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Use of a rep-PCR system to predict species in the Aspergillus section Nigri

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ABSTRACT

The Aspergillus niger aggregate within the A. section Nigri is a group of black-spored aspergilli of great agroeconomic importance whose well defined taxonomy has been elusive. Rep-PCR has become a rapid and costeffective method for genotyping fungi and bacteria. In the present study, we evaluated the discriminatory power of a semi-automated rep-PCR barcoding system to distinguish morphotypic species and compare the results with the data obtained from ITS and partial calmodulin regions. For this purpose, 20 morphotyped black-spored Aspergillus species were used to create the A. section Nigri library in this barcoding system that served to identify 34 field isolates. A pair-wise similarity matrix was calculated using the cone-based Pearson correlation method and the dendrogram was generated by the unweighted pair group method with arithmetic mean (UPGMA), illustrating four different clustered groups: the uniseriate cluster (I), the Aspergillus carbonarius cluster (II), and. the two A. niger aggregate clusters (named III.A and III.B). Rep-PCR showed higher resolution than the ITS and the partial calmodulin gene analytical procedures. The data of the 34 unknown field isolates, collected from different locations in the United States, indicated that only 12% of the field isolates were >95% similar to one of the genotypes included in the A. section Nigri library. However, 64% of the field isolates matched genotypes with the reference library (similarity values >90%). Based on these results, this barcoding procedure has the potential for use as a reproducible tool for identifying the black-spored aspergilli.

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1. Introduction

Members of the Aspergillus section Nigri (also known as blackspored aspergilli) are an important group of fungi because of their impact on food safety, medical mycology, and in the biotechnology industry. They have been isolated mainly from soil, but they also have been found in several other substrates (Magnoli et al., 2006, 2007; Riba et al., 2008; Viswanath et al., 2007; Xavier et al., 2008; Zinedine and Manes, 2009), where they are known to cause detrimental effects, especially as food spoilage and opportunistic animal and human pathogens. On the other hand, some black-spored aspergilli are beneficial organisms, such as Aspergillus niger in the biotechnology industry, where this species has been granted the GRAS (Generally Recognized as Safe) status by the Food and Drug Administration (Schuster et al., 2002). Compounding the economical importance of black-spored aspergilli, are the recent studies that some members of the section Nigri are able to produce two mycotoxins, ochratoxin A (Abarca et al., 1994; Accensi et al., 2001; Amezqueta et al., 2008; Belli et al., 2006; Guzev et al., 2006), and the B2 fumonisin (Frisvad et al., 2007). Ochratoxin A is teratogenic, immunosuppressive, and is a

potential carcinogenic agent in animals and humans (Abarca et al., 2004). Ochratoxin A has been detected in several agricultural products and their derivatives including wine, coffee drinks, and beer (de Almeida et al., 2007; Mateo et al., 2007). The fumonisins are common mycotoxins of the *Fusarium*, but have recently been demonstrated as being produced by black-spored aspergilli. The fumonisins are very important carcinogenic mycotoxins that are produced by *Fusarium* species on maize and maize by-products worldwide, and are toxic to all livestock species, and poultry. They are also associated with human esophageal cancer and recently associated with human neural tube defects (Marasas et al., 2004; Voss et al., 2006; Waes et al., 2005). The frequency of their production by species of the *Aspergillus* section *Nigri* has not been established.

Black-spored aspergilli are difficult to classify and the taxonomy of this section is still unclear (Geiser et al., 2007). Traditionally, the classification of this section was based on morphological characteristics. However, recognition of phenotypic characters within this group by an inexperienced diagnostician is a tremendous challenge. A diagnostic phenotypic procedure based on biochemical traits on agar media along with some molecular approaches has been recently reviewed (Samson et al., 2007). Complicating identification of species within this section is the revelation by molecular examination that there exist several cryptic species within the major recognized morphospecies, creating at least 15 provisional species with this

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black-spored group (Samson et al., 2004). Even under molecular scrutiny recent examination has reduced several species to either synonyms or as a variety of *A. niger* (Peterson, 2008; Samson et al., 2006; Geiser et al., 2007), although the more recently described species (Samson et al., 2004) have not be so scrutinized.

Molecular tools such as RAPD's, RFLPs, ribosomal RNA and proteincoding gene sequences have been used to differentiate between taxa within the section Nigri, and some of them showed relative high biodiversity in the A. niger aggregate (Niessen et al., 2005; Perrone et al., 2006; Samson et al., 2006; Susca et al., 2007). The use of molecular tools such as the internal transcribed spacer (ITS) rDNA region and partial calmodulin gene sequencing analysis has been documented as a good approach. However, the main limitations of these techniques are their low discriminatory power to differentiate black-spored aspergilli, low reproducibility, high price and the requirement of highly specialized technicians to perform such analyses. A molecular-based approach known as rep-PCR is based on the amplification of intervening sequences located within short repetitive DNA sequences that are dispersed throughout the genome of prokaryotes and eukaryotes (Versalovic et al., 1991). Rep-PCR uses primers that target repetitive extragenic palindromic (rep) regions of bacterial and fungal genomes. Most of these approaches are ideal for phylogenetic analyses (Peterson, 2008).

Recently, a semi-automated rep-PCR barcoding system was developed that might overcome the low reproducibility and limitations of relative low discriminatory resolution at species level of taxonomic molecular tools (Healy et al., 2005a). Molecular techniques useful for fungal identification center on barcoding because it shows enormous promise for the very rapid identification of organisms at the species level, although there is considerable debate for this approach for phylogenetics due to uncertainty over the length of barcoding sequences that should be used. However, current data now indicate that standard short barcoding sequences are sufficient for species identification (Hajibabaei et al., 2006; Min and Hickey, 2006; Taylor et al., 2000). Rep-PCR was originally developed for bacterial typing, but it has also been recently used in fungi (Hansen et al., 2008; Healy et al., 2005b). While the work of Hansen et al. (2008) specifically addressed several Aspergillus species, it included only two blackspored species, and no type species of these. They concluded that there were multiple clusters of their A. niger isolates and suggested that these represented subspecies.

In this study, we assessed the discriminatory power of the semiautomated DiversiLab rep-PCR system for typing the *Aspergillus* section *Nigri* species compared to conventional DNA sequencing approaches. The species selected were the generally accepted species, and usually included the type of these. As a second objective, we created a black-spored aspergilli library that was used as a tool to identify field isolates of this section with this system.

2. Materials and methods

2.1. Fungi

Fifty-four black-spored aspegilli isolates were used in this study. Of these, 20 strains (Table 1) were previously identified to the species level by morphological characteristics (Klich, 2002b). These strains were used to construct a validated *Aspergillus* section *Nigri* library. Thirty-four black-spored aspergilli strains were isolated from surface-sterilized (Bacon et al., 1994) field maize (*Zea maize*) and peanuts (*Arachis hypogaea*) kernels. Field isolates were isolated maize and peanut meats on Czapek Yeast Agar (CYA) and single-spore cultures were made of each isolate (Table 2). All strains were stored as frozen stocks at $-80\,^{\circ}$ C in 0.01% Tween 5% glycerol solution or on working slants of CYA maintained for up to 6 months at room temperature. Cultural morphology of these isolates was examined on CYA following the procedure of Klich (2002b). The black-spored aspergilli isolated

Table 1Aspergillus section Nigri isolates used in this study.

Isolate ^a	Species	Source ^b
SRRC 13B	Aspergillus niger	NRRL 2042
SRRC 16	Aspergillus carbonarius	NRRL 2007
SRRC 60	Aspergillus niger	NRRL 3 (ATCC 9029)
SRRC 278	Aspergillus niger	NRRL 364, ATCC 16882
SRRC 320	Aspergillus foetidus	NRRL 337, ATCC 10254
SRRC 321	Aspergillus foetidus	NRRL 341 ^T
SRRC 325	Aspergillus japonicus	Soil, Panama, NRRL 1782(ATCC 16873)
SRRC 334	Aspergillus japonicus	NRRL 5118
SRRC 359	Aspergillus niger	Bran, NRRL 3112
SRRC 369	Aspergillus carbonarius	Honduras, NRRL 346
SRRC 370	Aspergillus carbonarius	NRRL 368, ATCC 6276
SRRC 462	Aspergillus heteromorphus	Culture contaminant, Brazil, NRRL 4747 ^T
SRRC 464	Aspergillus aculeatus	NRRL 5119
SRRC 476	Aspergillus japonicus	NRRL 359
SRRC 484	Aspergillus tubingensis	NRRL 4875, CBS 128.48
SRRC 487	Aspergillus tubingensis	NRRL 4700
SRRC 488	Aspergillus tubingensis	NRRL 4866
SRRC 2131	Aspergillus carbonarius	FRR 369 ^T
SRRC 2378	Aspergillus niger	NRRL 3536 ^T
SRRC 168	Aspergillus aculeatus	NRRL 5094

^a SRRC, obtained from culture collection of the USDA, ARS, Southern Regional Research Center, New Orleans, LA.

from seed were reported as relative isolation frequency and expressed as a percent.

2.2. DNA extraction

All fungal isolates were cultured in 125-ml baffle-bottom Erlenmeyer flasks containing 20-ml Yeast Extract Sucrose (YES) broth

Table 2Field collection of isolates with predicted species results.

Strain number ^a	Location	Similarity % of top match	Predicted species
RRC 453	Peanut, South Georgia	90.0	A. niger
RRC 454	Peanut, South Georgia	90.6	A. foetidus
RRC 455	Peanut, South Georgia	89.6	A. niger
RRC 456	Peanut, South Georgia	85.5	A. niger
RRC 457	Peanut, South Georgia	93.8	A. niger
RRC 458	Peanut, South Georgia	91.3	A. foetidus
RRC 459	Peanut, South Georgia	87.5	A. niger
RRC 460	Peanut, South Georgia	78.7	A. japonicus
RRC 475	Peanut slurries, Dawson, Georgia	79.3	A. niger
RRC 476	Peanut slurries, Dawson, Georgia	98.3	A. niger
RRC 477	Peanut slurries, Dawson, Georgia	98.4	A. foetidus
RRC 478	Peanut slurries, Dawson, Georgia	93.3	A. niger
RRC 479	Peanut slurries, Dawson, Georgia	75.1	A. japonicus
RRC 480	Peanut slurries, Dawson, Georgia	98.5	A. niger
RRC 481	Peanut slurries, Dawson, Georgia	93.1	A. niger
RRC 482	Maize kernels, Midwestern, USA	92.7	A. niger
RRC 483	Maize kernels, Midwestern, USA	93.3	A. niger
RRC 484	Maize kernels, Midwestern, USA	93.4	A. niger
RRC 485	Maize kernels, Midwestern, USA	93.4	A. niger
RRC 486	Maize, kernels, Midwestern, USA	90.7	A. niger
RRC 487	Maize kernels, Midwestern, USA	90.7	A. niger
RRC 488	Maize kernels, Midwestern, USA	89.9	A. niger
RRC 489	Maize kernels, Midwestern, USA	88.4	A. niger
RRC 490	Maize kernels, Midwestern, USA	90.1	A. niger
RRC 493	Maize kernels, Midwestern, USA	92.8	A. niger
RRC 494	Maize kernels, Midwestern, USA	92.9	A. niger
RRC 495	Maize kernels, Midwestern, USA	93.4	A. niger
RRC 497	Maize kernels, Midwestern, USA	82.2	A. niger
RRC 500	Maize kernels, Midwestern, USA	97.0	A. niger
RRC 501	Maize kernels, Midwestern, USA	88.6	A. niger
RRC 503	Maize kernels, Midwestern, USA	85.2	A. niger
RRC 504	Maize kernels, Midwestern, USA	89.4	A. niger
RRC 507	Maize kernels, Midwestern, USA	94.4	A. foetidus
RRC 510	Maize kernels, Midwestern, USA	92.1	A. niger

^a RRC field isolates from the culture collection, USDA, ARS, Russell Research Center, Athens, GA.

^b Type isolates are designated by a superscript T.

(Difco Laboratories, Detroit, Michigan, USA), and closed with Styrofoam plugs. The inoculum used was prepared from 6-day-old fungi grown on CYA. Inoculated flasks were incubated at 25 °C for 72 h as stationary cultures. Genomic DNA was extracted from a 10-µl loop of individual black-spored aspergilli hyphae using the UltraCleanTM Microbial DNA isolation Kit (Mo Bio Laboratories, Solana Beach, California) following the manufacturer's instructions. To increase yields, hyphal material was heated (65 °C for 10 min) and the bead mechanical lysis step was extended to 30 min. DNA concentration was determined using a NanoDropTM 1000 spectrophotometer (Nano-Drop Technologies, Wilmington, Delaware) at 260 nm and adjusted to approximately 25 ng/µl.

2.3. Rep-PCR DNA barcodes

DNA samples were amplified using the DiversiLab Aspergillus Kit for DNA fingerprinting (bioMerieux, Inc, Durham, North Carolina), following the manufacturer's instructions. Briefly, approximately 2 µl (or 50 ng) of genomic DNA was added to 0.5 µl (2.5 U) of AmpliTag polymerase (Applied Biosystems, Foster City, California), 2-ul kitsupplied primer mix, 2.5-µl GeneAmp 10× PCR Buffer (Applied Biosystems), and 18-ul kit-supplied rep-PCR mix (MM1). The PCR step was performed using a PTC 200 Peltier thermal cycler. (Bio-Rad, Hercules, California, USA) under the following thermocycler conditions: initial denaturation of 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 70 °C for 90 s, and a final extension of 70 °C for 3 min. The rep-PCR amplicons were separated using the chip-based technology LabChip® (Caliper Technologies Corp., Mountain View, California), and analyzed using an Agilent 2100 bioanalyzer version B.02.06 51418 (Agilent Techonologies, Palo Alto, California). Finally, the resulting DNA barcode patterns were reported as electropherograms and the genetic relationships among the isolates were analyzed using DiversiLab software version 3.3 and Pearson's correlation coefficient to generate the distance matrices and the unweighted pair group method with arithmetic average (UPGMA) dendrogram. All the analyses were automatically uploaded to the DiversiLab Microbial Typing system (www.diversilab.com).

2.4. DNA sequencing analysis

A two-phase DNA sequencing approach was utilized to confirm the identities of the black-spored isolates. First, a 688 bp DNA fragment of the partial calmodulin gene was amplified and sequenced using CL1 and CL2A primers, as described by O'Donnell et al. (2000). Second, a 710 bp DNA fragment of the internal transcribed spacer ribosomal region (ITS) was amplified using ITS-4 and ITS-5 primers (White et al., 1990). In both cases, the amplicons were purified using QIAquick® PCR purification Kit (QIAgen Sciences, Maryland). Bidirectional sequencing for each amplicon was performed at the USDA-ARS Eastern Regional Research Center Facility (Wyndmoor, PA) using the same primer sets. DNA contiguous sequences were constructed and edited using a Sequencer v. 4.7 software (Gene Codes Corporation, Ann Arbor, Michigan). DNA sequence alignments were generated using ClustalW2 software, and phylogenetic analyses for both the partial calmodulin gene and ITS region were conducted using MEGA version 4 software (Tamura et al., 2007). Phylogenetic trees were generated using the neighbor-joining algorithm, complete deletion, Nucleotide: Maximum Composite Likelihood method with 1000 bootstrap replications.

3. Results and discussion

The two main aims of this study were: i) to compare the taxonomic resolution of the semi-automated DiversiLab rep-PCR barcoding system with ITS and partial calmodulin gene DNA sequences analysis, and ii) to determine if this barcoding system can provide an additional taxonomic tool to differentiate between members of the *Aspergillus*

section *Nigri*, especially members of the *A. niger* aggregate. One feature of this barcoding system is the generation of gel-like images or virtual gel images of the DNA amplicons (Fig. 1). An *Aspergillus* section *Nigri* library was created using 20 black-spored aspergilli that were previously identified at species level by morphological characters (Table 1).

From the dendrogram analysis, four major clusters (shown as roman numbers) were identified: the uniseriate cluster (the Aspergillus aculeatus/Aspergillus japonicus, Fig. 1 cluster I), the Aspergillus carbonarius cluster (Fig. 1 cluster II), and two clusters from the A. niger aggregate (Fig. 1, clusters III.A and III.B). The rep-PCR system clearly differentiated between two morphological groups in Aspergillus section Nigri: the uniseriate (A. japonicus/aculeatus) and the biseriate group, which includes species within the A. niger aggregate and the A. carbonarius cluster. The uniseriate species formed a cluster (Fig. 1, cluster I) consisting of A. japonicus and A. aculeatus, that showed low similarity (<85%). A second A. aculeatus isolate, SRRC 168, formed another branch, however this branch is strongly associated with the biseriate section Nigri species (Fig. 1). The SRRC 168 isolate was analyzed in triplicate and using different frozen stock material, however, after performing the dendrogram analysis, this sample was always placed outside of the uniseriate cluster (I). One possible explanation might be that our stock material might be contaminated with material from other fungal/bacterial organism, which lead to a different barcoding pattern. The five A. carbonarius isolates formed a lineage with concordance and statistical support (90% cutoff) (Fig. 1, cluster II) and their genetic relatedness can be visualized using the gel-like images. The A. niger aggregate (clusters III.A and III.B in Fig. 1) showed high biodiversity among the analyzed isolates. The cluster III. A showed high similarity, with most of the isolates showing >90% similarity. Magnani et al. (2005) demonstrated that A. niger and A. tubingensis can be differentiated by RFLPs, and in our analysis using the DiversiLab dendrogram, 'A. niger' and 'A. tubingensis' clusters are clearly separate from each other. The 'A. tubingensis' cluster (III.B) formed a distinct group which, based on our analysis, is a separate group from the 'A. niger' cluster (III.A). The difference between clusters III.A and III.B is clearly evident when comparing the barcoding pattern of each cluster.

Thirty-four field black-spored isolates obtained from different substrates in the United States (Table 2) were scrutinized at the species level using the DiversiLab system using the Top-Match feature. This feature compares the queried sample (field isolate) fingerprints with the other ones included in the Aspergillus section Nigri library. As a result, the percentage of similarity between the gueried samples and the five most similar matches, based on the fingerprinting patterns, are reported (Fig. 2, Table 2). The data reflect the large diversity within the section, possibly reflecting subspecies and strains with large and varied relationships. For the interpretation, 95% similarities between the queried and library isolates were considered a positive designation. In our analysis, 4 (12%) of the field isolates fingerprints had >95% similarity (Table 2), with all the isolates classified in the A. niger aggregate. Twenty (59%) isolates were >90% similar to fingerprints in the database. From these data, only 3 (9%) of the field isolates were <80% similar to any of the black-spored aspergilli included in the A. section Nigri database. The data generated do show high promise as an aid in providing the quick and reproducible identification of this group. However, it is evident that an extended Aspergillus section Nigri database will be required to improve performance and potentially to replace the highly subjective morphological identification systems within this very difficult section.

The second objective of our study was to compare the resolution power of the rep-PCR approach with the conventional DNA sequencing approaches for species identification. A comparative analysis between the DiversiLab dendrogram results (Fig. 1) with both ITS and partial calmodulin gene sequence analysis (Fig. 3) showed high discriminatory power of the DiversiLab analysis relative to the ITS

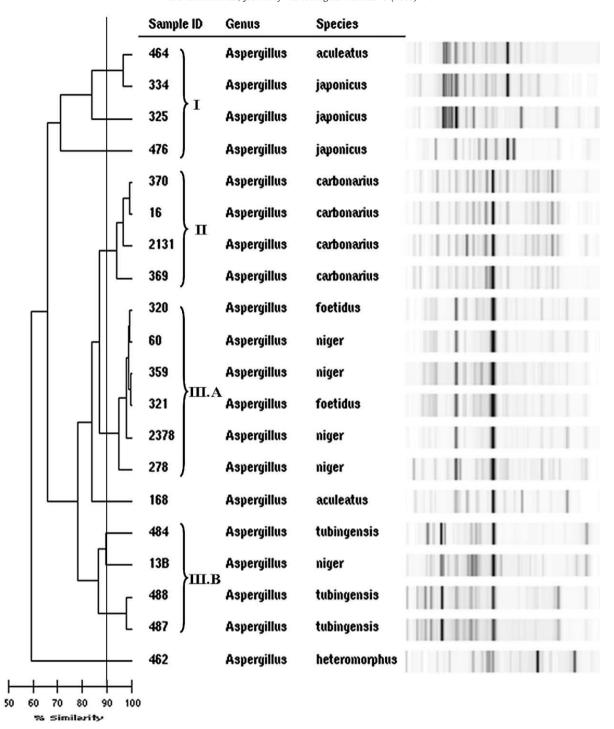


Fig. 1. Dendrogram and gel-like images resulting from rep-PCR amplicons of black-spored aspergilli used to create the *Aspergillus* section *Nigri* library in the DiversiLab system. A pairwise percent similarity matrix was generated using the Pearson's correlation coefficient, which was used to generate the UPGMA dendrogram. All isolates were identified previously by morphology. Roman numbers indicate the different clusters in the *A.* section *Nigri* analysis using rep-PCR. The vertical line on the dendrogram indicates 90% similarity cutoff.

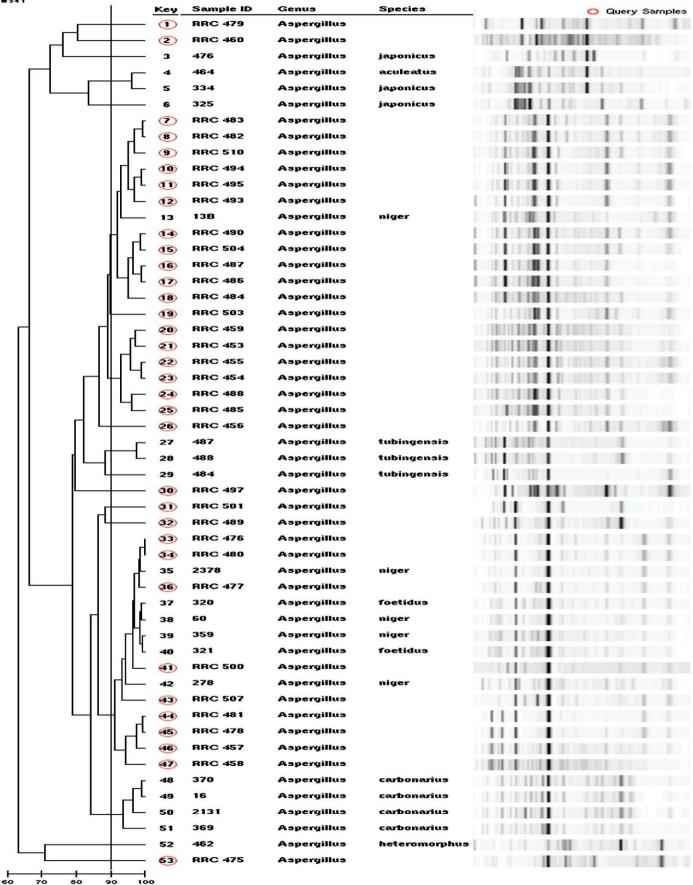
rDNA and the partial calmodulin gene sequencing analysis (Fig. 3). One of the limitations of the DNA sequencing analyses is their low discriminatory power for species belonging to the *A. niger* aggregate. In our analysis, it is clear that the *A. niger* aggregate showed high biodiversity using the DiversiLab system compared to the sequencing analyses. A small cluster within the *A. niger* aggregate, consisted of six isolates (SRRC 320, SRRC 60, SRRC 2378, SSRC 278, SSRC 321, and SSRC

359) showed consistent similarities using both ITS and partial calmodulin gene sequencing trees (Fig. 3). However, in the system described here, these isolates showed high variability. The discriminatory power of the rep-PCR approach technique is higher than with the ITS rDNA and calmodulin sequencing approaches, which is more evident in the cryptic species within the *A. niger* aggregate. Another advantage of this barcoding system is the use of a semi-automated

Fig. 2. Dendrogram and gel-like images illustrating the rep-PCR barcodes of all 54 Aspergillus section Nigri isolates. A pair-wise percent similarity matrix was generated using the Pearson's correlation coefficient, which was used to generate the UPGMA dendrogram. Queried sample numbers are indicated by circles and established identities of these are indicated in Table 2.

Diversilab v 3.3 PC # 34 1

% Similarity



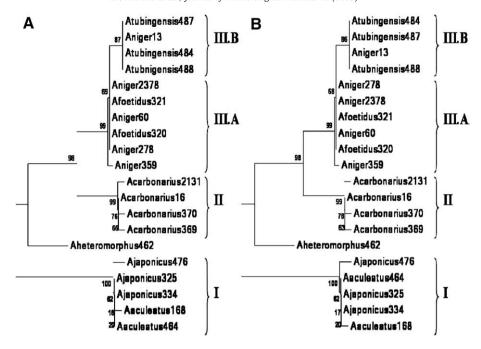


Fig. 3. Collapsed trees generated by the neighbor-joining algorithm of the ITS-4 rDNA ITS-5 (panel A) and partial calmodulin gene (panel B) DNA sequences. Bootstrap percentages were calculated from 1000 re-sampling replications and are indicated at nodes. In both panels A and B, I, II, IIIA, and IIIB indicate the different phylogenetic groups.

system for black-spored classification. This system allows, once a reference library has been created (i.e. the *Aspergillus* section *Nigri* library), the comparison of the DNA barcode pattern of the queried (field) isolate with the barcode pattern of the isolates included in the reference library.

The predicted species data (Table 2) offer some important suggestions for consideration. These data indicate that A. niger has the highest relative frequency of occurrence (82%) of all the predicted black-spored species, followed by Aspergillus foetidus (12.1%). This is in concordance with prior data that suggested A. niger is the species that is usually reported as being occurring in soils (Klich, 2002a) and the cause of seedling and kernel diseases of maize and peanuts. A. carbonarius is another species reported as being an ochratoxin A producer and somewhat cosmopolitan (Wicklow et al., 1996; Klich, 2002a) but was absent in our rather limited survey of maize and peanuts. We are not aware of a comprehensive survey of maize and peanuts for black-spored aspergilli. These data suggest that peanuts are infected by several black-spored species, primarily by A. niger, followed by A. foetidus. Maize is infected primarily by A. niger as the dominant species or 94% of the maize samples were infected by this species. Since the kernels were surfaced sterilized, the results indicate that in addition to being seed-borne, some species have the potential of being endophytic in their association with maize and peanut. A more comprehensive study of the black-spored species, both endophytic and non-endophytic, is warranted.

Of the accepted black-spored Aspergillus species (Samson et al., 2007), several were isolated from maize and peanuts in this study, and of these two, A. niger and A. foetidus, are reported as producers of ochratoxin A (Perrone et al., 2006; Samson et al., 2004). However, based on beta-tubulin analysis, these two species were considered synonyms (Peterson, 2008), i.e., A. foetidus was made a synonym of A. niger, which is supported by the data from our work. A. foetidus was erected in 1945 to include the black-spored fungi with persistently dark grayish brown to olive-brown heads, to distinguish it from the carbon black heads of A. niger. Similarly, both A. ficuum and A. awamori were made synonyms of A. niger. This indicates the highly superficial nature of morphology and reflects the complexity and problematic nature of the taxonomy of this section. However, this barcode procedure could distinguish A. japonicus, A. aculeatus, A. niger,

A. tubingensis, A. carbonarius, A. foetidus, and Aspergillus heteromorphus. Based on preliminary data, we believe that others within this section can also be differentiated using this procedure. Due to the great diversity observed in isolates of A. niger, and in other related black-spored species, our data suggest that studies must be conducted on the biology, potential for ochratoxin A and fumonisin production, and plant pathology of the black-spored species now identified on maize and peanuts, two commodities of great agronomic importance in the USA.

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References

Abarca, M.L., Bragulat, M.R., Castell, G., Cabañes, F.J., 1994. Ochratoxin A production by strains of *Aspergillus niger* var. *niger*. Appl. Environ. Microbiol. 60, 2650–2652.

Abarca, M.L., Accensi, F., Cano, J., Cabañes, F.J., 2004. Taxonomy and significance of black aspergilli. Antonie van Leeuwenhoek 86, 33–49.

Accensi, F., Abarca, M.L., Cano, J., Figuera, L., Cabañes, F.J., 2001. Distribution of ochratoxin A producing strains in the *A. niger* aggregate. Antoine van Leeuwenhock 79, 365–370.

Amezqueta, S., Gonzalez-Penas, E., Dachoupakan, C., Murillo-Arbizu, M., de Cerain, A.L., Guiraud, J.P., 2008. OTA-producing fungi isolated from stored cocoa beans. Lett. Appl. Microbiol. 47, 197–201.

Bacon, C.W., Hinton, D.M., Richardson, M.D., 1994. A corn seedling test for resistance to Fusarium moniliforme. Plant Dis. 78, 302–305.

Belli, N., Bau, A., Marin, S., Abarca, M.L., Ramos, A.J., Bragulat, M.R., 2006. Mycobiota and ochratoxin A producing fungi from Spanish wine grapes. Int. J. Food Microbiol. 111, S40–S45.

de Almeida, A.P., Alaburda, J., Shundo, L., Ruvieri, V., Navas, S.A., Lamardo, L.C.A., Sabino, M., 2007. Ochratoxin A in Brazilian instant coffee. Braz. J. Microbiol. 38, 300–303.

Frisvad, J.C., Smedsgaard, J., Samson, R.A., Larsen, T.O., Trane, U., 2007. Fumonisin B2 Production by Aspergillus niger. J. Agric. Food Chem. 55, 9727–9732.

Geiser, D.M., Klich, M.A., Frisvad, J.C., Peterson, S.W., Samson, R.A., 2007. The current status of species recognition and identification in *Aspergillus*. Stud. Mycol. 59, 1–10.
 Guzev, L., Danshin, A., Ziv, S., Lichter, A., 2006. Occurrence of ochratoxin A producing fungi in wine and table grapes in Israel. Int. J. Food Microbiol. 111, S67–S71.

Hajibabaei, M., Smith, M.A., Daniel, J., Rodriguez, J.J., Whitfield, J.B., Herbert, P.D.N., 2006. A minimalist barcode can identify a specimen whose DNA is degraded. Mol. Ecol. Notes 6, 959–964.

- Hansen, D., Healy, M., Reece, K., Smith, C., Woods, G.L., 2008. Repetitive-sequence-based PCR using the DiversiLab system for identification of Aspergillus species. J. Clin. Microbiol. 46, 1835–1839.
- Healy, M., Huong, J., Bittner, T., Lising, M., Frye, S., Raza, S., Schrock, R., Manry, J., Renwick, A., Nieto, R., Woods, C., Versalovic, J., Lupski, J.R., 2005a. Microbial DNA typing by automated repetitive-sequence-based PCR. J. Clin. Microbiol. 43, 199–207.
- Healy, M., Reece, K., Walton, D., Huong, J., Frye, S., Raad, I.I., Kontoyiannis, D.P., 2005b.
 Use of the DiversiLab system for species and strain differentiation of *Fusarium* species isolates, I. Clin. Microbiol. 43, 5278–5280.
- Klich, M.A., 2002a. Biogeography of *Aspergillus* species in soil and litter. Mycologia 94, 21–27.
- Klich, M.A., 2002b. Identification of Common Aspergillus Species. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
- Magnani, M., Fernandes, T., Prete, C.E.C., Homechim, M., Ono, E.Y.S., Vilas-Boas, L.A., Sartori, D., Furlaneto, M.C., Fungaro, M.H.P., 2005. Molecular identification of *Aspergillus* spp. isolated from coffee beans. Scientia Agricola. 62, 45–49.
- Magnoli, C., Astoreca, A., Ponsone, M.L., Fernández-Juri, M.G., Barberis, C., Dalcero, A.M., 2007. Ochratoxin A and Aspergillus section Nigri in peanut seeds at different months of storage in Córdoba, Argentina. Int. J. Food Microbiol. 119, 213–218.
- Magnoli, C., Hallak, C., Astoreca, A., Ponsone, L., Chiacchiera, S., Dalcero, A.M., 2006. Occurrence of ochratoxin A-producing fungi in commercial corn kernels in Argentina. Mycopathologia 161, 53–58.
- Marasas, W.F.O., Riley, R.T., Hendricks, K.A., Stevens, V.L., Sadler, T.W., Gelineau-van Waes, J., Missmer, S.A., Cabrera, J., Torres, O., Gelderblom, W.C.A., Allegood, J., Martínez, C., Maddox, J., Miller, J.D., Starr, L., Sullards, M.C., Roman, A.V., Voss, K.A., Wang, E., Merrill Jr., A.H., 2004. Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and in vivo: a potential risk factor for human neural tube defects among populations consuming fumonisin-contaminated maize. J. Nutri. 134, 711–716.
- Mateo, R., Medina, A., Mateo, E.M., Mateo, F., Jimenez, M., 2007. An overview of ochratoxin A in beer and wine. Int. J. Food Microbiol. 119, 79–83.
- Min, X.J., Hickey, D.A., 2006. Assessing the effect of varying sequence length on DNA barcoding of fungi. Molecular Ecol. Notes 7, 365–373.
- Niessen, L., Schmidt, H., Muhlencoert, E., Farber, P., Karolewiez, A., Geisen, R., 2005. Advances in the molecular diagnosis of ochratoxin A-producing fungi. Food Addit. Contam. 22, 324–334.
- O'Donnell, K., Nirenberg, H., Aoki, T., Cigelnik, E., 2000. A multigene phylogeny of the *Gibberella fujikuroi* species complex: detection of additional phylogenetically distinct species. Mycoscience 41, 61–78.
- Perrone, G., Mule, G., Susca, A., Battilani, P., Pietri, A., Logrieco, A., 2006. Ochratoxin A production and amplified fragment length polymorphism analysis of Aspergillus carbonarius, Aspergillus tubingensis, and Aspergillus niger strains isolated from grapes in Italy. Appl. Environ. Microbiol. 72, 680–685.

- Peterson, S.W., 2008. Phylogenetic analysis of *Aspergillus* species using DNA sequence from four loci. Mycologia 100, 205–226.
- Riba, A., Mokrane, S., Mathieu, F., Lebrihi, A., Sabaou, N., 2008. Mycoflora and ochratoxin A producing strains of *Aspergillus* in Algerian wheat. Int. J. Food Microb. 122, 85–92.
- Samson, R.A., Houbraken, J.o.s., Kuijpers, A., Frank, J.M., Frisvad, J.C., 2004. New ochratoxin A or sclerotium producing species in *Aspergillus* section *Nigri*. Stud. Mycol. 50, 45–61.
- Samson, R.A., Noonim, P., Meiger, M., Houbraken, J., Frisvad, J.C., Varga, J., 2007. Diagnostic tools to identify black aspergilli. Stud. Mycol. 59, 129–145.
- Samson, R.A., Hong, S.B., Frisvad, J.C., 2006. Old and new concepts of species differentiation in *Aspergillus*. Med. Mycol. 44, S133–S148.
- Schuster, E., Dunn-Coleman, N., Frisvad, J.C., van Dijck, P.W.M., 2002. On the safety of Aspergillus niger — a review. Appl. Microbiol. Biotech. 59, 426–435.
- Susca, A., Stea, G., Mule, G., Perrone, G., 2007. Polymerase chain reaction (PCR) identification of *Aspergillus niger* and *Aspergillus tubingensis* based on the calmodulin gene. Food Addit. Contam. 24, 1154–1160.
- Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 10, 1093.
- Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbet, D.S., Fisher, M.C., 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genet. Biol. 31, 21–32.
- Versalovic, J., Koeuth, T., Lupski, J.R., 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. 19, 6823–6831.
- Viswanath, P., Kavitha, S., Ayesha, F., Appaiah, K.M., 2007. A survey of ochratoxin a in wheat and barley in India. J. Food Safety 27, 111–123.
- Voss, K.A., Gelineau-van Waes, J., Riley, R.T., 2006. Fumonisins: current research trends in developmental toxicology. Mycotoxin Res. 22, 61–69.
- Waes, G., Starr, L., Maddox, J., Aleman, F., Voss, K.A., Wilberding, J., Riley, R.T., 2005. Maternal fumonisin exposure and risk for neural tube defects: Mechanisms in an in vivo mouse model. Teratology 73, 487–497.
- Wicklow, D.T., Dowd, P.T., Alfatafta, A.A., Gloer, J.B., 1996. Ochratoxin A: an antiinsectan metabolite from the sclerotia of Aspergillus carbonarius NRRL 369. Can. J. Microbiol. 42, 1100–1103.
- White, T., Burns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: a Guide to Methods and Applications. Academic Press, San Diego, CA, pp. 315–322.
- Xavier, M.O., Sales, M.D.U., Camargo, J.D.P., Pasqualotto, A.C., Severo, L.C., 2008. Aspergillus niger causing tracheobronchitis and invasive pulmonary aspergillosis in a lung transplant recipient: case report. Rev. Soc. Bras. Med. Trop. 41, 200–201.
- Zinedine, A., Manes, J., 2009. Occurrence and legislation of mycotoxins in food and feed from Morocco. Food Control 20, 334–344.