Antonie van Leeuwenhoek 53: 147–158 (1987)

Aspergillus nomius, a new aflatoxin-producing species related to *Aspergillus flavus* and *Aspergillus tamarii*

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Key words: *Aspergillus*, aflatoxin, DNA relatedness

Abstract. *Aspergillus nomius* is described and represents a new aflatoxigenic species phenotypically similar to *A. flavus*. Strains examined were isolated from insects and agricultural commodities. Separation from *A. flavus* is based on the presence of indeterminate sclerotia and a lower growth temperature. Comparisons of DNA relatedness show *A. nomius* to have only relatively recently evolved from *A. flavus* and *A. tamarii*.

Introduction

An understanding of relationships among species associated with *Aspergillus flavus* Link:Fr. has been difficult because of the considerable morphological and physiological variation exhibited by these taxa. There has been controversy concerning whether *A. flavus*, *A. oryzae* (Ahlburg) Cohn, *A. parasiticus* Speare and *A. sojae* Sakaguchi & Yamada ex Murakami represent a single species or distinct taxa (Raper & Fennell 1973). *Aspergillus flavus* and *A. parasiticus* are aflatoxigenic species common to cereal grains and peanuts (*Arachis hypogaea* L.) while *A. oryzae* and *A. sojae* are not known to produce aflatoxin and are found, possibly exclusively, in food fermentations in the Orient (Wang & Hesseltine 1982). Blochwitz (1929), Saito (1943) and Wicklow (1984) have proposed that *A. oryzae* and *A. sojae* represent domesticated forms of *A. flavus* and *A. parasiticus*, respectively. Chemotaxonomic studies generally support the view that all four taxa are closely related (Christensen 1981; Kulik & Brooks 1970; Murakami 1971; Nasuno 1974; Vincent & Kulik, 1970). Kurtzman et al. (1986) addressed this problem through comparisons of deoxyribonucleic acid (DNA) relatedness and found sufficiently high complementarity among the four taxa to conclude that they were conspecific. Because some divergence was detected between *A. flavus*/

[1] The mention of firm names or trade products does not imply that they are endorsed or recommended by the US Department of Agriculture over other firms or similar products not mentioned.

A. oryzae and *A. parasiticus*/*A. sojae*, Kurtzman et al. (1986) proposed that the taxon be divided into two subspecies and four varieties resulting in the following designations: *A. flavus* Link:Fr. subsp. *flavus* var. *flavus*, *A. flavus* Link:Fr. subsp. *flavus* var. oryzae (Ahlburg) Kurtzman et al., *A. flavus* Link:Fr. subsp. *parasiticus* (Speare) Kurtzman et al. var. *parasiticus* (Speare) Kurtzman et al., and *A. flavus* Link:Fr. subsp. *parasiticus* (Speare) Kurtzman et al. var. *sojae* (Sakaguchi & Yamada ex Murakami) Kurtzman et al.

During the course of the foregoing DNA comparisons, a group of isolates was detected that showed relatively low relatedness with *A. flavus* and its varieties. All of these strains produce aflatoxin and were initially identified as atypical forms of *A. flavus* (Hesseltine et al. 1970). However, once the genetic difference was detected through comparisons of DNA complementarity, closer scrutiny revealed these strains to produce indeterminate sclerotia and to have a lower temperature optimum for growth than does *A. flavus* and its varieties. As a consequence of our molecular comparisons, we are describing this group of isolates as a new species.

Materials and methods

Strains and culture conditions

Cultures of the strains studied are maintained by the Agricultural Research Service Culture Collection (NRRL), Northern Regional Research Center, and they are listed in Table 1 along with their sources and guanine plus cytosine (G+C) contents. For DNA extraction, all strains were grown at 25 C in 2800-ml Fernbach flasks with 1500 ml of Wickerham's (1951) YM broth on a rotary shaker at 200 rpm. Each flask was inoculated with 2 ml of an aqueous suspension of conidia obtained by suspending the conidia from a 10-day-old Czapek's agar slant in 5 ml of sterile distilled water. Mycelial growth was harvested by vacuum filtration at stationary growth phase (ca. 36 hr) and suspended in SSE + 0.5 buffer (Timberlake 1978). Colony diameters for growth determinations (7 da, 42 C) were obtained from three-point inoculated Petri plates (15 × 100 mm) of Czapek's and malt extract agars. Petri plates were incubated in plastic bags to prevent desiccation.

DNA extraction, purification and characterization

Mycelial growth was initially homogenized in a Waring blendor for ca. 1 min, and the cells were then broken in a Braun homogenizer with 0.5-mm glass beads. Whole-cell DNAs were extracted and purified as previously described (Price et al. 1978; Kurtzman et al. 1980b). Analytical ultracentrifuge scans ($A_{262\ nm}$)

Table 1. Intraspecific DNA relatedness, nuclear guanine plus cytosine content and source of the aspergilli examined.

Species designation	NRRL no.	Percent intra-specific DNA relatedness[1]	Mol% Guanine plus cytosine content[2]	Source
Aspergillus nomius	13137[3]		50.0 ± 0.11	Moldy wheat (*Triticum vulgare* Vill.), U.S.A.
	3353	92	50.0 ± 0.25	Diseased alkali bee (*Nomia melanderi* Cockerell), Wyoming
	3161	85	50.4 ± 0.19	*Cycas circinalis* L., Polynesia
	5919	100	49.7 ± 0.10	Diseased alkali bee, Wyoming
	6343	99	49.8 ± 0.00	Diseased alkali bee, Wyoming
	6552	97	50.1 ± 0.08	Pine sawfly [*Diprion similis* (Hartig)], Wisconsin
	13138	91	49.7 ± 0.21	F. A. Hodge, strain M 90. Peanuts (*Arachis hypogaea* L.)?
Aspergillus flavus var. *flavus*	1957[4]		49.8 ± 0.14	Moldy cellophane, South Pacific
	482	95	49.2 ± 0.04	Wehmer's *A. flavus* reference strain
	3251	96	49.5 ± 0.19	Walnuts (*Juglans regia* L.), U.S.A.
	13135	97	49.1 ± 0.56	Moldy peanuts, U.S.A.
	13136	97	49.9 ± 0.02	Kangaroo rat (*Dipodomys merriami* subsp. (?) *merriami* Mearns) cheek pouch, Arizona
Aspergillus flavus var. *oryzae*	447[4]		49.6 ± 0.09	Sake koji, Japan
	451	100	49.3 ± 0.05	Soy sauce, China
Aspergillus flavus var. *parasiticus*	502[3]		49.1 ± 0.13	Mealy bug (*Pseudococcus* sp.?), sugarcane, Hawaii
	465	100	50.0 ± 0.06	J. Takamine
Aspergillus flavus var. *sojae*	5595[4]		49.3 ± 0.05	Koji, Japan?
	5594	100	49.2 ± 0.14	Koji, Manchuria?
Aspergillus tamarii	429[4]		48.6 ± 0.13	Soy Sauce, China
	13139	100	48.8 ± 0.25	Kangaroo rat cheek pouch, Arizona
Aspergillus leporis	3216[3]		49.2 ± 0.04	Dung of jack rabbit (*Lepus townsendii* Bachman), Wyoming

[1] Standard deviation ≤ 5% calculated from 2–4 determinations. Type or representative strain served as the basis for comparison.

[2] ± standard deviation calculated from 3 determinations.

[3] Type strain.

[4] Representative strain.

showed each preparation to contain less than 3% mitochondrial DNA. Hyperchromicity of the preparations as the percentage of initial absorbency at 260 nm ($A_{260\,nm}$) ranged from 36–37%. In preparation for reassociation experiments, DNA was mechanically sheared in a French press to a fragment size of approximately 400 nucleotides. Extent of DNA relatedness was determined spectrophotometrically (Seidler & Mandel 1971; Seidler et al. 1975) in a Gilford Model 250 recording spectrophotometer with a Model 2527 thermoprogrammer as previously described (Kurtzman et al. 1980b).

The guanine plus cytosine (G + C) content of the nuclear DNA was calculated from buoyant density in cesium chloride gradients (Schildkraut et al. 1962). Determinations were made in a Beckman Model E analytical ultracentrifuge equipped with an electronic scanner. *Micrococcus luteus* (Schroeter) Cohn (synonym *M. lysodeikticus* Fleming) DNA with a buoyant density of 1.7311 g/ml served as a reference (Price et al. 1978).

Genome sizes for all species were determined from comparisons with the reassociation rate constant of DNA from *Escherichia coli* (Migula) Castellani & Chalmers (Zimmerman & Goldberg 1977) using the methods cited above.

Scanning electron microscopy

Fresh conidia were collected on Nucleopore filters of 0.4 μm pore size, passed through a graded acetone series and critical-point dried as described earlier (Kurtzman et al. 1974). Specimens were gold-coated under vacuum and viewed in an ISI Model SS130 scanning electron microscope.

Results

Comparisons presented in Table 1 show the extent of intraspecific DNA relatedness for all taxa to be 85% or greater. As we previously reported, complementarity between *A. flavus* var. *flavus*, *A. flavus* var. *oryzae*, *A. flavus* var. *parasiticus* and *A. flavus* var. *sojae* (Table 2) ranges from 69–100%, and we have interpreted these data to indicate that the four taxa are conspecific (Kurtzman et al. 1986). Relatedness between this complex and NRRL 13137, the type strain of our proposed new species, averages 38%. Similarly, relatedness between *A. tamarii* Kita and various strains of the *A. flavus* complex averages 47% while the extent of complementarity between *A. tamarii* and NRRL 13137 is 42%. By contrast, *A. leporis* States & Christensen, as well as *Neurospora crassa* Shear & Dodge, which was included as a control, showed less than 10% similarity with the other species. This latter value is typical of the background complementarity shown between species having no close relatedness (Kurtzman 1985).

Estimates of genome size were made to assess the possibility that these species

Table 2. Pairwise comparisons showing the percent of DNA complementarity between *Aspergillus nomius* and other phenotypically similar aspergilli.[1]

Species designation NRRL no.	*A. flavus* var. *flavus* 1957	*A. flavus* var. *oryzae* 447	*A. flavus* var. *parasiticus* 502	*A. flavus* var. *sojae* 5595	*A. tamarii* 429	*A. nomius* 13137	*A. leporis* 3216
Aspergillus flavus var. *flavus* 1957		100	70	74	52	34	0
Aspergillus flavus var. *oryzae* 447			69	73	55	32	8
Aspergillus flavus var. *parasiticus* 502				91	41	39	7
Aspergillus flavus var. *sojae* 5595					40	47	0
Aspergillus tamarii 429						42	7
Aspergillus nomius 13137							7
Neurospora crassa[2] 13141	1	8	1	0	2	3	3

[1] Standard deviation $\leq 5\%$ calculated from 2–4 determinations.

[2] *N. crassa* was included as an unrelated control of similar genome size.

were genetically isolated from one another because of aneuploidy or amphidiploidy. Genome sizes calculated from reassociation rates were the following: *A. flavus* var. *flavus* = 1.00, *A. flavus* var. *oryzae* = 1.00, *A. flavus* var. *parasiticus* = 1.10, *A. flavus* var. *sojae* = 0.97, *A. tamarii* = 0.92, *A. nomius* = 0.88 and *A. leporis* = 0.93, where $1.00 = 2.2 \times 10^{10}$ daltons (s.d. = 0.06, $n = 7$). Subjecting these data to an analysis of variance suggests *A. flavus* var. *parasiticus* and *A. nomius* to have genome sizes significantly different from *A. flavus* var. *flavus*. However, experimental variables, such as the difficulty of controlling DNA fragment size, may bias measurements of reassociation kinetics, and therefore argue against over interpretation of the noted differences. It should be emphasized that even differences in ploidy cannot be resolved by this methodology.

As we will discuss, we believe our data demonstrate the presence of a new species of *Aspergillus*, and it is described accordingly.

Aspergillus nomius Kurtzman, Horn & Hesseltine, sp. nov. Figs. 1, 3–6.

Coloniae in agare soluto Czapeki crescunt usque ad diametrum 4–7 cm intra dies septem, temperatura 25 C; temperatura 42 C crescunt non plus quam ad 0–1.5 cm intra idem tempus (Table 3, Fig. 1). Superficies coloniarum levis aut floccosa, a mycelio constans vegetali albo aut subaurantio, in quo sunt structur-

Table 3. Growth of *Aspergillus nomius* and other members of the *Aspergillus flavus* group after 7 days at 42 C on Czapek's and malt extract agars.

Taxon/no. of strains tested	Colony diam. (cm)[1] on	
	Czapek's agar	Malt extract agar
Aspergillus nomius	0 –1.5	0 –0.7
Aspergillus flavus var. *flavus*	2.4–3.6	1.5–2.9
Aspergillus flavus var. *oryzae*	1.8–3.2	0.7–2.1
Aspergillus flavus var. *parasiticus*	1.8–3.3	1.9–3.0
Aspergillus flavus var. *sojae*	1.5–2.7	1.1–2.3
Aspergillus tamarii	0.2–1.0	0.1–0.3
Aspergillus leporis	0	0

[1] Colony diameters represent the range obtained from the measurement of 9 colonies (3 colonies/ Petri plate) for each strain.

ae conidiales rarae sive aliquando copiosiores, saepe ortae iuxta margines, et aliquando non nisi post incubationem longam. Capita conidialia in toto visa luteocitrina (XVI, Ridgway 1912) sive paene vireonica (IV, Ridgway 1912) post dies septem, postremo se mutantia, aetate provecta, in colorem lapidis serpentinae (XVI, Ridgway 1912), floris callae (V, Ridgway 1912) vel veratri (XVII, Ridgway 1912). Parte aversa flava vel lutea; in isolatis sclerotialibus adest exudatum fulvum. *Sclerotia* fulva usque ad nigra, apicibus albis, ad perpendiculum elongata in longitudinem inconstantem sed plerumque 500–800 × 1200–2100 µm, aliquando ad 6000 µm (Fig. 3). *Capita conidialia* biseriata aut uniseriata, radiata, saepe fissa in plures columnas, 150–600 µm diametro; capita minora aliquando solute columnaria, 20–80 × 100–250 µm. *Conidiophori* colore carentes, echinulati; sub vesiculo ipso diametro 8–22 µm; longitudine variantes, plerumque 300–1100 µm, usque ad 3000 µm. *Vesiculae* globosae aut subglobosae, 25–65 µm diametro; *metulae* 4.0–8.6 × 8.1–17.3 µm; *phialides*, 3.8–6.5 × 7.6–11.3 µm (Fig. 4). *Conidia* globosa aut subglobosa, echinulata; diametro variantia, 3.7–8.1 µm, plerumque 4.5–6.5 µm (Figs. 5 and 6).

Cultura typica: NRRL 13137, isolata a tritico mucido, exsiccatus in National Fungus Collection, Beltsville, Maryland, et vivus in Collectione Culturarum, Officina Investigationum Tractus Borealis, Peoria, IL, United States.

Colonies on Czapek's solution agar attaining a diameter of 4–7 cm in 7 days at 25 C; growth at 42 C in 7 days restricted, 0–1.5 cm (Table 3, Fig. 1). Colony surface velvety to floccose, consisting of white or light orange-brown vegetative mycelium and sparse to moderately abundant conidial sturctures, often produced in marginal areas and occasionally only after extended incubation. Conidial heads *en masse* yellowish citrine (XVI, Ridgway 1912) or near warbler green (IV, Ridgway 1912) at 7 days, eventually shifting with age to serpentine green (XVI, Ridgway 1912), calla green (V, Ridgway 1912) or hellebore green (XVII,

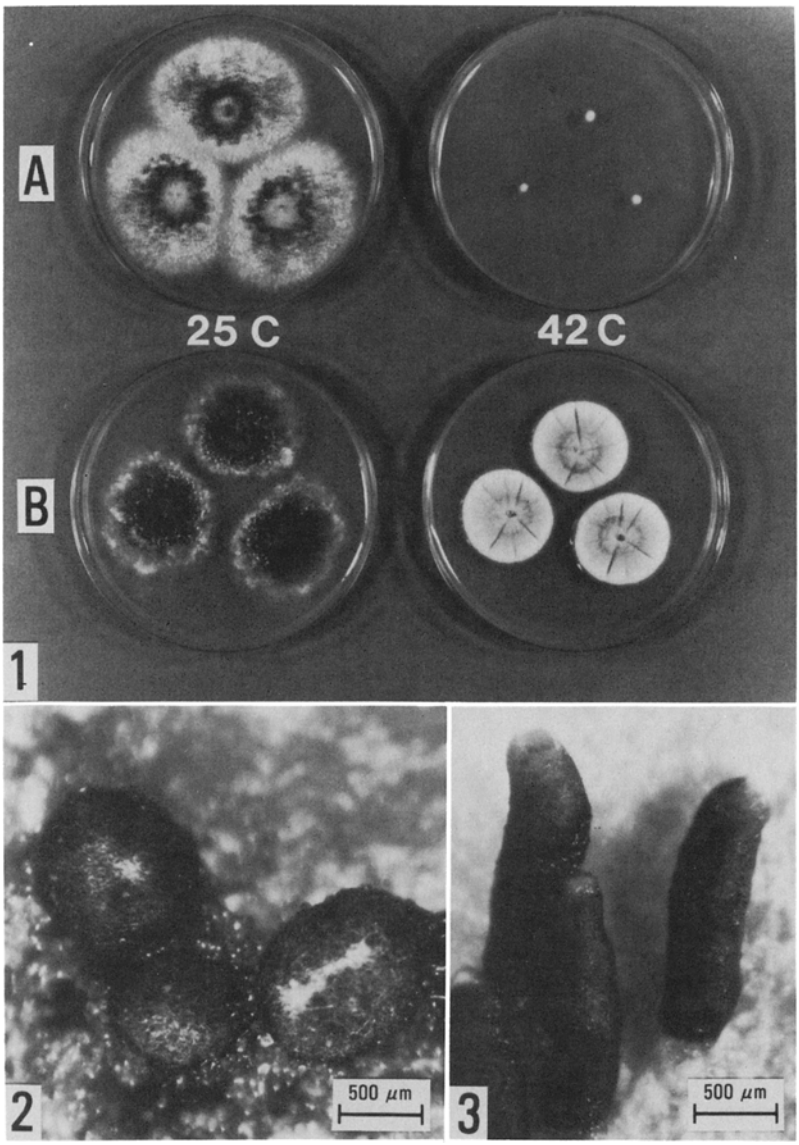

Fig. 1. Growth of *A. nomius* and *A. flavus* var. *flavus* at 25 C and 42 C on Czapek's agar. A. *A. nomius* NRRL 13137. B. *A. flavus* var. *flavus* NRRL 1957.
Fig. 2. Sclerotia of *A. flavus* var. *flavus* NRRL 1957.
Fig. 3. Sclerotia of *A. nomius* NRRL 13137.

Ridgway 1912). Reverse light yellow- or orange-brown; red-brown exudate present in sclerotial isolates. Sclerotia dark brown to black and white-tipped, vertically elongate, indeterminate, mostly 500–800 × 1200–2100 μm but up to 6000 μm in length (Fig. 3). *Conidial heads* biseriate or uniseriate, radiate, often split-

ting into several columns, 150–600 μm in diameter; smaller heads may be loosely columnar, 20–80 × 100–250 μm. *Conidiophores* uncolored, echinulate; diameter immediately below vesicles 8–22 μm; variable in length, mostly 300–1100 μm but up to 3000 μm. *Vesicles* globose to subglobose, 25–65 μm diameter; *metulae*, 4.0–8.6 × 8.1–17.3 μm; *phialides*, 3.8–6.5 × 7.6–11.3 μm (Fig. 4). *Conidia* globose to subglobose, echinulate; variable in diameter, 3.7–8.1 μm, mostly 4.5–6.5 μm (Figs. 5 and 6).

Colonies on malt extract agar attaining a diameter of 4–7 cm in 7 days; growth at 42 C in 7 days restricted, 0–0.7 cm. Colony surface mostly floccose; sporulation often occurring at marginal areas. Conidial heads *en masse* near warbler green (IV, Ridgway 1912), cerro green (V, Ridgway 1912) or elm green (XVII, Ridgway 1912). Sclerotia when present smaller and less abundant than on Czapek's medium.

Type culture. NRRL 13137, isolated from moldy wheat, USA. Dried holotypic material has been deposited with the National Fungus Collection, US Department of Agriculture, Beltsville, Maryland. Living cultures were deposited in the ARS Culture Collection, Peoria, Illinois.

The species name of this new taxon was derived from the genus name of the alkali bee, *Nomia melanderi* Cockerell, from which many of the strains were isolated. The type strain, from moldy wheat, appears to be phenotypically the most representative strain of this species in terms of sclerotia formation, colony morphology, and microscopic characteristics.

Discussion

Comparison of nuclear DNA base sequence complementarity as a means for defining taxa among the filamentous fungi has not yet been exploited. However, this technique has proved to be a reliable means for recognizing yeast species, and such studies have demonstrated many phenotypic characters to be unreliable indicators of kinship (Mendonça-Hagler & Phaff 1975; Price et al. 1978; Kurtzman 1984). Among heterothallic yeasts, correlation of fertility with extent of DNA complementarity has provided a basis for assigning biological significance to various degrees of DNA relatedness. Such comparisons showed that mating reactions did not totally disappear until DNA complementarity reached background levels, i.e., 10–15% or less (Kurtzman et al. 1980a; Kurtzman 1985), and fertile F_2 progeny resulted from one pair showing only 25% relatedness. Although this particular pairing seems exceptional, it should not be unusual to find fertile crosses between strains showing as little as 60% DNA relatedness. Consequently, it is suggested, assuming the absence of major chromosome rearrangements and ploidy differences, that strains showing 60% or greater DNA relatedness be considered conspecific. While the studies providing these guide-

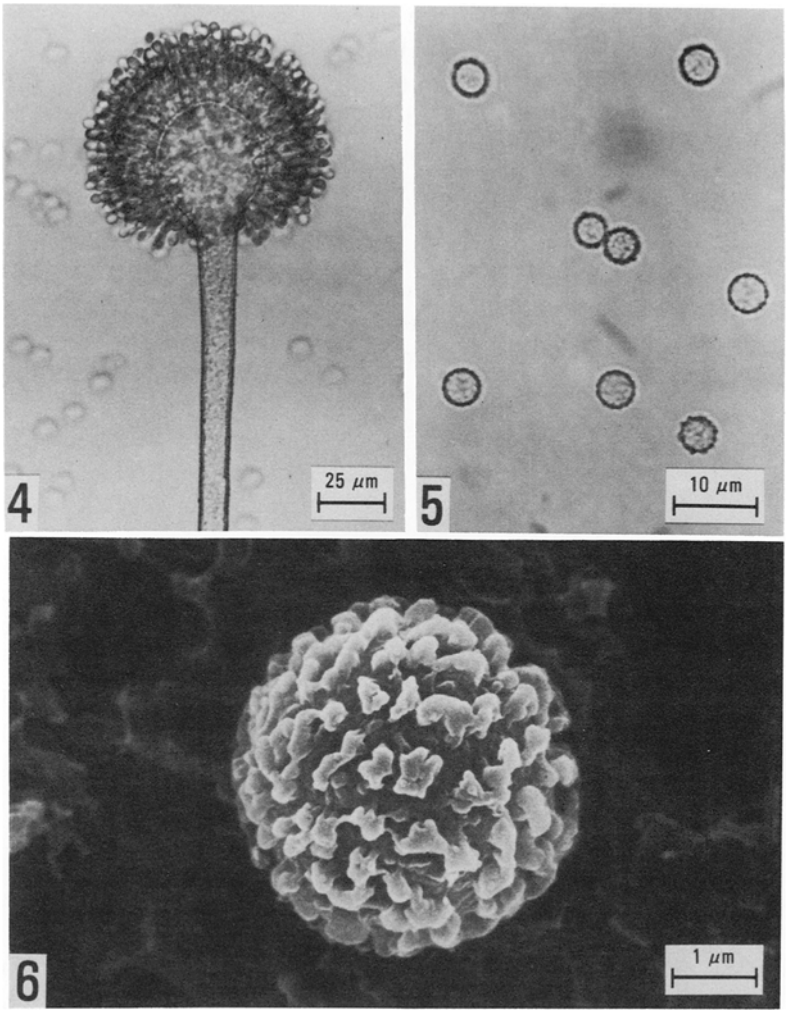

Fig. 4. Conidiophore of *A. nomius* NRRL 13137.
Fig. 5. Brightfield micrograph of conidia from *A. nomius* NRRL 13137.
Fig. 6. Scanning electron micrograph of a conidium from *A. nomius* NRRL 13137.

lines have focused on yeasts, we propose that the findings are also relevant for estimates of biological relatedness among filamentous fungi including the aspergilli examined in the present work.

Aspergillus nomius, *A. flavus* and *A. tamarii* show greater DNA relatedness than is usually found between species and this suggests that these three taxa only relatively recently evolved from a common ancestor. The evolutionary forces causing this divergence are unknown but differences in such metabolic functions as temperature tolerance, mycotoxin production or pathogenicity to

insects may have isolated strains of the ancestral species into distinctive though poorly recognized habitats that lead to their genetic isolation.

The relatively recent divergence of *A. nomius* from other members of the *A. flavus* group seems to have allowed the development of few phenotypic characters useful for its discrimination in the laboratory. The indeterminate nature of the sclerotia (Fig. 3) provides a ready contrast with the determinant sclerotia formed by *A. flavus* (Fig. 2). However, not all strains of either *A. nomius* or *A. flavus* produce sclerotia, so this distinction is not always practical. A comparison by scanning electron microscopy of the surface ornamentation of conidia from all strains listed in Table 1 revealed no consistent differences that would allow separation of any of the taxa from one another. By contrast, growth of *A. flavus* at elevated temperatures does provide a conclusive means for separation of this species from *A. nomius* (Fig. 1, Table 3). A 7-day incubation period proved convenient for rapid recognition of the two taxa. Growth at elevated temperatures was shown earlier to be a useful taxonomic character for aspergilli (Christensen 1981) and penicillia (Pitt 1979).

Aspergillus nomius shares with *A. flavus* var. *flavus* and *A. flavus* var. *parasiticus* the unique ability to produce aflatoxin, but this carcinogen is not a metabolite of *A. tamarii* (Hesseltine et al. 1970). On the other hand, most strains of *A. tamarii* produce cyclopiazonic acid, an indole-tetramic acid mycotoxin also formed by *A. flavus* var. *flavus*, as well as many species of *Penicillium*, but not detected in cultures of *A. flavus* var. *parasiticus* (Dorner 1983). These comparisons raise interesting questions concerning the origin of the genes coding for mycotoxin biosynthesis and on the mechanisms controlling their expression.

Acknowledgements

We thank D. T. Wicklow for helpful discussions, C. J. Robnett, M. J. Smiley and F. L. Baker for their competent technical assistance, and H. M. Howe for providing the Latin diagnosis of *A. nomius*.

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Received 27 June 1986; accepted 14 January 1987