DNA fingerprinting analysis of vegetative compatibility groups in *Aspergillus caelatus*

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Abstract: Forty-three isolates of *Aspergillus caelatus*, whose vegetative compatibility groups (VCGs) have been identified, were assessed by DNA fingerprinting using a repetitive sequence DNA probe (pAF28) cloned from *A. flavus*. Thirteen distinct DNA fingerprint groups or genotypes were identified among the 43 isolates. Twenty-four isolates belonging to VCG 1 produced identical DNA fingerprints and included isolates from the United States and Japan. Four other DNA fingerprint groups had multiple isolates sharing identical fingerprints corresponding to VCGs 2, 3, 12 and 13. Eight of the 13 fingerprint groups corresponding to VCGs 4–11 were represented by a single isolate with a unique fingerprint pattern. These results provide further confirmation that the pAF28 probe can distinguish VCGs of species within *Aspergillus* section *Flavi* based on DNA fingerprint patterns and that the probe can be used to estimate the number of VCGs in a sample population. Most of the *A. caelatus* isolates produced fewer restriction fragments and weakly hybridized with the repetitive DNA probe pAF28 compared to hybridization patterns obtained with *A. flavus*, suggesting less homology of the probe to *A. caelatus* genomic DNA.

Key words: genotypic diversity, hybridization patterns, probe, Southern blot, vegetative compatibility group

INTRODUCTION

*Aspergillus caelatus* is a newly described species belonging to *Aspergillus* section *Flavi* (Horn 1997). The species has been isolated from agricultural soils across the southern United States (Horn and Dorner 1998) from tea plantation soils in Japan (Peterson et al 2000) and from peanut seeds associated with insect-damaged pods in southwestern Georgia (Horn and Greene 1995, Horn et al 1996). *A. caelatus* shares several morphological characteristics with *A. tamarii*, but the metabolite profiles of the two species are quite different. All *A. caelatus* strains that have been tested produce kojic acid and no detectable cyclopiazonic acid, whereas *Aspergillus tamarii* produces cyclopiazonic acid and lesser amounts of kojic acid; neither species produces aflatoxins (Horn et al 1996).

*A. caelatus* and *A. tamarii* show 65% DNA complementarity, and the DNA sequences differ at three internal transcribed spacer (ITS) regions and at two nucleotides of the 5′ end of the large subunit ribosomal DNA (lsu-rDNA) (Peterson 2000).

Vegetative compatibility groups (VCGs) and molecular analysis techniques such as restriction fragment length polymorphism (RFLP) have become widespread as a means of studying genetic diversity of fungal populations and as useful tools for tracking isolates in nature. VCG analysis based on complementation between nitrate-nonutilizing (nit) mutants was used to study *Aspergillus flavus* populations in a Georgia peanut field (Horn and Greene 1995, Horn et al 1996), in an Arizona cotton field (Bayman and Cotty 1991, 1993) and in corn from Georgia (Papa 1986).

McAlpin and Mannarelli (1995) constructed the repetitive sequence DNA probe pAF28 from *A. flavus* NRRL 6541 that could hybridize to a homologous region of the *A. flavus* genome. Strains of *A. flavus* producing unique DNA fingerprints with the pAF28 probe are distinguished as different genotypes. The DNA probe was used successfully by McAlpin et al (2002) in identifying *A. flavus* strains as belonging to the VCGs previously determined by Horn and Greene (1995) and Papa (1986). The probe also has been used to characterize the genotypic diversity within a population of *A. flavus* (Wicklow et al 1998) and *Aspergillus parasiticus* (McAlpin et al 1998) from an Illinois cornfield as well as the diversity of *A. fla-
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**MATERIALS AND METHODS**

**Fungal strains.**—The *A. caelatus* strains used in this study were obtained from the Agricultural Research Service Culture Collection (National Center for Agricultural Utilization Research, Peoria, Illinois) and are listed in Table I with their corresponding VCG designations. The cultures include 32 isolates from soil and peanut seed samples collected from a field in southwestern Georgia (Horn and Greene 1995); eight isolates from soil samples collected from tea fields in Japan (Peterson et al 2000); two isolates from soil collected in agricultural fields in northern Louisiana (NRRL 26015) and central Mississippi (NRRL 26017) received from P.J. Cotty; and NRRL 26303 isolated from a peanut seed in Texas, and received from H.W. Schroeder. All 43 isolates were analyzed by DNA fingerprinting using a repetitive sequence DNA probe pAF28 cloned from *A. flavus* (McAlpin and Mannarelli 1995). The DNA fingerprint group assignment of each isolate was compared with its VCG designation as determined by complementation tests using *nit* mutants (Horn and Greene 1995).

**Media and cultural conditions.**—Myelia of *A. caelatus* were obtained by inoculating 500 mL flasks containing 200 mL of yeast extract-peptone-dextrose broth (YEPD) (McAlpin and Mannarelli 1995) with a conidial/hyphal suspension (ca. 10⁶ CFU/mL) from 5-7 d old cultures, then incubating 24 h at 32°C on a rotary shaker at 200 rpm. Myelia were harvested by filtering through sterile Whatman No. 1 filter paper in a Büchner funnel, rinsing twice with sterile distilled water, transferring to a 50 mL Sarstedt tube, freezing at −80°C and lyophilizing at least 24 h.

**DNA isolation and fingerprinting.**—DNA was isolated and purified based on the method of Raeder and Broda (1985) which was slightly modified (McAlpin and Mannarelli 1995). DNA from each of the *A. caelatus* isolates was digested with *Pst I* (Roche Molecular Biochemicals, Indianapolis, Indiana) as specified by the manufacturer and Southern blots were made with a vacuum blotter (Model 785, BioRad Laboratories, Hercules, California). Probes were labeled and DNA fingerprints were observed using the DIG Nonradioactive Nucleic Acid Labeling, Hybridization and Detection System (Roche Molecular Biochemicals). Membranes were exposed to X-ray films (BioMax MR, Eastman Kodak Imaging Systems, Rochester, New York) at room temperature 1–3 h.

**DNA fingerprint analyses.**—DNA hybridization bands representing different molecular weights were compared based on the presence or absence of fragments at a specific position. Banding patterns of strains were compared to determine the similarity or dissimilarity of the bands using the Dice coefficient (Nei and Li 1979). Using the NTSYS Numerical Taxonomy and Multivariate Analysis System (Rohlf 1997), the similarity matrix was obtained and cluster analysis with the SAHN program was made. The SAHN program identifies which strains show identical fingerprints but does not necessarily imply phylogenetic relationships. Phenetic analysis was performed to generate a phenogram using the unweighted-pair-group arithmetic average (UPGMA).

**Vegetative compatibility group analyses.**—The 32 isolates of *A. caelatus* from a single Georgia peanut field (Table I) previously were categorized into 10 VCGs (Horn and Greene 1995). For the additional 11 isolates from Japan, Louisiana, Mississippi and Texas, *nit* mutants were created and complementary *nit* mutants within each isolate were paired on a nitrate medium to ensure self compatibility (Horn and Greene 1995). Complementary *nit* mutants of the 11 strains were paired in all combinations and also were paired with tester *nit* mutants representing VCGs 1–10.

**RESULTS**

The pAF28 DNA probe was evaluated for its ability to distinguish among strains of *A. caelatus* and was found to differentiate successfully the 32 Georgia iso-
lates according to the 10 VCGs reported by Horn and Greene (1995). Three new VCGs (11–13) were identified among five isolates from Japan, Louisiana, Mississippi and Texas based on pairings of complementary nit mutants (Table I); VCGs 11–13 also demonstrated unique fingerprint groups. All strains belonging to the same VCG produced nearly identical DNA fingerprints. VCG 1 (Lanes 2–7, 9–19, 21–24) was the dominant genotype or VCG, which included strains from the United States and a tea field in Kochi Prefecture, Japan (Fig. 1A). Two isolates from a tea field in Shizuoka Prefecture, Japan, NRRL 25576 and 25577, had very similar fingerprints with strains in VCG 1 except for a few minor bands equivalent to 82% similarity but were incompatible vegetatively with VCG 1; therefore, VCG 12 was created to accommodate these strains (Fig. 1B).

VCG 2 (Lanes 2–5) was represented by four isolates (NRRL 26115, 26116, 26118, 26127), VCG 3 (Lanes 6–8) by three isolates (NRRL 26124, 26126, 26130), VCG 12 (Lanes 9–10) by two isolates (NRRL 25576, 25577), and VCG 13 (Lanes 11–12) by two isolates.
Fig. 1. A. DNA fingerprints of *Aspergillus caelatus* isolates belonging to the same vegetative compatibility group have nearly identical fingerprints. (Lane 1 = Lambda; Lanes 2-7, 9-19, 21-24 = VCG 1; Lanes 8 and 20 = NRRL 19997, reference strain.) B. *Aspergillus caelatus* isolates belonging to vegetative compatibility groups 2-13 and size marker (Lambda). (Lane 1 = Lambda; Lanes 2-5 = VCG 2; Lanes 6-8 = VCG 3; Lanes 9-10 = VCG 12; Lanes 11-12 = VCG 13; Lanes 13-20 = VCGs 4-11).
bands were difficult to decipher, requiring repeated Southern blots with more genomic DNA and longer film exposures to get darker bands.

**DISCUSSION**

*Aspergillus caelatus* isolates belonging to the same fingerprint group exhibited 100% similarity and were considered to be of the same genotype. Isolates within the same fingerprint group and sharing DNA profiles at greater than or equal to 80% similarity have been considered to belong to the same VCG (McAlpin et al. 1998, McAlpin et al. 2002). In this study, NRRL 25576 and 25577 from Japan were compatible and shared 82% similarity with the VCG 1 isolates. These two isolates belong to the same DNA fingerprint group based on the criterion used previously (Xia et al. 1993, Wicklow et al. 1998) and were clustered together in the same phenetic subgroup with VCG 1 (Fig. 2); however, they were incompatible with all of the VCG 1 strains. Isolates belonging to the same fingerprint group (similarity coefficient of 80% or more) may not necessarily be compatible because vegetative compatibility is governed by multiple vegetative incompatibility (het) loci. Heterokaryon formation can occur only between strains with identical alleles at each of the loci controlling vegetative compatibility (Caten 1971, Croft 1987). *A. caelatus* strains within a VCG are usually quite similar with respect to morphology and other characters (Horn et al. 1996). Therefore it is possible that the morphologically and genotypically similar isolates NRRL 25576 and 25577 have developed genetic changes in vegetative compatibility and that enough genetic divergence has occurred to impede heterokaryon formation (Anagnostakis and Waggoner 1981, Gordon and Okamoto 1991). Isolation due to differences in ecological niches may be responsible for differences in compatibility systems between genotypically similar strains as a result of accumulated mutations that restrict gene flow (Brasier and Hansen 1992, Harlton et al. 1995). Such highly similar clonal genotypes can be inferred to represent the same clonal lineage (Anderson and Kohn 1995).

Most of the *A. caelatus* isolates produced fewer restriction fragments and weakly hybridized with the repetitive DNA probe pAF28 compared to hybridization patterns obtained with *A. flavus*, suggesting less homology of the probe to *A. caelatus* genomic DNA. The pAF28 probe has been sequenced and it was determined that this 6.3 kb genomic insert from *A. flavus* encodes a retrotransposon-like element designated as ARTL-1 (Okubara et al. 2003), which includes motifs and open reading frames characteristic of mobile transposable elements of the gypsy class and similar retrotransposons reported in other fungi. The re-
duced ability of the pAF28 probe to bind with altered genomic DNA resulting from deletions and other types of mutations might explain the low intensity of the banding patterns in *A. caelatus*, as has been proposed for *A. bombycis* genotypes with faint banding patterns (Goto et al 2003).

VCG 1 was the dominant genotype of *A. caelatus* in this study and included isolates from both the United States and Japan. The presence of identical genotypes among isolates from two geographically distant countries suggests a strong clonal component in its lineage (Kistler 1997). The six *A. caelatus* isolates from a mature tea plantation in Kochi Prefecture belong to VCG 1, while two genotypically similar isolates from a tea plantation in Shizuoka Prefecture belong to VCG 12. These *A. caelatus* cultures were included among 57 isolates of *Aspergillus* section *Flavi* obtained from 27 of the 136 soil samples collected from tea fields in 10 Prefectures of Japan (Ito et al 1999). The prevalent microbial (fungal) populations in tea field soils are presumed to have become adapted to the edaphic environment of established tea plantations, which includes a negative rhizosphere effect, inhibitory compounds from decomposing leaf litter (Pandey and Palni 1996), and decades of annual NPK fertilizer applications resulting in soil acidification (Ito et al 1999). Clonal populations of *A. caelatus* VCG 1 and VCG 12 likewise might have become adapted to this long established and relatively undisturbed edaphic environment. The failure to demonstrate the presence of more than one *A. caelatus* genotype (VCG) among isolates from tea plantations in Kochi and Shizuoka Prefectures could point to: (i) an overall lack of *A. caelatus* genotypic diversity in Japan; (ii) the superior competitive ability of a founding population of VCG 1. The production of sclerotia would contribute to long-term survival while *Aspergillus* conidia function in dispersal and substrate colonization, thus precluding the establishment of potential competitors. Papa (1986) theorized that while wind dispersal of *A. flavus* conidia would allow for considerable mixing of genotypes within a given area, the amount of inoculum associated with measures of genotypic diversity could be maintained through the formation and overwintering of sclerotia. Persistent asexual reproductive structures such as conidia or sclerotia may remain stationary, producing an uneven distribution of genotypes in uncultivated native soils, but become spatially rearranged or mixed within fields when soil is cultivated (Gordon et al 1992, Anderson and Kohn 1995). While only *A. caelatus* VCG 1 was recorded from soil samples collected at different points with a tea plantation in Kochi Prefecture, Horn and Greene (1995) recorded VCG 1 along with nine other genotypes (VCGs) of *A. caelatus* from a single field cultivated to peanuts in southwestern Georgia. Fields where peanuts are grown also are planted with rotation crops such as corn, cotton and soybean, which require different tillage practices, fertilizers and pesticides. When considered together, these agronomic practices represent major and varied disturbances affecting soil microbial communities. The disruption of established microbial communities and the presence of a greater variety of crop residues for fungal colonization could present opportunities for less competitive genotypes to enter such crop fields. *A. caelatus* is not known from any natural ecosystem. Additional collections of *A. caelatus* from other areas of the world are necessary to characterize more fully the distribution of VCGs.

This research provided evidence that DNA fingerprinting using the repetitive DNA probe pAF28 can match DNA fingerprints with VCGs in *A. caelatus*, a species of *Aspergillus* from section *Flavi*. Some strains belonging to the same fingerprint group were incompatible with one another and therefore only 12 of the 13 VCGs were detected in *A. caelatus* by RFLP analysis. Species-specific retrotransposon-like repeated sequences represented by pAF28 have been shown to be highly reproducible and reliable for characterizing genetic populations in *A. fumigatus* Fresenius (Girardin et al 1993, Nuveeglise et al 1996), *A. flavus* (Wicklow et al 1998, James et al 2000, McAlpin et al 2002), *A. parasiticus* (McAlpin et al 1998), domesticated koji strains of *A. flavus* and *A. parasiticus* (Wicklow et al 2002) and *A. bombycis* isolates from silkworm cultivation (Goto et al 2003).

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**LITERATURE CITED**


