotamarii Y. Ito, S. W. Peterson, Wicklow & T. Goto, **sp. nov.** (Figs 6–11)

Coloniae in agaro Czapekii post septem diebus in 25 °C diametrum 6–7 cm attingentes; post septem diebus in 37 ° diametrum 3.0–3.5 cm cresecentes; in 42 ° conidia non germinantia. Superficies coloniarum maxima ex parte velutinae, ex capitulis conidialibus abundantibus composita. Capitula conidialia septem diebus aurantiaco-fusca, aetate pallide fuscescentia. Pars aversa coloniarum pallide flavido-brunnea, pigmento diffluento concoloris agarum visum. Sclerotia fusca vel atra, globosa vel subglobosa, 1000–2000 µm diametrum. Capitula conidialia globosa vel radiata, plerumque in nonulli columnas findentia, 500–770 µm diametrum. Stipe hyalini, scabriusculi. Vesiculae globosae vel subglobosae, 26–38 µm diametrum, metulae 8.1–9.6 × 3.1–3.9 µm; phialides 4.5–6.1 × 3.1–4.5 µm. Conidia globosa vel subglobosa, echinulata, diametro variabili, 3.9–9.9 µm, plerumque 6.1–7.8 µm; paries internus firmus pariete externo laxo circumdatus. Coloniae in agaro malti post septem diebus in 25 °C diametrum 6–7 cm attingentes. Superficies coloniarum praecipue floccosae. Capitula conidialia olivaceo-viridia.

Typus: **Japan**: Miyazaki Prefecture, isol. ex soil in fields of *Camellia sinensis*, 1993, *Y. Ito* (BPI 746098 – holotypus; NRRL 25517 – cultura viva).

Colonies on Czapek–Dox Agar (Fig. 6) 6–7 cm diam in 7 d at 25 °C; colonies at 37 ° in 7 days of 3.0–3.5 cm diam; at 42 ° spores do not germinate. Colony surface is mostly velvety consisting of abundant conidial heads. *Conidial heads* orange brown at 7 d, eventually shifting to light brown in mature cultures. Colony reverse pale yellow brown; diffusible pigment of the same colour seen in the agar medium. *Sclerotia* dark brown to black, globose to subglobose, 1000–2000 µm diam. Conidial heads globose to radiate, often splitting into several columns, 500–770 µm diam. *Stipe* hyaline, finely roughed (Figs 7, 10). Vesicles globose to subglobose, 26–38 µm diam (Fig. 7), metulae 8.1–9.6 × 3.1–3.9 µm; phialides, 4.5–6.1 × 3.1–4.5 µm. *Conidia* globose to subglobose, echinate; variable in diameter, 3.9–9.9 µm, mostly 6.1–7.8 µm; loose outer wall surrounds a firm inner wall (Figs 8–9, 11). *Colonies* on malt extract agar 6–7 cm diam in 7 d (Fig. 6), with colony surface mostly floccose and conidial heads olive green.

The holotypes of colonies of NRRL 25517 grown 7 d in darkness on Czapek's agar and dried.

DISCUSSION

Species concepts range from the morphological (Greuter *et al.* 1994) that requires only recognizable morphological differences, to the biological species concept (Dobzhansky 1937) that invokes interfertility as the limit of a species, to the phylogenetic species concept that Eldridge & Cracraft (1980) defined as the smallest diagnosable group where a pattern of ancestry and descent exists.

The morphological characters of *Aspergillus pseudotamarii* isolates are similar to those in the bronze series of *A. tamarii* (Thom & Raper 1945) which was a group based mainly on colony colour. Colony colour of *A. paeudotamarii* isolates is initially green or yellowish green (Pantone 397, 138) but after 7 days the colony colour is orange-brown, and after 14 days is close to peal brown or medal bronze (Pantone 105, 469). By comparison, *A. tamarii* isolates are brown or greenish-brown when young and are very dark brown in maturity. *Aspergillus caelatus* cultures are initially olive, becoming brownish-olive after 14 days. The colony reverse of *A. pseudotamarii* grown on Cz is pale yellow, and a diffusible pale yellow pigment is seen in the agar (also seen in *A. caelatus*), while *A. tamarii* cultures are colourless or slightly pinkish in reverse, and have

no diffusible pigments. Metulae and phialides of *A. pseudotamarii* are smaller than those in *A. tamarii*, but are within the described size range of *A. caelatus* (Horn 1997). Conidia of *A. pseudotamarii* and *A. tamarii* are not distinguishable by size or ornamentation.

Two physiological characters, aflatoxin production and maximal growth temperature, distinguish *A. pseudotamarii*, *A. caelatus* and *A. tamarii*. Isolates of *A. pseudotamarii* produce B aflatoxins, while isolates of *A. caelatus* and *A. tamarii* do not. *A. pseudotamarii* and *A. caelatus* grow at 37 °C, with no growth or conidial germination at 42 °, while isolates of *A. tamarii* grow at both 37 ° and 42 °. The other aflatoxin producing species, *Aspergillus flavus*, *A. parasiticus* and *A. nomius* typically grow moderately at 42 ° and quite rapidly at 37 ° and have much smaller conidia than those of *A. pseudotamarii*, *A. tamarii* or *A. caelatus*. Kurtzman, Horn & Hesseltine (1987) used maximum growth temperature to distinguish *A. nomius* from *A. flavus*. Horn (1997) used mycotoxin production as a non-morphological distinction of the new species *A. caelatus*. This suite of characters distinguishes *A. pseudotamarii* from *A. tamarii* and *A. caelatus*.

There is no overt meiotic recombination known among isolates from *Aspergillus* section *Flavi*, thus mating data for a biological species concept are not available. Each of the species in *Aspergillus* section *Flavi* is apparently clonal in nature, but Geiser, Pitt & Taylor (1998a) showed that *A. flavus* is composed of two recombining, genetically isolated, cryptic species. Because we could not obtain a reasonably large population sample of *A. pseudotamarii* isolates, it was not possible to perform multi-locus polymorphism studies to determine the reproductive mode of these isolates (Taylor *et al*. 1999). Koufopanou, Burt & Taylor (1997) interpreted concordant gene trees as evidence of reproductive isolation. However, until a better sampling of *A. pseudotamarii* is available, the reproductive mode of the species will remain subject to speculation, and a biological species concept will not be applicable.

The gene trees based on calmodulin or β-tubulin sequences agree in topology, and the partition homogeneity test confirms that these data can be combined. The tree based on the combined data has strong statistical support based on bootstrap values (Fig. 5). These genetic data are concordant with the phenotypic analysis and mycotoxin analysis.

The genetic distance (reflected in branch lengths; Fig. 5) between *A. tamarii*, *A. caelatus* and *A. pseudotamarii* is similar to the distance found between *A. flavus* and *A. parasiticus*, two species difficult to differentiate morphologically (Klitch & Pitt 1988). Given the genetic distance and the additional morphological and aflatoxin production differences between *A. tamarii* and *A. pseudotamarii*, we feel justified in naming this new species.

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