29

Intraspecific Variation in *Claviceps africana*

Paul W. Tooley and Nichole R. O'Neil

Abstract

We analyzed genetic variability within a global collection of the sorghum ergot pathogen *Claviceps africana* by using DNA sequence analysis, random amplified microsatellite (RAM) analysis, and amplified fragment length polymorphism (AFLP) analysis. Distinct groups of *Claviceps* species could be resolved based on the sequence of β-tubulin and elongation factor 1α (EF-1α) gene sequences, and PCR primers were designed for specific detection of *C. africana* and other species. AFLP analysis allowed assessment of intraspecific variation in *C. africana*, and high levels of genetic similarity were observed among Australian/Indian/Japanese isolates and U.S./Mexican/African isolates. Within individual populations, including the one in the United States, AFLP polymorphisms were observed, which indicates that multiple *C. africana* genotypes are present.

Introduction

Sorghum ergot, caused by *Claviceps africana*, has recently become established in the United States. The pathogen initially was restricted to Asia and Africa, but has since spread to South America, Australia, Japan, the United States, and other regions (2, 18). In 1997, ergot was found in Puerto Rico as well as the lower Rio Grande Valley of Texas (11) and spread northward as far as Nebraska and eastward to Mississippi (25). Sorghum ergot threatens sorghum production in a number of states and is of concern to the sorghum industry.

Population genetic analysis of the sorghum ergot pathogen using molecular markers could help elucidate which species may be present in the United States, enable comparisons of isolates found in the United States with those from other regions, and lead to a rapid means of pathogen detection. However, *Claviceps* is not a widely studied genus, and the number of studies of the genetics and population structure of *C. africana* is not large.

Claviceps purpurea is the most widely studied species in the genus *Claviceps* (6, 7, 12). Levels of genetic variation within *C. purpurea* (12) and *C. africana* (18) have been assessed with random amplified polymorphic DNA (RAPD). In *C. purpurea*, DNA fragments resulting from PCR amplification of genomic DNA with a single RAPD primer distinguished more than 20 *C. purpurea* isolates, with most strains from specific hosts grouping together. In 28 geographically diverse *C. africana* isolates, 65 RAPD primers amplified uniform, species-specific patterns, and seven primers could be used to distinguish four groups of isolates, but little variation was observed within groups (18). In terms of molecular taxonomy, phylogenetic analyses comparing ribosomal DNA sequences place the Clavicipitales within the unitunicate perithecial ascomycetes (8, 14, 20).

The amplified fragment length polymorphism (AFLP) technique (24) is a relatively new DNA fingerprinting technique that has become a valuable and powerful method to study population structure in fungi. The AFLP technique selectively amplifies a small portion, usually < 0.4%, of the DNA fragments resulting from a complete double restriction enzyme digest of genomic DNA. AFLPs have several advantages relative to other tools commonly used to assess genetic variation, including high reproducibility, large information content per reaction, theoretical neutrality of the markers detected, and dispersal of markers detected apparently randomly throughout the entire genome (15). AFLP analysis has become a valuable tool for identifying previously undetected levels of genetic diversity, especially among isolates within a species of fungal pathogens, and for identifying genetic markers associated with important biological, ecological, and phylogenetic characters (1, 17, 19). We analyzed strains of *C. africana* with AFLPs and found that the technique provided significant information about genetic variation within the ergot pathogen that was consistent with the results of RAPD analysis (18) in reconstructing recent patterns of pathogen migration.

Cultural Variation in *C. africana*

We have 233, mostly single conidial, isolates of *C. africana* in our collection, with 80 from the mainland United States, 33 from Puerto Rico, 51 from India, 14 from Australia, 17 from Africa, 9 from Japan, 8 from Mexico, 11 from Brazil, 5 from El Salvador, and 5 from Thailand, that we can work with in specialized containment facilities (16) not available elsewhere in the United States. Isolates are stored cryogenically at the Central Cell Repository of the National Cancer Institute at Ft. Detrick, Maryland both as dry mycelial plugs (from PDA cultures) and in 10% dimethyl-sulfoxide cryoprotectant. As outgroup species, we use *C. purpurea* (ATCC 34699, ATCC 34501), *Claviceps fusiformis* (ATCC 26019), *Claviceps sorghicola* (23), and *Claviceps paspali* from *Paspalum* grasses.

C. purpurea and *C. fusiformis* grow faster than *C. africana*, and *C. fusiformis* has "feathery" colony edges. *C. sorghicola* has a "smooth" appearance and produces a brown pigment, which is especially prominent on the underside of the colony. Cultures of *C. africana* from different geographic locations grown on potato dextrose agar at 22-24°C in darkness vary in growth rate (0.79-2.54 mm/day) and colony morphology. Cultures from Texas grow slowly, with irregular margins and lumpy colony surfaces, while those from Kansas include some with similar morphology to those from Texas as well as others with a "raised" colony morphology and/or feathery colony margins. Indian and Australian isolates generally have smooth colony surfaces and less restricted growth. Thai cultures have slow-growing, hard, dark, brown areas interspersed with smooth or lumpy, white areas of growth. We also have cultures that are intermediate between the smooth and lumpy phenoltypes and/or that appear to sector for this phenotype.

Molecular Analysis of *Claviceps africana*

We used three different molecular techniques to characterize genetic variation in *C. africana* including DNA sequence analysis, RAM analysis, and AFLP analysis. We cloned and sequenced the β-tubulin intron 3 region of 40 *Claviceps* isolates representing five species, and the EF-1α intron 4 region (21) of 11 isolates from the same five species. A very small amount of intra-specific variation was observed in the two regions, and the differences in sequence could be used to separate the five *Claviceps* species.

We also used RAM and AFLP analyses to assess intraspecific variation in *C. africana*. RAM analysis (9, 10) is a PCR-based method that uses primers containing microsatellite sequences and degenerate anchors at the 5′ ends, and has been used to evaluate variation in several fungal species (9, 10). We used the primers described by Hantula *et al.* (9) and named by the target repeat sequences: ACA, CCA, CGA, and GT. PCR was performed in a Perkin-Elmer 9600 thermal cycler with previously described reaction conditions (9). The annealing temperature used depended upon the 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 × TBE buffer and stained with ethidium bromide. Three sets of RAM primers gave reproducible banding patterns and could be used to differentiate species. Amplified DNA fragments ranged from ~200-2000 bp in length; characteristic bands were observed for each species (22).

AFLPs were generated for *C. africana* (22) with the AFLP Analysis System II (Life Technologies Inc., Gaithersburg, MD). The AFLP reaction products were labeled with ^{32}P and separated by electrophoresis through a denaturing 6% (w/v) polyacrylamide DNA sequencing gel containing 7.5 M urea. Autoradiographs were obtained by exposing Kodak BioMax MR-2 film (Eastman Kodak Co., Rochester, NY) to the dried gel overnight at room temperature. Autoradiographs were scanned and each lane scored for the presence or absence of bands. Bands of 75-295 bp were scored by using Proscore version 2.36 (DNA Proscan Inc., Nashville, TN). Binary matrices consisting of "0"s (denoting absence of a band) and "1"s (denoting presence of the band) were analyzed to obtain simple matching coefficients among the isolates by using NTSYS-pc, version 2.0 (Exeter Biological Software, Setauket, New York). Simple matching coefficients were clustered to generate similarity trees using the SAHN clustering option and the unweighted pair-group method with arithmetic average (UPGMA) algorithm in NTSYS-pc. We analyzed 20 *C. africana* isolates plus isolates of *C. purpurea*, *C. fusiformis*, *C. sorghicola*, and *Pleospora papaveraceae*. Selective primers used were *Mse*I+C and *Eco*RI+AG, and the resulting AFLP patterns were highly reproducible.

When AFLP data were analyzed using the UPGMA algorithm within NTSYS-pc (Fig. 29-1), different *Claviceps* species ranged in similarity from ≤ 60% to approximately 75%. Based on AFLP analysis, *C. sorghicola*, which causes sorghum ergot in Japan, was more closely related to *C. africana* than were either *C. purpurea* or *C. fusiformis* (22). Indian and Australian isolates also were approximately 90% similar, and shared several polymorphic bands. These findings and those from the RAM data and the RAPD data (18) all are consistent with the hypothesis that the Indian and Australian isolates are closely related. *C. africana* isolates from South Africa (Cla-56 and Cla-58) were nearly identical to those from the United States, and shared a 111 bp band not present in either the Australian (Cla-2 and Cla-3) or Indian (Cls-1 through Cls-4) isolates.

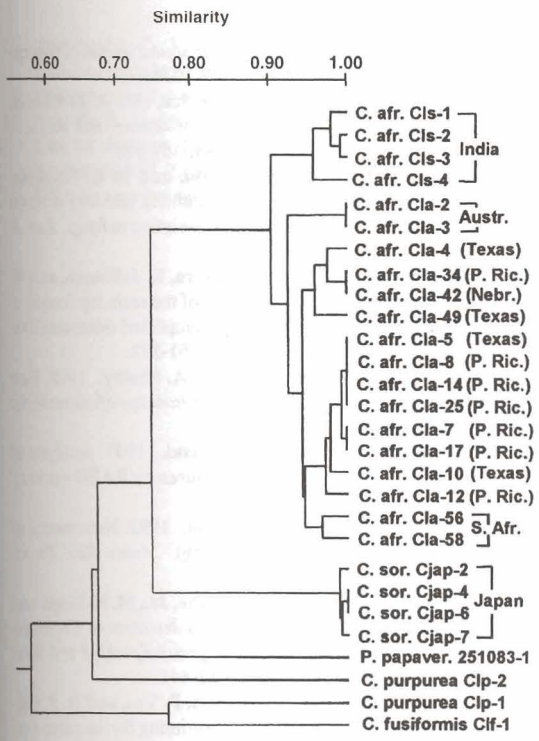

FIGURE 29-1. Dendrogram showing similarities among isolates and species of *Claviceps* based on AFLP analysis and simple matching coefficients. The tree was generated using the SAHN clustering program with the unweighted pair-group method with arithmetic average (UPGMA) algorithm in NTSYS-pc, version 2.0. PCR products between molecular sizes 75 and 295 bp were scored for the analysis. Species names are abbreviated as: C. afr., *Claviceps africana*; C. sor., *Claviceps sorghicola*; P. papaver, *Pleospora papaveracea* (5). Reprinted from (22) with the permission of the American Phytopathological Society.

The UPGMA dendrogram generated from the AFLP fingerprints has the four *C. africana* isolates from India in one cluster, which, as a group, had approximately 90% similarity to *C. africana* isolates from Australia, the United States, Puerto Rico, and South Africa (Fig. 29-1). The first isolate we obtained from infected sorghum in Nebraska in 1997 (Cla-42) was identical to Cla-34, which originated from the South Coast region (Guayanilla) of Puerto Rico in 1998. Isolates Cla-4, Cla-5, Cla-10, and Cla-49 from three different locations in Texas were 95-100% similar to isolates Cla-7, Cla-8, Cla-12, Cla-14, Cla-17, and Cla-25 from Isabela, in northwestern Puerto Rico (Fig. 29-1).

Indian *C. africana* isolates Cls-1 through Cls-4 clustered with > 95% similarity in the AFLP dendrogram, and were approximately 90% similar to other *C. africana* isolates. These Indian isolates were identical to other *C. africana* isolates based on β-tubulin and EF-1α sequences (21). Thus, we consider these isolates to be *C. africana* rather than *C. sorghi*. These Indian isolates also were identified as *C. africana* by Bogo and Mantle (3) based on morphology and the presence of the alkaloid dihydroergosine.

Some isolates had unique AFLP bands that distinguished them from other isolates from the same geographic location. Thus, a substantial amount of genetic variation exists within *C. africana*. Even amongst the isolates from the United States, there is some variation (isolates are approximately 95% similar), which indicates that several clonal genotypes of the pathogen are present.

In 1997, ergot was observed in Mexico prior to being found in the mainland United States. Puerto Rican isolates could have originated from the mainland United States following its establishment there, or from a third location, which could have been the source for the epidemics in Mexico and the mainland United States. The high (95%) similarity between isolates from South Africa and the United States implicates Africa, rather than India or Australia, as the original source of the isolates responsible for the 1997 epidemics in Puerto Rico and the mainland United States. Support for this hypothesis includes the recovery of isolates with identical RAPD patterns from both South Africa and the Americas (18). Based on RAPDs (18), isolates from the United States all represented a single clone, although the isolates tested were collected in Texas in a single year.

Conclusions and Future Research

AFLP analysis of *C. africana* isolates shows that Indian, Australian, and Japanese isolates, and U.S., Mexican, and African isolates are related to one another but fall into two groups. These results are consistent with those obtained by RAPD analysis (18) and reflect existing patterns in sorghum seed trade. Within each of these groups, AFLP variation exists, which indicates the presence of multiple genotypes in each region. Mechanisms responsible for the observed variation could include mutation, or more recently characterized modes of genetic change such as chromosome alterations, cytoplasmically transmitted genetic elements, or transposon activity (13). The parasexual cycle can occur in *Claviceps* sp. (4), but it is not known if it occurs in *C. africana*. Greater knowledge of the role of the sexual cycle, and whether it occurs in areas of recent introduction, *e.g.*, the United States, could help estimate the pathogen's ability to rearrange existing genetic variation and form new genotypes that could complicate the deployment of long-term control measures.

The AFLP method should be useful in further studies of ergot species diversity and in the monitoring of pathogen migrations. Further analysis of greater numbers of isolates from diverse regions including Africa, India, Australia, South and Central America, and the United States will allow associations of AFLP patterns with pathotype diversity, host of origin, and/or geographic origin to be made. The resulting associations may help predict future migrations and changes in the population composition of this potentially rapidly spreading pathogen. Furthermore, analysis of genotypes that are present regionally over a period of years can be used to determine whether the same genotypes cause disease every year, whether the pathogen can survive between seasons on alternative hosts, *e.g.*, johnsongrass, and whether initial inoculum is composed of immigrant genotypes from other regions. These data will provide a better overall picture of the population genetics and dynamics of *C. africana* in regions of new introduction and lead to predictions regarding the epidemiology of the disease.

Acknowledgements

We thank Richard E. Fredericksen, Debra E. Frederickson, Tom Isakeit, Jeff A. Dahlberg, Ranajit Bandyopadhyay, Gary N. Odvody, Malcolm J. Ryley, T. Tsukiboshi, Stan Jensen, and Neal McClaren for supplying infected sorghum and/or *Claviceps* cultures.

References

1. **Baayen, R. P., K. O'Donnell, P. J. M. Bonants, E. Cigelnik, L. P. N. M. Kroon, E. J. A. Roebroeck, and C. Waalwijk.** 2000. Gene geneologies and AFLP analyses in the *Fusarium oxysporum* complex identify monophyletic and nonmonophyletic formae speciales causing wilt and rot disease. *Phytopathology* **90:** 891-900.
2. **Bandyopadhyay, R., D. E. Frederickson, N. W. McLaren, G. N. Odvody, and M. J. Ryley.** 1998. Ergot: A new disease threat to sorghum in the Americas and Australia. *Plant Dis.* **82:** 356-367.
3. **Bogo, A., and P. G. Mantle.** 1999. *Claviceps africana* discovered in India. *Plant Dis.* **83:** 79.
4. **Brauer, K. L., and J. E. Robbers.** 1987. Induced parasexual processes in *Claviceps* sp. strain SD58. *Appl. Environ. Microbiol.* **53:** 70-73.
5. **Farr, D. F., N. R. O'Neill, and P. B. van Berkum.** 2000. Morphological and molecular studies on *Dendryphion penicillatum* and *Pleospora papaveracea*, pathogens of *Papaver somniferum*. *Mycologia* **92:** 145-153.
6. **Garre, V., U. Muller, and P. Tudzynski.** 1998. Cloning, characterization, and targeted disruption of *cpcat1*, coding for an *in planta* secreted catalase of *Claviceps purpurea*. *Molec. Plant-Microbe Interact.* **11:** 772-783.
7. **Giesbert, S., H. B. Lepping, K. B. Tenberge, and P. Tudzynski.** 1998. The xylanolytic system of *Claviceps purpurea*: Cytological evidence for secretion of xylanases in infected rye tissue and molecular characterization of two xylanase genes. *Phytopathology* **88:** 1020-1030.
8. **Glenn, A. E., C. W. Bacon, R. Price, and R. T. Hanlin.** 1996. Molecular phylogeny of *Acremonium* and its taxonomic implications. *Mycologia* **88:** 369-383.
9. **Hantula, J., M. Dusabenyagasani, and R. C. Hamelin.** 1996. Random amplified microsatellites (RAMs) a novel method for characterizing genetic variation in fungi. *Eur. J. For. Pathol.* **26:** 159-166.
10. **Hantula, J., E. M. Niemi, J. Kaitera, R. Jalkanen, and T. Kurkela.** 1998. Genetic variation of the resin top fungus in Finland as determined by random amplified microsatellites (RAMs). *Eur. J. For. Pathol.* **28:** 361-372.
11. **Isakeit, T., G. N. Odvody, and R. A. Shelby.** 1998. First report of sorghum ergot caused by *Claviceps africana* in the United States. *Plant Dis.* **82:** 592.
12. **Jungehulsing, U., and P. Tudzynski.** 1997. Analysis of genetic diversity in *Claviceps purpurea* by RAPD markers. *Mycol. Res.* **101:** 1-6.
13. **Kistler, H. C., and V. P. W. Miao.** 1992. New modes of genetic change in filamentous fungi. *Annu. Rev. Phytopathol.* **30:** 131-152.
14. **Kuldau, G. A., J.-S. Liu, J. F. White, Jr., M. R. Siegel and C. L. Schardl.** 1997. Molecular systematics of Clavicipitaceae supporting monophyly of genus *Epichloë* and form genus *Ephelis*. *Mycologia* **89:** 431-441.
15. **Majer, D., R. Mithen, B. G. Lewis, P. Vos, and R. P. Oliver.** 1996. The use of AFLP fingerprinting for the detec-tion of genetic variation in fungi. *Mycol. Res.* **100:** 1107-1111.
16. **Melching, J. S., K. R. Bromfield, and C. H. Kingsolver.** 1983. The plant pathogen containment facility at Frederick, Maryland. *Plant Dis.* **67:** 717-722.
17. **O'Neill, N. R., P. van Berkum, J. J. Lin, J. Kuo, G. N. Ude, W. Kenworthy, and J. A. Saunders.** 1997. Application of amplified restriction fragment length polymorphism for genetic characterization of *Colletotrichum* pathogens of alfalfa. *Phytopathology* **87:** 745-750.
18. **Pažoutová, S., R. Bandyopadhyay, D. E. Frederickson, P. G. Mantle, and R. A. Frederiksen.** 2000. Relations among sorghum ergot isolates from the Americas, Africa, India, and Australia. *Plant Dis.* **84:** 437-442.
19. **Pongam, P., T. C. Osborn, and P. H. Williams.** 1998. Genetic analysis and identification of amplified fragment length polymorphism markers linked to the *alm1* avirulence gene of *Lepotsphaeria maculans*. *Phytopathology* **88:** 1068-1072.
20. **Spatafora, J. W., and M. Blackwell.** 1993. Molecular systematics of unitunicate perithecial ascomycetes: the Clavicipitales–Hypocreales connection. *Mycologia* **85:** 912-922.
21. **Tooley, P. W., E. D. Goley, M. M. Carras, R. D. Frederick, E. L. Weber, and G. Kuldau.** 2001. Characterization of *Claviceps* species pathogenic on sorghum by sequence analysis of the β-tubulin gene intron 3 region and EF-1α gene intron 4. *Mycologia* **93:** 541-551.
22. **Tooley, P. W., N. R. O'Neill, E. D. Goley, and M. M. Carras.** 2000. Assessment of diversity in *Claviceps africana* and other *Claviceps* spp. by RAM and AFLP analyses. *Phytopathology* **90:** 1126-1130.

23. **Tsukiboshi, T., T. Shimanuki, and T. Uematsu.** 1999. *Claviceps sorghicola* sp. nov., a destructive ergot pathogen of sorghum in Japan. *Mycol. Res.* **103:** 1403-1408.
24. **Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, K. A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau.** 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* **23:** 4407-4414.
25. **Zummo, N., L. M. Gourley, L. W. Trevanthan, and M. S. Gonzalez.** 1998. Occurrence of ergot (sugary disease) incited by a *Sphacelia* sp. on sorghum in Mississippi in 1997. *Plant Dis.* **82:** 590.