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The Phylogenetics of Mycotoxin and Sclerotium Production in *Aspergillus flavus* and *Aspergillus oryzae*

David M. Geiser,*,[1] Joe W. Dorner,† Bruce W. Horn,† and John W. Taylor‡

**Department of Plant Pathology, The Pennsylvania State University, 204 Buckhout Laboratory, University Park, Pennsylvania 16802-4507; †National Peanut Research Laboratory, USDA, ARS, Dawson, Georgia 31742-0509; and ‡Department of Plant and Microbial Biology, University of California, Berkeley, California 94720-3102*

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Geiser, D. M., Dorner, J. W., Horn, B. W., and Taylor, J. W. 2000. The phylogenetics of mycotoxin and sclerotium production in *Aspergillus flavus* and *Aspergillus oryzae*. *Fungal Genetics and Biology* 31, 000–000. *Aspergillus flavus* is a common filamentous fungus that produces aflatoxins and presents a major threat to agriculture and human health. Previous phylogenetic studies of *A. flavus* have shown that it consists of two subgroups, called groups I and II, and morphological studies indicated that it consists of two morphological groups based on sclerotium size, called "S" and "L." The industrially important non-aflatoxin-producing fungus *A. oryzae* is nested within group I. Three different gene regions, including part of a gene involved in aflatoxin biosynthesis (omt12), were sequenced in 33 S and L strains of *A. flavus* collected from various regions around the world, along with three isolates of *A. oryzae* and two isolates of *A. parasiticus* that were used as outgroups. The production of B and G aflatoxins and cyclopiazonic acid was analyzed in the *A. flavus* isolates, and each isolate was identified as "S" or "L" based on sclerotium size. Phylogenetic analysis of all three genes confirmed the inference that group I and group II represent a deep divergence within *A. flavus*. Most group I strains produced B aflatoxins to some degree, and none produced G aflatoxins. Four of six group II strains produced both B and G aflatoxins. All group II isolates were of the "S" sclerotium phenotype, whereas group I strains consisted of both "S" and "L" isolates. Based on the omt12 gene region, phylogenetic structure in sclerotium phenotype and aflatoxin production was evident within group I. Some non-aflatoxin-producing isolates of group I had an omt12 allele that was identical to that found in isolates of *A. oryzae*.

Index Descriptors: aflatoxin; *Aspergillus* section *Flavi;* cyclopiazonic acid; cryptic species; gene genealogies.

[1] To whom correspondence should be addressed. Fax: (814) 863-7217. E-mail: dgeiser@psu.edu.

Aspergillus flavus Link is a cosmopolitan, filamentous fungus that is known to occur mostly in soils, but it is also found in plant products, particularly oil-rich seeds, and in living plants. Concerns about its association with plant products, particularly corn, cotton, peanuts, and tree nuts, center around its ability to produce aflatoxins, polyketide secondary metabolites that are potentially carcinogenic.

Phenotypic variation in *A. flavus* has been well documented. It has been long noted that certain isolates of *A. flavus* produced abundant small sclerotia and that some of these isolates produced both B- and G-type aflatoxins (Hesseltine *et al.*, 1970; Saito *et al.*, 1986, 1989), whereas isolates that produced fewer and larger sclerotia produced only B aflatoxins. B and G aflatoxins differ in their ring structures and have different properties in thin-layer chromatography, fluorescing blue-green and green, respectively (Singh *et al.*, 1991). Later workers divided *A. flavus* isolates into "S" and "L" types based on whether they

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produced small or large sclerotia (Cotty, 1989). Some small sclerotial isolates were named *A. flavus* var. *parvisclerotigenus* Saito and Tsuruta (1993). The type isolate of this taxon was noted to be a nonproducer of G aflatoxins.

Genetic variation has also been noted in *A. flavus* isolates, and some connections have been made between genetic and phenotypic variation. *A. flavus* populations tend to be extremely diverse in terms of vegetative compatibility groups (Cotty *et al.*, 1994). Variation in isozymes (Yamatoya *et al.*, 1989), electrophoretic karyotypes (Keller *et al.*, 1992), randomly amplified polymorphic DNA (RAPD) (Bayman and Cotty, 1991, 1993; Tran-Dinh *et al.*, 1999), nuclear and mitochondrial DNA RFLPs (Moody and Tyler, 1990a,b), RFLPs of nuclear coding genes (Egel *et al.*, 1994; Geiser *et al.*, 1998a), and DNA sequences (Geiser *et al.*, 1998a) have been characterized. Bayman and Cotty (1993) analyzed randomly amplified polymorphic DNAs in S and L isolates and found that S isolates formed a single clade derived from within the L type. Tran-Dinh *et al.* (1999) analyzed RAPDs among toxigenic and nontoxigenic isolates of *A. flavus* and found that neither type was monophyletic. Egel *et al.* (1994) characterized taka-amylase gene RFLPs from two G aflatoxin-producing, small sclerotial isolates from Nigeria previously referred to as an unnamed taxon by Hesseltine *et al.* (1970) and found that they took an intermediate phylogenetic position between *A. flavus* and *A. parasiticus.* Geiser *et al.* (1998a) characterized DNA sequences from five different coding gene regions in isolates from Australian peanut fields and found two clear groupings of isolates, termed group I and group II.

A close phylogenetic association between *A. flavus* and *A. oryzae* (Ahlburg) Cohn has been clearly demonstrated (Yamamoto *et al.*, 1989; Kurtzman *et al.*, 1986; Egel *et al.*, 1994; Geiser *et al.*, 1998a). Although *A. oryzae* has "generally regarded as safe" status for use in industry, concerns about the potential for mycotoxin production have emerged in recent decades. Its relationship to *A. flavus* has been long recognized (Thom and Church, 1921). Molecular methods, including isozyme analyses, DNA/DNA hybridization studies, and DNA sequencing, have confirmed a very strong phylogenetic connection between *A. flavus* and *A. oryzae* (Kurtzman *et al.*, 1986; Yamatoya *et al.*, 1989; Egel *et al.*, 1994). Geiser *et al.* (1998a) found that *A. oryzae* isolates were derived from within *A. flavus* group I, with very strong bootstrap support. Wicklow (1983) proposed redefining *A. oryzae* as a variant of *A. flavus* based on morphological characteristics, and Kurtzman *et al.* (1986) found 100% DNA/DNA hybridization between *A. flavus* and *A. oryzae.* Proposals to taxonomically meld a food-producing fungus and a major toxin producer have not been enthusiastically embraced by the food mycology community.

Despite the strong phylogenetic connection, a number of nondiscrete phenotypic differences exist between *A. oryzae* and *A. flavus,* although distinguishing them in culture is not always easy (Klich and Pitt, 1988). *A. oryzae* isolates tend to have a floccose colony texture, producing abundant aerial mycelium, whereas *A. flavus* isolates tend not to be floccose. *A. oryzae* tends to produce colonies of an olive-green color, whereas *A. flavus* tends to produce more yellow-green shades. The conidia in *A. oryzae* tend to be larger than those of *A. flavus* and possess a smooth outer wall. *A. oryzae* isolates rarely produce sclerotia in culture, whereas *A. flavus* isolates often produce them. Aflatoxins have not been reported to be produced by *A. oryzae.* Some of the genes in the aflatoxin biosynthetic pathway appear to be present in *A. oryzae* isolates, although large deletions have been detected in the cluster in different *A. oryzae* isolates (Kusumoto *et al.*, 2000). *A. flavus* also produces cyclopiazonic acid (CPA), a toxic metabolite produced by an unrelated pathway. *A. oryzae* produces no CPA or very low levels (Orth, 1977). Toxin production is known to vary greatly among *A. flavus* isolates, with aflatoxin and CPA levels ranging from undetectable to quite high (Horn *et al.*, 1996).

We sought to test whether two of the variable phenotypes known to exist in *A. flavus,* toxin and sclerotium production, correlated with known phylogenetic groupings. We performed a phylogenetic analysis of portions of three nuclear genes on 29 geographically and phenotypically diverse isolates of *A. flavus* and on 3 isolates of *A. oryzae.* We also analyzed the production of B and G aflatoxins and CPA and characterized isolates as S or L based on their colony characteristics.

MATERIALS AND METHODS

Growth and culturing of Aspergillus strains. A list of isolates analyzed in this study is given in Table 1. Isolates were chosen based on their availability from our previous studies (Geiser *et al.*, 1998a) and on being representatives of known phenotypic subgroups of *A. flavus* (*A. oryzae,* S and L phenotypes) and from different geographic origins (California, Texas, Nigeria). Isolates are available from the first author at the Fusarium Research Center at The Pennsylvania State University. Strains were

TABLE 1
Strains of *A. flavus*, *A. oryzae*, and *A. parasiticus* Used in This Study

Strain	Location	Substrate	amdS12 Sequence	omt12 Sequence	trpC13 Sequence	Sclerotia	Taxon
1-9	Qld., Australia	Soil, peanut field	AF036768	AF036808	AF036851	L	*A. flavus* group I
1-22	”	”	AF036769	AF036809	AF036852	S	*A. flavus* group II
1-26	”	”	AF036770	AF036810	AF036853	L	*A. flavus* group I
1-29	”	”	AF036771	AF036811	AF036854	L	*A. flavus* group I
3-2	”	”	AF036772	AF036812	AF036855	L	*A. flavus* group I
4-2	”	”	AF036773	AF036813	AF036856	S	*A. flavus* group II
5-1	”	”	AF036774	AF036814	AF036857	L	*A. flavus* group I
7-2	”	”	AF036775	AF036815	AF036858	L	*A. flavus* group I
7-3	”	”	AF036776	AF036816	AF036859	L	*A. flavus* group I
7-4	”	”	AF036777	AF036817	AF036860	S	*A. flavus* group I
11-4	”	”	AF262376	AF262377	AF262378	L	*A. flavus* group I
12-4	”	”	AF036780	AF036820	AF036863	S	*A. flavus* group II
13-4	”	”	AF036781	AF036821	AF036864	S	*A. flavus* group II
14-1	”	”	AF036782	AF036822	AF036865	L	*A. flavus* group I
14-2	”	”	AF036783	AF036823	AF036866	L	*A. flavus* group I
17-4	”	”	AF036784	AF036824	AF036867	L	*A. flavus* group I
A55	California	Pistachio field	AF261827	AF261845	AF261861	S	*A. flavus* group I
A111	”	”	AF261838	AF261856	AF261872	L	*A. flavus* group I
A120	”	”	AF261828	AF261846	AF261862	S	*A. flavus* group I
A130	”	”	AF261827	AF261857	AF261873	L	*A. flavus* group I
A150	”	”	AF261839	AF261858	AF261874	L	*A. flavus* group I
A399	”	”	AF261829	AF261847	AF261863	L	*A. flavus* group I
TX12-10-2S	Texas	Soil, peanut field	AF261843	AF261859	AF261877	S	*A. flavus* group II
TX18-11S	”	”	AF261830	AF261848	AF261864	S	*A. flavus* group I
TX19-21S	”	Soil, corn field	AF261831	AF261849	AF261865	S	*A. flavus* group I
TX20-32S	”	Soil, cotton field	AF261832	AF261850	AF261866	S	*A. flavus* group I
TX21-1S	”	”	AF261833	AF261851	AF261867	S	*A. flavus* group I
F14	Georgia	Soil, peanut field	AF261834	AF261852	AF261868	L	*A. flavus* group I
F15	”	”	AF261835	AF261853	AF261869	L	*A. flavus* group I
F35	”	”	AF261836	AF261854	AF261870	L	*A. flavus* group I
F60	”	Peanut seed	AF261837	AF261855	AF261871	L	*A. flavus* group I
NRRL A11611	Nigeria	Soil, peanut field	AF261844	AF261860	AF261878	S	*A. flavus* group II
NRRL448	?	?	AF261841	AF036826	AF261875	N/A	*A. oryzae*
NRRL449	?	?	AF261842	AF036827	AF261876	N/A	*A. oryzae*
NRRL469	?	?	AF036785	AF036828	AF036868	N/A	*A. oryzae*
CA1-05	Dixon, CA	Pistachio	AF036786	AF036829	AF036869	N/A	*A. parasiticus*
CA3-01	”	”	AF036787	AF036830	AF036870	N/A	*A. parasiticus*

Note. Location and substrate from which each strain was isolated is given. GenBank accession numbers are listed for each sequence analyzed. Sclerotia for each strain are characterized as “S” or “L” according to criteria described by Cotty (1989), and *A. flavus* strains are listed as group I or group II based on the results of Geiser *et al.* (1998a) and this study.

grown on slants or plates containing potato dextrose agar (PDA) for observation of growth characteristics, including sclerotium production. Isolates were identified as “S” or “L” by gross colony morphology: L isolates produce large (>400-μm-diameter) sclerotia typically in a sparse fashion, whereas S isolates produce abundant small (<400-μm-diameter) sclerotia. For DNA extraction, masses of mycelium and conidia were transferred to liquid media (yeast extract–glucose (YEG) or PDA) and incubated without agitation at 37°C (30°C for *A. oryzae* strains). After 1–3 days, undifferentiated mycelium was harvested, frozen, and stored at −80°C.

DNA extraction, PCR, and sequencing. Methods for DNA extraction and PCR are described elsewhere (Geiser *et al.*, 1998a,b). Three DNA regions were chosen for analysis: a ca. 437-bp portion of the acetamidase gene *amd*S, which includes three introns; a ca. 423-bp portion of the *O*-methyltransferase gene within the aflatoxin gene

TABLE 2
Variable, Phylogenetically Informative, and Total Nucleotide Sites Sampled in the amdS12, omt12, trpC13, and Combined Data Sets

Gene region	Total sites	Variable sites within *A. flavus*	Phyl. inf. sites within *A. flavus*	Unshared poly. among subgroups of group I	Shared poly. among subgroups of group I	Fixed diffs. among subgroups of group I	Monomorphic in group I	Unshared poly. between groups I/II	Shared poly. between groups I/II	Fixed poly. between I/II
amdS12	438	44	28*	1	5	0	22	25*	2*	1
Non-cod	137	21	14	0	2	0	12	15	1	1
Syn			11	1	1	0	9	11	0	0
Repl			3	0	2	0	1	2	1	0
omt12	443	72	64	10	0	21	33	38	1	25
Non-cod	162	38	37	6	0	11	20	22	1	14
Syn			19	2	0	8	9	9	0	10
Repl			8	2	0	2	4	7	0	1
trpC13	470	17	11	2	5	0	4	7	0	4
Non-cod	437	15	10	2	4	0	4	6	0	4
Syn			0	0	0	0	0	0	0	0
Repl			1	0	1	0	0	1	0	0
All 3	1131	133	103	13	10	21	59	70	3	30
Non-cod	736	74	61	8	6	11	36	43	2	19
Syn			30	3	1	8	18	20	0	10
Repl			12	2	3	2	5	10	1	1

* 20/27 Site polymorphisms within group II at this locus are explained by isolate 1-22 possessing the common group I character state.

cluster, *omt*, also with three introns; and a ca. 470-bp region of the 5′ untranslated region of the *trp*C gene (Geiser *et al.,* 1998a). These three regions are named amds12, omt12, and trpc13, respectively, and have been shown in previous studies to harbor informative variation at the intra- and interspecific levels in *Aspergillus* species (Geiser *et al.*, 1998a,c). DNA sequences were generated on an Applied Biosystems Model 377 automated DNA sequencer, as described previously (Geiser *et al.*, 1998a,b), edited, and added to previously generated alignments available at the TREEBASE database (http://herbaria.harvard.edu/treebase/).

DNA sequence analysis. All phylogenetic analyses were performed using v4.0b2 and b4 of PAUP* (Phylogenetic Analysis Using Parsimony; Swofford, 1999). Gaps were encoded as missing data and were thus excluded from the analyses. To increase the chance of finding the most-parsimonious (MP) trees, 100-replicate heuristic searches were performed using random addition of sequences. For bootstrap analyses, 1000 resampled data sets were used. The partition homogeneity test (PHT) was performed on parsimony-informative sites only with 1000 randomized data sets, using heuristic searches with simple addition of sequences. Phylogenetic analyses using neighbor-joining were performed using the Kimura two-parameter model. To compare the likelihood of different tree topologies, the Kishino–Hasegawa test was employed (Kishino and Hasegawa, 1985), using a transition:transversion ratio of 2:1 and the assumption that all sites evolve at the same rate. The program SITES v.1.1 (Hey and Wakeley, 1997) was used to classify polymorphic sites in sequences.

Toxin analyses. Fungal isolates were grown for 7 days at 30°C in 4-ml vials containing 1 ml of liquid medium (three replicates per isolate) as described by Horn and Dorner (1999). Vial cultures were analyzed for aflatoxins and CPA by high-performance liquid chromatography (HPLC) according to the methods of Horn *et al.* (1996), except that a Shimadzu Class VP chromatography laboratory automated software system was used to quantify aflatoxins.

RESULTS

Phylogenetic analyses. Characteristics of the amds12, omt12, and trpC13 data sets are presented in Table 2. Parsimony analysis of the three data sets produced the following phylogenetic trees: four most-parsimonious trees of 88 steps for the amdS12 data set, eight trees of 92 steps for the omt12 data set, and one tree of 34

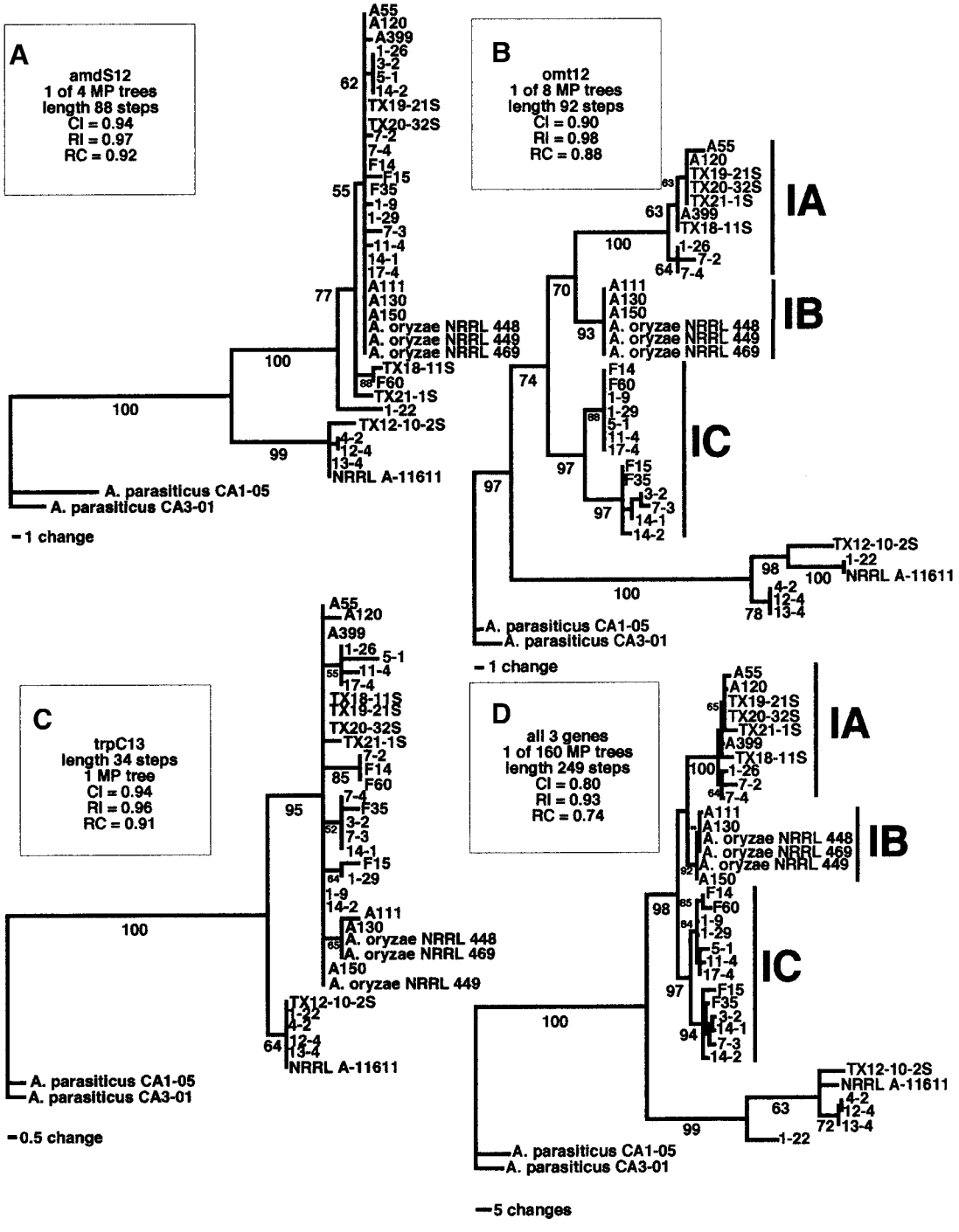

FIG. 1. One of the most-parsimonious trees from each of the three data sets (A, amdS12; B, omt12; C, trpC13) and from the combined data (D). Numbers below branches represent bootstrap percentages of 1000 replications. CI, consistency index; RI, retention index; RC, rescaled consistency index.

steps for the trpC13 data set (Fig. 1). The topologies of these single-gene parsimony trees and those produced by neighbor-joining were identical where internal branches were strongly supported (neighbor-joining analysis not shown). Coding versus noncoding sequences were also analyzed separately, and no strongly supported topology

differences were identified between them. Parsimony analysis for each individual data set showed few homoplasies, indicated by high (>0.9) consistency indices, rescaled consistency indices, and retention indices. When each gene was subjected to the Archie–Faith–Cranston permutation test (PTP), the null hypothesis of recombination could be rejected for each gene ($P < 0.01$ for all three genes; data not shown; Burt *et al.*, 1996). Although these results do not necessarily exclude recombination, they provide no evidence for it within the three genes.

All three genes supported the distinction between group I and group II. One minor difference is that the amdS12 sequence from isolate 1-22 forms a strongly supported clade with group I sequences, making group II paraphyletic, whereas the omt12 and trpC13 sequences from this isolate group strongly with those from group II isolates. Two other genes also indicated that 1-22 was related to group II in a previous analysis (Geiser *et al.*, 1998a).

One of the three genes, omt12, showed highly supported structure within group I, whereas the other two genes showed little structure. At least three well-supported clades within group I were evident from the omt12 data, labeled IA, IB, and IC on Fig. 1B. Analysis of polymorphisms in the three data sets shows that these three subgroups are evident in the omt12 gene region only. None of the sites that are parsimony-informative within group I are fixed among these three subgroups in the amdS12 and trpC13 regions (Table 2). Five of 6 informative sites in the amdS12 region are shared polymorphisms among the three omt12-defined subgroups of group I, and 5 of 7 in the trpC13 region are shared. In contrast, 21 of 31 informative omt12 sites are fixed among the three subgroups, and 10 of 31 show unshared polymorphism, with no polymorphisms in the omt12 region being shared among subgroups of group I. The omt12 region contained the most parsimony-informative sites (64/103 in A. *flavus*) of the three data sets, particularly within group I (31/44). Our past analysis of fewer isolates (Geiser *et al.*, 1998a) showed conflict among the topologies of these genes (and two others) among the group I isolates. Comparison of the topologies of amdS12, omt12, and trpC13 for the expanded collection of isolates analyzed in this report again showed conflict by the partition homogeneity test or the incongruence length difference test, with the actual sum of tree lengths for the three genes (171 steps) at least 22 steps shorter than that from any of the 1000 replicates. Two nonparametric tests, the Templeton–Wilcoxon signed-ranks test (Templeton, 1983) and the winning-sites test (Prager and Wilson, 1988), were also employed to test for congruence among the three data sets. The former method tests the relative number of steps required for each character on different trees, whereas the latter compares the number of characters that favor different trees on a binomial distribution. Both tests showed significant incongruence between all of the three sets of MP trees produced by the three individual data sets and between all three pairs of data sets except amdS12 and trpC13 (not shown). A combined parsimony analysis of the three data sets produced 160 most-parsimonious trees of 249 steps, 35 steps longer than the minimum length, also indicating incongruence among the three data sets. A consensus of these trees is shown in Fig. 2.

One of the three strongly supported omt12 subgroups of group I (IB) contained the three *A. oryzae* isolates analyzed, plus three Californian *A. flavus* isolates: A111, A130, and A150. The other two gene regions did not indicate a strong relationship between these six isolates, other than their inclusion within group I. To test whether the amdS12 and trpC13 data sets conflicted with the omt12 data set with regard to the strong relationship between *A. oryzae* and these three Californian isolates, the PHT was applied to these six isolates plus members of their "sister clade" (group IA) as indicated by the omt12 data: isolates A55, A120, TX 18—11S, TX19-21S, TX20-32S, TX21-1S, A399, 1-26, 7-2, and 7-4. The analysis did not indicate significant conflict between the three data sets with regard to the relationships between these isolates (not shown). This suggests that whereas the amdS12 and trpC13 gene regions do not add support to the distinction between groups IA and IB, neither do they conflict with it.

Two newly analyzed isolates, one collected from peanuts in Texas and the other from Nigeria, were found to be members of group II. The addition of more isolates from around the world in this study added further strength to the inference that group II isolates are phylogenetically divergent with respect to other *A. flavus* isolates (Geiser *et al.*, 1998a).

Small sclerotial S isolates were found to occur in two distinct phylogenetic groups, both of which received strong bootstrap support in the omt12 data set, indicating nonmonophyly of the S phenotype (Fig. 2). All group II isolates were of the S type. According to the omt12 data set and the combined data set, group I S isolates formed a paraphyletic group with two L isolates, 7-2 and 1-26. To test whether strains with the S phenotype were nonmonophyletic, a Kishino–Hasegawa test was performed (Kishino and Hasegawa, 1989). The likelihoods of the 160 MP trees made from the combined data were compared to the likelihoods of the trees made with the constrains of S phenotype monophyly (Table

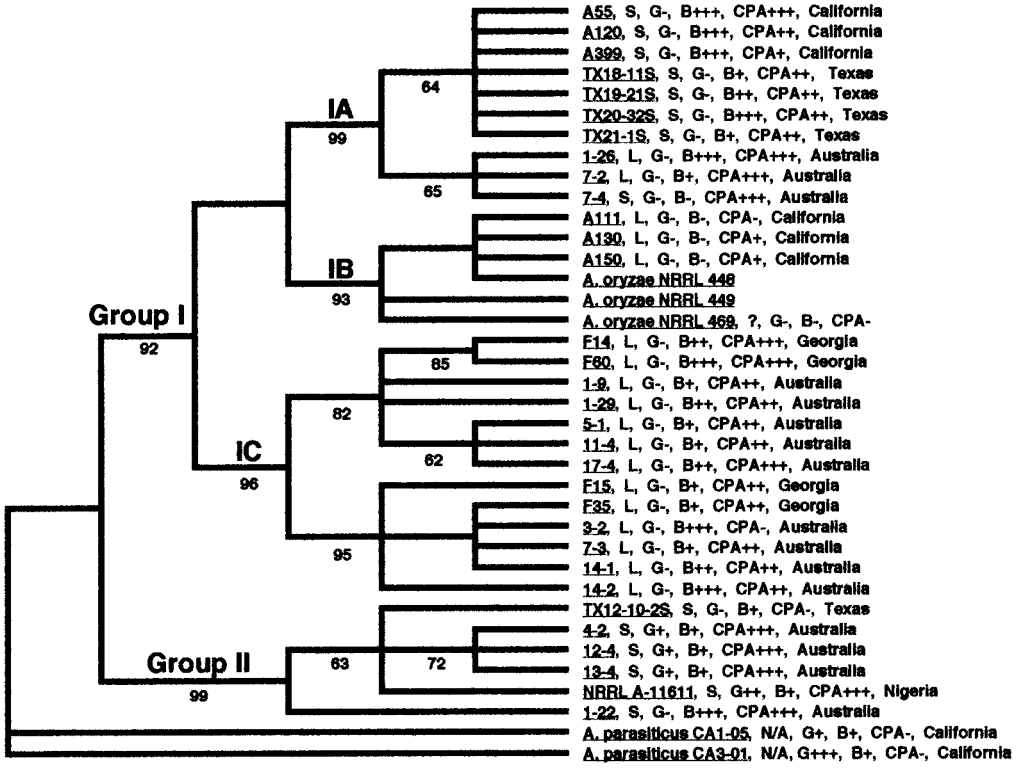

FIG. 2. The strict consensus of 160 MP trees obtained from analysis of the combined data. Numbers below branches represent bootstrap percentages of 1000 replications. Information is listed for each isolate in the following order: sclerotium phenotype (S for small and L for large); G and B aflatoxin and cyclopiazonic acid (CPA) production (−, none detected, +, 0.01–50 μg/ml; ++, 50–100 μg/ml; +++, >100 μg/ml); and geographic origin. Strongly supported clades within group I (IA, IB, IC) are shown.

3). The most likely of the 16,253 MP constrained trees was 2.38 standard deviations less likely than the most likely unconstrained tree ($P \sim 0.017$).

Toxin analyses. Levels of aflatoxins B_1, B_2, G_1, and G_2 and those of CPA are listed in Table 4. Most group I isolates produced moderate to high levels ($\geq$55.5 μg/mL) of CPA in all replicates, except for the three Californian isolates in omt12 clade IB, which produced average CPA levels of 0 (A111), 5.0 (A130), and 15.9 (A150) μg/mL, and the Australian isolate 3-2, which produced no detectable CPA. Group I isolates produced levels of B aflatoxins ranging from barely detectable (0.02 μg/ml) to high levels, with the highest levels found in members of omt12 clade IA. No group I isolates produced G aflatoxins. No B or G aflatoxins were detected in the three Californian *A. flavus* isolates in omt12 clade IB, nor in the one *A. oryzae* isolate (NRRL 469) tested, as was expected based on previous studies. An additional group I S isolate in clade IA, 7-4, did not produce detectable levels of aflatoxin.

All group II isolates produced detectable levels of CPA, and both B and G aflatoxins, with two exceptions: Texas isolate TX12-10-2S did not produce CPA or G aflatoxins and Australian isolate 1-22 produced high levels of CPA but no G aflatoxins were detected.

TABLE 3
Results of Kishino–Hasegawa Test for Monophyly of S Phenotype

Tree	-InL	Diff.-InL	SD	T	*P*
Best of 160 MP trees-unconstrained	3467.93	BEST			
Best of 16,253 MP trees-constrained	3524.06	56.13	23.50	2.39	0.017

DISCUSSION

Phylogenetic patterns. All three genes showed a branch separating group I strains from group II strains,

TABLE 4
Mycotoxin Production (μg/mL) of *A. flavus*, *A. oryzae*, and *A. parasiticus* Isolates

Strain	CPA	Aflatoxin B1	Aflatoxin B2	Aflatoxin G1	Aflatoxin G2	Mean total aflatoxin
A. flavus group II						
1-22	145.4 ± 26.0	118.41 ± 26.13	2.51 ± 0.79	0.00	0.00	120.92
4-2	103.4 ± 6.8	2.19 ± 0.64	0.03 ± 0.01	4.39 ± 2.11	0.08 ± 0.04	6.68
12-4	124.4 ± 5.1	0.75 ± 0.05	0.01 ± 0.00	1.02 ± 0.01	0.02 ± 0.01	1.80
13-4	128.4 ± 2.6	3.05 ± 0.27	0.03 ± 0.01	8.35 ± 1.24	0.12 ± 0.02	11.55
TX12-10-2S	0.00	32.27 ± 9.35	1.77 ± 0.74	0.00	0.00	34.04
NRRL A11611	135.0 ± 9.9	19.12 ± 1.24	0.75 ± 0.14	64.08 ± 6.81	1.95 ± 0.41	85.90
A. flavus group I						
1-9	98.5 ± 5.6	23.90 ± 7.06	0.89 ± 0.30	0.00	0.00	24.79
1-26	100.3 ± 11.4	137.62 ± 49.59	3.55 ± 1.25	0.00	0.00	141.17
1-29	60.0 ± 3.9	61.63 ± 7.16	2.18 ± 0.43	0.00	0.00	63.82
3-2	0.00	105.37 ± 26.62	1.48 ± 0.71	0.00	0.00	106.86
5-1	74.6 ± 8.7	40.67 ± 12.55	1.66 ± 0.55	0.00	0.00	42.33
7-2	101.1 ± 6.8	42.09 ± 2.80	0.57 ± 0.15	0.00	0.00	42.66
7-3	94.9 ± 6.0	3.83 ± 0.30	0.08 ± 0.01	0.00	0.00	3.91
7-4	191.7 ± 19.8	0.00	0.00	0.00	0.00	0.00
11-4	87.3 ± 8.2	30.46 ± 5.16	0.57 ± 0.13	0.00	0.00	31.03
14-1	93.6 ± 10.4	98.35 ± 22.41	1.05 ± 0.38	0.00	0.00	99.40
14-2	68.9 ± 3.8	123.01 ± 30.58	1.96 ± 0.44	0.00	0.00	124.97
17-4	117.0 ± 8.0	55.99 ± 9.98	2.02 ± 0.37	0.00	0.00	58.01
A55	117.8 ± 21.2	383.09 ± 20.57	13.03 ± 1.08	0.00	0.00	396.12
A120	64.4 ± 4.6	306.68 ± 31.18	7.38 ± 1.33	0.00	0.00	314.06
A399	90.7 ± 12.5	130.82 ± 66.22	5.07 ± 2.37	0.00	0.00	135.89
TX18-11S	80.5 ± 16.0	36.25 ± 8.85	0.95 ± 0.39	0.00	0.00	37.20
TX19-21S	89.1 ± 17.1	86.02 ± 13.92	2.33 ± 0.69	0.00	0.00	88.35
TX20-32S	67.5 ± 3.1	544.72 ± 54.48	23.20 ± 1.76	0.00	0.00	567.92
TX21-1S	75.7 ± 12.8	0.02 ± 0.01	~0.00	0.00	0.00	0.02
F14	114.8 ± 7.2	86.28 ± 21.66	1.51 ± 0.52	0.00	0.00	87.79
F15	93.3 ± 6.2	0.71 ± 0.13	0.03 ± 0.01	0.00	0.00	0.74
F35	66.0 ± 3.5	11.33 ± 3.23	0.35 ± 0.13	0.00	0.00	11.68
F60	158.4 ± 21.5	124.88 ± 35.51	4.23 ± 1.80	0.00	0.00	129.11
A111	0.00	0.00	0.00	0.00	0.00	0.00
A130	5.0 ± 0.6	0.00	0.00	0.00	0.00	0.00
A150	15.9 ± 1.3	0.00	0.00	0.00	0.00	0.00
A. oryzae						
469	0.00	0.00	0.00	0.00	0.00	0.00
A. parasiticus						
CA1-05	0.00	8.81 ± 1.05	0.15 ± 0.02	2.77 ± 0.39	0.04 ± 0.00	11.77
CA3-01	0.00	24.32 ± 2.93	1.05 ± 0.16	195.53 ± 24.03	6.67 ± 0.77	227.57

Note. Mean values of three replicates are shown, ± standard error.

and two of three (omt12 and trpC13) showed the two groups to be reciprocally monophyletic (Fig. 1). The inference that the two groups are separate is also strong in the combined analysis. However, some conflict between the three data sets was evident from the partition homogeneity test and the nonparametric tests and from the fact that the combined analysis yielded 160 MP trees that were 35 steps longer than the minimum length. According to the amdS12 data, strains TX18-11S and F60 form a clade with 88% bootstrap support, whereas these two strains are in different strongly supported clades in the omt12 dataset. Strain 1-22 is a strongly supported member of group II in the omt12 dataset and is identical to the other five members of group II in the trpC13 dataset, but forms a strongly supported clade with group I in the amdS12 dataset. Recombination among isolates of group I and the retention of ancestral polymorphism between group I and group II are probable explanations for these inconsisten-

cies. Given the evidence for recombination presented by Geiser *et al.* (1998a), conflict among different gene genealogies would be expected due to the presence of many group I isolates.

The trees produced by the combined data sets look very much like those from the omt12 data set, probably because 62% (64/103) of the phylogenetically informative sites in *A. flavus* and 70% (31/44) of the phylogenetically informative sites in group I came from this one locus. There are several possible explanations for the increased phylogenetic signal observed in the omt12 data set compared to the other two data sets. First, selection may be driving diversification of the aflatoxin gene cluster in the three major inferred lineages within group I. Second, a relaxation of selection in the non-aflatoxin-producing lineage may lead to an excess of substitutions on that lineage. This explanation seems unlikely because of the similarly large number of omt12 synapomorphies supporting the toxin-producing lineages in group I and group II (Fig. 1). Third, the omt12 gene region may evolve at a faster rate than other genes in the genome due to other factors. Effective tests of selection would require more data than presently available in this sample (14 polymorphic codon nucleotide sites, 4 of which were amino acid replacements).

B aflatoxin evolution. Most members of group I are B aflatoxin producers, with several exceptions. Three of the four non-B-aflatoxin-producing isolates identified in this study had an omt12 sequence identical to that found in isolates of *A. oryzae,* suggesting that the aflatoxin biosynthetic pathway in *A. oryzae* was derived from a non-aflatoxin-producing *A. flavus* progenitor. These three non-aflatoxin-producing isolates were isolated from pistachio orchards in California and are morphologically typical of *A. flavus,* producing yellow-green colony shades, occasional large sclerotia, and finely roughened conidia. Recently, Kusumoto *et al.* (2000) demonstrated that variation in the aflatoxin biosynthetic gene cluster in *A. oryzae* is characterized by three classes of directed deletions from the 5′ end. The omt12 gene region is the fifth-most 3′-distal open reading frame in the aflatoxin biosynthetic cluster and appears to be present in all of the *A. oryzae* isolates analyzed in that study. Because almost no DNA sequence variation has been identified among *A. oryzae* isolates, it is possible that *A. oryzae* isolates have a common origin of domestication in *A. flavus,* perhaps from a strain with the clade IB-type aflatoxin gene cluster. The process of domestication may have generated the novel phenotypes associated with *A. oryzae,* including floccose colony texture and olive-green, smooth conidia. Because of relaxed selection on the aflatoxin biosynthetic gene cluster, directed deletions also may have occurred in domestication (Kusumoto *et al.,* 2000). This model predicts that each of the three classes of directed deletions identified by Kusumoto *et al.* (2000) would have the clade IB-type omt12 allele. Whereas weak aflatoxin producers were found throughout the three omt12 group I clades, only one isolate outside of clade IB was found to produce no detectable aflatoxin (7-4).

G aflatoxin evolution. Four of six group II strains were found to be G aflatoxin producers, and no group I strains produced detectable levels. There have been several reports in the literature regarding G-aflatoxin-producing strains of *A. flavus* and their connection with the S phenotype (Hesseltine *et al.,* 1970; Saito *et al.,* 1986, 1989; Cotty and Cardwell, 1999). Hesseltine *et al.* (1970) referred to small sclerotial G aflatoxin producers as part of a "new taxon." Saito *et al.* (1993) applied the name *A. flavus* var. *parvisclerotigenus* to strains with this phenotype, although these authors included non-G-aflatoxin producers in this taxon, including the type strain. Egel *et al.* (1994) found that strains corresponding to Hesseltine's "new taxon" were phylogenetically intermediate between most *A. flavus* isolates and *A. parasiticus,* based on RAPD data and sequence from a portion of the taka-amylase gene. In this study, we analyzed one of the strains (NRRL A-11611) analyzed by Hesseltine *et al.* (1970) and Egel *et al.* (1994) and found that it is strongly included within *A. flavus* group II, using all three gene regions tested. This result strongly suggests a correlation between group II and *A. flavus* var. *parvisclerotigenus* Saito and Tsuruta (1993), although this name could also be applied to small sclerotial members of clade IA in group I. The fact that the type strain of this species does not produce G aflatoxin suggests that it is more likely a member of clade IA than of group II. Unfortunately, a culture of this type strain is unavailable. In a survey of North American and West African S isolates, Cotty and Cardwell (1999) found that 40% of West African S isolates were G aflatoxin producers, whereas no G-aflatoxin-producing strains were found in North America. We think that it is likely that these African G aflatoxin producers correspond to group II. It appears that G-aflatoxin-producing group II strains of *A. flavus* occur with high frequency in Australia, southeast Asia, and Africa, but not in North America (Geiser *et al.,* 1998a; Cotty and Cardwell, 1999; Carter and Pitt, 1999; D. M. Geiser, unpublished data). We have found only a single North American isolate that is a member of group II (TX12-10-2S), but it did not produce detectable levels of G aflatoxins. This isolate was one of two group II strains

that did not produce detectable levels of G aflatoxin. Group II isolates were generally not heavy aflatoxin producers in comparison to many group I isolates, particularly those belonging to the small sclerotium producing omt12 clade IA (Fig. 2; Table 4). The observation that the outgroup species *A. parasiticus* produces G aflatoxin, as does the more distantly related species *A. nomius,* suggests that the lack of G aflatoxin production in group I and in some isolates of group II represents a loss of function.

The results of this study further demonstrate that *A. flavus,* as it is currently defined, represents a nonmonophyletic assemblage, including at least two major groups (I and II) of strains that show evidence for a long history of reproductive isolation (Fig. 2). Additional cryptic evolutionary units may exist within the assemblage known as *A. flavus* group I, detected as clades with high bootstrap values in the omt12 data set, including the clade that connects *A. oryzae* with the non-aflatoxin-producing *A. flavus* isolates (clade IB) and the mostly S-type clade IA. Whether these clades represent divergent allelic classes within a species or three different reproductively isolated subgroups can only be determined by the analysis of additional, independent variable sequence data sets.

Evolution of the S phenotype. The two phenotyes of *A. flavus* isolates, large sclerotium-producing L isolates and small sclerotium-producing S isolates (Cotty, 1989) were well represented in this sample. Two distinct groups of isolates, one in group I and the other in group II, possess the small sclerotial S phenotype. All group I S isolates were part of omt12 clade IA, a clade strongly supported (99–100% bootstrap) by the omt12 and combined data sets (Figs. 1 and 2), whereas the second group corresponded to group II, which also received strong support. Isolates in omt12 clade IA were not exclusively small sclerotial because they included two Australian L isolates, 7-2 and 1-26, and monophyly of the S phenotype was rejected both by bootstrap analysis and by the Kishino–Hasegawa tests (Fig. 2, Table 3). Members of this clade with the S phenotype produced CPA and B aflatoxins, with no G aflatoxins produced, whereas group II S isolates often produced CPA and both B and G aflatoxins. It is important to note that the small sclerotial S phenotype is not necessarily predictive with respect to phylogenetic relationship or aflatoxin production.

Taxonomic recommendations. These results show that the name *A. flavus* is currently applied to a paraphyletic group and that taxonomic changes are necessary. While it is clear that a phylogenetic connection exists between group II and *A. flavus* var. *parvisclerotigenus* and that group II deserves recognition as a species, it is not clear whether *A. flavus* var. *parvisclerotigenus* ought to be elevated to species status and applied to this clade because the type strain is not available. We now have evidence of genetic distinctiveness between *A. oryzae* and most aflatoxin-producing strains of *A. flavus,* providing support for maintaining the species name *A. oryzae.* Additional DNA sequence analyses will be necessary to determine whether *A. oryzae* represents a distinct organismal lineage within the *A. flavus/parasiticus* species complex or whether it simply possesses a divergent class of alleles in the aflatoxin gene cluster.

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