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Aspergillus bombycis, a new aflatoxigenic species and genetic variation in its sibling species, *A. nomius**

Stephen W. Peterson[1]

Microbial Properties Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, 1815 N. University St., Peoria, Illinois 61604-3999 USA

Yoko Ito

National Research Institute for Vegetables, Ornamental Plants and Tea, Ministry of Agriculture Forestry and Fisheries, Ano, Mie, 514-2392 Japan

Bruce W. Horn

National Peanut Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, 1011 Forrester Dr. SE, Dawson, Georgia 31742 USA

Tetsuhisa Goto

National Food Research Institute, Ministry of Agriculture Forestry and Fisheries, Kannondai, Tsukuba, 305-8642 Japan

Abstract: A new aflatoxigenic species of *Aspergillus, A. bombycis,* was discovered during isolation of fungi from insect frass collected in silkworm rearing houses in Japan. The new species resembles *A. flavus,* but produces B and G aflatoxins. It is distinguished from *A. flavus* and *A. nomius* by differences in growth rates at 37 and 42 C, from *A. nomius* by roughness of the stipe, and from both of these species by differences in the nucleotide sequences in the beta-tubulin, calmodulin, norsolorinic acid reductase, ITS, and lsu-rDNA genes. *Aspergillus bombycis* is known from nine isolates, eight collected in silkworm-rearing houses in Japan and one collected in a silk-worm rearing house in Indonesia. Phylogenetic analysis of the DNA sequences shows that *A. bombycis* is a phylogenetically distinct species which is most closely related to *A. nomius* and which belongs in *Aspergillus* section *Flavi.* Analysis by partition homogeneity did not reveal evidence of genetic recombination in *A. bombycis,* but in *A. nomius* the patterns of polymorphisms in different genes strongly suggest cryptic genetic recombination.

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[1] Corresponding author, E-mail: peterssw@ncaur.usda.gov

* Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

Key Words: aflatoxin, fungi, molecular systematics, ribosomal DNA sequence

INTRODUCTION

Aflatoxin has been the subject of many studies because of its deadly toxicity to certain domesticated animals, including turkeys, ducks and trout (Hesseltine et al 1966). In addition increased incidence of human hepato-carcinoma is associated with ingestion of sublethal doses of aflatoxin (Scholl and Groopman 1995). Because of aflatoxin's effects on animal and human health, it is essential to determine which species produce this toxin, as well as details about their life histories and distribution in nature. Currently, there are three known aflatoxigenic species from *Aspergillus* section *Flavi*: *A. flavus, A. parasiticus,* and *A. nomius* (Cotty et al 1994). An additional species, *A. ochraceoroseus* (section *Circumdati*) has recently been reported as aflatoxigenic (Klich et al 1998). *Aspergillus ochraceoroseus* is phylogenetically part of the *Aspergillus versicolor* group (Peterson unpubl).

During a study of the incidence of aflatoxigenic fungal isolates from soil (Peterson et al 2000), we encountered some isolates that produced aflatoxins and whose phylogenetic position (based on ribosomal DNA sequences) among the other species of *Aspergillus* section *Flavi* suggested that they might be undescribed species. One of these unusual fungal types (Goto et al 1996) has recently been described as the new aflatoxigenic species *Aspergillus pseudotamarii* Ito et al on the basis of its unique morphology and phylogenetic distinction from the other species of section *Flavi* (Ito et al 2001).

We also isolated fungi from the dust and insect frass found in silk worm-rearing houses in eastern Asia. Some of these isolates have been identified as *A. nomius,* whereas others resemble *A. nomius* but differ genetically and morphologically from the typical *A. nomius* strain. Species from the *A. flavus* clade can have overlapping character states, making identification of some isolates by phenotype problematical (Klich and Pitt 1988, Horn et al 1996), and several species are not identifiable phenotypically, such as the cryptic species found (but not named) by Geiser et al (1998). In order to determine the phylogenetic placement of these isolates and whether they

might represent a new species, we have sequenced portions of the large subunit rDNA and ITS regions as well as the genes for beta-tubulin, calmodulin and norsolorinic acid reductase. In addition, morphological and physiological comparisons of these new isolates were made with other species in *Aspergillus* section *Flavi*.

MATERIALS AND METHODS

Isolation data, permanent accession numbers, and provenance for the fungi used in this study are listed in TABLE I. These fungi are permanently preserved in the Agricultural Research Service Culture Collection (NRRL), Peoria, Illinois.

Isolation of fungi.—Fungi were isolated by dispersing 1 g of substrate (insect frass, dust, etc.) in 100 mL sterile 0.01% Tween 80 and mixing 0.03–1.0 mL of the substrate dilution with 15 mL of isolation medium, then pouring the molten (ca 60 C) agar into a 90-mm petri plate. The isolation medium contained (per liter): 45 g malt extract (Difco); 30 g NaCl; 30 mg chloramphenicol; 30 mg rose bengal; and 1 mg DDVP (Diclorvos) (King et al 1979). Petri plates were incubated in darkness at 27 C for 1–6 d and checked daily for colony development. Individual colonies from isolation plates were subcultured on Czapek agar (Cz) (Raper and Fennell 1965) or other suitable agar media.

Growth and examination of cultures.—For temperature tolerance and morphological examination studies, the isolates were inoculated at three points on 90-mm petri plates (Pitt 1979) containing 30 ml of Blakeslee's malt agar (MA). Fungal material was viewed with a Zeiss microscope equipped with phase and differential interference contrast. For SEM, blocks of agar and fungal material (ca 5 mm × 5 mm) were cut from a petri plate culture, fixed overnight in 1% osmium tetroxide, dehydrated in increasingly concentrated ethanol baths, critical point dried, and sputter coated with gold-paladium (Peterson 1992). For DNA extraction, 100 mL of malt extract (ME) broth (Raper and Fennell 1965) was autoclaved for 15 min in a 500-mL cotton stoppered flask. An agar slant culture was flooded with 2–3 mL of 0.1% sterile Triton X-100, spores were dislodged with a wire loop, and the spore suspension was pipetted into the malt broth. Flasks were incubated at 25 C on a rotary shaker (200 rpm) for 1–2 d until 1–2 g biomass had accumulated.

DNA extraction and purification.—Biomass was separated from the medium by vacuum filtration over cheesecloth or Whatman number 54 filter disks, and mycelium was fractured by vortex mixing with glass beads using the method of Peterson et al (2000). Proteins were extracted with phenol-chloroform. The DNA was further purified by adsorption to a silica matrix (GeneClean, Bio101, La Jolla, California) in the presence of concentrated NaI, then eluted into 1/10th strength TE. The DNA was stored at −20 C until used.

PCR amplifications.—The ITS regions and about 600 bases of the 5′ end of lsu rDNA (collectively referred to as ID region) were amplified by means of polymerase chain reaction (PCR). Conditions and buffers were those of White et al (1990) except the primers used were ITS1 (White et al 1990) and D2R (Peterson et al 2000) and the thermal profile (96 C, 30 s; 51 C, 45 s; 72 C, 120 s) was repeated 30 times, followed by 7 min at 72 C. The amplified fragment was purified using GeneClean according to manufacturer's instructions, eluted in 1/10th strength TE and stored at −20 C. Part of the beta-tubulin gene (referred to as BT) was amplified using primers Ben0 (5′-ATGCGTGAGATTGTATGTT) or Ben0b (5′-ATGCGTGAGATTGTATG) that are identical to the first exon of the gene from *A. flavus* (GenBank M38265) as well as several bases in the first intron, and Ben2 (5′-ATCTGGAAACCCTGGAGGC) which is complementary to part of the gene sequence in exon 6. The thermal profile for beta-tubulin amplification was 96 C for 2 min followed by 30 cycles (96 C, 20 s; 51 C, 45 s; 72 C, 2 min) then 72 C for 5 min. Part of the norsolorinic acid reductase gene (referred to as NOR) was amplified using the primers and conditions developed by Geisen (1996). A portion of the calmodulin gene (referred to as CAL) was amplified using the primers and conditions described by Feibelman et al (1998).

DNA sequencing and analysis.—DNA sequencing reactions were carried out using Taq polymerase, ABI fluorescent dye labeled dideoxy nucleotides, and DNA template and oligonucleotide primers. For the ID templates, primers ITS1, ITS2, ITS3, ITS4 (White et al 1990) and D1, D1R, D2, D2R (Peterson et al 2000) were used for sequencing. For CAL and NOR, the primers used for amplification were also used for sequencing. For BT, sequences were determined using Ben0, Ben0b, Ben2, br (5′CCAGAAAGCGGCACC) and bf (5′-GAGCCCGGTACCATGGA). Unincorporated dye was removed from sequencing reactions by spun-column chromatography (Maniatis et al 1982) over Pharmacia ultra-fine sephadex G-50. The column eluate was dried in a rotary vacuum drier and dissolved in 1.2 μL ultrapure formamide, with or without 20 mg/mL high molecular weight blue dextran. The nucleotide sequences were determined by electrophoresis on an Applied Biosystems 377 DNA sequencer. The sequence of each fragment was determined from the base sequences of both DNA strands.

Sequences were aligned using ClustalW (Thompson et al 1994) and alignments were checked visually using an ASCII editor. DNA analysis was performed using PAUP* ver. 4.0β4a (Swofford 1998) for parsimony, partition homogeneity test and bootstrap analysis. Trees were redrawn from PAUP* tree files using TREEVIEW (Page 1996).

Mycotoxin analyses.—Aflatoxin production was assayed from isolates grown on glucose yeast extract broth, and detection was performed using thin layer chromatography and high performance liquid chromatography (Manabe et al 1978, Wright et al 1982). Cyclopiazonic acid production was assayed from isolates grown on modified Czapek's broth (Ito and Goto 1994, Goto et al 1996) using high performance liquid chromatography.

RESULTS

Aspergillus bombycis S. W. Peterson, Y. Ito, B. W. Horn, et T. Goto, sp. nov.

Coloniae in agaro "Czapek" post 7 dies temperatura 25 C diam 65 mm atting entes; colonia post 7 dies temperatura 37 C diam 15 mm attingens; germinatio praesens sed incrementum nullum repertum post 7 dies temperatura 5 C. Conidiophora (300–500 μm × 10–20 μm) exasperata, aequabiliter sed non nimis dense in colonia disposita, texturam profundam et laxam facientia. Vesicula globosa, 30–50 μm diam, biseriata, per superficiem totam fertilis. Metulae cylindricae 5 × 12 μm, phialidibus 4 × 8 μm, ampulliformibus. Conidia laevia, globosa vel subglobosa (3.5–) 4–7 (–8.5) μm diam.

HOLOTYPE. BPI. 745225, from colonies of NRRL 26010 grown 7 da in darkness on Czapek's agar then dried. Deposited in the National Fungus Herbarium, U.S. Department of Agriculture, Beltsville, Maryland, USA.

Etymology. bombycis is derived from the Latin name for the silkworm, *Bombyx mori.*

Colonies on Czapek agar (FIG. 1) attain a diam of 65 mm after 7 d at 25 C; colony diam 15 mm after 7 d at 37 C; germination but no growth at 5 C after 7 d. Colony texture loose and deep, usually in shades near lettuce green or calla green (Ridgway 1912, plate V), rarely brownish, near medal bronze (Ridgway plate IV); colony reverse color ranges from uncolored to a light yellow (cream color or Naples yellow, R-XVI) and no diffusible pigments were noted; stipes (FIGS. 2, 3, 6) smooth-walled, 300–500(–1000) × 10–20 μm. Vesicles (FIGS. 2, 3) globose 30–50 μm diam, fertile over entire surface. Metulae cylindrical 4–5 × 10–12 μm, phialides 3–4 × 8 μm, flask-shaped. Conidia (FIGS. 4, 5) roughened, globose to subglobose, (3.5)–4–7–(8.5) μm diam. Conidial heads 100–200 diam × 300–600 μm, with the dry chains of conidia organized into loose columns. Columns mostly split into three or four smaller columns in mature heads.

On ME agar, colony growth is similar to that on Cz agar, but the color has a greater component of yellow and is approximately mignonette green (Ridgway XXXI) to courge green (Ridgway XVII). Colonies are 1–3 mm deep; stipes are uncrowded and are primarily located in the peripheral half of each colony. Immature conidial heads are yellowish and mature to a deeper green. Sclerotia were not found in cultures of *A. bombycis.*

All isolates were grown on Cz and MEA petri plates at 5, 25, 37, 42, and 45 C for 7 d in darkness. Colony diameters for growth on MEA are listed in TABLE II. *Aspergillus bombycis* isolates grew well at 25 C ($\bar{x}$ = 65.1 mm diam, SD = 0.35), grew moderately well at 37 C ($\bar{x}$ 19.3 mm diam, SD = 11.8), and failed to grow at 42 C. Conidia of *A. bombycis* often germinated at 42 C but fail to grow further. However germination of conidia was prevented when the agar plates were warmed to 42 C prior to inoculation. Most *A. nomius* isolates grew well at 25 C ($\bar{x}$ = 59.8 mm diam, SD = 4.9) and 37 C ($\bar{x}$ = 51.3 mm diam, SD = 10.4), and to some extent at 42 C ($\bar{x}$ = 19.8 mm diam, SD = 7.7).

Aspergillus bombycis and *A. nomius* isolates are distinguishable in several ways. *Aspergillus bombycis* isolates grow restrictedly (ca 15 mm diam) at 37 C and do not grow at 42 C, compared to *A. nomius* isolates that grow vigorously at 37 C (65 mm diam) and also grow at 42 C. *Aspergillus bombycis* isolates have smooth stipes compared to the roughened stipes of *A. nomius* isolates. Isolates of the two groups occur on distinct and strongly supported branches in gene trees calculated from all loci (FIGS. 9–12). Colony color, sclerotium formation, conidium characters (size, shape and ornamentation), and other morphological characteristics of *A. bombycis* overlap those of *A. nomius.* The production of aflatoxins B and G and the lack of cyclopiazonic acid production are the same in these two species (TABLE III). Growth rate at 42 C, stipe roughness and DNA sequences are the most valuable characters for distinguishing *A. bombycis* and *A. nomius.*

Aspergillus parasiticus is distinguished from *A. bombycis* by its typically dark ivy green colony color on CZ agar, and the production of AFB and AFG but no production of CPA. The conidial heads also are predominantly uniseriate in *A. parasiticus,* and isolates typically grow at 42 C. *Aspergillus flavus* is distinguished from *A. bombycis* on the basis of smooth versus rough conidiophores, respectively, and *A. flavus* isolates typically grow at up to 45 C, while *A. bombycis* isolates fail to grow at 42 C. Distinguishing *A. flavus* and *A. nomius* has always been difficult (Kurtzman et al 1987). These species share many characteristics of morphology and physiology. Both species having roughened stipes, and while *A. flavus* usually produces AFB, it may also produce AFG (Cotty and Cardwell 1999). *Aspergillus flavus* usually, but not always, produces cyclopiazonic acid. *Aspergillus nomius* can produce both AFB and AFG but does not produce cyclopiazonic acid. Other characteristics of these species also tend to intergrade. Genetically *A. flavus* and *A. nomius* are distinct species with many nucleotide substitutions present in the comparisons of their genes (FIGS. 9–12). The genetic difference is the most consistent and reliable means of distinguishing isolates of these two species.

All isolates of *A. bombycis* produced aflatoxins B

TABLE I. Fungal isolates examined, their locale, substrate and date of isolation. Alleles present at four loci are given for the *A. nomius* isolates

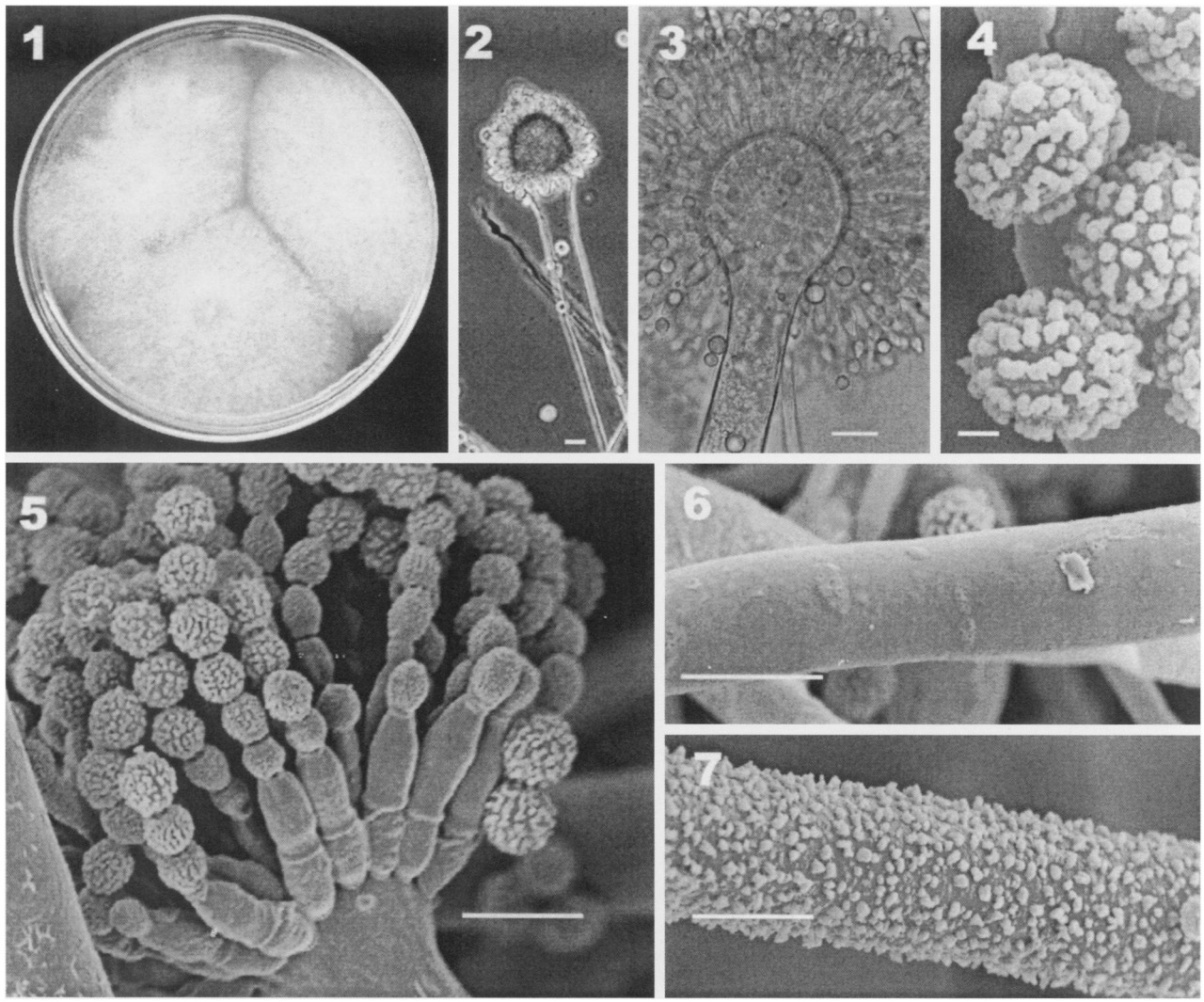
NRRL	Origin of isolate	Genotype[ab] at locus: ID	BT	CAL	NOR
Aspergillus avenaceus G. Smith					
517	GREAT BRITAIN. Received from G. Smith as isolate BB155, 1940.				
Aspergillus bombycis S.W. Peterson, Y. Ito, B.W. Horn, & T. Goto					
25593	JAPAN. Isolated from frass in a silkworm rearing house, 1987.				
26010	JAPAN. Isolated from frass in a silkworm rearing house, 1987. *ex type*				
28900	JAPAN. Isolated from frass in a silkworm rearing house, 1997 by Dr. H. Takahashi.				
28901	JAPAN. Isolated from frass in a silkworm rearing house, 1997 by Dr. H. Takahashi.				
29235	JAPAN. YAMAGATA PREFECTURE. Isolated from frass in a silkworm rearing house, 1983, by Dr. K. Tanaka, = MAFF 235209.				
29236	JAPAN. IBARAKI PREFECTURE. Isolated from frass in a silkworm rearing house, 1983, by Dr. K. Tanaka, = MAFF 235213.				
29237	JAPAN. IBARAKI PREFECTURE. Isolated from frass in a silkworm rearing house, 1983, by Dr. K. Tanaka, = MAFF 235218.				
29241	JAPAN. OITA PREFECTURE. Isolated from frass in a silkworm rearing house, 1983, by Dr. K. Tanaka, = MAFF 111301.				
29253	INDONESIA. Isolated from frass in a silkworm rearing house, 1999.				
Aspergillus caelatus B. W. Horn					
25528	USA. GEORGIA: Terrell Co. Isolated from soil by B. W. Horn, 1992. *Ex type.*				
Aspergillus carbonarius (Bainier) Thom					
67	BRAZIL. Received as *Aspergillus niger* from Da Fonseca, 1923.				
Aspergillus flavus Link					
1957	SOUTH PACIFIC. Isolated from cellophane, 1944. *Ex type.*				
Aspergillus leporis States & M. Christensen					
3216	USA. WYOMING. Isolated from dung of *Lepus townsendii*, 1966. Martha Christensen isolate RMF 99. *Ex type.*				
Aspergillus nomius Kurtzman, Horn & Hesseltine					
13137	USA. Isolated by A. F. Schindler from wheat, 1965. U. S. FDA number M93. *Ex type.*	A	A	A	A
3161	GUAM. Isolated by A. C. Keyl, from *Cycas circinalis*, 1965.	A	A	A	A
3353	USA. WYOMING. Isolated by D. Shimanuki, from diseased alkali bee (*Nomius* sp.), 1965. Number 129.	D	E	A	C
5919	USA. WYOMING. Isolated by D. Shimanuki, from diseased alkali bee (*Nomius* sp.), 1965. Number 130.	C	E	B	C
6107	GUAM. Isolated from *Cycas circinalis*, sent by J. Forgacs. Possibly the same strain as NRRL 3161.	A	A	A	A
6108	USA. Sent by F. A. Hodges, as U.S. FDA number M93. Possibly the same strain as NRRL 13137.	A	A	A	A
6343	USA. WYOMING. Isolated by D. Shimanuki, from diseased alkali bee (*Nomius* sp.), 1965. Number 126.	C	C	B	C
6552	USA. WISCONSIN. Isolated by C. R. Benjamin, from a diseased pine sawfly, 1967.	C	F	B	C
13138	Sub-isolate from a mixed culture sent by U. Diener, 1967.	A	A	A	A
20745	INDIA. Isolated from dried *Curcuma longa.*	A	H	B	B
25393	JAPAN. OKINAWA. Isolated from tea-field soil, 1994.	A	G	A	D
25585	JAPAN. Isolated from silk worm frass, 1987.	F	J	C	A
26450	USA. LOUISIANA: Natchitoches Parish. Isolated by B. W. Horn, from cotton field soil, 1996.	B	B	B	C
26451	USA. LOUISIANA: Natchitoches Parish. Isolated by B. W. Horn, from cotton field soil, 1996.	B	B	B	C
26452	USA. LOUISIANA: Natchitoches Parish. Isolated by B. W. Horn, from cotton field soil, 1996.	B	B	B	C

TABLE I. Continued

NRRL	Origin of isolate	Genotype[ab] at locus: ID	BT	CAL	NOR
26453	USA. LOUISIANA: Natchitoches Parish. Isolated by B. W. Horn, from cotton field soil, 1996.	A	D	A	A
26454	USA. LOUISIANA: Natchitoches Parish. Isolated by B. W. Horn, from cotton field soil, 1996.	B	B	B	C
26455	USA. LOUISIANA: Rapides Parish. Isolated by B. W. Horn, from cotton field soil, 1996.	C	E	B	D
26878	USA. TEXAS: Gaines Co. Isolated by B. W. Horn, from uncultivated soil with mesquite, 1997.	C	C	B	C
26879	USA. TEXAS: Gaines Co. Isolated by B. W. Horn, from uncultivated soil with mesquite, 1997.	C	C	B	C
26880	USA. LOUISIANA: Grant Parish. Isolated by B. W. Horn, from soybean field soil, 1997.	C	C	B	C
26881	USA. LOUISIANA: Grant Parish. Isolated by B. W. Horn, from soybean field soil, 1997.	C	C	B	C
26882	USA. LOUISIANA: Grant Parish. Isolated by B. W. Horn, from soybean field soil, 1997.	C	C	B	C
26883	USA. LOUISIANA: Grant Parish. Isolated by B. W. Horn, from soybean field soil, 1997.	C	E	B	D
26884	USA. LOUISIANA: Grant Parish. Isolated by B. W. Horn, from soybean field soil, 1997.	C	C	B	C
26885	USA. LOUISIANA: Concordia Parish. Isolated by B. W. Horn, from cotton field soil, 1997.	B	F	B	C
26886	USA. LOUISIANA: Concordia Parish. Isolated by B. W. Horn, from cotton field soil, 1997.	A	I	B	C
26887	USA. MISSISSIPPI: Adams Co. Isolated by B. W. Horn, from soybean field soil, 1997.	E	D	A	A
29212	USA. CALIFORNIA: Five Points. Isolated by M. A. Doster from pecans, 1990.	A	A	A	A
29213	USA. CALIFORNIA: Madeira Co. Isolated by M. A. Doster from pistachio nuts, 1991.	A	A	A	A
29234	JAPAN. YAMAGATA PREFECTURE. Isolated from dust in a silkworm rearing house, 1999.	A	G	D	A
29238	JAPAN. KUMAMOTO PREFECTURE. Isolated from dust in a silkworm rearing house, 1999.	A	D	A	A
29239	JAPAN. KUMAMOTO PREFECTURE. Isolated from dust in a silkworm rearing house, 1999.	A	A	D	A
Aspergillus parasiticus Speare					
502	USA. HAWAII. Isolated from mealy bug on sugarcane by Speare, 1913. *Ex lectotype.*				
Aspergillus pseudotamarii Y. Ito, S. W. Peterson, D. T. Wicklow & T. Goto					
25517	JAPAN. MIYAZAKI PREFECTURE. Isolated from tea-field soil, 1993. *Ex type.*				
443	ARGENTINA. Received from Da Fonseca, 1923. Substrate unknown.				
Aspergillus tamarii Kita					
20818	Isolated from activated carbon. *Ex lectotype.* Received as *Aspergillus tamarii* QM 9374.				
Petromyces alliaceus Malloch & Cain					
4181	AUSTRALIA. Isolated from soil by J. H. Warcup. *Ex type.*				

[a] ID = ITS region and 5′ portion of large subunit rDNA; BT = the partial beta tubulin gene sequence; CAL = the partial calmodulin gene sequence; and NOR = the partial norsolorinic acid reductase gene sequence, as described in the text.

[b] Each letter under a gene label represents a particular sequence at that locus which differs from isolates that have a different letter. Isolates with the same letter have an identical DNA sequence at that locus. Genotypes are presented only for *A. nomius* isolates.



FIGS. 1–7. *Aspergillus bombycis*. 1. Colony grown for 7 d in darkness at 25 C on Czapek's agar. 2. Immature stipe and vesicle; note the smooth stipe. 3. Vesicle with metulae and acuminate phialides covering the entire surface. 4. Conidia showing the roughened surface ornamentation. 5. Scanning electron micrograph of a mature conidial head. 6. A portion of a typical smooth stipe. 7. *Aspergillus nomius* stipe. Note the roughened stipe that differentiates *A. nomius* and *A. bombycis*. Scale: FIGS. 2, 3, 5, 6, 7, bar = 10 μm; FIG. 4 bar = 1 μm.

and G, but not cyclopiazonic acid. The highest levels of aflatoxin production were found in isolate NRRL 28901 which produced 9.6 ppm AFB_1, 0.2 ppm AFB_2, 33.1 ppm AFG_1, and 0.5 ppm AFG_2. The lowest aflatoxin production was in NRRL 25593 with 1.0, 0.5, 1.5, and 0.5 ppm of aflatoxins AFB_1, AFB_2, AFG_1, and AFG_2 respectively. Other isolates produced between 1.5 and 8.1 ppm of AFB_1 in the GY (Wongurai et al 1990) liquid culture medium. Isolates of *A. nomius* typically also produce detectable amounts of aflatoxins AFB_1, AFB_2, AFG_1, and AFG_2, but not cyclopiazonic acid.

The amplified ID fragments varied from 1153–1156 base pairs in length, with an aligned length of 1173. Homologous DNA sequences from *A. flavus* NRRL 1957, *A. parasiticus* NRRL 502, *A. tamarii* NRRL 20818, *A. caelatus* NRRL 25528, *A. pseudotamarii* NRRL 25517, *A. leporis* NRRL 3216, *Petromyces albertensis* NRRL 20602, *A. avenaceus* NRRL 517, and *A. carbonarius* NRRL 67 were aligned with sequences from *A. nomius* and *A. bombycis* isolates. Seventy-four positions were eliminated from consideration due to uncertain alignment or indels, 71 characters were variable but parsimony uninformative, 52 characters were parsimony informative, and all other characters were constant. The data were analyzed using parsimony criterion and in an heuristic search, and a single most parsimonious tree of 176 steps was found (FIG. 9).

The amplified portion of the BT from different

TABLE II. Colony diam, in mm, after 7 d growth on Blakelee's malt agar, in darkness, at the specified temperature. Presence or absence of sclerotia is also noted

NRRL	25 C	37 C	42 C	Sclerotia
Aspergillus bombycis				
25593	65	15	0	−
26010	65	15	0	−
28900	65	5	0	−
28901	66	8	0	−
29235	65	30	0	−
29236	65	nd[1]	0	−
29237	65	37	0	−
29253	65	25	0	−
Aspergillus nomius				
13137	65	65	32	+
5919	65	40	5	−
6107	65	65	15	+
6108	65	65	18	+
13138	65	65	22	+
20745	65	65	21	+
25393	60	56	27	−
25585	59	48	11	+
26450	54	45	21	−
26451	nd	45	23	+
26453	65	54	29	+
26454	53	42	11	+
26455	54	54	25	+
26878	60	52	24	−
26879	57	47	14	−
26880	56	40	8	−
26881	60	49	11	+
26882	58	49	14	+
26883	60	57	25	+
26884	59	50	21	+
26885	45	52	25	−
26886	53	56	27	+
26887	63	58	30	+
29212	60	56	20	−
29213	60	55	15	−
29234	62	27	8	−
29238	63	56	32	−
29239	61	24	nd	−

[1] nd = value not determined.

TABLE III. Major mycotoxins produced[ab] by species in *Aspergillus* section *Flavi*

Species	AFB	AFG	CPA
A. bombycis	+	+	−
A. parasiticus	+	+	−
A. nomius	+	+	−
A. flavus	+/−	−	+/−
A. pseudotamarii	+	−	+
A. tamarii	−	−	+
A. caelatus	−	−	−

[a] Data abstracted from Goto et al, 1996, Horn et al, 1996, and the current study.

[b] AFB = aflatoxin B, AFG = aflatoxin G, CPA = cyclopiazonic acid.

organisms was aligned into a data set of 818 base pairs that included introns 1, 2, 3, 4 and 5 and exons 2, 3, 4, 5 and part of 6, based on the complete sequence of the *A. parasiticus* beta tubulin gene (GenBank L49386; FIG. 8). Of the aligned nucleotides, 42 were eliminated due to indels and uncertain alignment, 39 positions were variable but uninformative, 650 positions were constant, and 87 nucleotide positions were phylogenetically informative. Translation of the sequences to amino acids showed that most of the isolates had identical amino acid sequences, with *A. carbonarius* differing at one AA site, *P. alliaceus* varying at one AA site, and *A. leporis* differing from other species at two AA positions. Heuristic search of the DNA sequence data set resulted in a single most parsimonious tree of 147 steps (FIG. 10).

The aligned portion of the CAL contained 580 nucleotide positions. Three positions were eliminated due to an indel, 480 positions were constant, 31 positions were variable but not phylogenetically informative, and 66 positions were phylogenetically informative. The amplified portion of the gene included introns 3, 4, and 5, and exons 3, 4, and part of 5 (based on the complete *A. oryzae* sequence GenBank D44468; FIG. 8). After alignment, the putative coding regions were translated to amino acid sequences. No amino acid differences were noted among the species sequenced. Heuristic search of the aligned data resulted in two equally parsimonious trees that differed only in the branching order of *A. tamarii, A. caelatus* and *A. pseudotamarii.* One of those trees is shown in FIG. 11.

A portion of the coding region of exon 2 of NOR (based on the *A. parasiticus* gene sequence, Genbank L27801; FIG. 8) was amplified from the aflatoxigenic species and from *A. caelatus,* a non-aflatoxigenic species. No product was obtained when the DNA of *A. tamarii* was amplified using the NOR primers. The aligned dataset included 300 nucleotide positions. Of those, 257 were constant, 3 were variable but uninformative and 40 were parsimony informative. A single most parsimonious tree of 49 steps resulted from heuristic search of the data (FIG. 12).

The maximum parsimony trees calculated for each of the loci had a branch that contained all of the *A. nomius* isolates and another branch that contained all of the *A. bombycis* isolates (FIGS. 9–12). In bootstrap analysis, the *A. nomius* isolates and the *A. bombycis* isolates occurred on branches that were present in 100% of the bootstrap samples for the three pro-

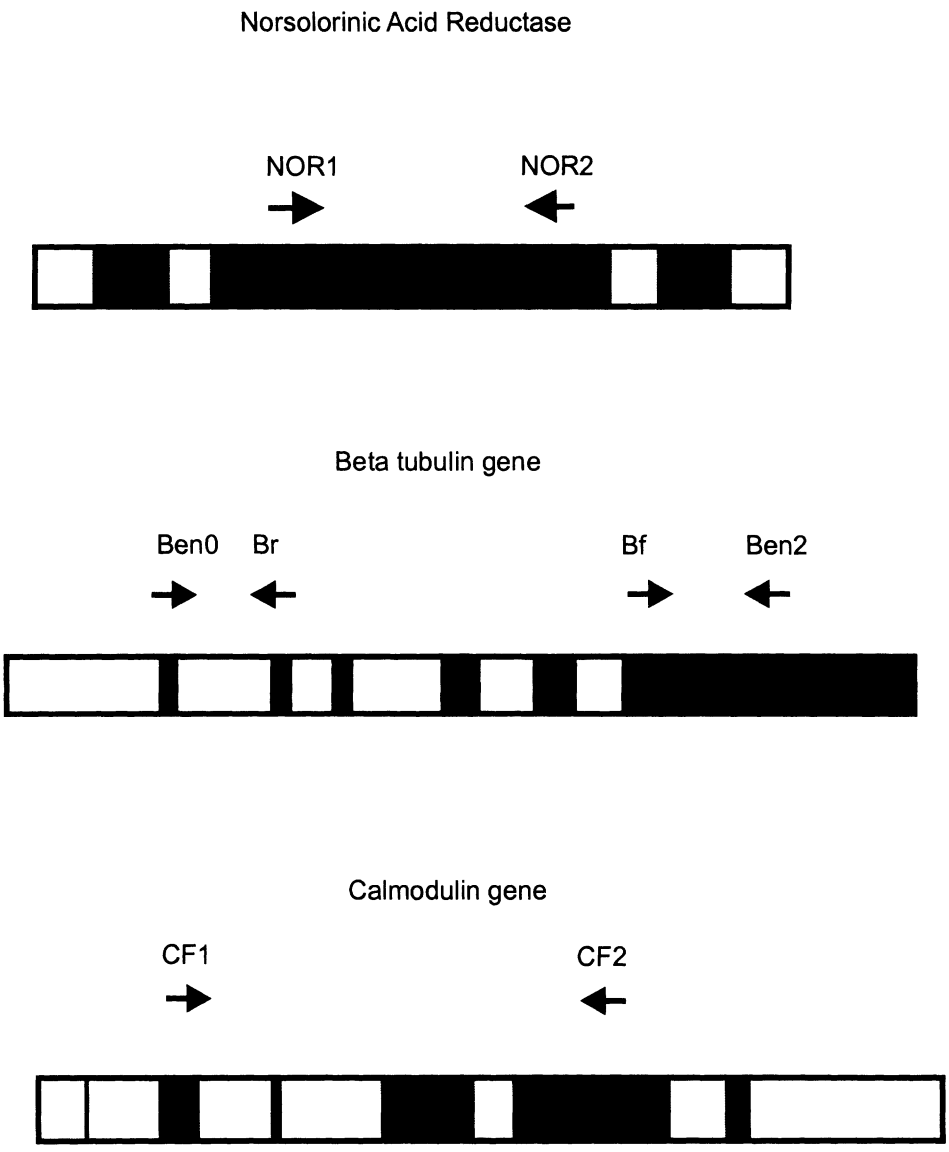


FIG. 8. Location of primers on the single-copy protein coding genes. Filled bars represent protein coding regions and open bars represent introns.

teins coding genes and in 97% and 74%, respectively, of the ID samples. The congruence of the gene trees leading to these two groups of isolates shows that they are genetically isolated groups and under phylogenetic theory they represent distinct species (Koufopanou et al 1997).

Aspergillus bombycis has no sexual state (teleomorph) and in our observations reproduces solely by means of mitotically formed conidia. However, Geiser et al (1998) concluded that cryptic genetic recombination could be inferred in anamorphic fungi by comparing gene trees from different genetic loci. We analyzed the gene trees generated from each of the four genetic loci to determine congruence using the partition homogeneity test (PHT). The ID and CAL loci were identical in all *A. bombycis* isolates (uninformative), and comparison of the gene trees based on BT and NOR revealed no significant deviation from congruence.

Aspergillus nomius isolates were also analyzed to detect cryptic recombination. In the ID region, the 34 *A. nomius* isolates have 1 of 3 different genotypes (labeled A–C in TABLE I), in the BT region there are 10 genotypes (labeled A–J in TABLE I), in CAL there are 4 genotypes (labeled A–D in TABLE I), and in NOR there are 4 genotypes (labeled A–D in TABLE I). The letter designations for genotypes are arbitrary and signify difference in DNA sequences with no additional meaning. The PHT was performed in all pair-wise combinations on the four data sets (TABLE IV). For most pair-wise comparisons, the PHT generated trees were significantly longer than the most

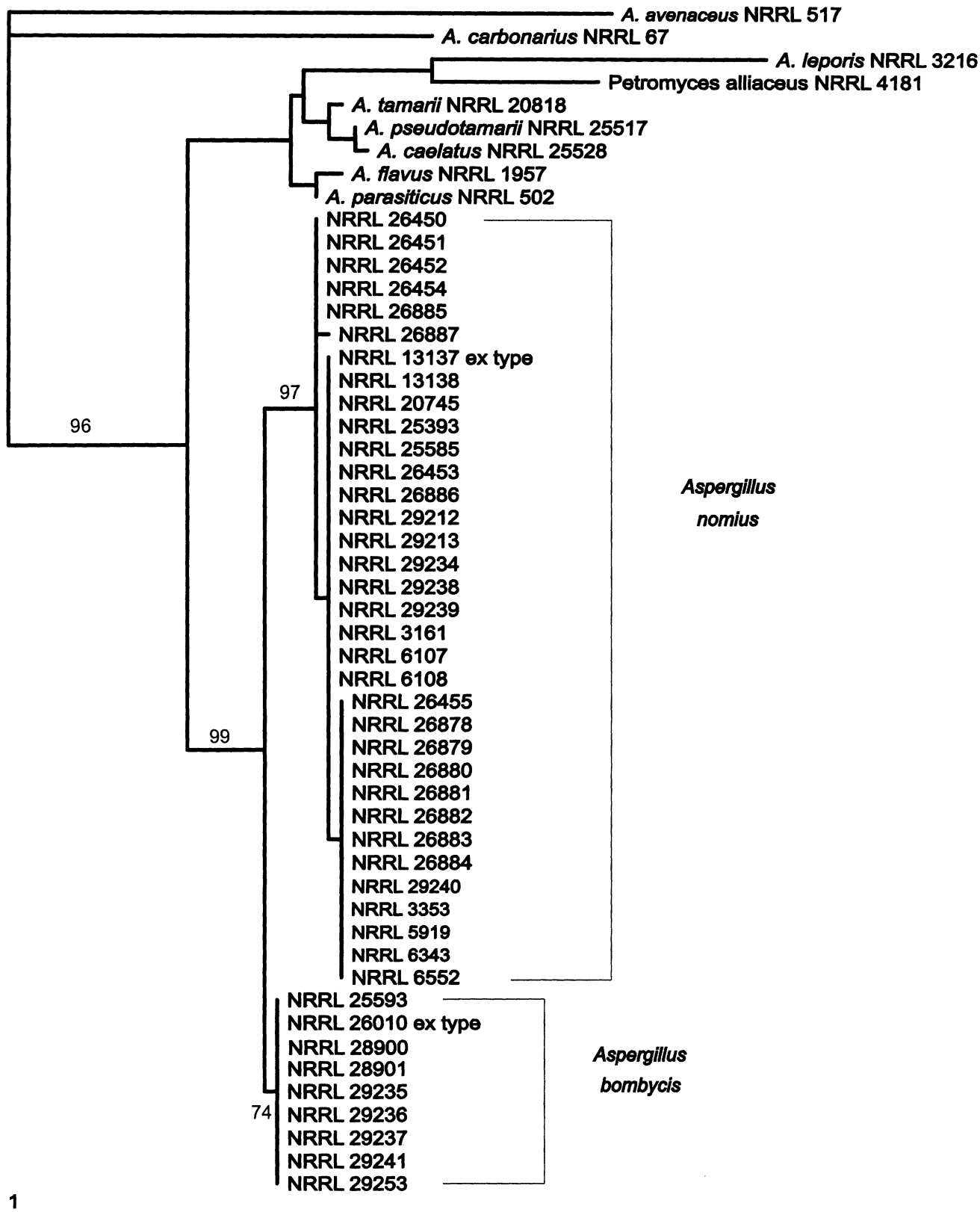


FIG. 9. Single most parsimonious tree calculated using PAUP* 4.0 (β4a) in an heuristic search of the ITS and LSU rDNA sequence data. Tree length is 176 steps, consistency index (CI) = 0.8125, homoplasy index (HI) = 0.1875, retention index (RI) = 0.8596 and rescaled consistency index (RC) = 0.6984. *Aspergillus carbonarius* and *A. avenaceus* are used as outgroup species to root the tree on the basis of comprehensive trees of the genus *Aspergillus* (Peterson 2000). Numbers above the internodes are bootstrap values based on 1000 replicate samples. DNA sequences are deposited in GenBank as accessions AF004929, AF004931, AF027860, AF027862–AF027864, AF104444, AF104445, AF104447, AF272575 and AF338611–AF338647, and in Treebase as accession M896.

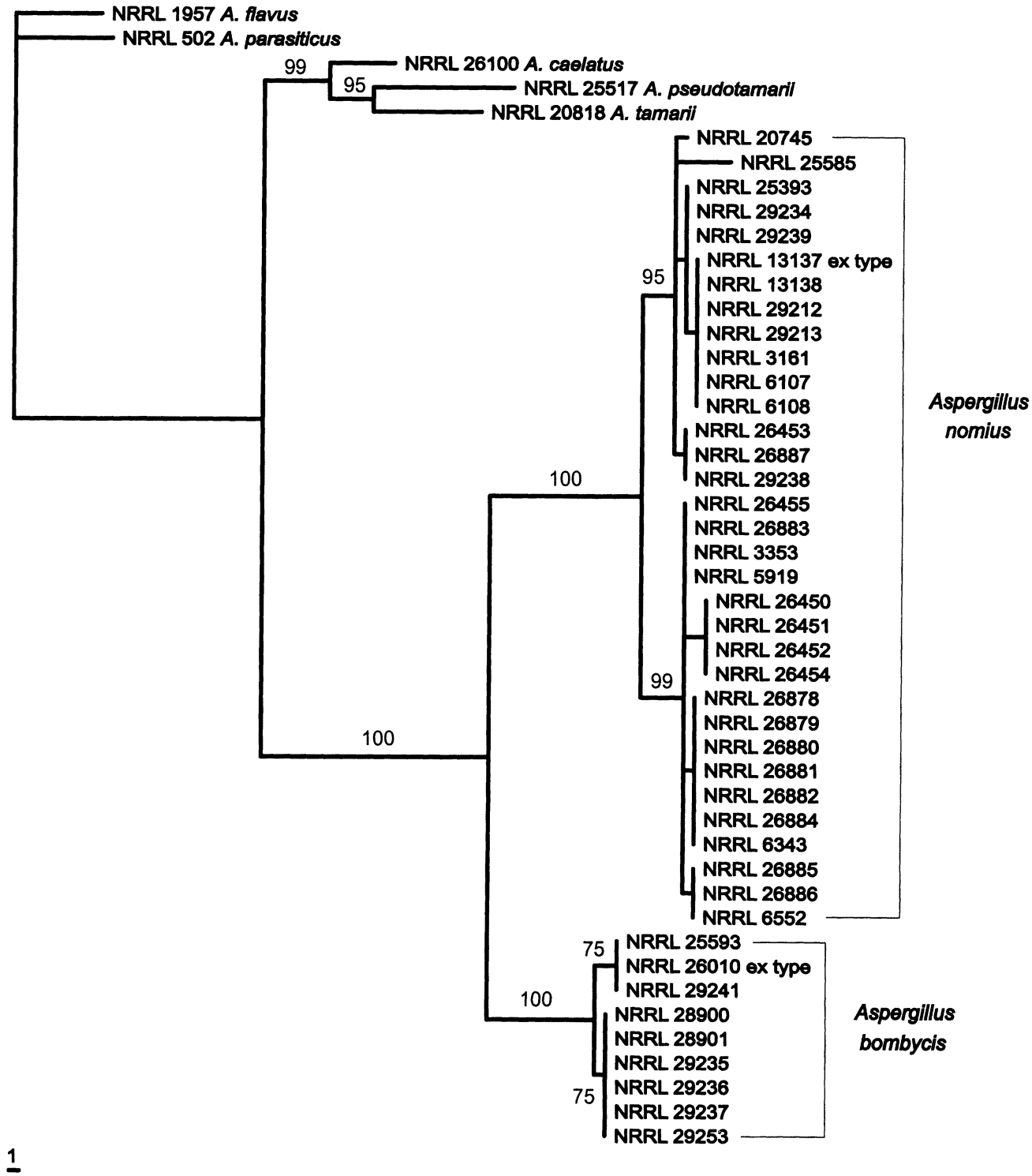


FIG. 10. Single most parsimonious tree calculated using PAUP* 4.0 (β4a) in an heuristic search of the β-tubulin sequence data. Tree length is 147 steps, CI = 0.9320, HI = 0.0680, RI = 0.9807 and RC = 0.9139. *Aspergillus flavus* and *A. parasiticus* are used as outgroup species to root the tree on the basis of FIG. 9. Bootstrap values are placed above the nodes. DNA sequences are deposited in GenBank as accessions AY017536–AY017582, and in Treebase as accession M893.

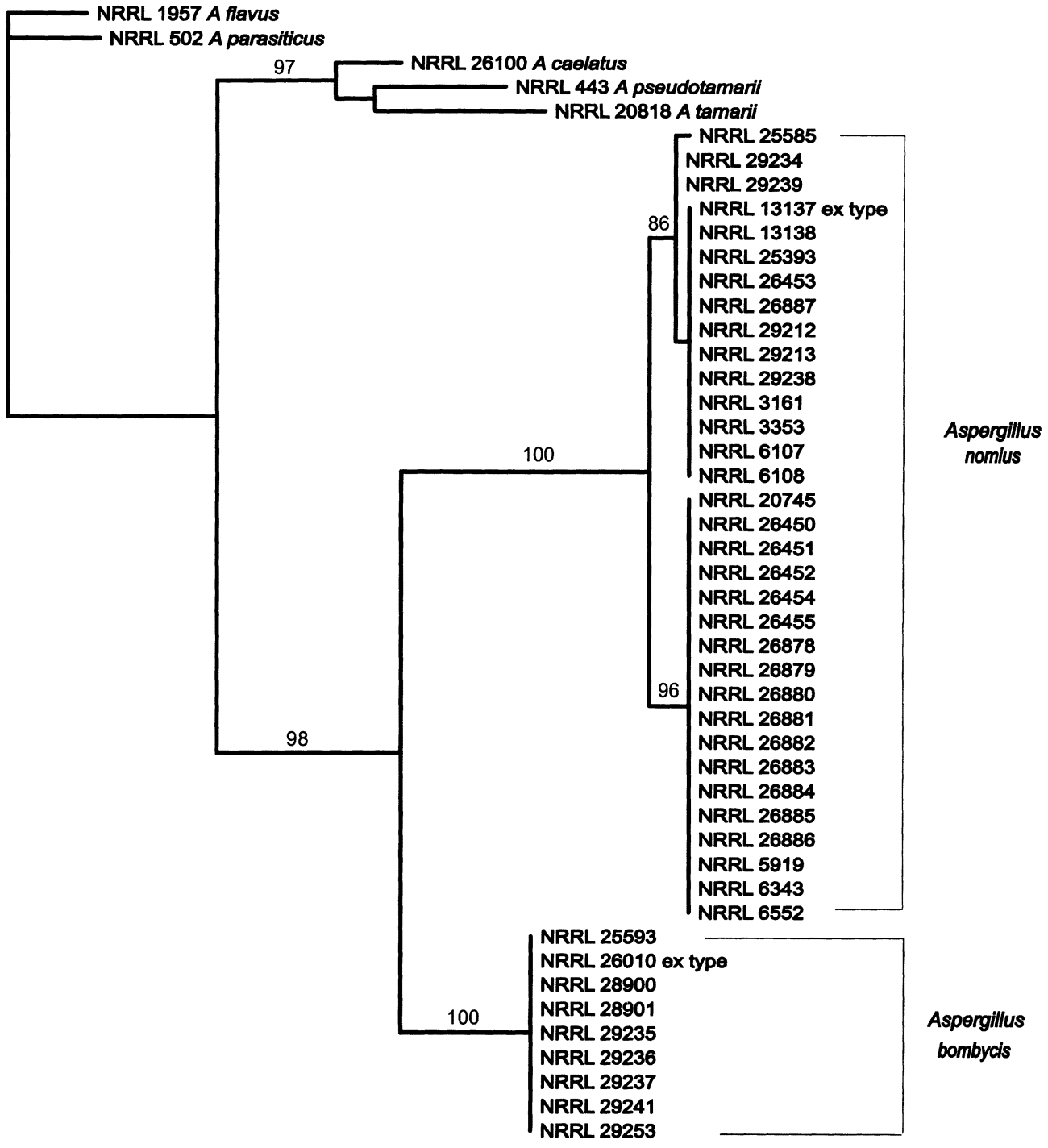


FIG. 11. One of two most parsimonious tree calculated using PAUP* 4.0 (β4a) in an heuristic search of the calmodulin sequence data. Tree length is 119 steps, CI = 0.8992, HI = 0.1008, RI = 0.9753 and RC = 0.8770. Bootstrap values are placed above the nodes. DNA sequences are deposited in GenBank as accessions AY017583–AY017629, and in Treebase as accession M895.

parsimonious tree (MPT) suggesting recombination. NOR data versus ID or BT produced trees that were not significantly longer that the MPT. There was no observed constant branch from the four loci that divided the *A. nomius* isolates into definable subgroups.

There does not appear to be a significant geographic component to the distribution of genotypes

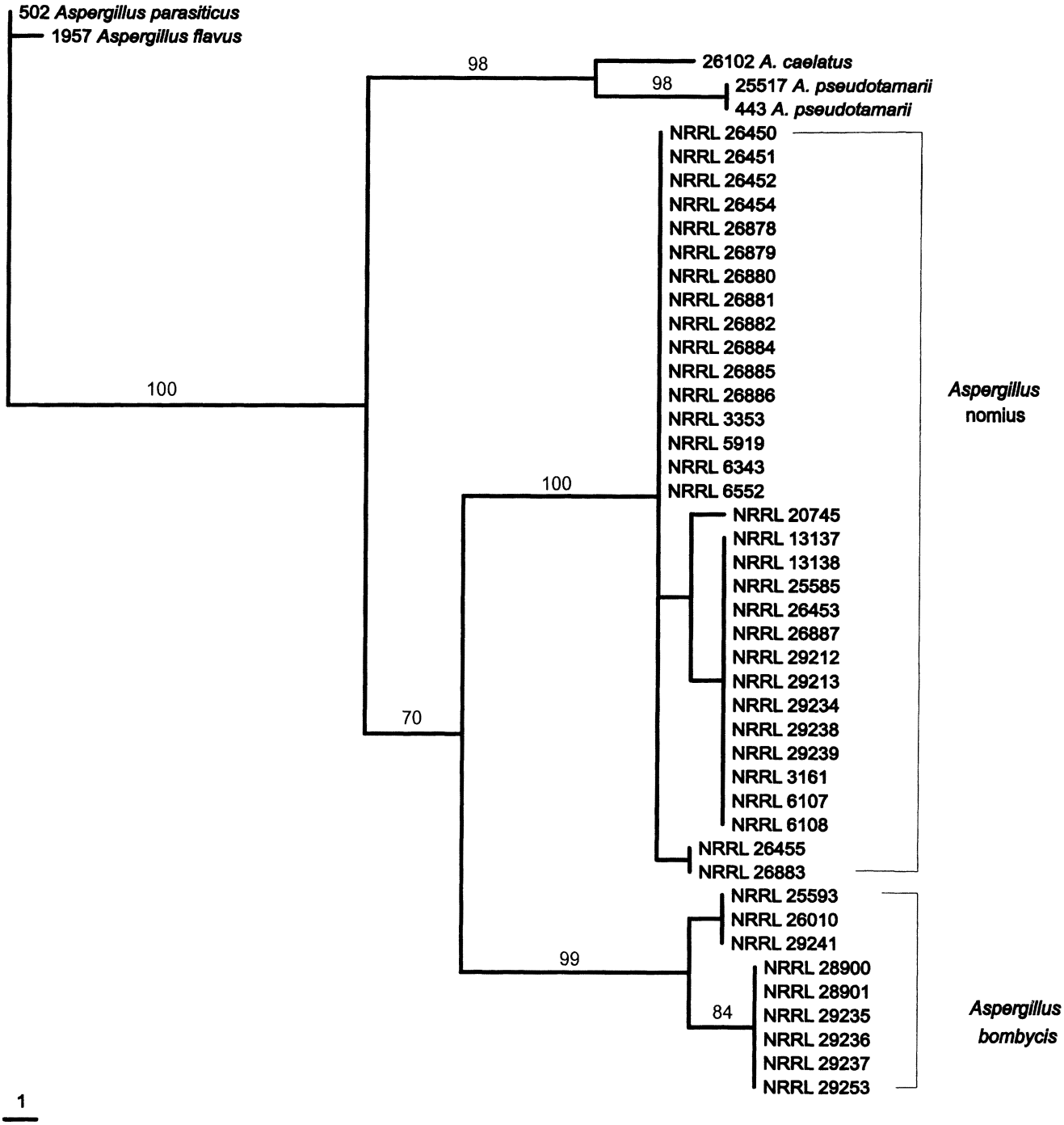


FIG. 12. Single most parsimonious tree calculated using PAUP* 4.0 (β4a) in an heuristic search of the norsolorinic acid reductase sequence data. Tree length is 49 steps, CI = 0.9388, HI = 0.0622, RI = 0.9858 and RC = 0.9254. Numbers above the internodes are bootstrap. DNA sequences are deposited in GenBank as accessions AY017630–AY017675, and in Treebase as accession M894.

(TABLE I). For example, four isolates from Grant Parish, Louisiana, two isolates from Gaines County, Texas, and one isolate from an alkali bee in Wyoming share the same genotype at all four loci. Four isolates from a single collection in Natchitoches Parish, Louisiana share the same genotype and may be clonally related, but a fifth isolate from the same collection shares its genotypes with an isolate from soil in Honshu, Japan. Seven isolates share the same genotype as the *ex type* isolate, but three of the cycad isolates may have originated from a single culture, having come to the ARS Culture Collection via different routes, and one of the isolates is probably a subculture of the *ex type* isolate.

TABLE IV. P-values obtained in pair-wise comparisons of loci[a], using the partition homogeneity test in PAUP* among the *A. nomius* isolates. Uninformative sites were excluded, and the p-values are based on 5000 replicates

	ID	BT	CAL
BT	0.004		
CAL	0.009	0.001	
NOR	0.309	1.0	0.013

[a] Loci are: ID = ITS and LSU rDNA; BT = beta tubulin; CAL = calmodulin; NOR = norsolorinic acid reductase, as described in the text.

DISCUSSION

Aspergillus bombycis and *A. nomius* isolates are distinguishable in several ways. *Aspergillus bombycis* isolates grow restrictedly (ca 15 mm diam) at 37 C and do not grow at 42 C, compared to *A. nomius* isolates that grow vigorously at 37 C (65 mm diam) and also grow at 42 C. *Aspergillus bombycis* isolates have smooth stipes compared to the roughened stipes of *A. nomius* isolates. Isolates of the two groups occur on distinct and strongly supported branches in gene trees calculated from all loci compared (FIGS. 9–12). Colony color, conidium characters (size, shape and ornamentation), and other morphological characteristics of *A. bombycis* overlap those of *A. nomius*. The production of aflatoxins B and G and the lack of cyclopiazonic acid production are the same in these two species. Growth rate at 42 C, stipe roughness and DNA sequences are the most useful characters for distinguishing *A. bombycis* from *A. nomius*.

Koupafanou et al (1997) pointed out that gene trees based on unlinked loci should produce congruent gene trees in clonally reproducing organisms. If organisms are meiotically recombining, the patterns of descent will be non-congruent because of gene flow between populations, generation of new genotypes by crossing over and extinction of some lineages (Taylor et al 1999). Among *A. bombycis* isolates, the patterns of descent are congruent and this observation argues for clonal propagation. The number of loci examined and the low level of polymorphism present in them make it impossible to conclude whether or not genetic recombination is occurring among isolates of *A. bombycis*.

Egel et al (1994) and Feibelman et al (1998) studied restriction endonuclease length variations of genes among isolates of *A. nomius* but did not determine the reproductive strategy of the species from those data. Using the more sensitive technique of DNA sequencing, non-congruence of gene trees at the four loci sampled (FIGS. 9–12) is seen among the *A. nomius* isolates. Non-congruence suggests that *A. nomius* possesses a cryptic sexual stage.

The table of genotypes (TABLE I) shows a very local element of clonal propagation (for example isolates from Grant Parish, Louisiana) but also distinct genotypes from the same locale, similar to the spatial and temporal patterns of *A. flavus* vegetative compatibility groups in cotton field in Arizona (Bayman and Cotty 1991).

Horn and Dorner (1998) isolated *Aspergillus* sect. *Flavi* isolates from 166 agricultural fields, plus some non-agricultural fields in the eastern and southern US and found *A. nomius* in one uncultivated and five cultivated fields. In soil samples where *A. nomius* was found, it accounted for from 1–20% of all *Aspergillus* sect. *Flavi* isolates in the sample. The cause of this heterogeneity is unknown, but we know that *A. nomius* is often isolated from dead or diseased insects (TABLE I). On the basis of spotty soil distribution and recorded isolation from insects, it is possible that insects are a common growth substrate for this species and that *A. nomius* persists in the soil environment until it can infect insects or grow on insect cadavers. Rare isolation from agricultural products suggests that these are not the preferred substrate of *A. nomius*. The spotty distribution and sometimes high local soil density of *A. nomius* are consistent with arrival of conidia in the soil via sporulation from dead insects or other point sources. The sister species to *A. nomius* is *A. bombycis* (FIGS. 9–12), a species that has been isolated only from insects or insect-associated materials.

Aspergillus nomius is known from collections in Asia and North America, and *A. bombycis* is known only from Asian locales. As seen in TABLE I, both *A. nomius* and *A. bombycis* have been isolated from insect frass in silkworm-rearing houses. If allopatric speciation occurred, then the ranges of the two species have converged post speciation. If speciation was sympatric, a potential isolating factor would be host specialization, but the only germane data are the limited isolation records of these two species.

Keller and Hohn (1997) reviewed clustering of functionally related genes in fungi. The aflatoxin biosynthetic genes are clustered on a relatively short (ca 60 KB) fragment of DNA (Yu et al 1995). Cluster organization of functionally related genes may allow simple regulation of the entire pathway (Yu et al 1995). It has also been pointed out that if all the genes of a functional pathway are clustered on a short segment of DNA, it might be possible for a complete functional pathway to be transferred laterally between species (see Keller and Hohn 1997). There has been little evidence to support or refute the hypothesis of lateral inter-species transfer of gene

clusters. However, if lateral transfer of the aflatoxin biosynthetic pathway genes had occurred, one would expect to see non-congruence among gene trees based on AF pathway genes and unlinked genes of the organisms. FIGURES 9–12 show congruence between norsolorinic acid reductase (an aflatoxin biosynthetic pathway gene) and the other three unlinked loci. These data suggest that lateral gene transfer has not occurred in this aflatoxin biosynthetic pathway gene and suggests that this gene was present in the common ancestor of the *A. flavus* group, just as the beta-tubulin, calmodulin and rDNA genes were.

The uniformly present and congruent branches leading to *A. bombycis* and *A. nomius* indicate the genetic isolation of the two clades and provide evidence that they are distinct species under the phylogenetic species concept (Taylor et al 1999).

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