# Relationships Among Isolates of *Aspergillus* sect. *flavi* that Vary in Aflatoxin Production

Daniel S. Egel, Peter J. Cotty, and Karol S. Elias

U.S. Department of Agriculture, Agricultural Research Service, Southern Regional Research Center, P.O. Box 19687, New Orleans, LA 70179.
Current address of first author: American Sunmelon, P.O. Box 153, Hinton, OK 73047.
We thank Darlene Downey for technical assistance.
Accepted for publication 12 April 1994.

## ABSTRACT

Egel, D. S., Cotty, P. J., and Elias, K. S. 1994. Relationships among isolates of *Aspergillus* sect. *flavi* that vary in aflatoxin production. Phytopathology 84:906-912.

Relationships among 47 fungal isolates belonging to *Aspergillus flavus* sect. *flavi* were inferred from restriction site variability in a portion of the Taka-amylase A gene. Portions (1,168 bp) of the gene were amplified by polymerase chain reaction from genomic DNA of *A. flavus, A. oryzae, A. parasiticus, A. sojae, A. nomius,* and *A. tamarii.* The resulting amplification products were subjected to restriction analysis. A UPGMA (unweighted paired group method with arithmetic averaging) dendrogram based on the analysis divided *A. flavus* into two clusters that were 98% similar. Both clusters included atoxigenic and toxigenic isolates, and one cluster contained all four isolates of *A. oryzae* examined. Isolates within the highly toxigenic S strain, which produced only B aflatoxins, were not differentiated from certain L strain isolates. However, two S strain isolates, which produced both B and G aflatoxins, differed by 18% from other *A. flavus* isolates and were almost as closely related to *A. parasiticus* isolates as to other *A. flavus* isolates. All isolates of both *A. sojae* and *A. parasiticus* were identical at all restriction sites tested. However, *A. nomius* isolates were highly variable. Conspecific variability was far greater within *A. nomius* species than within other aflatoxin-producing species. However, one isolate of *A. tamarii* differed from the three other *A. tamarii* isolates studied (which were identical at all tested sites) by 35%. Parsimony analyses of the restriction site data suggest that both *A. flavus* and *A. parasiticus* arose from an ancestor shared with *A. nomius* via an *A. flavus* S strain intermediate. The results support previous suggestions that *A. sojae* and *A. oryzae* are forms of *A. parasiticus* and *A. flavus,* respectively.

*Additional keywords*: mycotoxins, population genetics.

Continuing concern about food safety and potential effects of mycotoxins in the human diet have stimulated increasing interest in fungi within *Aspergillus* sect. *flavi.* This interest stems from three species within this section, *A. flavus* Link:Fr., *A. parasiticus* Speare, and *A. nomius* Kurtzman et al, which have the ability to produce aflatoxins (9,14). Aflatoxins are notorious fungal metabolites that are carcinogenic and, in many countries, regulated at part-per-billion levels in foods and feeds (22). These compounds contaminate agricultural products when the aflatoxin-producing fungi infect plants or plant products, either during crop development or in storage (8). These fungi also have several industrial uses, and isolates of *A. oryzae* (Ahlburg) Cohn and *A. sojae* Sakagushi & Yamada have been used in the traditional fermented food industry in the Orient for hundreds of years (3). Certain isolates of *A. flavus* are used for production of enzymes for industrial and medicinal uses (16).

Fungal isolates within section *flavi* are variable in several characters, and considerable variability occurs even among isolates within individual species (1,2,19). Isolates belonging to the species *A. flavus* vary in aflatoxin-producing ability, sclerotial morphology, virulence to plants, enzyme-producing ability, and a variety of other physiological characteristics (5,7). Relationships among morphologically and physiologically diverse isolates are not clear. Interest in this variability has increased because of recent suggestions that atoxigenic strains of *A. flavus* might be applied to agricultural fields in order to reduce the risk of aflatoxin contamination (6) and because of increased recognition of the potential importance of the S strain of *A. flavus* (2,5). The relationship of *A. nomius* to other aflatoxin-producing species is also a source of interest and even controversy (15,18). We have been interested in a better understanding of diversity within this group of fungi and the factors dictating retention and loss of various characters. Knowledge of the relative phylogenetic distances among isolates and strains may facilitate assessment of the rate at which characters are acquired and lost and thus the relative stability of characters (i.e., aflatoxin-producing ability and type of sclerotia) among subgroups and species within this section.

Phylogenetic relationships among strains might best be investigated through direct genetic techniques. Such a technique, DNA reassociation, has been used to suggest that *A. flavus, A. oryzae, A. parasiticus,* and *A. sojae* belong to the same species and that *A. oryzae* and *A. sojae* are domesticated strains derived from *A. flavus* and *A. parasiticus,* respectively (15). Also on the basis of DNA reassociation and certain physiological traits, the aflatoxin-producing species *A. nomius* was separated from both of the other aflatoxin-producing members of section *flavi* and *A. tamarii* (14). Nuclear DNA restriction fragment length polymorphisms (RFLPs) supported a greater similarity between *A. flavus* and *A. nomius* than between *A. flavus* and *A. parasiticus* in one study (18). In contrast, mitochondrial DNA sizes were identical in *A. flavus* and *A. parasiticus,* while *A. nomius* differed markedly from these two species (17). Production of relatively large, elongate sclerotia is one distinguishing characteristic of *A. nomius* (14). Sclerotia have also been used to distinguish groups of isolates within the species *A. flavus* (5). *A. flavus* S strain or "atypical" isolates produce numerous small (<300 μm average diameter) sclerotia and, on the average, produce much greater quantities of aflatoxins than typical (L strain) isolates (5). In 1970, three isolates with S strain sclerotia were placed in a separate unnamed taxon by Hesseltine et al (12). The division of *A. flavus* into S and L strains has been supported by one genetic study of both vegetative compatibility analysis and random amplified polymorphic DNA (RAPD) (2).

Physiological differences, especially in aflatoxin production, have often been noted among strains of *Aspergillus* sect. *flavi*

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1994.

(5,9,12). All isolates classified as *A. tamarii, A. oryzae,* and *A. sojae* are atoxigenic. Most isolates of *A. parasiticus* and *A. nomius* produce both B and G aflatoxins (9). Typical isolates (L strain isolates) of *A. flavus* may produce only B aflatoxins or no aflatoxins at all, and all *A. flavus* S strain isolates (the atypical isolates of Saito et al [21]) reported to date (75 isolates) produce relatively large quantities of aflatoxins, some producing only B aflatoxins and others producing both B and G aflatoxins (5,12,21). Aflatoxin production and genetic relatedness did not correlate on the basis of either nuclear DNA RFLPs or restriction enzyme analysis of mitochondrial DNA (17,18). However, in some cases, both RAPDs and vegetative compatibility tests associated isolates of *A. flavus* with similar toxin-producing ability (2).

Although RAPDs were useful in differentiating certain closely related isolates of *A. flavus,* the exact nature of the variability detected by the RAPD technique is unknown. Furthermore, in our hands, RAPDs were occasionally variable and had reduced reliability as relatedness among strains increased (2). In order to avoid these difficulties, confirm the RAPD results, and further examine the relationship of *A. nomius* to other taxa within section

TABLE 1. Isolates of *Aspergillus* spp. used in this study

| Isolate number | Isolate designation[a] | Origin | | | Sclerotia type[b] | Aflatoxins[c] | Reference[d] |
|---|---|---|---|---|---|---|---|
| | | Location | Substrate | Year | | | |
| *A. flavus* | | | | | | | |
| 1 | LA 2-5 | Louisiana | Soil | 1989 | S | B | ... |
| 2 | 42 | Louisiana | Soil | 1987 | S | B | 5 |
| 3 | MR 3-15 | Arizona | Soil | 1989 | S | B | ... |
| 4 | YV 5-12 | Arizona | Soil | 1989 | S | B | ... |
| 5 | MR 5-11 | Arizona | Soil | 1989 | L | B | 1 |
| 6 | LA 2-9 | Mississippi | Soil | 1989 | L | B | ... |
| 7 | 13 | Arizona | Soil | 1987 | L | B | 5 |
| 8 | MR 5-35 | Arizona | Soil | 1989 | L | ND | ... |
| 9 | YV 2-29 | Arizona | Soil | 1989 | L | ND | ... |
| 10 | 36 | Arizona | Cottonseed | 1987 | L | ND | 5 |
| 11 | NRRL 5917 = A-4018b | Unknown | Bran | 1952 | ND | ND | 12 |
| 12 | NRRL 5918 = A-14152 | Minnesota | Corn | 1966 | L | ND | 12 |
| 13 | NRRL 1957[T] | South Pacific | Cellophane | Unknown | L | ND | 12 |
| 14 | AL 1-20 | Alabama | Soil | 1989 | L | B | ... |
| 15 | YV 4-9 | Arizona | Soil | 1989 | ND | ND | ... |
| 16 | NRRL 5565 = A-12268 | Unknown | Turkey feed mix | 1963 | L | ND | 12 |
| *A. oryzae* | | | | | | | |
| 17 | NRRL 447 | Japan | Unknown | Unknown | ND | ND | 14 |
| 18 | NRRL 451 | China | Unknown | Unknown | ND | ND | 14 |
| 19 | NRRL 3485 | Japan | Unknown | Unknown | ND | ND | 14 |
| 20 | NRRL 6271 | Taiwan | Unknown | Unknown | ND | ND | 14 |
| *A. parasiticus* | | | | | | | |
| 21 | NRRL 2999 = ATCC 26691 | Uganda | Peanut | Unknown | ND | B, G | 12 |
| 22 | SU-1 = ATCC 56775 | Unknown | Unknown | Unknown | ND | B, G | ATCC |
| 23 | SRRC 2043 = ATCC 62882 | Georgia | Peanut | Unknown | ND | ND | ATCC |
| 24 | AL 1-16 | Alabama | Soil | 1989 | L | B, G | ... |
| 25 | AL 1-22 | Alabama | Soil | 1989 | L | B, G | ... |
| 26 | AL 1-26 | Alabama | Soil | 1989 | ND | B, G | ... |
| 27 | AL 1-27 | Alabama | Soil | 1989 | ND | B, G | ... |
| 28 | AL 1-31 | Alabama | Soil | 1989 | ND | B, G | ... |
| 29 | SRRC 75[T] = ATCC 1018 | Hawaii | Mealybug | 1913 | ND | ND | 12 |
| *A. sojae* | | | | | | | |
| 30 | NRRL 5594 | China | Unknown | Unknown | ND | ND | 14 |
| 31 | NRRL 5595 | Japan | Unknown | Unknown | ND | ND | 14 |
| 32 | NRRL 5596 | Japan | Unknown | Unknown | ND | ND | 14 |
| 33 | NRRL 1988 | China | Unknown | Unknown | ND | ND | 14 |
| *A. nomius* | | | | | | | |
| 34 | IMI 358751 | Louisiana | Soil | 1989 | O | B, G | ... |
| 35 | IMI 358752 | Louisiana | Soil | 1989 | O | B, G | ... |
| 36 | IMI 358749 | South America | Brazil nut | 1990 | O | B, G | ... |
| 37 | NRRL 5919 =A-13838 | Wyoming | Alkali bee | 1965 | O | B, G | 12, 15 |
| 38 | NRRL 6107 = A-13570 | Guam | *Cycas circinalis* | 1965 | O | B, G | 12, 15 |
| 39 | NRRL 13137 = A-13794[T] | Illinois | Wheat | 1965 | O | B, G | 12, 15 |
| 40 | NRRL 6552 = A-15619 | Wisconsin | Pine sawfly | 1967 | O | B, G | 12, 15 |
| 41 | IMI 358750 | Mississippi | Cottonseed | 1991 | O | B, G | ... |
| Unnamed | | | | | | | |
| 42 | A-11611 | Nigeria | Peanuts | Unknown | S | B, G | 12 |
| 43 | A-11612 | Nigeria | Peanuts | Unknown | S | B, G | 12 |
| *A. tamarii* | | | | | | | |
| 44 | AL 1-12 | Alabama | Soil | 1989 | ND | ND | ... |
| 45 | A-28668 | Arizona | Soil | 1989 | ND | ND | ... |
| 46 | NRRL 20818[T] | Unknown | Activated carbon | Unknown | ND | ND | 14 |
| 47 | A-28672 | Mississippi | Soil | 1989 | ND | ND | ... |

[a]NRRL or A = Culture Collection of National Center for Agricultural Utilization Research (formerly Northern Regional Research Laboratory); ATCC = American Type Culture Collection, Rockville, MD; SRRC = Southern Regional Research Center; IMI = International Mycological Institute; and [T] = ex type of species.

[b]The largest sclerotia formed after 30 days incubation (32 C) on Czapek-Dox agar were examined at 30× for gross morphological characters. S = sclerotia typical of the S strain of *A. flavus*; L = sclerotia typical of the L strain of *A. flavus*; ND = none detected; and O = ovoid sclerotia.

[c]Aflatoxins produced in liquid fermentation. B = B aflatoxins; G = G aflatoxins; and ND = none detected.

[d]Article in which isolate is cited. ... = Isolate collected by the authors and not previously referred to in an article, and ATCC = current catalog of the American Type Culture Collection.

*flavi*, we examined variability within a specific genomic segment. Preliminary data were obtained by restriction analysis of a polymerase chain reaction (PCR)-amplified mitochondrial rDNA segment previously used for similar studies (24). This technique detected only enough variability to differentiate *A. tamarii* from the other members of the group (D. Egel, P. Cotty, and K. Elias, *unpublished observations*). Therefore, we developed a technique to amplify and restrict what we hoped would be a less conserved region, a portion of the Taka-amylase A gene (23,26) of which approximately one-third is intervening sequence (which should be selectively neutral). Parts of this work have been presented in preliminary form (10,11).

## MATERIALS AND METHODS

**Fungal isolates and growth conditions.** Sources and geographical origins of isolates used in this study are listed in Table 1. Isolates were kept for long periods by placing 3-mm plugs of sporulating culture grown on 5/2 agar (5% V8 juice and 2% agar) in 4-dram vials containing 5 ml of sterile distilled water and maintaining them at 8 C (5). Conidia from 7- to 10-day-old cultures grown on 5/2 agar unilluminated at 32 C were suspended in distilled water. Erlenmeyer flasks (250 ml) containing 70 ml Czapek broth (Difco, Detroit, MI) were seeded with 100 $\mu$l of conidial suspension (10,000–100,000 spores) and incubated unilluminated at 32 C on a rotary shaker (150 rpm) for 4 days. Mycelia were harvested by vacuum filtration through Whatman no. 4 filter paper.

**DNA extraction and amplification conditions.** DNA was extracted with a DNA extraction kit (Stratagene, La Jolla, CA) according to manufacturer's instructions. Initial DNA pellets were further purified by extraction with 1 ml of phenol-chloroform-isoamyl alcohol (25:24:1) followed by extraction with 1 ml of chloroform-isoamyl alcohol (24:1). DNA pellets were dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at −20 C. The amount and purity of DNA extracted was determined by 260/280-nm absorption spectra (UV-160, Shimadzu Scientific Instruments, Columbia, MD). Genomic DNA preparations were used for amplifications only if 260:280 ratios exceeded 1.75.

Oligonucleotide primers (20 mers) were designed to amplify a 1,168-bp fragment of the Taka-amylase A gene; sequence data from Tada et al (23) were used. A second set of primers nested within the first set were developed in order to demonstrate that the intended fragment had indeed been amplified (Fig. 1). Primers were named by the number of the base at the 5′ end as numbered by Tada et al (23). Names and orientations of primers are indicated in Figure 1. Primer 111 has the sequence GGATCGATTTGCAAGGACGG, primer 237 is AACATGGCAGGGCATCATCG, primer 1250 is TTGACTTGAAGGCGTTGAGG, and primer 1280 is TAGAGGTCGTCCATGCTGCC. Amplification conditions were as follows: a TC1 thermocycler (Perkin-Elmer Cetus, Norwalk, CT) was used; and amplification temperatures were 94 C for 3 min for initial denaturation, after which the first three cycles consisted of 94 C (denaturation), 58 C (annealing), and 72 C (extension), each for 1 min. The next 22 cycles were identical to the first three, except the annealing temperature was 54 C.

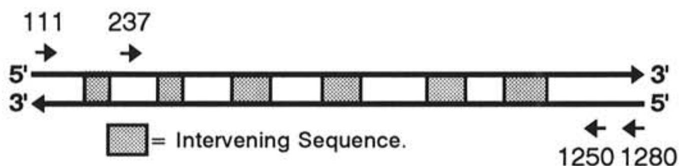


**Fig. 1.** Strategy for amplification of a Taka-amylase A gene fragment. Primers 111 and 1280 were used for initial amplifications and subsequent restriction analyses. Primers 237 and 1250, nested within the initial primer set, were used in amplifications where the products of amplifications with primers 111 and 1280 served as template. Amplifications with primers 237 and 1250 confirmed the identity of the initial amplifications according to sequence data of Tada et al (23).

After the final cycle (25 cycles total) was complete, a 5-min extension period at 72 C was added. Amplifications were carried out in a final reaction volume of 100 $\mu$l consisting of 200 mM PCR buffer (Perkin-Elmer Cetus; 500 mM KCl, 100 mM Tris-HCl, pH 8.3), 3 mM $MgCl_2$, 200 $\mu$M dNTP solution (Boehringer Mannheim, Indianapolis, IN), 2.5 U of *Taq* polymerase (Amplitaq; Perkin-Elmer Cetus), 10 pmoles each of the appropriate oligonucleotide primers, and 1 $\mu$g of genomic DNA template. Reamplification with primers 237 and 1250 was accomplished as above, except 1 $\mu$l of the solution resulting from the initial amplification with primers 111 and 1280 was used instead of 1 $\mu$g of genomic DNA. Ampliwax (Perkin-Elmer Cetus) was used to increase specificity and yield of reactions according to the manufacturer's specifications. Amplification products were separated on 0.75% agarose gels, stained in ethidium bromide (0.5 mg/L), and photographed with type 55 film (Polaroid, Cambridge, MA).

**Restriction endonuclease analysis.** Amplification products, approximately 1.2 kb in length, were restricted with each of the five restriction endonucleases (*Ava*II, *Hae*III, *Sau*3A, *Sau*96I, and *Taq*I) individually according to manufacturer's specifications (Boehringer Mannheim). Restriction products were separated in 3.5% NuSieve agarose (FMC, Rockland, ME) at approximately 5 V/cm. Products from *Taq*I restrictions tended to be smaller and were separated on acrylamide 4–20% gels (Novex, San Diego, CA) at 15 V/cm. Each restriction was repeated at least once, and all restriction fragment patterns observed for each enzyme were compared at least once on the same gel. This was done by first examining restriction products from each genomic DNA on initial gels and then comparing representatives of each pattern.

**Sclerotia.** Sclerotia were observed after 30 days of growth (unilluminated, 32 C) on Czapek-Dox broth (Difco) supplemented with a 2× concentration of the vitamins of Nitsch and Nitsch (Sigma, St. Louis, MO) and solidified with 2% agar. Sclerotia were washed with 95% ethanol to remove conidia (5). The presence and shape of sclerotia were noted after examination with a dissecting microscope at 15× magnification. On the basis of sclerotial morphology, isolates of *A. flavus* were classified according to previously described procedures (5) as S strain isolates if numerous small (average diameter <300 $\mu$m) sclerotia were produced and as L strain if fewer larger sclerotia (average diameter >300 $\mu$m) were produced. Sclerotia of isolates not classified as *A. flavus* were similarly classified as either L- or S-type sclerotia. L-type sclerotia were further classified as spherical or ovoid. An ovoid designation was given if 25% or more of the sclerotia produced had a length-width ratio greater than 2.

**Aflatoxin production.** Aflatoxin production in the liquid medium of Adye and Matales was determined as previously described (5). Erlenmeyer flasks (250 ml) containing 70 ml of medium were inoculated with 100 $\mu$l of a conidial suspension containing 5,000–7,000 spores. Flasks were incubated in the dark on an orbital shaker (150 rpm) for 5 days, after which time 70 ml of acetone was added to each flask to lyse fungal cells and extract the aflatoxins from the mycelium.

Culture filtrates containing 50% acetone (v/v) were filtered through Whatman no. 4 filter paper. One hundred milliliters of filtrate was added with an equal volume of water to a 250-ml separatory funnel, and the solution was extracted twice with 25 ml of methylene chloride. The methylene chloride extracts were filtered through 50 g of anhydrous sodium sulfate to remove residual water, and the sodium sulfate was rinsed with an additional 25 ml of methylene chloride after filtration. The rinse and extracts were combined and evaporated at room temperature. Extracts and aflatoxin standards were separated on thin-layer chromatography plates (silica gel 60, 250 mm) by development with diethyl ether-methanol-water (96:3:1) (24). Aflatoxins were not quantified, but limits of detection for aflatoxins $B_1$ and $G_1$ were 300 ng/70 ml fermentation and 450 ng/70 ml fermentation, respectively.

**Data analysis.** Similarity among isolates was estimated with the SIMQUAL (simple matching coefficient) and SAHN (UPGMA [unweighted paired group method with arithmetic

averaging] cluster analysis) programs on NTSYS-pc 1.7 (Exeter Software, Setauket, NY). Restriction sites within the amplified gene segment were identified in the *A. oryzae* sequence of Tada et al (23) with the program Oligo (National Biosciences, Plymouth, MN). Sizes of restriction fragments of the *A. oryzae* amplification product were consistent in all cases with the identified sites. For other taxa, variability in DNA fragment patterns was used to infer loss or gain of restriction sites. Restriction sites were treated as unordered characters with two states, 1 or 0. To further explore possible phylogenetic relationships, cladistic analysis and bootstrapping were performed with the PAUP (phylogenetic analysis using parsimony) program, version 3.1.1 (Center for Biodiversity, Illinois Natural History Survey, Champaign).

## RESULTS

**Amplifications.** The products of amplifications with primers 111 and 1280 were of a size consistent with that predicted from the sequence data (23) (Fig. 2). A single amplification product with no size polymorphisms was observed. Genomic DNA of *A. nidulans* did not amplify with the same primers and under the same conditions. When products of amplifications with primers 111 and 1280 were used as templates for amplifications employing primers 237 and 1250, again a single amplification product was observed on agarose gels. The size of this second amplification product was consistent with both the nested position of primers 237 and 1250 within primers 111 and 1280 and the Taka-amylase sequence data (23) (Fig. 2).

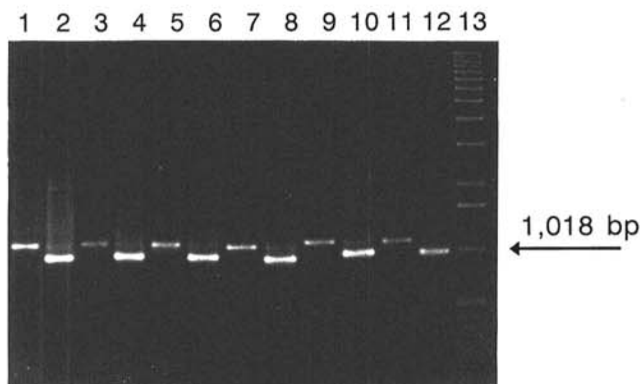


**Fig. 2.** Amplification products of Taka-amylase A gene fragments from several *Aspergillus* sect. *flavi* species. Lanes 1, 3, 5, 7, 9, and 11 contain products of approximately 1,168 bp, which resulted from polymerase chain reaction (PCR) analyses with genomic DNA and primers 111 and 1280. Lanes 2, 4, 6, 8, 10, and 12 contain products of approximately 1,013 bp, which resulted from PCR analyses with the 1,160-bp products and primers 237 and 1250. Lanes 1 and 2, NRRL 447 *A. oryzae;* lanes 3 and 4, NRRL 5594 *A. sojae;* lanes 5 and 6, isolate 13 *A. flavus;* lanes 7 and 8, NRRL 13137 *A. nomius;* lanes 9 and 10, NRRL 2999 *A. parasiticus;* lanes 11 and 12, A-28672 *A. tamarii;* and lane 13, 1-kb DNA ladder.

**Restriction analysis.** DNA fragments of various sizes were resolved on either agarose or polyacrylamide gels after enzymatic digestion of products amplified with primers 111 and 1280. Fragment number and size were dependent on both enzyme and isolate. One to six bands were resolved from *Ava*II and *Sau*96I digestions; five to seven bands resulted from *Sau*3A and *Hae*III digestions (Fig. 3); and seven to nine bands were identified in *Taq*I digestions (Fig. 4). No polymorphisms were observed among isolates of *A. flavus* and *A. oryzae*, except with *Taq*I (Fig. 4), and even with *Taq*I, isolates of *A. oryzae* could not be distinguished from four isolates of *A. flavus*. Isolates of *A. parasiticus* and *A. sojae* could not be differentiated with any of the enzymes used. Considerable variation was detected among isolates of *A. nomius*; only four of the eight isolates examined were identical at all restriction sites tested. Although all isolates of *A. tamarii* were readily differentiated from other *A. flavus* group isolates, *A. tamarii* isolates AL1-12, A-28668, and NRRL 20818 were indistinguishable; however, these isolates differed from *A. tamarii* isolate A-28672 at several restriction sites. Locations and frequencies of examined restriction sites are indicated in Table 2.

**Phenetic analysis.** A phenogram was constructed from the Taka-amylase A gene restriction site data by using the simple matching coefficient in the SAHN subprogram of NTSYS-pc (Fig. 5) (20). Sixteen *A. flavus* isolates, which produced either B aflatoxins only or no aflatoxins, and four *A. oryzae* isolates formed a cluster of isolates at least 98% similar. This cluster included both S and L strain isolates of *A. flavus*, as determined by sclerotial morphology. Isolates that produced both S strain sclerotia and B and G aflatoxins (reported by Hesseltine as an unnamed new taxon [12]) formed a separate group intermediate between the *A. flavus*-*A. oryzae* cluster and the *A. parasiticus*-*A. sojae* cluster. The two isolates in Hesseltine's unnamed taxon were only 82% similar to each other. *A. parasiticus*-*A. sojae* isolates (13 total), which produced both B and G aflatoxins or no aflatoxins and had either

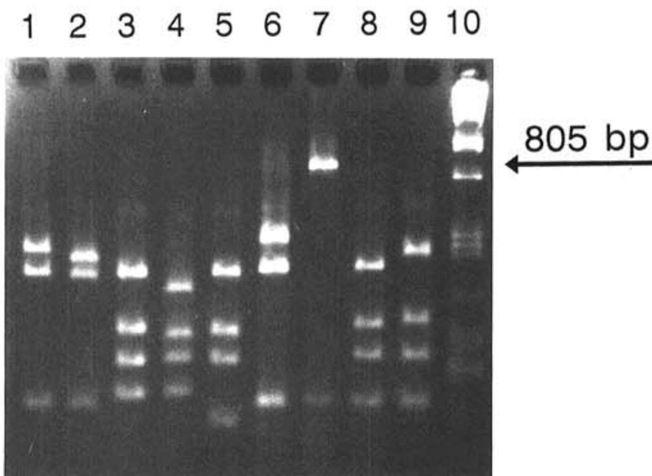


**Fig. 3.** Restriction with *Hae*III of a Taka-amylase A gene fragment that had been amplified from several *Aspergillus* sect. *flavi* species. Lane 1, NRRL 1957 *A. flavus;* lane 2, SRRC 75 *A. parasiticus;* lane 3, IMI 358751 *A. nomius;* lane 4, IMI 358749 *A. nomius;* lane 5, NRRL 13137 *A. nomius;* lane 6, A-11611 unnamed; lane 7, A-11612 unnamed; lane 8, A-28668 *A. tamarii;* lane 9, A-28672 *A. tamarii;* and lane 10, phage λ restricted with *Pst*I.

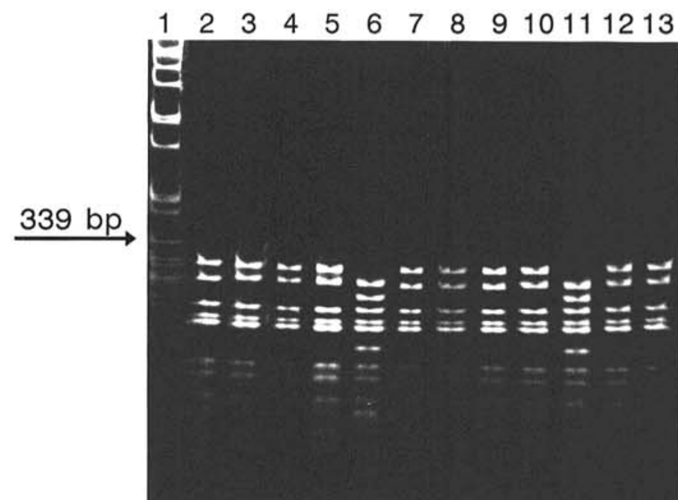


**Fig. 4.** Restriction with *Taq*I of a Taka-amylase A gene fragment amplified from strains of *Aspergillus flavus* representing both toxigenic and atoxigenic strains and two sclerotial morphs. Lane 1, Phage λ restricted with *Pst*I; lane 2, LA 2-5; lane 3, 42; lane 4, MR 3-15; lane 5, YV 5-12; lane 6, AL 1-20; lane 7, MR 5-11; lane 8, LA 2-9; lane 9, isolate 13; lane 10, MR 5-35; lane 11, YV 4-9; lane 12, YV 2-29; and lane 13, isolate 36.

sclerotia similar to the L strain of *A. flavus* or no sclerotia, were homologous at all restriction sites tested and 80% similar to *A. flavus*. *A. nomius* isolates (eight total) formed a complex cluster 60% similar to *A. flavus*. The four *A. tamarii* isolates were equally similar. One *A. tamarii* isolate (isolate A-28672 from soil collected in Mississippi) differed as much from the three homologous *A. tamarii* isolates (which included the ex type) as it did from the eight *A. nomius* isolates examined (65% similar). All *A. tamarii* isolates failed to produce both aflatoxins and sclerotia. *A. nomius* formed the most complex cluster, with only two of the eight isolates 100% similar. One *A. nomius* isolate, IMI 358749, from a Brazil nut gathered in South America differed from the other *A. nomius* isolates by more than *A. parasiticus* did from *A. flavus*. All *A. nomius* isolates produced both B and G aflatoxins and ovoid sclerotia.

**Parsimony analysis.** PAUP, version 3.1.1, was used to generate the most parsimonious trees. Both the branch and bound search and heuristic search methods yielded the same five most parsimonious trees. Four of these trees are illustrated in Figure 6.

TABLE 2. Locations and frequencies of restriction sites used for phenetic and cladistic analyses of examined *Aspergillus* sect. *flavi* fungi

| Enzyme[a] | Restriction site | Location[b] | Frequency (%) |
|---|---|---|---|
| *Ava*II | 1 | 649 | 91.5 |
| | 2 | ~290 | 2.1 |
| | 3 | ~410 | 29.8 |
| | 4 | ~780 | 2.1 |
| *Hae*III | 5 | 588 | 97.9 |
| | 6 | 1,003 | 100.0 |
| | 7 | 1,046 | 100.0 |
| | 8 | 1,107 | 97.9 |
| | 9 | ~160 | 29.8 |
| | 10 | ~320 | 25.5 |
| | 11 | ~1,150 | 4.3 |
| | 12 | ~630 | 2.1 |
| *Sau*3A | 13 | 164 | 74.5 |
| | 14 | 387 | 100.0 |
| | 15 | 810 | 89.4 |
| | 16 | 864 | 93.6 |
| | 17 | 916 | 91.5 |
| | 18 | 974 | 76.6 |
| | 19 | 1,102 | 83.0 |
| | 20 | ~1,010 | 27.7 |
| | 21 | ~435 | 14.9 |
| *Sau*96I | 22 | 588 | 91.5 |
| | 23 | 1,046 | 44.7 |
| | 24 | ~330 | 29.8 |
| | 25 | ~470 | 2.1 |
| | 26 | ~220 | 2.1 |
| | 27 | ~550 | 8.5 |
| *Taq*I | 28 | 115 | 91.5 |
| | 29 | 137 | 91.5 |
| | 30 | 470 | 97.9 |
| | 31 | 620 | 70.2 |
| | 32 | 736 | 100.0 |
| | 33 | 868 | 46.8 |
| | 34 | 927 | 74.5 |
| | 35 | 976 | 74.5 |
| | 36 | 1,013 | 72.3 |
| | 37 | 1,094 | 51.1 |
| | 38 | ~325 | 27.7 |
| | 39 | ~210 | 14.9 |
| | 40 | ~1,050 | 2.1 |
| | 41 | ~395 | 4.3 |
| | 42 | ~195 | 8.5 |

[a]Enzyme used to restrict the Taka-amylase A gene segment amplified by polymerase chain reaction.

[b]Locations were extrapolated from band patterns observed in electrophoresis gels and the sequence of *A. oryzae* reported by Tada et al (23). Unqualified numbers indicate the first base of the restriction site, and ~ indicates approximate locations of restriction sites absent in *A. oryzae* but present in some of the examined taxa.

In the fifth tree, the portion of the tree in inset A was rearranged so that a common ancestor of the *A. flavus-A. oryzae* clade and the clade containing *A. parasiticus, A. sojae*, and the unnamed taxon was derived from an ancestor shared with the other *A. flavus* clade. In all the most parsimonious trees, a common ancestor of one isolate assigned to the unnamed taxon (12) and the *A. parasiticus-A. sojae* clade shared a common ancestor with the second isolate of the unnamed taxon. The strict consensus tree contained a clade with all *A. oryzae, A. sojae, A. flavus*, and *A. parasiticus* isolates and the two isolates in the unnamed taxon. All the *A. nomius* isolates, except IMI 358749, belonged to a second clade. The three *A. tamarii* isolates that were identical at all tested restriction sites, *A. nomius* IMI358749, and *A. tamarii* each occupied separate, single-member clades. A bootstrap majority rule consensus tree was generated to estimate probabilities of the most frequent tree branches. Branch probabilities were labeled on the most parsimonious tree when these values exceeded 50% (Fig. 6).

## DISCUSSION

Populations of *A. flavus* group fungi are highly diverse. This diversity is reflected in aflatoxin-producing ability, vegetative compatibility groups, and morphology (2,12,19). However, relationships among the various *A. flavus* group members are not clear. Several molecular techniques have been applied to study these relationships. On the basis of DNA complementarity, Kurtzman et al (15) collapsed *A. oryzae, A. sojae*, and *A. parasiticus* into varieties of *A. flavus*. Later, also on the basis of DNA complementarity, Kurtzman and his colleagues designated *A. nomius* a separate species (14). Removal of *A. parasiticus* and creation of *A. nomius* has led to some controversy and confusion. This stems partially from production of B and G aflatoxins by the ex type culture of *A. nomius*, a character traditionally associated with *A. parasiticus* (9,14). The isozyme studies of Yamatoya et al support delineation of *A. nomius* but not reduction of *A. parasiticus* into a variety of *A. flavus* (27). Similarly, Moody and Tyler found that restriction analysis of both mitochondrial (17) and genomic (18) DNA supported delineation of *A. parasiticus, A. flavus*, and *A. nomius*. The mitochondrial variability Moody and Tyler observed (17) supported Kurtzman's observations (14) that *A. nomius* was more distant from *A. flavus* and *A. parasiticus* than the latter two species were from each other. Genomic variability (with heterologous

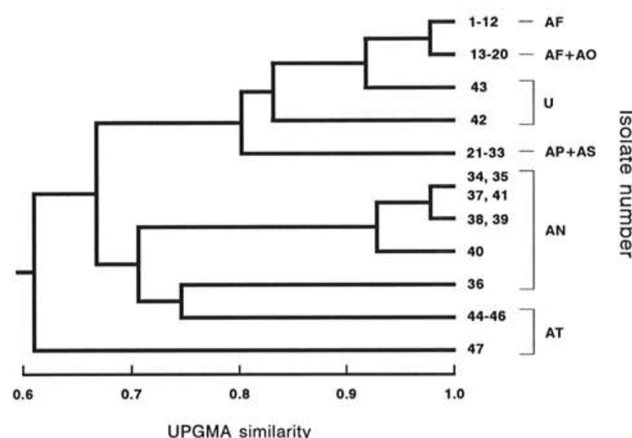


**Fig. 5.** Phenogram based on cluster analysis of pairwise similarity coefficients between various *Aspergillus* sect. *flavi* isolates using the unweighted paired group method with arithmetic averaging (UPGMA) of Taka-amylase A restriction site data. Similarities among the various isolates studied is indicated by distance along the x-axis. Distances along the y-axis are arbitrary. Numbers on the y-axis identify isolates according to Table 1. Species is indicated for each monophyletic group: AF = *A. flavus;* AO = *A. oryzae;* AP = *A. parasiticus;* AS = *A. sojae;* AN = *A. nomius;* AT = *A. tamarii;* and U = isolates assigned to an unnamed taxon by Hesseltine et al (12).

probes) suggested that *A. nomius* was much more closely related to *A. flavus* than to *A. parasiticus* (18), contrary to the observations of Kurtzman et al (14). Using RAPDs, Bayman and Cotty (2) found the ex type of *A. nomius* clustered with certain *A. flavus* isolates when analyzed cladistically. All these studies used very few (one or two) *A. nomius* isolates for interspecific comparisons.

In the current study, variability in the Taka-amylase A gene clearly distinguished several species within the *A. flavus* group. This study included eight *A. nomius* isolates, and these clustered in a single clade in both phenetic and phylogenetic analyses. *A. nomius* isolates were equally different from *A. flavus* and *A. parasiticus*. These observations are consistent with the observations of Kurtzman et al (14) and with their delimitation of the species. Taka-amylase A variability did not distinguish either *A. flavus* from *A. oryzae* or *A. parasiticus* from *A. sojae*, and thus this study supports the hypothesis (15,25) that *A. oryzae* and *A. sojae* are domesticants of *A. flavus* and *A. parasiticus*, respectively. The Taka-amylase A technique did differentiate two isolates intermediate between *A. flavus* and *A. parasiticus* by phenetic analysis and ancestral to *A. flavus* and *A. parasiticus* by cladistic analysis. These intermediate isolates were placed by Hesseltine et al (12) in an unnamed new taxon. They are further distinguished from other isolates examined in the current study by production of S strain sclerotia and both B and G aflatoxins. From four of five most parsimonious trees (Fig. 6), it may be inferred that isolates similar to these intermediate isolates were ancestral to both *A. parasiticus* and *A. flavus*. In the fifth tree, the inference applies only to *A. parasiticus*.

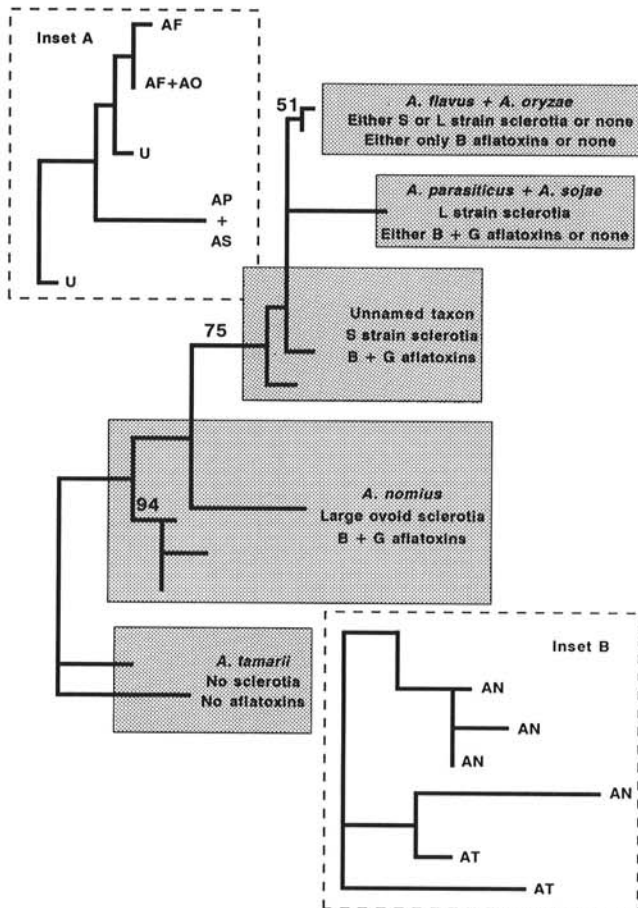


**Fig. 6.** One of five most parsimonious trees. Insets A and B illustrate rearrangements of the taxa present in other most parsimonious trees. *Aspergillus* species are indicated in the insets: AF = *A. flavus;* AO = *A. oryzae;* AP = *A. parasiticus;* AS = *A. sojae;* AN = *A. nomius;* AT = *A. tamarii;* and U = isolates assigned to an unnamed taxon by Hesseltine et al (12). Numbers on branches indicate the percentage of bootstrap trees (500 replicates) in which the monophyletic group to the right of that branch occurred. Distances along the x-axis are relative to distances inferred from the Taka-amylase A technique. Distances along the y-axis are arbitrary.

*A. flavus* clearly has considerable morphological variability, and sclerotial type can be used to separate the L and S strains of *A. flavus* (5). However, although polymorphisms in the RAPD profile supported this division (2), variation in the Taka-amylase A gene was not sufficient to separate the two strains. As previously mentioned, Hesseltine et al suggested that S strain isolates of *A. flavus* that produce only B aflatoxins or B and G aflatoxins might be combined and named a new taxon (12). However, Saito and coworkers (21) considered both S strain types to be variants of *A. flavus*. In the current study, the Taka-amylase A technique did distinguish S strain isolates that produce only B aflatoxins from those that produce both B and G aflatoxins. Cladistic analysis suggested that S strain isolates that produce both B and G aflatoxins are ancestral to both *A. parasiticus* and *A. flavus*. Thus, if all S strain isolates are morphologically assignable to the species *A. flavus*, as suggested by Saito et al (21) and others, the current study supports reduction of *A. parasiticus* to variety status, as suggested by Kurtzman et al (15). This does not alter the fact that isolates currently classified as *A. parasiticus* are readily distinguishable by clear morphological, physiological, and, as demonstrated by the current study, genetic criteria (2,9,12,13).

The cladistic analysis illustrated in Figure 6 suggests a sequence through which *A. flavus* group species and phenotypes may have evolved. In this analysis, *A. tamarii* was designated the outgroup. Outgroup status is supported by the phenetic analysis (Fig. 5), differences in aflatoxin-producing abilities among the groups (Table 1; 9,14), our experience with polymorphisms in the mitochondrial rDNA, and morphological criteria (13). Derivation of *A. flavus* and *A. parasiticus* from isolates that produce both sclerotia typical of the S strain of *A. flavus* and G aflatoxins is suggested by cladistic analysis, regardless of whether *A. nomius* or *A. tamarii* is assigned outgroup status. The ancestral type leading to both S strain sclerotia and the large ovoid sclerotia of *A. nomius* is not clear. However, the cladistic analysis does suggest that certain isolates that produce relatively large sclerotia (>300 $\mu$m average diameter; i.e., certain *A. parasiticus* isolates and isolates belonging to the L strain of *A. flavus*) may be derived from isolates that produce small S strain sclerotia. Similarly, certain isolates producing echinulate spores (i.e., *A. parasiticus* isolates) may be derived from isolates that produce smooth-walled conidia (S strain isolates [21]) and not directly from *A. tamarii* isolates that produce conidia with very rough, thickened walls (13). Also, strains that produce only B aflatoxins are apparently derived from strains that produce both B and G aflatoxins. This course of speciation suggests a series of adaptations that may explain the ability of certain *Aspergillus* sect. *flavi* fungi to parasitize distantly related animal and plant hosts and utilize diverse substrates. Identifying the path of adaptation and the role adaptations play in niche occupation may eventually permit development of techniques to avoid problems caused by these fungi. Such information would undoubtedly support efforts to develop techniques to prevent aflatoxin contamination by altering *Aspergillus* sect. *flavi* populations to be less toxigenic (6).

Taka-amylase may have differential importance to niche occupation by the various members of *Aspergillus* sect. *flavi*, and this may result in less pressure to conserve gene structure within certain taxa. Thus, if Taka-amylase is not important to the survival of *A. nomius* but is important to the other taxa within *Aspergillus* sect. *flavi*, the greater variability observed in the *A. nomius* Taka-amylase A gene might be expected. However, the DNA reassociation data of Kurtzman et al (14) also showed a relatively large amount of variation among isolates of *A. nomius* compared with variation observed among isolates of either *A. flavus, A. parasiticus*, or *A. tamarii*. The variability detected within *A. nomius* may thus reflect a relatively early divergence from the other aflatoxin-producing members of the *A. flavus* sect. *flavi*. *A. nomius* is relatively infrequent in nature, but this asexual species may be composed of several clones that diverged relatively early and that have similar fitness and thus occur at similar frequencies.

Since divergence, *A. tamarii* and *A. flavus* have similarly developed variability, as exemplified by *A. tamarii* isolate A-28672 (isolate 47) and the isolates that produce both S strain sclerotia and G aflatoxins (isolates 42 and 43). However, these clones are relatively rare and may be less frequently detected in niches related to agriculture to which the more frequently detected clones (which include the ex type of each species) may be better adapted. Isolates that produce both B and G aflatoxins and echinulate spores (i.e., *A. parasiticus*) may have diverged more recently, and thus little variability is detected among isolates of *A. parasiticus* and its probable domesticant, *A. sojae* (15,25). *A. tamarii* A-28672 may represent a distinct division in the species. Examination of more representatives of *A. tamarii* is needed to better describe the variability within this taxon and the possible importance of this division.

The relative genetic similarities presented here may be biased because the data are based on a single, very small DNA fragment. Other regions of the genome may be more or less conserved and therefore show more or less variation. However, because asexuality apparently arose prior to divergence of the taxa involved, it seems unlikely that analysis of another region of the genome would produce an entirely different cladogram. The cladogram (Fig. 6) presented here is in agreement with the DNA complementarity studies of Kurtzman et al (14,15), the RAPD study of Bayman and Cotty (2), and the mitochondrial DNA study of Moody and Tyler (17). A cladogram was not generated for the chromosomal RFLP study of Moody and Tyler, which used heterologous probes (18). However, phenetic analysis in that study suggested *A. nomius* was intermediate between *A. flavus* and *A. parasiticus*. That result is different from the results of all the other studies to date. We have no explanation for lack of agreement between the current work and the RFLP chromosomal study.

The use of PCR to characterize and determine relationships among fungal strains is a relatively recent development (24). By altering the target of amplification, the ability of PCR techniques to resolve different fungal taxa can be altered. Nondirected techniques that use small primers (less than 20 bp; i.e., RAPD techniques) can often resolve very closely related strains, and thus RAPDs can differentiate vegetative compatibility groups and the S and L strains of *A. flavus* (2). However, such techniques may be inappropriate when more distantly related taxa are compared. Indeed, through Southern hybridizations with RAPD products, Bayman and Cotty (2) found that certain RAPD products with identical electrophoretic mobilities were not homologous among species. Most molecular fungal characterizations have used rDNA genes (4,24). However, these genes have too little variability to differentiate certain taxa by restriction analysis or even sequencing (4). As mentioned above, our results indicate that this is the case for most comparisons within the *Aspergillus* sect. *flavi*. Restriction analysis of the Taka-amylase gene provides an intermediate level of resolution that, although not useful for differentiating individual strains, does permit clear differentiation of major subgroups within the *Aspergillus* sect. *flavi*. The ability of the Taka-amylase gene technique described here to distinguish subgroups within the *Aspergillus* sect. *flavi* demonstrates the potential benefit of investigating variability within nontraditional amplification targets.

## LITERATURE CITED


1. Bayman, P., and Cotty, P. J. 1991. Vegetative compatibility and genetic diversity in the *Aspergillus flavus* population of a single field. Can. J. Bot. 69:1707-1711.
2. Bayman, P., and Cotty, P. J. 1993. Genetic diversity in *Aspergillus flavus*: Association with aflatoxin production and morphology. Can. J. Bot. 71:23-31.
3. Beuchat, L. R. 1978. Traditional fermented food products. Pages 224-253 in: Food and Beverage Mycology. L. R. Beuchat, ed. AVI, Westport, CT.
4. Carbone, I., and Kohne, L. M. 1993. Ribosomal DNA sequence divergence within internal transcribed spacer 1 of the Sclerotinaceae. Mycologia 85:415-427.
5. Cotty, P. J. 1989. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology 79:808-814.
6. Cotty, P. J. 1990. Effect of atoxigenic strains of *Aspergillus flavus* on aflatoxin contamination of developing cottonseed. Plant Dis. 74:233-235.
7. Cotty, P. J. 1991. Effect of harvest date on aflatoxin contamination of cottonseed. Plant Dis. 75:312-314.
8. Cotty, P. J., Cleveland, T. E., Brown, R. L., and Mellon, J. E. 1990. Variation in polygalacturonase production among *Aspergillus flavus* isolates. Appl. Environ. Microbiol. 56:3885-3887.
9. Dorner, J. W., Cole, R. J., and Diener, U. L. 1984. The relationship of *Aspergillus flavus* and *Aspergillus parasiticus* with reference to production of aflatoxins and cyclopiazonic acid. Mycopathologia 87:13-15.
10. Egel, D. S., and Cotty, P. J. 1992. Restriction of DNA amplified from aflatoxin producing and nonproducing strains of *Aspergillus*. (Abstr.) Phytopathology 82:1080.
11. Egel, D. S., and Cotty, P. J. 1992. Relationships among strains in the *Aspergillus flavus* group which differ in toxin production, morphology, and vegetative compatibility group. Page 27 in: Proc. Aflatoxin Elimination Workshop. J. F. Robens, ed. U.S. Department of Agriculture, Agricultural Research Service, Beltsville, MD.
12. Hesseltine, C. W., Shotwell, O. L., Smith, M., Ellis, J. J., Vandegraft, E., and Shannon, G. 1970. Production of various aflatoxins by strains of the *Aspergillus flavus* series. Pages 202-210 in: Proc. U.S.-Jpn. Conf. Toxic Micro-Org., 1st.
13. Klich, M. A., and Pitt, J. I. 1988. Differentiation of *Aspergillus flavus* from *A. parasiticus* and other closely related species. Trans. Br. Mycol. Soc. 91:99-108.
14. Kurtzman, C. P., Horn, B. W., and Hesseltine, C. W. 1987. *Aspergillus nomius*, a new aflatoxin-producing species related to *Aspergillus favus* and *Aspergillus tamarii*. Antonie van Leeuwenhoek 53:148-158.
15. Kurtzman, C. P., Smiley, M. J., Robnett, C. J., and Wicklow, D. T. 1986. DNA relatedness among wild and domesticated species in the *Aspergillus flavus* group. Mycologia 78:955-959.
16. Leplotois, P., Le Douarin, B., and Loison, G. 1992. High-level production of a peroxisomal enzyme: *Aspergillus flavus* uricase accumulates intracellularly and is active in *Saccharomyces cerevisiae*. Gene 122:139-145.
17. Moody, S. F., and Tyler, B. M. 1990. Restriction enzyme analysis of mitochondrial DNA of the *Aspergillus flavus* group: *A. flavus*, *A. parasiticus*, and *A. nomius*. Appl. Environ. Microbiol. 56:2441-2452.
18. Moody, S. F., and Tyler, B. M. 1990. Use of nuclear DNA restriction fragment length polymorphisms to analyze the diversity of the *Aspergillus flavus* group: *A. flavus*, *A. parasiticus*, and *A. nomius*. Appl. Environ. Microbiol. 56:2453-2461.
19. Raper, K. B., and Fennell, D. I. 1965. The Genus *Aspergillus*. Williams & Wilkins, Baltimore.
20. Rolf, F. J. 1992. NTSYS-pc. Numerical Taxonomy and Multivariate Analysis System, Version 1.70. Exeter Publishing, Setauket, NY.
21. Saito, M., Tsuruta, O., Siriacha, P., Kawasugi, S., Manabe, M., and Buangsuwon, D. 1986. Distribution and aflatoxin productivity of the atypical strains of *Aspergillus flavus* isolated from soils in Thailand. Proc. Jpn. Assoc. Mycotoxicol. 24:41-46.
22. Stoloff, L., van Egmond, H. P., and Park, D. L. 1991. Rationales for the establishment of limits and regulations for mycotoxins. Food Addit. Contam. 8:213-222.
23. Tada, S., Iimura, Y., Gomi, K., Takahashi, K., Hara, S., and Yoshizawa, K. 1989. Cloning and nucleotide sequence of the genomic Taka-amylase A gene of *Aspergillus oryzae*. Agric. Biol. Chem. 53:593-599.
24. White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols: A Guide to Methods and Applications. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, New York.
25. Wicklow, D. T. 1984. Conidium germination rate in wild and domesticated yellow-green aspergilli. Appl. Environ. Microbiol. 47:299-300.
26. Wirsel, S., Lachmund, A., Wildhardt, G., and Ruttkowski, E. 1989. Three α-amylase genes of *Aspergillus oryzae* exhibit identical intron-exon organization. Mol. Microbiol. 3:3-14.
27. Yamatoya, K., Sugiyama, J., and Kuriashi, H. 1990. Electrophoretic comparison of enzymes as a chemotaxonomic aid among *Aspergillus* taxa: (2) *Aspergillus* section *Flavi*. Pages 395-405 in: Modern Concepts in *Penicillium* and *Aspergillus* Classification. R. A. Samson and J. I. Pitt, eds. Plenum Press, New York.