Assessment of Diversity in *Claviceps africana* and Other *Claviceps* Species by RAM and AFLP Analyses

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ABSTRACT

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Genetic diversity among isolates of *Claviceps africana*, the sorghum ergot pathogen, and isolates of other *Claviceps* spp. causing ergot on sorghum or other hosts, was analyzed by random amplified microsatellite (RAM) and amplified fragment length polymorphism (AFLP) analyses. Of the RAM primer sets tested, one revealed polymorphism in *C. africana* isolates, with Australian and Indian isolates possessing a unique fragment. AFLP analysis, in addition to clearly distinguishing *Claviceps* spp., revealed polymorphisms in *C. africana*. A group of isolates from the United States, Puerto Rico, and South Africa exhibited 95 to 100%

similarity with one another. Several isolates from Isabela, Puerto Rico were 100% similar to an isolate from Texas, and another isolate from Puerto Rico was identical with one from Nebraska. Australian and Indian isolates showed greater than 90% similarity with isolates from the United States, Puerto Rico, and South Africa. A number of polymorphisms existed in the United States group, indicating that the recently introduced population contains multiple genotypes. Isolates of *C. sorghicola*, a newly described sorghum pathogen from Japan, were very distinct from other species via RAM and AFLP analyses, as were isolates from outgroups *C. purpurea* and *C. fusiformis*. Both RAM and AFLP analysis will be useful in determining future patterns of intercontinental migration of the sorghum ergot pathogen, with the AFLP method showing greater ability to characterize levels of intraspecific variation.

Ergot of sorghum (*Sorghum bicolor* (L.) Moench) caused by *Claviceps africana* Frederickson, Mantle, and de Milliano (9) was first discovered in the Western Hemisphere in Brazil in 1995 (22), after initially being restricted to Asia and Africa (1,2,21). In the United States, sorghum ergot was found in the lower Rio Grande Valley of Texas in March 1997 (13) and was reported to occur in Mississippi in October 1997 as well (27). In that year, the disease spread rapidly through Texas and northward as far as Nebraska. The disease is currently a threat to sorghum production in several states including Texas, Kansas, Oklahoma, and Nebraska and has caused heavy losses in some areas.

The pathogen present in the United States was diagnosed as *C. africana* based on morphological characters and ergot alkaloid analysis (13). These criteria differentiate the sorghum ergot pathogen described from India as *C. sorghi* Kulkarni, Seshadri, and Hegde (15,18), and the African pathogen described as *C. africana* (9). *C. sorghicola* Tsukiboshi, Shimanuki, and Uematsu (24) is a third distinct species causing sorghum ergot recently described from Japan. *C. sorghicola* differs from *C. sorghi* and *C. africana* in the color and texture of sclerotia, color and morphology of stromata, and size of ascospores and conidia.

Concern for the production of hybrid seed as well as sorghum grain production has led to extensive efforts to better understand the biology of *C. africana* and determine ways to limit its destructive potential. The ergot pathogen infects sorghum at flowering and can substantially reduce seed set in commercial hybrid

seed-production fields. In grain production fields, ergot can attack tillers late in the season and present serious problems at harvest. Sticky honeydew (an exudate containing high concentrations of fungal conidia) is present on harvested seed and can foul equipment and interfere with harvest operations and further distribution of harvested seed.

Little is known of the genetic relationship among *Claviceps* spp. and particularly *C. africana* isolates causing sorghum ergot. Mycological studies have been performed with *C. africana* and *C. sorghi* from Africa and India (1,9,15,17), but molecular studies have been few. Recently, Pažoutová et al. (21) studied intraspecific variation in sorghum ergot isolates by random amplified polymorphic DNA (RAPD) analysis.

In 1997, we began collecting *C. africana* isolates from outbreaks in the United States and in other countries (Table 1). Using this collection, we analyzed the sequence of the internal transcribed spacer (ITS) regions and intron 3 of the β -tubulin gene (23) and determined that U.S. isolates could not be differentiated from *C. africana* isolates from other regions. *C. africana* isolates as a group were distinguished clearly from four other *Claviceps* spp., and polymerase chain reaction (PCR) primers were used for identification of the five species. Although these tools allowed interspecific comparisons of *Claviceps* spp., knowledge of intraspecific diversity in *C. africana* is needed to determine the relationships among U.S. and other isolates of this rapidly emerging pathogen. This information would help determine possible origins of U.S. isolates and to assess the level of genetic variation present in the U.S. *C. africana* population.

A wide variety of molecular methods exist to assess levels of intraspecific variation in plant pathogenic fungi including restriction fragment length polymorphisms (RFLPs), RAPDs, mitochondrial DNA polymorphisms, microsatellite analysis, amplified fragment length polymorphism (AFLP) analysis, and others (5,6,19). Random amplified microsatellite (RAM) analysis is

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PCR-based with primers containing microsatellite sequences and degenerate anchors at the 5' ends. This method was useful in describing variation in several groups of fungi (10), including *Peridermium pini*, the causal agent of the resin top disease of Scots pine (*Pinus sylvestris* L.) (11).

The AFLP technique (16,26) has been used for genetic characterization of a variety of organisms including bacteria, plants, and nematodes (3,8,12). Recent applications include genetic characterization of plant pathogenic fungi (7,20,25). Within the fungal genera *Colletotrichum*, *Pleospora*, and *Dendryphion*, the AFLP method allowed assessment of pathogen diversity and species determination (7,20).

In this study, we have applied RAM and AFLP techniques to *C. africana* isolates collected from diverse sources to determine relationships among isolates and to assess levels of genetic variation in this new pathogen.

MATERIALS AND METHODS

Isolates of *Claviceps* spp. were obtained from various sources or isolated directly from diseased sorghum submitted to our laboratory (Table 1). Cultures were established by diluting honeydew (≈ 100 -fold) in sterile-distilled water and streaking them on plates of 2% water agar containing 100 μ g/ml of ampicillin and 100 μ g/ml of streptomycin sulfate. Plates were incubated at 22°C in the dark and individually germinated conidia were transferred to plates of potato dextrose agar where they grew colonies. When samples had low conidial viability, a conidial suspension made in sterile-distilled water was used to inoculate flowering heads of susceptible sorghum plants (male sterile lines aTX 623 and aTX 2752, obtained from G. Odvody and D. Frederiksen, Texas A&M University, Corpus Christi) grown in the USDA-ARS containment greenhouse complex at Ft. Detrick, MD. Fresh honeydew produced on inoculated heads was used to establish single-conidial cultures as described previously. *C. africana* isolates were sent in pure culture from Australia by M. Ryley (Queensland Department of Primary Industries). Pure cultures of isolates of *C. purpurea*, *C. fusiformis*, and *Pleospora papaveracea* (de not.) Sacc. (7) used for outgroup comparisons were obtained from the American Type Culture Collection and collected by the authors.

RAM analysis. The primers used were as described in Hantula et al. (10) and designated by target repeat sequences ACA, CCA, CGA, and GT. PCR was performed in a thermal cycler (Model 9600; Perkin-Elmer, Foster City, CA) with temperature profiles as described in Hantula et al. (10). Annealing temperature was dependent on the specific primer used: 64°C for CCA, 61°C for CGA, 58°C for GT, and 49°C for ACA. PCR products were separated in 1.4% agarose gels in 0.5 $\times$ Tris-borate-EDTA buffer and stained with ethidium bromide.

AFLP analysis. AFLPs were generated with the AFLP Analysis System II (Life Technologies Inc., Gaithersburg, MD) with a modified protocol (16). The primary template was prepared by digesting 250 ng of fungal genomic DNA with *EcoRI* and *MseI* to completion and heating to 70°C for 15 min to inactivate the enzymes. The DNA fragments were ligated to the *EcoRI* and *MseI* adapters provided in the kit for 2 h at 200°C. The ligation mixture was diluted 10-fold with sterile-distilled water, and the fragments were preamplified by 20 PCR cycles with primers in preamp primer mix II (Life Technologies) (26). The preselective amplification was performed in a Perkin-Elmer 2400 thermal cycler with a temperature profile of 94°C for 30 s, 56°C for 60 s, and 72°C for 60 s.

Selective primers were obtained from Life Technologies. Preliminary experiments using *MseI* + C combined with *EcoRI* + AG or *EcoRI* + AT resulted in the generation of many amplified DNAs, and *EcoRI* + AG was selected for further use. Five-microliter subsamples of the preamplified fragments were diluted 50-fold and used with unlabeled *MseI* + C primers, and primer *EcoRI* + AG ³²P-labeled by the AFLP Analysis System I protocol (Life

Technologies). The PCR amplification temperature profile was one cycle at 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s. The annealing temperature was lowered for each cycle by 0.7°C for 12 additional cycles; the remainder of the amplification was 23 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s. The PCR products in 2.5- μ l subsamples were separated by electrophoresis with a denaturing (6%, wt/vol) polyacrylamide DNA sequencing gel containing 7.5 M urea. Autoradiographs were obtained by exposing BioMax MR-2 film (Eastman Kodak Co., Rochester, NY) to the dried gel overnight at room temperature.

Autoradiographs were scanned on a computer to score each lane for the presence or absence of bands between molecular sizes of 75 and 295 bp using Proscore (Version 2.36; DNA Proscan Inc., Nashville, TN). Only polymorphisms that could be scored unequivocally for presence or absence of bands were included in the analyses; faint bands were never scored as absent. Binary matrices consisting of 0's (denoting absence of a given band) and 1's (denoting presence of a given band) were analyzed to obtain simple matching coefficients among the isolates using NTSYS-pc,

TABLE 1. *Claviceps* spp. and *Pleospora* sp. used in this study

Species and isolate ^a	Source	Host	Date ^b
<i>C. purpurea</i>			
Clp-1 (34699)	Montana	<i>Hordeum vulgare</i>	NF
Clp-2 (34501)	East Germany	<i>Secale cereale</i>	NF
<i>C. fusiformis</i>			
Clf-1 (26019)	Africa	<i>Pennisetum typhoideum</i>	NF
<i>C. africana</i>			
Cls-1	Patancheru, India	<i>Sorghum bicolor</i>	1997
Cls-2	Akola, India	<i>Sorghum bicolor</i>	1997
Cls-3	Dharwar, India	<i>Sorghum bicolor</i>	1997
Cls-4	Patancheru, India	<i>Sorghum bicolor</i>	1997
Cla-1	Queensland, Australia	<i>Sorghum bicolor</i>	1996
Cla-2	Queensland, Australia	<i>Sorghum bicolor</i>	1996
Cla-3	Queensland, Australia	<i>Sorghum bicolor</i>	1997
Cla-4	College Station, Texas	<i>Sorghum bicolor</i>	1997
Cla-5	College Station, Texas	<i>Sorghum bicolor</i>	1997
Cla-7	Isabela, Puerto Rico	<i>Sorghum bicolor</i>	1997
Cla-8	Isabela, Puerto Rico	<i>Sorghum bicolor</i>	1997
Cla-9	Weslaco, Texas	<i>Sorghum bicolor</i>	1998
Cla-10	Weslaco, Texas	<i>Sorghum bicolor</i>	1998
Cla-12	Isabela, Puerto Rico	<i>Sorghum bicolor</i>	1998
Cla-14	Isabela, Puerto Rico	<i>Sorghum bicolor</i>	1998
Cla-17	Isabela, Puerto Rico	<i>Sorghum bicolor</i>	1998
Cla-25	Isabela, Puerto Rico	<i>Sorghum bicolor</i>	1998
Cla-34	Guayanilla, Puerto Rico	<i>Sorghum bicolor</i>	1998
Cla-42	Virginia, Nebraska	<i>Sorghum bicolor</i>	1997
Cla-49	Hale Center, Texas	<i>Sorghum bicolor</i>	1998
Cla-56	Potchefstroom, South Africa	<i>Sorghum bicolor</i>	1998
Cla-58	Potchefstroom, South Africa	<i>Sorghum bicolor</i>	1998
<i>C. sorghicola</i>			
Cjap-2	Nishinasuno, Tochigi, Japan	<i>Sorghum bicolor</i>	1996
Cjap-4	Nishinasuno, Tochigi, Japan	<i>Sorghum bicolor</i>	1997
Cjap-6	Nishinasuno, Tochigi, Japan	<i>Sorghum bicolor</i>	1996
Cjap-7	Nishinasuno, Tochigi, Japan	<i>Sorghum bicolor</i>	1996
<i>P. papaveracea</i>			
251083-1	Afghanistan	<i>Papaver somniferum</i>	NF

^a Isolates Cls-1 to Cls-4 provided by R. Bandyopadhyay (ICRISAT, Patancheru, India) and isolates Cla-2 and Cla-3 provided by M. Ryley (Queensland Dept. of Primary Industries, Australia). Other *C. africana* isolates were obtained from ergot-infected sorghum provided by R. Frederiksen, G. Odvody, J. Dahlberg, R. Bandyopadhyay, S. Jensen, N. McClaren, and T. Isakeit. Isolates Cjap-2, Cjap-4, Cjap-6, and Cjap-7 were provided by T. Tsukiboshi (National Institute of Agro-Environmental Sciences, Tsukuba, Ibaraki, Japan).

^b Not found.

Fig. 1. Polymerase chain reaction of isolates of four *Claviceps* spp. by **A**, random amplified microsatellite (RAM) primer set CCA; **B**, RAM primer set CGA; and **C**, RAM primer set GT. For **A** through **C**, Lane 1 contains size marker ϕ X174 RF DNA/*Hae*III digest; Lane 2 indicates isolate Clp-1 (*C. purpurea*); Lane 3 indicates isolate Clf-1 (*C. fusiformis*); Lane 4 indicates isolate Cls-1 (*C. africana*, India); Lane 5 indicates isolate Cla-2 (*C. africana*, Australia); Lane 6 indicates isolate Cla-5 (*C. africana*, TX); and Lane 7 indicates isolate Cjap-2 (*C. sorghicola*, Japan).

(version 2.0; Exeter Biological Software, Setauket, NY). Simple matching coefficients were clustered to generate similarity trees using the SAHN clustering program, selecting the unweighted pair-group method with arithmetic average (UPGMA) algorithm in NTSYS-pc.

RESULTS

All four sets of RAM primers provided reproducible banding patterns that allowed specie differentiation (Fig. 1). Experiments were repeated at least three times with identical results. Bands ranged from ≈ 200 to $\approx 2,000$ bp, and characteristic bands were observed for each species (Fig. 1). Using RAM primer set CGA, only a few band differences were observed in *C. africana*, notably a DNA fragment $\approx 1,200$ bp that was present in U.S. isolates but not present in isolates from India or Australia (Fig. 2).

AFLP analysis provided a large number of polymorphic bands for isolate and species comparisons (Fig. 3). The total number of scorable DNA fragments across isolates within the range of 75 to 295 bp was 125 for the AFLP analyses with primer *Eco*RI + AG in combination with *Mse*I + C, and resulted in a matrix with 3,500 data points. The PCR products of molecular sizes larger than 295 bp were not scored, because resolution was insufficient to discriminate between bands of different molecular sizes. AFLP patterns were highly reproducible, and experiments were repeated at least twice in two laboratories with identical results.

Claviceps spp. showed widely divergent AFLP banding patterns (Fig. 3), and when AFLP data were analyzed by the UPGMA algorithm in NYSYS-pc, *Claviceps* spp. showed similar values ranging from less than 60 to $\approx 75\%$ (Fig. 4). *C. sorghicola*, the recently described species causing sorghum ergot in Japan (24), was more closely related by AFLP analysis to *C. africana* than were *C. purpurea* or *C. fusiformis*, which attack other hosts (Fig. 4).

AFLP variation within the four *C. sorghicola* isolates analyzed was less than that observed in *C. africana*, which ranged from 90 to 100%. *C. africana* isolates from Australia (Cla-2 and Cla-3) shared a very prominent DNA fragment ≈ 120 bp in size with isolates from India (Cls-1 to Cls-4 [Fig. 3, arrow]). *C. africana* isolates from South Africa (Cla-56 and Cla-58) were nearly identical to those from the United States and shared an 111-bp band not present in either Australian (Cla-2 and Cla-3) or Indian (Cls-1 through Cls-4) isolates (Fig. 3, arrow). The four *C. africana* isolates from India clustered together and showed $\approx 90\%$ similarity with *C. africana* isolates from Australia, the United States, Puerto

Fig. 2. Polymerase chain reaction of isolates of *Claviceps africana* by random amplified microsatellite primer set CGA. Lane 1 contains size marker ϕ X174 RF DNA/*Hae*III digest; Lanes 2-5 contain Indian isolates Cls-1, Cls-2, Cls-3, and Cls-4; Lanes 6-9 contain Australian isolates Cla-1, Cla-2, and Cla-3; Lanes 10-19 contain U.S. isolates Cla-4, Cla-5, Cla-7, Cla-8, Cla-9, Cla-10, Cla-12, Cla-14, and Cla-17, respectively. Specific band indicated by arrow in U.S. isolate group and not present in Indian or Australian isolates.

Rico, and South Africa (Fig. 4). The first isolate obtained from infected sorghum in Nebraska in 1997 (Cla-42) showed 100% similarity with isolate Cla-34, which originated from the South Coast region (Guayanilla) of Puerto Rico in 1998, and isolates Cla-4, Cla-5, Cla-10, and Cla-49 from three different locations in Texas showed high similarity (95 to 100%) with isolates Cla-7, Cla-8, Cla-12, Cla-14, Cla-17, and Cla-25 from Isabela in northwestern Puerto Rico (Fig. 4).

DISCUSSION

We used RAM and AFLP analysis to perform inter and intraspecific comparisons among isolates of *Claviceps* spp. causing sorghum ergot in the United States and elsewhere (1,2,21,24). Both RAM and AFLP analyses yielded specific and reproducible banding patterns for isolates and species. Thus, both methods were useful in distinguishing *Claviceps* spp. from one another and in assessing similarity among isolates. The use of RAM primer sets allowed clear discrimination of species, whereas use of the CGA RAM primer set allowed separation of *C. africana* isolates into two groups based on presence or absence of a specific band. Indian and Australian isolates lacked the DNA fragment of $\approx 1,200$ bp that was present in U.S. isolates. The small number of polymorphic bands in *C. africana* limited genetic variation assessment for RAM analyses.

Claviceps isolate and species relationships observed by RAM analysis were supported by AFLP analysis at a higher level of resolution. *C. purpurea*, *C. fusiformis*, and *C. sorghicola* showed less than 75% similarity with *C. africana* isolates. *C. sorghicola*, the sorghum pathogen recently described from Japan is distinct from *C. africana* in terms of morphological (24) and molecular (23) characters. It showed a higher degree of similarity with *C. africana* ($\approx 75\%$) based on AFLP analysis than did *C. purpurea* or *C. fusiformis*, which attack other hosts.

Fig. 3. Gel electrophoresis autoradiograph resulting from amplified fragment length polymorphism analysis of 27 isolates of *Claviceps* spp. and outgroup species *Pleospora pappaveracea*. Selective amplification was performed with primer combination *EcoRI* + AG and *MseI* + C. Molecular sizes (base pairs) are indicated at the left. Arrows indicate polymorphic bands that distinguish Australian and Indian isolates from U.S. and South African isolates.

In *C. africana* isolates collected from different parts of the world there was 90 to 100% similarity according to AFLP analysis (Fig. 4), indicating a close taxonomic affinity. Indian *C. africana* isolates (Cls-1 through Cls-4) clustered with $>95\%$ similarity in the AFLP analysis, and showed $\approx 90\%$ similarity with other *C. africana* isolates. Previously, the Indian isolates showed identity with other *C. africana* isolates in terms of β -tubulin and ITS sequences (23). Thus, they represent *C. africana* rather than *C. sorghi*. The same Indian isolates we analyzed were also identified as *C. africana* by Bogó and Mantle (4) in describing the occurrence of *C. africana* in India based on morphology and presence of the alkaloid dihydroergosine.

Many isolates showed unique AFLP bands that distinguished them from other isolates with the same geographic origin. Thus, a substantial amount of genetic variation exists in *C. africana* from diverse sources. Even in the U.S. population of isolates, some variation (above 95% similarity) was observed indicating several clonal genotypes of the pathogen. Variation in U.S. isolates was not observed with RAPDs (21), although all U.S. isolates tested in that study were collected in Texas in the same year.

Indian and Australian isolates showed $\approx 90\%$ similarity with one another and shared several polymorphic bands (Fig. 3, arrows). This result supports the relationship between isolates from these two locations as observed by RAM analysis and in agreement with the results of Pažoutová et al. (21), who found that Indian and Australian isolates grouped together according to RAPD analysis.

Isolates from different parts of Texas, as well as a single isolate from Nebraska showed high levels of similarity (at or near 100%) with isolates from Puerto Rico. Puerto Rican samples were ob-

Fig. 4. Dendrogram showing similarities among isolates and species of *Claviceps* based on amplified fragment length polymorphism analysis using simple matching coefficients. The tree was generated using the SAHN clustering program with the unweighted pair-group method with arithmetic average algorithm in NTSYS-pc. Polymerase chain reaction products between molecular sizes of 75 and 295 bp were scored for the analysis. Species names: C. afr. = *C. africana*; C. sor. = *C. sorghicola*; P. papaver. = *P. pappaveracea*.

tained from two areas—Isabela in the Northwest part of the country and Guayanilla on the South Coast. Cla-34 from Guayanilla showed an identical AFLP pattern with Cla-42, which originated from Nebraska during the first ergot outbreak in that state in 1997, whereas Cla-8 and Cla-14 from Isabela showed an identical pattern with Cla-5 from the original 1997 epidemic in Texas. These samples may represent the genotypes first responsible for infecting U.S. sorghum in 1997. The data suggest that ergot could have spread from Puerto Rico to Texas and other parts of the U.S. in 1997 causing the initial U.S. infestation or that the two 1997 epidemics (Puerto Rico and mainland United States) were initiated by the same clonal genotype originating from a third location. The fact that the U.S. isolates were more similar (95%) to isolates from South Africa than to Indian or Australian isolates may implicate Africa as the original source of isolates causing the 1997 epidemics in the mainland United States and Puerto Rico (2,13). Supporting this theory is the fact that isolates with identical RAPD patterns were found in South Africa and the Americas (21).

The degree of resolution we obtained with AFLPs for intraspecific analysis parallels results of the population analysis performed on *C. purpurea* by others using RAPDs (14). These results showed that a single RAPD primer was able to distinguish more than 20 *C. purpurea* isolates and that most strains from specific hosts grouped together.

Our studies assessing population diversity and comparing isolates of the sorghum ergot pathogen by RAM and AFLP analyses provide knowledge of the genetic makeup of the *C. africana* population now present in the United States. Furthermore, the methods should prove useful in additional studies of ergot species diversity and in monitoring pathogen isolates in the future. Further analysis of greater numbers of isolates from diverse regions including Africa, India, Australia, South and Central America, and the United States will help determine the potential source of sorghum ergot in the United States and provide more data for assessing levels of genotypic diversity in this rapidly emerging pathogen.

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