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DNA RELATEDNESS AMONG WILD AND DOMESTICATED SPECIES IN THE *ASPERGILLUS FLAVUS* GROUP

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ABSTRACT

The aflatoxin-producing fungi *Aspergillus flavus* and *A. parasiticus* show many phenotypic similarities with the nonaflatoxigenic species *A. oryzae* and *A. sojae* which are widely used in food fermentations. Absence of teleomorphic states has prevented clarification of relationships through conventional genetic means and molecular comparisons have not been previously reported. Our data show that all four species have high (69–100%) nuclear DNA complementarity and similar genome size. These findings indicate that the four taxa represent a single species. The data support the concept that *A. oryzae* and *A. sojae* were derived (domesticated) from the naturally occurring *A. flavus* and *A. parasiticus* through their adaptation to a koji environment.

Key Words: *Aspergillus*, aflatoxin, DNA relatedness, fermented foods.

The molds *Aspergillus flavus* Link : Fr. and *A. parasiticus* Speare commonly infest cereal grains and peanuts (*Arachis hypogaea* L.) where they may produce the potent carcinogen aflatoxin. Because of this, these fungi have had a significant worldwide impact on agriculture, requiring governments to monitor toxin levels and to alter agronomic practices to limit toxin formation. *Aspergillus oryzae* (Ahlb.) Cohn and *A. sojae* Sakaguchi et Yamada : Murakami, also members of the *A. flavus* group, are widely used throughout the Orient as koji molds for fermentation of sake, miso, and soy sauce, and *A. oryzae* has been used in the West as a source of food grade amylase. However, these latter two species have never been found to produce aflatoxin (Wang and Hesseltine, **1982**). Mycologists treat the four taxa as separate species, although Thom and Raper (**1945**) observed that cultural and morphological characteristics of the four species tend to merge, and that among these taxa "any variant from the dwarf and deep green *A. parasiticus* to the longest stalked and palest greenish-yellow *A. oryzae* may be found if we look for it." Additionally, chemotaxonomic studies tend to support the supposition that all four taxa are closely related (Christensen, **1981**; Kulik and Brooks, **1970**; Murakami, **1971**; Nasuno, **1974**; Vincent and Kulik, **1970**). Blochwitz (**1929**) and Saito (**1943**) also have discussed the possibility that *A. oryzae* may have been derived as a natural variant of *A. flavus* through longterm successive cultivation on rice (*Oryza sativa* L.). Wicklow (**1984a**) proposed that koji strains differ from "wild" strains primarily because longterm domestication results in the loss of certain taxonomic characters having adaptive value in nature. We have sought to test this hypothesis by comparisons of the deoxyribonucleic acid (DNA) base sequence complementarity of these economically important species.

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

MATERIALS AND METHODS

Organisms and culture conditions.—The strains examined are listed in TABLE I 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 h) and suspended in SSE + 0.5 buffer (Timberlake, **1978**).

DNA extraction and characterization.—Mycelial suspensions were initially homogenized in a

TABLE I

INTRASPECIFIC DNA RELATEDNESS, NUCLEAR GUANINE + CYTOSINE CONTENT AND SOURCE OF THE ASPERGILLI EXAMINED

Species designation	NRRL No.	Percent intraspecific DNA relatedness[a]	Mol % G + C[b]	Source
A. flavus	1957[c]		49.8 ± 0.14	Moldy cellophane, South Pacific
	482	95	49.2 ± 0.04	Wehmer's *A. flavus* reference strain (in Raper and Fennell, **1973**)
	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
A. oryzae	447[c]		49.6 ± 0.09	Sake koji, Japan
	451	100	49.3 ± 0.05	Soy sauce koji, China
	3485	98	49.4 ± 0.22	Miso koji, Japan
	6271	100	49.3 ± 0.03	Soy sauce koji, Taiwan
A. parasiticus	502[d]		49.1 ± 0.13	Mealy bug (*Pseudococcus* sp.?), sugarcane, Hawaii
	465	100	50.0 ± 0.06	J. Takamine, substrate unknown
A. sojae	5595[c]		49.3 ± 0.05	Koji, Japan?
	5594	100	49.2 ± 0.14	Koji, Manchuria?
	5596	100	49.1 ± 0.04	Koji, Japan?
	1988	100	49.4 ± 0.13	Soy sauce koji, China
A. leporis	3216[d]		49.2 ± 0.04	Dung of jack rabbit (*Lepus townsendii* Bachman), Wyoming

[a] Standard deviation ≤ ±5% calculated from 2–4 determinations.
[b] Standard deviation calculated from 3 determinations.
[c] Representative strain.
[d] Type strain.

Waring blender for *ca.* 1 min, and the cells were then broken in a Braun homogenizer. Whole-cell DNAs were extracted as previously described (Price *et al.*, **1978**; Kurtzman *et al.*, **1980b**). Following spooling, all DNAs were further treated with ribonucleases (RNases) and then chromatographed on hydroxylapatite for additional purification (Price *et al.*, **1978**). Analytical ultracentrifuge scans ($A_{262\ nm}$) 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 and Mandel, **1971**; Seidler *et al.*, **1975**; Kurtzman *et al.*, **1980b**) in a Gilford model 250 recording spectrophotometer with a model 2527 thermoprogrammer. Reaction mixtures contained 75 μg/ml (A_{260} = 1.5) of sheared double-stranded DNA in 5 × SSC (SSC = standard saline citrate: 150 mM NaCl, 15 mM sodium citrate, pH 7.0) with 20% dimethylsulfoxide. Mixtures were heated in the spectrophotometer 10 min at 95 C to allow the DNA to melt to single strands and following rapid cooling (12 min), were allowed to reassociate at 58 C (T_m − 25 C) (Kurtzman *et al.*, **1980b**). Reassociations were allowed to proceed to $C_ot_{0.5}$ (C_ot = moles of nucleotides per liter times seconds) as determined from decreasing absorbance, and the extent of relatedness was calculated using the formula $\{1 - [\text{obs. } C_ot_{0.5}^{mix} + (C_ot_{0.5}^{100} - C_ot_{0.5}^{0})]/C_ot_{0.5}^{100}\} \times 100$ (Seidler and Mandel, **1971**). For intraspecific relatedness, pairings included the type or representative strain as reference and each of the other strains of that species (SD ≤ 5%, n = 2–4). Earlier comparisons showed that the extent of DNA relatedness determined spectrophotometrically was comparable to results from nitrocellulose filter binding (Seidler and Mandel, **1971**) and from free-solution reactions of isotope-labelled sequences partitioned by hydroxylapatite (Kurtzman *et al.*, **1980b**).

TABLE II

MATRIX OF PAIRWISE COMPARISONS SHOWING THE EXTENT OF DNA COMPLEMENTARITY BETWEEN *ASPERGILLUS FLAVUS* AND OTHER PHENOTYPICALLY SIMILAR ASPERGILLI[a]

Species designation NRRL No.	*A. flavus* 1957	*A. oryzae* 447	*A. parasiticus* 502	*A. sojae* 5595	*A. leporis* 3216
A. flavus 1957		100	70	74	0
A. oryzae 447			69	73	8
A. parasiticus 502				91	7
A. sojae 5595					0
Neurospora crassa[b] 13141	1	8	1	0	3

[a] Standard deviation ≤ ±5% calculated from 2–4 determinations.
[b] *N. crassa* was included as an unrelated control of similar genome size.

The guanine plus cytosine (G + C) content of the nuclear DNA was calculated from buoyant density ($n = 3$) 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 and Goldberg, **1977**) using the methods cited above.

RESULTS

The Aspergilli that we compared included two or more strains of *A. flavus, A. parasiticus, A. oryzae,* and *A. sojae,* as well as a representative of the phenotypically similar species *A. leporis* States & Christensen. Intraspecific DNA relatedness was 95% or greater (TABLE I). The extent of DNA complementarity between *A. flavus* and *A. oryzae* was 100% (TABLE II). Similarly, *A. parasiticus* and *A. sojae* showed 91% relatedness. Complementarity between these two groups, demonstrated in the *A. flavus–A. parasiticus* pairing, was 70%. By contrast, *A. leporis,* as well as *Neurospora crassa* Shear & Dodge which was included as a control, exhibited 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**).

In order to preclude the possibility that these species were genetically isolated from one another through major differences in genome size, we calculated the proportional genome sizes from reassociation rates to be: *A. flavus* = 1.00, *A. oryzae* = 1.00, *A. parasiticus* = 1.10, *A. sojae* = 0.97, *A. leporis* = 0.93, where $1.00 = 2.2 \times 10^{10}$ daltons (SD = 0.11, $n = 6$). These measurements show no significant difference in genome size, although the methodology cannot resolve an even difference in ploidy.

As we will discuss, we believe our data to support the concept that *A. flavus, A. oryzae, A. parasiticus,* and *A. sojae* represent morphological and physiological variants of a single species. Because the term aflatoxin was derived from the binomial *A. flavus,* the oldest valid name of this group, use of *A. flavus* as the sole descriptor to taxonomically describe nontoxic industrially imporant koji molds is undesirable. Further, the morphological differences found among *A. flavus, A. oryzae, A. parasiticus,* and *A. sojae* are generally sufficiently recognizable to allow taxonomic separation (Murakami and Suzuki, **1970**; Raper and Fennell, **1973**). In view of this, we propose that the four taxa be regarded as varieties of *A. flavus.* Owing to the lesser extent of DNA relatedness between *A. flavus* and *A. parasiticus,* we suggest that they be designated as subspecies with *A. oryzae* and *A. sojae* as varieties of their respective subspecies. This proposal is consistent with our molecular data and takes into account taxonomic rules as well as commercial and legislative practicalities.

ASPERGILLUS FLAVUS Link : Fr. [subsp. *flavus* var. *flavus*] *Mag. Ges. Naturf. Freunde,* Berlin **3**: 16. 1809. (: Fries. Syst. Mycol. **3**: 386. 1826).

SYNONYMS: Listed by Raper and Fennell (**1973**).

Aspergillus flavus subsp. **flavus** var. **oryzae** (Ahlburg) Kurtzman, Smiley, Robnett & Wicklow, *comb. nov.*

BASIONYM: *Eurotium oryzae* Ahlburg. *Dingler's Polytechnisches Jour.* **230**: 330. 1878.
SYNONYMS: Listed by Raper and Fennell (**1973**).

Aspergillus flavus subsp. **parasiticus** (Speare) Kurtzman, Smiley, Robnett & Wicklow [var. **parasiticus**] *comb. nov.*

BASIONYM: *Aspergillus parasiticus* Speare. *Hawaiian Sugar Planters' Exp. Sta., Path. & Physiol. Ser. Bull.* **12**, p. 38. 1912.
SYNONYMS: Listed by Raper and Fennell (**1973**).

Aspergillus flavus subsp. **parasiticus** var. **sojae** (Sakaguchi & Yamada : Murakami) Kurtzman, Smiley, Robnett & Wicklow, *comb. nov.*

BASIONYM: *Aspergillus sojae* Sakaguchi & Yamada : Murakami. *J. Gen. Appl. Microbiol.* **17**: 302. 1971.
SYNONYMS: Listed by Murakami (**1971**), Raper and Fennell (**1973**) and Wicklow (**1983**).

DISCUSSION

The interpretation of the data presented rests on the correlation of DNA relatedness with the definition of a fungus species. While guidelines are lacking for the filamentous fungi, this issue has been addressed among heterothallic yeasts. When comparisons were made using nuclear DNA, as in the present study, mating reactions did not totally disappear until DNA complementarity reached background levels, *i.e.*, 10–15% or less (Kurtzman *et al.*, **1980a**; Kurtzman, **1985**). One allopatric pair showing only 25% DNA relatedness produced a few fertile F_2 progeny, but this may be exceptional. Although the lower limit of DNA homology indicating species delimitation among these fungi varies somewhat, it appears to be less than 70% as previously thought (Brenner, **1973**; Martini and Phaff, **1973**; Price *et al.*, **1978**). We suggest that the findings from the yeast studies are relevant for estimates of biological relatedness among filamentous fungi. In view of this, we propose that *A. flavus, A. oryzae, A. parasiticus,* and *A. sojae* represent a single species in which divergence is beginning to occur between *A. flavus/A. oryzae* and *A. parasiticus/A. sojae*. For example, *A. flavus* has been shown to produce cyclopiazonic acid while no *A. parasiticus* strains are known to synthesize this toxic indole metabolite (Dorner *et al.*, **1984**). Whether this divergence results from separation of the toxigenic strains solely through habitat specificity (ecological adaptation) or from chromosomal changes such as translocations or inversions is unknown.

Our data provide strong support for the concept (Blochwitz, **1929**; Saito, **1943**; Wicklow, **1984a**) that *A. oryzae* and *A. sojae* are domesticated variants of the naturally occurring *A. flavus* and *A. parasiticus*. In general, the domesticated strains show reduced sporulation (*i.e.*, fewer and smaller uniseriate conidial heads), produce conidiophores and conidia that are often less conspicuously roughened and more variable in size than those of the naturally occurring isolates, and do not produce sclerotia or aflatoxin. This is in keeping with the proposed transition from survival in nature to survival in koji fermentations. For example, Wicklow (**1984a**) has argued that large conidial apparati, survival structures such as sclerotia, and mechanisms of defense against arthropod predators are of no adaptive value to the fungus in a koji environment. In contrast, improved fitness in koji would depend more on rapid conidium germination and an increased ability to efficiently hydrolyze starches and proteins, characteristics which are more prevalent in *A. oryzae* and *A. sojae* (Wicklow, **1984b**). Adaptation of naturally occurring strains to koji environments would seem to require little change. For example, many strains of *A. flavus* produce no aflatoxin and some form no sclerotia. Furthermore, prolonged laboratory cultivation of *A. flavus* strains tends to produce colonies with more aerial mycelium which are similar to *A. oryzae*.

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