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Population Biology

Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*

(population genetics/mycotoxin/biological species/microbial evolution)

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ABSTRACT ***Aspergillus flavus*, like approximately one–third of ascomycete fungi, is thought to be cosmopolitan and clonal because it has uniform asexual morphology. *A. flavus* produces aflatoxin on nuts, grains, and cotton, and assumptions about its life history are being used to develop strategies for its biological control. We tested the assumptions of clonality and conspecificity in a sample of 31 Australian isolates by assaying restriction site polymorphisms from 11 protein encoding genes and DNA sequences from five of those genes. *A. flavus* isolates fell into two reproductively isolated clades (groups I and II). The lack of concordance among gene genealogies among isolates in one of the clades (group I) was consistent with a history of recombination. Our analysis included five strains of the closely related industrial fungus *A. oryzae*, all of which proved to be clonally related to group I.**

We applied the tools of evolutionary biology to analyze three closely related fungal species of the genus *Aspergillus* that are important in food safety and production, addressing questions about reproductive mode and phylogenetic relationships that relate to biological control and regulation in the food industry. *Aspergillus flavus* and *A. parasiticus* produce aflatoxins, among the most carcinogenic compounds known (1), which are a serious problem worldwide in agricultural commodities such as maize, peanuts, tree nuts, and cotton seed. On the other hand, *A. oryzae* is a nontoxigenic species that has been used for over 4,000 years to produce Asian foods and beverages such as soy sauce and sake, as well as for the production of industrial enzymes. *A. flavus* and *A. oryzae* are distinguished only by subtle morphological differences (2) and appear nearly identical by DNA/DNA hybridization (3), but their identity as the same species or sister species is still controversial, partly because of the regulatory confusion that conspecificity would generate.

In many countries, strict controls are placed on aflatoxin levels in food and feed products, but the cost of lost crops and animals, reduced yield, and monitoring aflatoxin levels is staggering (4). In countries without strict controls, aflatoxin certainly remains a significant public health problem (5). Our ability to reduce or eliminate aflatoxin contamination via biological control methods requires an understanding of the life history of the fungus, which is currently rudimentary. In the laboratory, *A. flavus* is known to reproduce exclusively asexually, with some potential for nonsexual recombination among isolates that are highly similar (6). In nature, populations are highly polymorphic (including for the production of aflatoxin; refs. 7 and 8), with nothing known about whether the fungus is recombining or completely clonal. Biological control methods currently being tested, including seeding fields with large quantities of natural nontoxigenic strains (9), either assume that the fungus is clonal or fail to consider the effects of potential outcrossing. The introduced strains necessarily must be effective competitors with respect to the native strains. In addition, aflatoxin production is a complex secondary metabolite phenotype that involves a large number of genes. Most of the specific enzymatic activities required for aflatoxin production are encoded in a gene cluster (10), but additional unlinked loci also are required (11). Because aflatoxin is a polygenic, variable trait, outcrossing between the introduced strains and native strains could produce competitive progeny with novel aflatoxin phenotypes, which could then spread to new locations.

MATERIALS AND METHODS

Strain Isolation. Isolates were taken from agricultural soils near Kingaroy, Qld, Australia, a major peanut growing region. Fourteen fields were sampled, seven adjacent to each other ≈3 km southeast of Kingaroy (fields represented by strain prefixes 1–7) and seven randomly selected within ≈20 km to the south of Kingaroy (prefixes 11–17). Soils were dilution-plated on a medium designed to select for *A. flavus* growth and incubated at 30°C for 3–5 days. Colonies producing a characteristic orange/yellow reverse were isolated and identified by standard morphological techniques (2). *A. parasiticus* isolates CA1–05 and CA3–01 were collected from tree nut orchards near Winters, CA. *A. oryzae* isolates were provided by Steven Peterson of the United States Department of Agriculture Northern Region Research Laboratory, Peoria, IL.

Identification and Analysis of Polymorphisms. Oligonucleotide primers were designed to PCR-amplify regions of 11 different protein-encoding loci (Table 1). The primer sequences used were as follows, 5′–3′: amdS1: ccatcggtataggaactga, amdS2: agggtgccacggtatgtc; pecA1: atctcagacattttctcc, pecA2: accctcccactccttgtag; omt1: ggagtatcagaggattta, omt2: agtgctgtaatagtcaaa; niaD1: tcgtgaatggagaagtgt, niaD2: gaaattggggtgtatgag; benA5: ctcttccgtcccgacaactt, benA6: ggggttggagagcttgagg; pgkA1: cttgccatcaccgatgtcg, pgkA2: tcagccttgaccttcttgc; facB3: gaaaagatcctgtggttggc, facB4: cctgggcgatacgactttgg; glaA1: caatcttgaataatatcg, glaA2: gtccgtcgctatgcttgt; trpC1: gacgggaaataggcttcc, trpC3: cgccttggtgggatggtg; cmdA7: gccaaaatcttcatccgtag, cmdA8: atttcgttcagaatgccagg; and gsdA1: cacaatagcacgcactgagg, gsdA2: cttggggaggaacttgttgc. PCRs were performed by using 1 μl of diluted genomic DNA template in 50-μl reactions. *Taq* DNA polymerase (0.5 unit), 0.2 mM deoxynucleotide triphosphates, and 0.2 mM of each primer were used, with the following temperature profile: 2

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Abbreviation: MP, most parsimonious.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AF036768-787 (amdS12), AF036788-807 (benA56), AF036808-830 (omt12), AF036831-850 (amdS12), and AF036851-870 (trpC13)].

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Table 1. The 11 protein-encoding loci analyzed

Locus	Encoding	Noncoding DNA	Total bp/noncoding bp	Restriction enzyme
1. amdS12	Acetamidase	3 introns	550/≈153	*Hin*fI
2. pecA12	Polygalacturonase	3 introns	533/≈211	*Dde*I
3. omt12	*O*-Methyltransferase	3 introns	458/≈171	*Taq*I
4. niaD12	Nitrate reductase	1 intron	465/≈56	*Dpn*II
5. benA56	β-Tubulin	none	411/0	*Dpn*II
6. pgkA12	Phosphoglycerate kinase	1 intron	482/≈72	*Dpn*II
7. facB34	Acetate regulation	5′ NT	469/≈402	*Sca*I
8. glaA12	Glucoamylase	2 introns	499/≈92	*Dpn*II
9. trpC13	Tryptophan synthesis	5′ NT	506/≈486	*Mse*I
10. cmdA78	Calmodulin	5′ NT	468/≈308	*Xba*I
11. gsdA12	Glucose-6-phosphate dehydrogenase	3 introns	623/≈467	*Taq*I

The numbers in each locus name represent the names of the primers used. 5′ NT, noncoding DNA just 5′ to the first amino acid of the gene.

min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at the annealing temperature of 56°C [except for loci pecA12 (53°C), glaA12 (46°C), and trpC13 (60°C)], and 1 min at 72°C, followed by 5 min at 72°C. Variation within PCR products was assayed first by single-strand conformation polymorphism analysis. ^{35}S-labeled dATP was incorporated into the PCR products with an additional 12 PCR cycles, and the radiolabeled products were separated on a nondenaturing 0.5X MDE polyacrylamide gel (FMC). Representative alleles were sequenced to identify polymorphic restriction sites by using an Applied Biosystems automated sequencer, model 377, according to the manufacturer's instructions. Sequences were generated from both strands and edited and initially aligned by using the SEQUENCE NAVIGATOR (v1.0.1, Applied Biosystems) software package, and the alignments then were optimized visually. Ten microliters of PCR product was used for restriction digestion, which was performed according to the manufacturer's instructions.

Data Analysis. Phylogenetic analyses (both parsimony and distance) and the partition homogeneity test were performed by using PAUP*, a prerelease version generously provided by D. Swofford, Smithsonian Institute of Natural History. Parsimony analysis was performed by using heuristic searches, with random addition in 1,000 replicates used to find the most parsimonious (MP) trees for each gene region. Distance analysis (UPGMA; ref. 12) was performed by using PAUP* with an uncorrected "p" measure of genetic distance. Small insertion/deletion polymorphisms were encoded as absent or present (0/1).

RESULTS AND DISCUSSION

To address the question of reproductive mode in *A. flavus*, we assayed single restriction site polymorphisms in portions of 11 different protein-encoding loci (Table 1) in 31 Australian *A. flavus* isolates, producing a multilocus genotype for each isolate (Table 2). Sixteen different genotypes were identified, with no obvious geographic pattern apparent in their distribution. Parsimony analysis of the 16 different genotypes produced 72 MP trees, the strict consensus of which contained an internal branch separating four of the genotypes (A, G, H, and N) from the other 12 (Fig. 1), effectively forming two groups (groups I and II). Distance analysis of the data showed this to be the longest branch on the tree. Although there was no strong geographic or morphological basis on which to separate these two groups, this result suggested that the collection of isolates comprised two reproductively isolated groups that should be considered separately. The genotypes of five *A. oryzae* isolates with a range of morphologies also were determined. Four of them possessed genotype "C" as seen in three *A. flavus* isolates, and the fifth isolate had a unique

Table 2. List of multilocus genotypes

	Locus												Locus											
	1	2	3	4	5	6	7	8	9	10	11			1	2	3	4	5	6	7	8	9	10	11	
Strain												Genotype	Strain												Genotype
1-7	1	1	0	1	0	1	1	1	0	1	0	A	11-4	1	1	1	1	0	0	1	0	1	0	1	L
1-9	1	1	1	1	1	0	1	0	1	0	1	B	12-1	1	1	1	1	1	0	1	0	1	0	1	B
1-10	1	1	1	1	0	1	1	0	1	0	1	C	12-3	1	0	0	1	1	0	1	0	1	0	1	M
1-18	1	1	1	1	1	0	1	0	1	0	1	B	12-4	0	1	0	1	0	1	1	0	0	0	0	H
1-22	1	1	0	1	0	1	1	1	0	1	0	A	13-4	0	1	0	1	0	1	1	0	0	1	0	N
1-26	1	1	0	1	1	0	0	0	1	0	1	D	14-1	1	1	1	1	1	0	1	0	0	0	1	O
1-27	1	1	0	1	0	1	1	1	0	1	0	A	14-2	1	0	1	1	1	0	1	0	1	0	1	P
1-29	1	1	1	1	1	0	0	0	1	0	1	E	15-2	1	1	1	1	1	0	1	0	1	0	1	B
2-4	1	1	1	1	1	0	1	0	1	0	1	B	16-1	1	1	1	1	1	0	1	0	1	0	1	B
3-2	1	1	1	1	0	0	1	0	0	0	1	F	17-1	0	1	0	1	0	1	1	0	0	1	0	N
4-2	0	1	0	1	0	1	1	0	1	0	0	G	17-2	1	1	1	1	1	0	1	0	1	0	1	B
5-1	1	1	1	1	1	0	1	0	1	0	1	B	17-3	0	1	0	1	0	1	1	0	0	1	0	N
5-2	1	1	1	1	0	1	1	0	1	0	1	C	17-4	1	1	1	1	0	1	1	0	1	0	1	C
6-4	0	1	0	1	0	1	1	0	0	0	0	H													
7-2	1	0	0	0	1	0	0	0	1	0	1	I	447	1	1	1	1	0	1	1	0	1	0	1	C
7-3	0	1	1	1	0	0	1	0	0	0	1	J	448	1	1	1	1	0	1	1	0	1	0	1	C
7-4	1	1	0	1	1	0	1	0	0	0	1	K	449	1	1	1	1	0	1	1	0	1	0	1	C
11-1	1	1	1	1	0	0	1	0	1	0	1	L	469	1	1	1	1	0	1	1	0	1	0	1	C
													483	1	0	1	1	0	1	1	0	1	0	1	Q

Multilocus genotypes were defined based on the presence (allele 1) or absence (allele 0) of each site (see Table 1 for information about each locus). The first 31 strains are Australian *A. flavus* isolates (hyphenated); the last five are *A. oryzae* isolates.

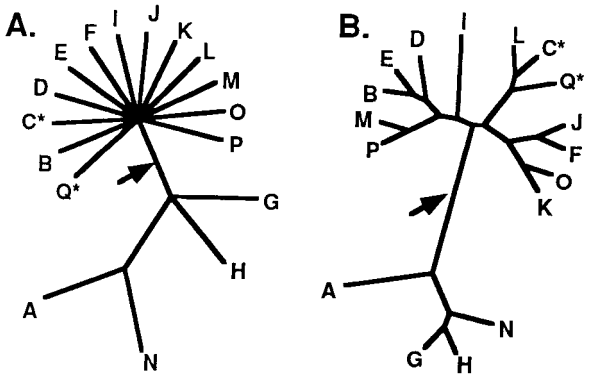

FIG. 1. (*A*) Strict consensus of the 72 MP trees inferred from the restriction site data for the 17 different multilocus genotypes (A–Q). The arrow indicates the internal branch separating groups I and II. (*B*) UPGMA (10) distance analysis of the same data. The arrow indicates the same branch highlighted in *A*, which is the longest branch on the tree (branch lengths shown are proportional to those inferred from the data). ∗, Genotype "C" represents *A. flavus* isolates 1–10, 5–2, and 17–4, as well as *A. oryzae* isolates NRRL 447, 448, 449, and 469. "Q" represents that of *A. oryzae* isolate NRRL 483.

genotype "Q," differing from genotype "C" only at the pecA12 locus.

We tested this result by sequencing five of the loci (amdS12, benA56, omt12, pecA12, and trpC13) in 17 of the *A. flavus* isolates, including one isolate from each of the 16 previously identified genotypes and a second isolate from genotype B. In addition, two strains of the related aflatoxin producer *A. parasiticus* were sequenced to be used as an outgroup, as well as one isolate of *A. oryzae*. The most variable locus, omt12, was sequenced from an additional three *A. oryzae* isolates with a range of morphologies. A total of 2,195 nucleotides was sequenced in each isolate, 144 of which were polymorphic in *A. flavus*, 47 of which were unique, and 97 of which were parsimony-informative. Each locus produced from 1 to 30 MP trees, with consistency indices ranging from ≈0.90 to 1, indicating that there was little or no homoplasy within loci and no evidence for intragenic recombination. The MP trees for each of the five loci showed a topology consistent with the existence of the two groups inferred from the restriction sites and consistent with the two groups being reproductively isolated (Fig. 2). Of the 97 polymorphic and informative sites within *A. flavus*, only two were polymorphisms shared between the two groups (Table 3). Of the remaining 95 sites, 37 were fixed in group I, 23 were fixed in group II, and 35 were fixed for differences in both groups. Furthermore, the *A. oryzae* isolate grouped consistently with isolates from group I, and all four *A. oryzae* isolates had identical sequences for the most variable locus, omt12.

Tests for reproductive mode can be confounded if individuals are sampled from different biological species, but with the knowledge that *A. flavus* isolates fell into two groups, we could limit the test to the group for which we had a larger sample, group I. To address this question, we asked whether the gene genealogies for the five different loci were significantly different from each other (13, 14). Under a model of clonality, the topologies of the five gene trees should be congruent, as should those of trees made from any combination of variable sites from the five genes, because all genes and all sites are inherited

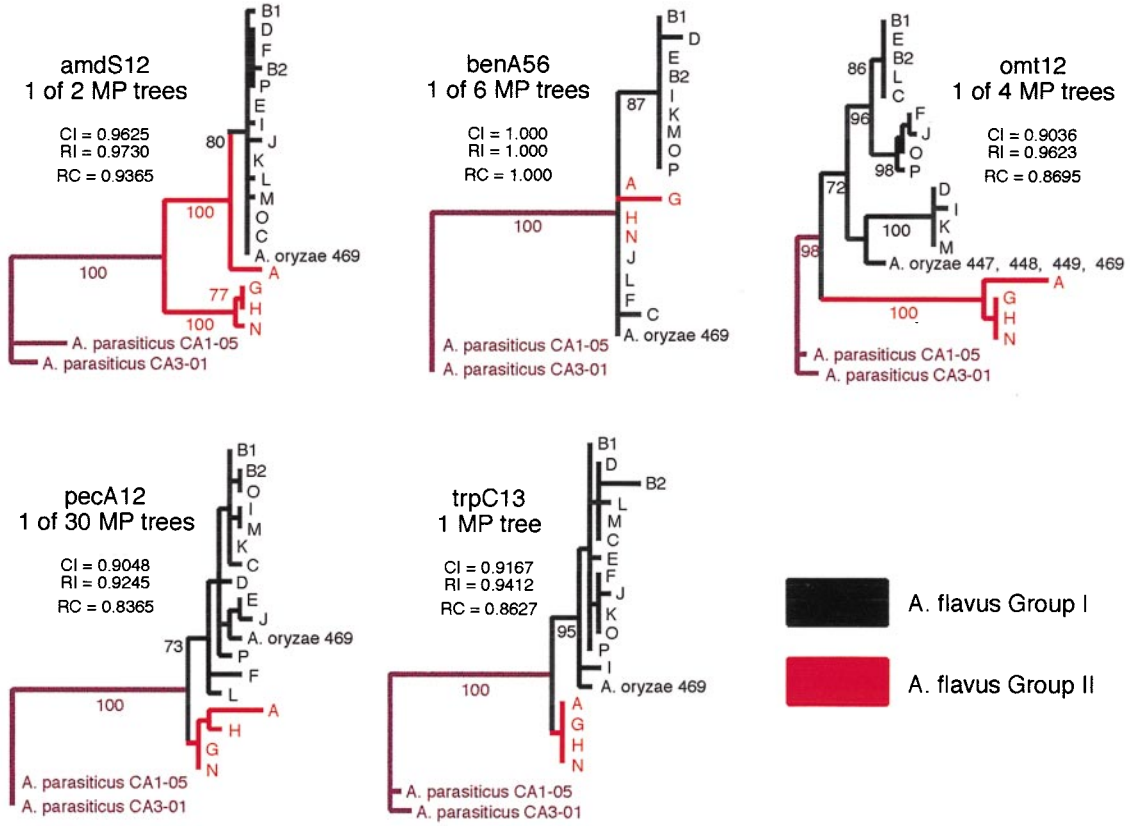

FIG. 2. One MP tree from each of the five gene regions sequenced. CI, consistency index; RI, retention index, RC, rescaled consistency index. Numbers below branches represent bootstrap values based on 500 replicates. Only values ≥70% are shown. The 16 different *A. flavus* multilocus genotypes are represented by letters. Isolates used representing each multilocus genotype: A, 1–22; B, 1–9 (B1) and 5–1 (B2); C, 17–4; D, 1–26; E, 1–29; F, 3–2; G, 4–2; H, 12–4; I, 7–2; J, 7–3; K, 7–4; L, 11–1; M, 12–3; N, 13–4; O, 14–1; and P, 14–2. GenBank accession numbers are AF036768-787 (amdS12); AF036788-807 (benA56); AF036808-830 (omt12); AF036831-850 (pecA12); and AF036851-870 (trpC13).

Table 3. Fixation of parsimony-informative sites in different genes in *A. flavus* isolates

Locus	Sites fixed in group I alone	Sites fixed in group II alone	Sites fixed in both	Shared polymorphisms	Polymorphic sites, total *n*
amdS12	1	21	4	1	27
benA56	2	0	0	0	2
omt12	26	2	24	0	52
pecA12	4	0	3	1	8
trpC13	4	0	4	0	8
All five genes	37	23	35	2	97

together. Under a model of recombination, genealogies of unlinked genes would be expected to conflict, and although sites in the same gene might all support the same genealogy, sites assembled from different genes should conflict because of recombination over larger physical distances and among different linkage groups. This conflict can be recognized by increased homoplasy and thus by increased phylogenetic tree lengths.

First, MP trees were constructed for each of the five loci, looking at group I isolates alone. Removal of group II reduced the number of informative sites from 97 to 37. The MP tree length for each locus is given in Table 4, along with the minimum number of steps possible. Two of the loci (amdS12 and benA56) produced trees of minimum length whereas omt12, pecA12, and trpC13 showed some homoplasy: they produced trees of 1, 3, and 1 excess steps, respectively, for a total of 5 extra steps. If the gene trees were perfectly concordant, the MP tree length for all five data sets combined should be the same as the minimum possible tree length: the number of informative sites (37 steps) plus the extra steps due to intralocus homoplasy (5 steps), or 42 steps. The actual MP tree length for the combined data was 57 steps, an excess of 15 steps that cannot be explained by homoplasy within loci. A phylogenetic test designed to assess congruence between gene trees, the Partition Homogeneity Test (15, 16), was used to examine the null hypothesis of recombination (14). In this test, the observed sites from all genes for each individual are pooled and resampled without replacement to give an artificial data set in which sites have been swapped randomly among loci. Many such artificial data sets are produced. MP trees are then made for each newly sampled "gene" in each artificial data set. Under clonality, the sums of the lengths of the gene trees for the observed and resampled data should be similar, but under recombination the sums of the tree lengths should be longer than that for the actual data because recombination among distant sites should introduce homoplasy into the data. In this case, significance was assigned by comparing the summed tree length from the actual data to those from 10,000 artificial data sets. The actual summed tree length of 42 steps was 2 steps shorter than that produced by any of the artificial data sets and 8 steps shorter than >95% of them ($P < 0.0001$; Fig. 3), indicating that the genes have significantly different topologies. The best explanation for these differences is recombination among loci at some time in the history of these isolates. Of the 37 informative sites used in this test, 26 came from one locus, omt12. It was possible that the dominance of omt12 in the data was somehow affecting the results, so we removed omt12 from the data set and found the MP tree for the remaining four loci combined. Again, the MP tree was 10 steps longer than the minimum (25 vs. 15 steps), demonstrating nonconcordance among these four loci alone.

Table 4. Actual and minimum MP tree lengths for each of five loci among group I isolates

Locus	MP tree length	Min. tree length	Excess steps
amdS12	1	1	0
benA56	2	2	0
omt12	27	26	1
pecA12	8	5	3
trpC13	4	3	1
All five genes	57	42	15

Tree lengths include phylogenetically informative sites only. Min., minimum.

Species concepts in fungi most frequently are based solely on morphology. Biological species concepts, which define species as groups of actually or potentially interbreeding individuals (17), have been applied in several cases in sexual fungi through mating tests. In each case, species barriers were discovered that were not first apparent based on morphology (18–20). However, if sex is lacking or covert, it is impossible to define biological species based on mating criteria. Genetic data provide a means for indirectly identifying reproductive isolation and can be applied to define biological species in organisms that are only cryptically outcrossing (13, 14) or clonal. The reproductive barriers that accompany biological speciation result in the conversion of genetic variability within a species to between-species differences (21). In this *A. flavus* population, 144 variable sites were identified, 97 of which were phylogenetically informative. Only 2/97 informative sites were polymorphic in both groups, 60 were fixed in one group and not the other, and 35 were fixed for differences in both groups. Under the most common model of gradual biological speciation, early in the speciation process a reproductive barrier arises within a species. At first, the two isolated groups share polymorphisms inherited from the ancestral species (22). In *A. flavus*, only 2/144 polymorphisms are consistent with this early degree of divergence between groups I and II, and these polymorphisms could easily have arisen by independent mutation after their isolation. Eventually, one of the two groups becomes fixed for a particular character state, and the other group remains polymorphic. Sixty of 97 phylogenetically informative polymorphisms reflect this state, many of which could have arisen after the divergence of the two groups. Then, fixation occurs in the other group, either for the same allele (making both groups identical) or for another allele (making

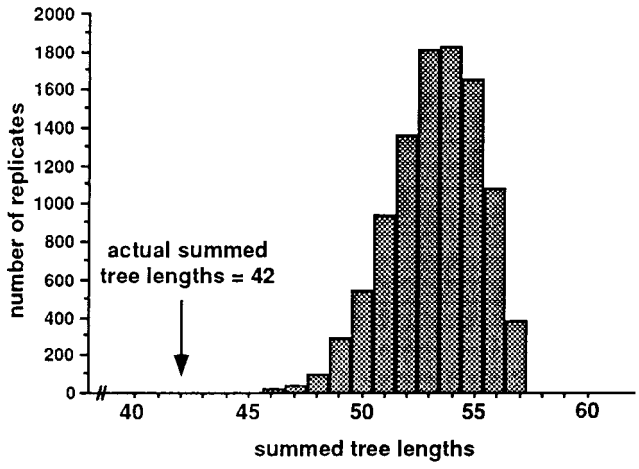

FIG. 3. Partition homogeneity test results.

fixed differences between the two groups). Thirty-five of 97 informative polymorphisms reflect this, a number that does not reflect polymorphisms that may have existed but were fixed for the same character state in both groups. As would be expected in an advanced state of biological speciation, the very low level of shared polymorphism between groups I and II is indicative of a long history of reproductive isolation.

Although there was strong evidence for divergence between groups I and II, the *A. oryzae* isolates analyzed appeared to be members of group I, and almost no variation was observed among *A. oryzae* isolates. We also added two additional sequences available in the GenBank database from two different *A. oryzae* strains, an amdS12 sequence from strain RIB40 and a pecA12 sequence from strain KBN616 (23, 24). Phylogenetic analysis showed that these sequences were very similar to those of strain 469 (one nucleotide difference for both) and that they were included in group I (data not shown). Our results are consistent with DNA/DNA hybridization studies that showed 100% complementarity between an *A. flavus* isolate and an *A. oryzae* isolate, suggesting that *A. oryzae* is a domesticated form of *A. flavus* (3). Clearly, there is little molecular genetic basis for separating the two species although there are morphological differences, and *A. oryzae* isolates do not make aflatoxin. Based on its pecA12 and omt12 sequences, the *A. flavus* isolate used most commonly in molecular genetic research (strain 70) also appears to belong in group I. This indicates that research on both organisms should be complementary because of the very high degree of genetic similarity between *A. oryzae* and *A. flavus* group I.

CONCLUSIONS

No fungus, including those with no known means of sexual reproduction, has passed strict population genetic tests for clonality, although isoenzyme studies of some rust populations argue against recombination (25). Clearly, there is a strong clonal component to *A. flavus'* life history, evident from the simple fact that the same clone can be isolated in different places. However, the lack of concordance observed among different gene genealogies in group I can be reasonably explained only by recombination between those loci. The human pathogenic fungus *Coccidioides immitis*, which also lacks a known sexual state, was found to have a recombining population structure (26) and to comprise two cryptic species (14). *Candida albicans*, another presumably asexual pathogen, was at first thought to be exclusively clonal (27), but a more comprehensive study showed evidence for recombination (28). *Emericella nidulans*, an *Aspergillus*-related species with a capability for sexual reproduction, also has a strongly clonal component to its life history superimposed on outcrossing (29).

Our results indicate a history of recombination in *A. flavus*, but we cannot say how or how often *A. flavus* recombines. Like many apparently asexual fungi, *A. flavus* has a close relative that is capable of sexual reproduction, *Petromyces alliaceus* (30–32). In culture, *A. flavus* produces hardened asexual structures called "sclerotia" that look very much like the sexual structures produced by *P. alliaceus* and may be homologous to them (33, 34). Perhaps under some conditions in nature the sclerotia of *A. flavus* harbor sexual reproduction as the homologous structures do in *P. alliaceus*. Nonmeiotic outcrossing (parasexuality) can be performed in *A. flavus* in the laboratory (6) but only between strains that are genetically very similar, and it has not been demonstrated in nature in any fungus. Furthermore, we cannot say how frequently *A. flavus* recombines, except that only a few individuals need to be produced sexually each generation to produce enough recombinant diversity to pass these kinds of tests (26).

The practice and utility of biological vs. phylogenetic species concepts has been debated heavily (see, e.g., ref. 35). Biological species concepts consider speciation to be inherently a process of reproductive isolation. The phylogenetic species concepts, in general, emphasize phylogenetic relationships in defining species as the smallest diagnosable taxonomic units with a clear pattern of parental ancestry (36). Thus, speciation is viewed as a process of cladogenesis and diversification. Proponents of biological species concepts argue that phylogenetic species concepts fail to consider reproductive isolation, an essential force in speciation (37). Proponents of phylogenetic species concepts argue that biological species concepts confuse information about the past with speculation about the future because they use reproductive isolation as the ranking and grouping criterion (36). We agree with Avise and Wollenberg (38), who explain that the two views are actually quite complementary, because phylogenetic and reproductive cohesiveness are strongly inter-related. The historical transmission routes of genes in sexual species are affected by reproductive barriers. Thus, comparisons of the transmission routes of different genes can be used to make inferences about reproductive isolation because of the differences in transmission predicted among interbreeding individuals (members of the same biological species) vs. reproductively isolated individuals (members of different biological species). The power of this integrated approach is particularly evident when applied to bacteria, fungi, and other organisms in which reproductive isolation cannot be ruled out by direct observation (13, 14).

It is clear from the level of fixation of polymorphisms among different loci that the two groups of *A. flavus* isolates have had a long history of reproductive isolation. Thus, they are best considered different biological species. The fact that the two groups appear sympatrically could easily reflect secondary contact because of the effects of agriculture. Although they are clearly divergent, we cannot say whether groups I and II retain the potential to interbreed because *A. flavus* isolates cannot be mated. If they do retain the potential, then their current apparent sympatry may lead to re-homogenization. Our sample of *A. flavus* individuals in this study is small and local and lacks representatives of morphologically distinguishable groups such as the North American large and small sclerotial forms (7). Investigation of a larger, world-wide sample of *A. flavus* may unravel a complex picture of different levels of allopatric speciation and secondary contact that is only hinted at by our data.

We conclude that *A. oryzae* is a species that evolved by domestication from *A. flavus* group I. Genetically, as assessed both by multilocus genotypes and gene sequences, the *A. oryzae* isolates are more similar to each other and to some *A. flavus* group I isolates than they are to other group I isolates. Phenotypically, they can be distinguished from *A. flavus* groups I and II based on continuous characters such as more olivaceous colony color and more floccose colony texture. In addition, *A. oryzae* isolates do not make aflatoxin. We conclude that *A. oryzae* is part of a monophyletic *A. flavus* clade, and its phenotypic distinctiveness is the result of strong selection associated with domestication. Given that 10/11 restriction sites that were polymorphic in *A. flavus* were found monomorphic in *A. oryzae* and that almost no sequence polymorphism was found among *A. oryzae* gene sequences, it is impossible to apply a biological species concept to *A. oryzae* vs. *A. flavus* group I, a concept that is probably moot for a domesticated species in any event. Although we found no evolutionary justification for maintaining *A. oryzae* as a separate species from *A. flavus*, there is a regulatory justification because *A. flavus* isolates are known to produce important mycotoxins and *A. oryzae* isolates are not. Our results indicate that industrial microbiologists should continue to be vigilant in their scrutiny of the mycotoxigenic potential of *A. oryzae* isolates.

These results have important implications for attempts to understand and control aflatoxin contamination in crops.

First, *A. flavus* comprises at least two reproductively isolated taxa, so these efforts have multiple targets that may have distinctive ecological niches. Second, at least one of these two groups has a history of recombination, which may indicate that recombination has an important role in the diversification of aflatoxin phenotypes. Further phylogenetic research is warranted to uncover other potential cryptic species, as well as further characterization of group II. Third, recombination in group I has important implications for biological control strategies, particularly adding nontoxigenic strains to fields where crops are grown, because of the potential for outcrossing between the introduced and native strains. With this knowledge, it would be prudent to characterize the quantitative genetics of aflatoxin synthesis in natural populations of *A. flavus*.

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