Differentiation of *Aspergillus parasiticus* from *Aspergillus sojae* by Random Amplification of Polymorphic DNA

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*Aspergillus parasiticus* and *Aspergillus sojae* are two morphologically similar species belonging to the *Aspergillus* section *flavi*. A new method to distinguish the two species, the aflatoxin producer *A. parasiticus* and the koji mold *A. sojae*, was developed. Single primers with arbitrary sequences were used to generate random amplified polymorphic DNA (RAPD) markers from strains of these two species. Three decamers, OPA-04, OPB-10, and OPR-01, allowed adequate discrimination between strains of *A. parasiticus* and *A. sojae* in RAPD analyses. *A. sojae* was further separated into group I and group II with the three primers. On the other hand, *A. parasiticus* was divided into group A and group B when amplified with OPA-04 and OPR-10 primers. The previously misidentified strain CCRC 32423 and the misclassified strain CCRC 30227 were identified as *Aspergillus flavus* and *A. sojae*, respectively, on the basis of RAPD patterns and morphological characteristics. We suggest that the RAPD technique is a rapid and reliable tool to distinguish *A. parasiticus* from *A. sojae*.

*Aspergillus parasiticus* and *Aspergillus sojae* are two morphologically similar species belonging to the *Aspergillus* section *flavi*. *A. parasiticus* commonly infects cereal grains and peanuts, in which it may produce aflatoxins, which are potent carcinogens. *A. sojae* is widely used throughout the Orient as a koji mold for the fermentation of miso and soy sauce. The same relationship of a toxin producer and a koji fungus for a pair of mold for the fermentation of miso and soy sauce. The same relationship of a toxin producer and a koji fungus for a pair of...

**MATERIALS AND METHODS**

**Fungal strains and morphological observation.** The fungal strains used are listed in Table 1. All the strains used that were not isolated in Taiwan were originally obtained from the American Type Culture Collection (ATCC) and then were collected in the Culture Collection and Research Center (CCRC) of the Food Industry Research and Development Institute. The Taiwan strains were isolated from local soil or fermented food. CCRC 30150 (= NRRL 465) (12) and CCRC 30103 (= NRRL 1988) were considered reference strains of *A. parasiticus* and *A. sojae*, respectively. *A. parasiticus* and *A. sojae* were grown on Czapek yeast extract agar (CYA) (6) and Czapek agar (Cz) (12) for morphological observation.

**Bleomycin test.** Test strains were inoculated onto modified minimal agar (MMA) (0.52 g of MgSO_4_, 0.52 g of KCl, 1.52 g of KH_2PO_4_, 0.5 ml of 2 N KOH, 10 g of glucose, 8 µg of biotin, 10 ml of 1 M proline solution, 1.0 ml of trace element solution, 15 g of agar, 1 liter of H_2O) supplemented with bleomycin (40 µg/ml) or without bleomycin (3). After growth at 25°C for 6 days, the diameter of colonies was measured.

**Preparation of fungal genomic DNA.** A modification of the rapid method of Leach et al. (9) for fungal DNA preparation was employed. Briefly, harvested mycelia were homogenized and then homogenized with LETS buffer (0.1 M LiCl, 10 mM EDTA, 10 mM Tris·Cl [pH 8.0], 0.5% sodium dodecyl sulfate). An equal

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differentiates the strains. After denaturation at 94°C, CTG, CTGCTGGGAC, and TGCGGGTCCT, respectively, were chosen to differentiate between the two species in the ATCC catalog. In addition, CCRC 30227 was recognized as an A. sojae strain but was listed as A. parasiticus in the ATCC catalog.

Restriction by bleomycin. The growth of both species on modified minimal medium supplemented with bleomycin was reduced, but A. sojae strains were much more restricted than A. parasiticus strains. Colony diameters of A. parasiticus on MMA-plus-bleomycin medium were larger than 16 mm, while those of A. sojae were smaller than 5 mm (Table 1). In contrast, colony diameters of both A. parasiticus and A. sojae were larger than 20 mm on MMA alone.

RAPD reaction. Seventy-six oligonucleotide decamers from Operon Technologies Inc. (OPA-01 to -20, OPB-01 to -20, OPC-01 to -12, OPR-01 to -12, and OPY-01 to -12) were tested for their abilities to generate RAPD markers from genomic DNAs of A. parasiticus and A. sojae. Three primers, OPA-04, OPB-10, and OPR-01, allowed adequate discrimination between strains of A. parasiticus and A. sojae (Fig. 1 to 3). Strain CCRC 32423 exhibited a RAPD pattern different from those of A. parasiticus and A. sojae strains with these three primers.
This strain was renamed *A. flavus* by rechecking the morphological observations as mentioned above. All strains of *A. parasiticus* except CCRC 30424 showed a significant 2.0-kb band and a minor 1.4-kb band when amplified with OPB-10 (Fig. 1). The significant 2.0-kb band was absent from *A. sojae* strains when amplified with OPB-10; in contrast, two kinds of polymorphic DNA patterns were produced from these strains and each pattern had specific bands distributed between 1.0 and 4.0 kb (Fig. 1). Therefore, strains of *A. sojae* could be separated into two groups (group I and group II) with OPB-10 in RAPD reactions. The two *A. sojae* groups were also differentiated when amplified with OPA-04 and OPR-01. A significant 0.6-kb band and a minor 2.2-kb band were present in group I but absent from group II *A. sojae* when amplified with OPA-04 (Fig. 2). On the other hand, a 0.7-kb band and a 1.8-kb band were present in group II but absent from group I *A. sojae* when amplified with OPR-01 (Fig. 3). Specific band patterns were also produced from group I with OPR-01 and group II with OPA-04.

Most strains of *A. parasiticus* produced similar patterns with the three primers and were designated group A. Two significant bands of 0.6 and 0.8 kb and a minor band of 1.6 kb were produced in all the strains of group A when amplified with OPR-01 (Fig. 3). Two strains of *A. parasiticus*, CCRC 30132 and 30150, were designated group B; in this group, the 0.6- and 1.6-kb bands were also produced but the 0.8-kb band was absent from the two strains in RAPD reactions with OPR-01 (Fig. 3). When strains of *A. parasiticus* group A were amplified with OPA-04, a significant 2.2-kb band and a minor 0.7-kb band were produced in all of these strains and a minor 3.1-kb band and a 0.5-kb band were amplified in most of these strains (Fig. 2). The significant 2.2-kb band was missing from group B; in contrast, three minor bands (1.7, 2.2, and 2.5 kb) were found in the two strains of group B when amplified with OPA-04 (Fig. 2). An aflatoxin-producing strain, CCRC 30424, showed patterns different from those of the other *A. parasiticus* strains in RAPD reactions when amplified with OPB-10 and OPR-01 but exhibited the same pattern as group A with OPA-04. These results suggest the possibility that a third group of *A. parasiticus* strains exists. CCRC 30227 was designated *A. parasiticus* by the ATCC (strain ATCC 11906) but designated *A. sojae* by the Institute of Fermentation, Osaka, Japan (strain IFO 4391). In RAPD reactions, this strain showed the pattern of *A. sojae* group I.

**DISCUSSION**

A new method to distinguish *A. sojae* from *A. parasiticus* was developed in this study. Three primers separated the two species in RAPD reactions clearly. Sakaguchi and Yamada isolated soy sauce molds and described the species *A. sojae* (13, 14) which was considered identical to *A. parasiticus* (12). Strain CCRC 30227 (= WB 4759) was originally obtained from a fermentation company in Japan and designated *A. sojae* but was changed to *A. parasiticus* in Raper and Fennell’s book (12). Although *A. sojae* has been separated from *A. parasiticus* (4), this strain is still listed under the name *A. parasiticus* by the ATCC. Our RAPD analysis showed clear results to indicate that CCRC 30227 and other strains of *A. sojae* are different from *A. parasiticus*. These results are important and valuable for industry to prove that the organisms used for making soy sauce or fermented foods are different from the aflatoxin producer *A. parasiticus*.

Both *A. sojae* and *A. parasiticus* strains were divided into at least two groups by RAPD. Most strains of *A. parasiticus* belong to group A. The only two strains of group B, CCRC 30132 and CCRC 30150, were originally isolated in Japan; in contrast, strains of group A were isolated mainly in the United States. More Japanese isolates of *A. parasiticus* may be required to prove the consistency between geography and genetic characteristics. No other characteristics with significant differences between the two *A. sojae* groups were found, except that
CCRC 30227, a member of A. sojae group A, was isolated a long time ago. In contrast, CCRC 38021, a member of A. sojae group B, is a strain currently used for making soy sauce. We suspect the microorganisms for making soy sauce were altered during adaptation to fermentation or forced to change by artificial selection.

The original name of CCRC 30424 was Aspergillus fusciculatum, which was recognized as a synonym for A. flavus (type strain WB 4743) or A. parasiticus (isolate strain WB 4742 = CCRC 30424) (12). By using primers OPB-10 and OPR-01, we separated this strain from the other A. parasiticus strains by RAPD analysis. Another interesting finding was that the RAPD pattern produced by CCRC 30424 became increasingly similar to those of the group I strains of A. sojae when amplified with OPR-01. Although these results suggest that this strain might be considered an intermediate between A. sojae and A. parasiticus, morphological behavior, bleomycin test results, and aflatoxin-producing characteristics (16) indicated that this strain should be designated A. parasiticus. It is possible to reclassify strains of the species A. fusciculatum or fusciculatum varieties under the species A. parasiticus by checking WB 4742 and WB 4743 by both morphological and molecular methods.

It is important to authenticate the test strains before running biochemical and genetic experiments, since misidentifications of these taxa occasionally happen. Our morphological observations confirmed that most of the test strains were correctly identified. Some strains with ambiguous morphology were confirmed by the bleomycin test and RAPD analysis. Strain CCRC 32423, which was identified as A. flavus, provides a significant example to demonstrate that a possibly misidentified strain can be authenticated by RAPD. The misidentified strain CCRC 32423 showed a pattern different from those of all the other strains of A. parasiticus and A. sojae in the RAPD reaction. These results suggest the possibility of using RAPD to distinguish the four similar taxa A. flavus, A. oryzae, A. sojae, and A. parasiticus.

The results of the bleomycin test have confirmed the sensitivity of A. parasiticus and A. sojae to this antimicrobial agent and can be used to differentiate these two species, but an economic and reliable method to distinguish A. parasiticus from A. sojae is still necessary. Furthermore, the characteristic of sensitivity to bleomycin cannot be used to distinguish A. flavus from A. oryzae (data not shown).

RAPD has been used to distinguish isolates below the species level, such as the human pathogen Aspergillus fumigatus (1) and the plant pathogen Fusarium solani f. sp. cucurbitae (2). The aflatoxin producer A. parasiticus and the kojih mold A. sojae are so closely related that Kurtzman et al. even indicated that they are same species (8). In this study, the RAPD method was proved to differentiate the two species successfully. Under accurate PCR conditions, the RAPD method was shown to be reproducible and less labor-intensive and to yield more-objective results than morphological observation. We thus suggest that the RAPD technique is a rapid and reliable tool to distinguish A. parasiticus from A. sojae and can be particularly used in choosing a safe strain for food fermentation.

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REFERENCES