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Identity and genetic diversity of the sorghum ergot pathogen in Australia

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Abstract. Sorghum ergot was first discovered in Australia in 1996. It affects seed production and grain usage in stock feed due to concerns of animal toxicity. Three species of *Claviceps* are known to cause ergot of sorghum with different epidemiological, animal toxicity, and management implications. *Claviceps africana* was identified as the causal agent but morphological differences between isolates raised the possibility of more than one species being involved. The major aim of this study was to identify the *Claviceps* species causing sorghum ergot and to determine the genetic diversity among isolates of the ergot pathogen from Australia and overseas. Symptom development, sequencing of the ITS1 region, and radiolabelled DNA amplification fingerprints (RAF) were used to confirm that ergot of sorghum in Australia is caused by *C. africana*. The morphology of sphacelia, microconidia, macroconidia, and secondary conidia of all 36 Australian isolates studied matched the description for *C. africana* and the DNA sequence of the ITS1 region of 2 selected Australian isolates was identical to that of *C. africana*. Based on RAF analysis of 110 Australian and overseas isolates of *Claviceps* spp., *C. africana* isolates could be clearly distinguished (<40% similarity) from *C. pusilla*, *C. sorghicola*, *C. sorghi*, and a *Claviceps* sp. isolated from *Panicum maximum*. The *C. africana* isolates formed 2 distinct clusters. Cluster 1 contained 72 Australian isolates and all 21 overseas isolates of *C. africana*. The 13 isolates in Cluster 2 were all from Australia and more diverse than those in Cluster 1. The high level of genetic diversity of *C. africana* isolates in Australia is unexpected given that ergot has only been reported recently. The most likely source of this diversity points to introductions from countries such as India.

Additional keywords: Claviceps africana, ITS1, molecular markers, RAF.

Introduction

Ergot of sorghum [*Sorghum bicolor* (L.) Moench] has emerged in recent years as a serious threat to sorghum production worldwide. Three *Claviceps* species can cause sorghum ergot, namely *C. sorghi* (Kulkarni *et al.* 1976), first described in India in 1917 as its anamorph *Sphacelia sorghi* (McRae 1917), *C. africana* (Frederickson *et al.* 1991), and *C. sorghicola* (Tsukiboshi *et al.* 1999). For over 75 years, sorghum ergot remained restricted to India and other parts of Asia and Africa. However, since 1995 it has spread within a few years to the Americas (Reis 1996) and Australia, where it was first discovered in 1996 (Ryley *et al.* 1996). In Australia, seed production is at greatest risk from sorghum ergot. In 1996, losses between 30 and 100% were experienced in nurseries and parent seed production blocks in Queensland, necessitating the regular use of foliar fungicides, and expensive changes to planting and harvesting procedures (Ryley and Henzell 1999). Honeydew infested panicles in grain sorghum increase the cost of harvesting and processing. Potential animal toxicity of ergot-infected grain, caused by alkaloids produced by *Claviceps* spp., has raised concerns for the livestock industries (Blaney *et al.* 2000).

Specific weather and host requirements for the infection of unfertilised gynoecia restrict the window of opportunity for the establishment of *Claviceps* spp. in sorghum and it is unclear how the pathogen has spread so rapidly between geographically isolated regions. Airborne secondary conidia are considered the main means of dissemination of *C. africana* (Bandyopadhyay *et al.* 1998), but their importance in long distance dispersal is unknown. Sclerotia play a vital role in the survival and perhaps dispersal of other *Claviceps* species, such as *C. purpurea* (Fr.) Tul. (Mantle and Shaw 1976). However, their role in survival and spread of *C. africana* has not been ascertained (Bandyopadhyay *et al.* 1998). In recent work in Australia, Bhuiyan *et al.* (unpublished data) have demonstrated that macroconidia can survive on honeydew-coated seed for at least 12 months at 4°C. The recent expansion in collaborative seed exchange programs between seed companies and the movement of genetic materials across national boundaries have been suggested as possible routes for the accidental introduction of this pathogen to Australia. It has also been suggested that the pathogen may have been introduced earlier than 1996 but remained at a low undetectable level due to unfavourable

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conditions such as drought following its introduction (Bandyopadhyay *et al.* 1996).

Based on sphacelial and spore morphology, Ryley *et al.* (1996) suggested that more than one *Claviceps* species might affect *Sorghum* spp. in Australia. Among the 3 *Claviceps* species infecting sorghum, only *C. africana* has the potential to cause widespread epidemics due to the production of wind-disseminated secondary conidia (Frederickson *et al.* 1989). In addition, only *C. africana* produces dihydroergosine and other alkaloids that can cause potential animal toxicity problems (Blaney *et al.* 2000). Because of the epidemiological and toxicological implications, there is a need to confirm the identity of the pathogen causing sorghum ergot in Australia. Separation of *Claviceps* species affecting *Sorghum* spp. is currently based on differences in the morphology of the anamorphic and teleomorphic stages of the fungus and the alkaloid profile. Pazoutová *et al.* (2000) described the sequence of the internal transcribed spacer (ITS) 1 region specific to *C. africana* and this offers a new tool in the molecular identification of this species.

Tracking pathogen genotypes from geographically isolated regions can offer clues to the movement and source of new infestations. Two recent studies have examined at a molecular level the intra-specific diversity in *C. africana* populations with a limited number of isolates. Pazoutová *et al.* (2000) used random amplified polymorphic DNA (RAPD) analysis to compare 28 isolates from Australia, Africa, India, and the Americas. Tooley *et al.* (2000) used random amplified microsatellite (RAM) and amplified fragment length polymorphism (AFLP) analysis to assess the diversity of 20 American, Indian, African, and Australian *C. africana* isolates. Both studies found a generally low level of diversity among *C. africana* isolates from different countries. Pazoutová *et al.* (2000) suggested that ergot in Australia was introduced from Asia but that the American isolates originated from Africa. Relatively few (3 and 5, respectively) Australian isolates originating only from south-east Queensland were used by Tooley *et al.* (2000) and Pazoutová *et al.* (2000). Sorghum ergot is now present in all grain and forage sorghum-growing regions in Australia and a comprehensive examination of *Claviceps* spp. affecting Australian sorghum is necessary. This study had 2 objectives: firstly, to establish the identity of the *Claviceps* spp. causing ergot in sorghum in Australia; and secondly, from a study of the genetic diversity of Australian and overseas isolates, to pinpoint possible sources of introduction of the pathogen into Australia.

Materials and methods

A total of 110 isolates of *Claviceps* species were studied. These included 106 putative *C. africana* isolates collected in Australia and overseas (Australia 85, India 15, America 4, Japan 1, Africa 1), one *C. sorghicola* isolate, one *C. sorghi* isolate, one *C. pusilla* isolate, and one unidentified *Claviceps* sp. (Table 1). *Claviceps africana* isolates from overseas and the *C. sorghicola* isolate were obtained as pure cultures. Australian *C. africana*, *C. pusilla*, and *C.* sp. isolates were obtained from single conidia or single sphacelia and sclerotia, respectively.

Isolation and cultivation of mycelial cultures

Isolates were cultured on T2 medium (Spalla 1973) or potato dextrose agar (PDA) (Sigma). The composition of 1L T2 medium is as follows: 100 g sucrose; 10 g asparagine; 0.1 g yeast extract; 0.25 g KH_2PO_4; 0.25 g $MgSO_4.7H_2O$; 0.02 g $FeSO_4.7H_2O$; 0.015 g $ZnSO_4.H_2O$; 0.12 g KCl; 1 g $Ca(NO_3)_2.4H_2O$; 20 g agar; pH 5.2.

To obtain single conidial isolates, honeydew collected from infected panicles in the field was streaked out on 2% water agar plates and incubated overnight at 22°C. Single germinated conidia were transferred to T2 agar plates with the aid of a fine needle and incubated at 27°C for 1 week and subsequently at 20–23°C until mycelium was visible. Isolates were subcultured on PDA or T2 plates as required. Alternatively, single sphacelia or sclerotia were surface-sterilised in 1% sodium hypochlorite for 3 min, rinsed 3 times in sterile water, and plated on T2 agar plates or PDA plates and incubated at 22°C.

All isolates were stored on agar discs in 15% glycerol in liquid nitrogen.

Morphological identification

Honeydew of 33 Australian isolates collected from various sorghum species was used to inoculate panicles of male sterile sorghum plants (*S. bicolor*, var. B70000ZV, Pacific Seeds, Australia) in a glasshouse. Fresh honeydew from infected flowers was streaked onto water agar to obtain single-spored isolates. These produced mycelia when grown on culture media. Mycelium was homogenised, suspended in water, and used to inoculate plants in the glasshouse to obtain honeydew from single-spored isolates. The honeydew containing conidia was used to record the shape, length, and width of 100 conidia for each isolate.

DNA preparation

Isolates of *C. africana* and *C. sorghi* were grown in sterile, clarified V8 juice. *Claviceps sorghicola* and *C. pusilla* were grown in potato dextrose broth. Conical flasks (250 mL) containing 150 mL of liquid medium were inoculated with mycelial fragments and incubated at room temperature (20–23°C) on an orbital shaker (150 rpm) for 7 days. Cultures were filtered through 2 layers of cheesecloth and freeze-dried. DNA was extracted using a modified method of Rogers and Bendich (1985). Up to 0.5 g of freeze-dried mycelium ground in liquid nitrogen was suspended in 4.8 mL of 1× CTAB buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 0.7 M NaCl; 1% CTAB) and 0.8 mL of 2× CTAB buffer (100 mM Tris-HCl, pH 8.0; 20 mM EDTA; 1.4 M NaCl; 2% CTAB) and incubated at 65°C for 5 min. The mixture was extracted twice with an equal volume of chloroform-isoamyl alcohol (24 : 1). After the second extraction, 1/10 volume of 10× CTAB buffer (0.7 M NaCl; 10% CTAB) was added to the supernatant and extracted with an equal volume of choroform-isoamyl alcohol. DNA was precipitated out from the supernatant with an equal volume of CTAB precipitation buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 1% CTAB). The pellet was resuspended in 2.5 mL of high salt TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA; 1 M NaCl) and the DNA was precipitated out for 1 h at 0°C with 2 volumes ice-cold 100% ethanol 1/10 volume of 3 M sodium acetate. The pellet was washed with 70% ethanol, resuspended in 0.1× TE buffer (1 mM Tris-HCl, pH 8.0; 0.1 mM EDTA), and treated with 2 µL RNAse (10 µg/µL) per 100 µL sample solution. All samples were cleaned using a DNA clean-up spin kit (Genomed, Germany).

The unidentified *Claviceps* sp. isolate was grown on PDA covered with sterile miracloth discs. Mycelium was scraped off and freeze-dried. DNA of *Claviceps* sp. was extracted in 1.5 mL centrifuge tubes using the same method in 1/10 of the volume. As a control for the purity and integrity of the genomic DNA, samples were run out on 0.8% agarose gels.

Table 1. Host plant, geographic origin, year of collection, and radiolabelled amplified fragment (RAF) cluster of *Claviceps* spp. isolates used in this study

Isolate	Species	Host plant	Year	RAF-cluster
		Japan		
Cjap3[A]	*C. sorghicola*	n.a.	1997	
Cla83[A]	*C. africana*	n.a.	1988	1
		India		
MH74[B]	*C. sorghi*	*S. bicolor*	2000	
APAU I[B]	*C. africana*	*S. bicolor*	2000	1
AP16[B]	*C. africana*	*S. bicolor*	2000	1
NI3[B]	*C. africana*	*S. bicolor*	2000	1
NI6[B]	*C. africana*	*S. bicolor*	2000	1
NI12[B]	*C. africana*	*S. bicolor*	2000	1
Cls1[A]	*C. africana*	n.a.	1997	1
MH70[B]	*C. africana*	*S. bicolor*	1997	1
MH71[B]	*C. africana*	*S. bicolor*	2000	1
MH72[B]	*C. africana*	*S. bicolor*	2000	1
MH73[B]	*C. africana*	*S. bicolor*	2000	1
KA53A[B]	*C. africana*	Forage sorghum	2000	1
KA61B[B]	*C. africana*	*S. bicolor*	2000	1
UASD I[B]	*C. africana*	*S. bicolor*	2000	1
SK5[B]	*C. africana*	*S. bicolor*	2000	1
SK7[B]	*C. africana*	*S. bicolor*	2000	1
		South Africa		
Cla58[A]	*C. africana*	n.a.	1998	1
		USA		
Cla5[A]	*C. africana*	n.a.	1997	1
Cla10[A]	*C. africana*	n.a.	1998	1
KG[C]	*C. africana*	n.a.	1997	1
		Puerto Rico		
Cla14[A]	*C. africana*	n.a.	1998	1
		Australia—south-east Queensland		
Dic	*C. pusilla*	*Dicanthium aristatum*	1999	
10959	*C.* sp.	*Panicum maximum*	1999	1
SE4[D]	*C. africana*	*S. bicolor*	1999	1
SE7	*C. africana*	*S. bicolor*	1999	1
SE8	*C. africana*	Forage sorghum	1999	1
SE9	*C. africana*	*S. bicolor*	1999	1
SE11[D]	*C. africana*	Forage sorghum	1999	1
SE14	*C. africana*	*S. bicolor*	1999	2
SE15	*C. africana*	*S. bicolor*	1999	1
SE19[D]	*C. africana*	*S. halepense*	1999	1
SE20[D]	*C. africana*	Forage sorghum	1999	2
SE21[D]	*C. africana*	*S. bicolor*	1999	1
SE25[D]	*C. africana*	*S. bicolor*	1999	1
SE26[D]	*C. africana*	*S. bicolor*	1999	1
SE36A	*C. africana*	*S. bicolor*	2000	1
SE38C	*C. africana*	*S. halepense*	2000	1
SE43[D]	*C. africana*	*S. bicolor*	2000	1
SE46	*C. africana*	*S. bicolor*	2000	1
SE48B	*C. africana*	*S. bicolor*	2000	1
SE51	*C. africana*	Forage sorghum	2000	1
SE52	*C. africana*	*S. bicolor*	2000	2
SE53[D]	*C. africana*	*S. bicolor*	2000	1
SE55A	*C. africana*	*S. bicolor*	2000	1
SE55C	*C. africana*	*S. bicolor*	2000	2
SE56B	*C. africana*	*S. halepense*	2000	1
SE58A[D]	*C. africana*	*S. bicolor*	2000	1
SE59[D]	*C. africana*	*S. bicolor*	2000	1
SE63	*C. africana*	*S. bicolor*	2000	1
SE66	*C. africana*	*S. halepense*	2000	1
SE68C	*C. africana*	*S. halepense*	2000	1
SE69[D]	*C. africana*	*S. bicolor*	2000	1
SE72A	*C. africana*	*S. bicolor*	2000	1
SE72C	*C. africana*	*S. bicolor*	2000	1
SE73	*C. africana*	*S. bicolor*	2000	1
SE73A	*C. africana*	*S. bicolor*	2000	1
SE73C	*C. africana*	*S. bicolor*	2000	1
SE77	*C. africana*	*S. bicolor*	2000	1
SE78[D]	*C. africana*	*S. bicolor*	2000	1
SE81B	*C. africana*	*S. bicolor*	2000	1
SE82[D]	*C. africana*	*S. bicolor*	2000	1
SE83A	*C. africana*	*S. bicolor*	2000	1
SE84A	*C. africana*	*S. bicolor*	2000	1
SE86	*C. africana*	*S. halepense*	2000	1
SE86S[D]	*C. africana*	*S. halepense*	2000	1
SE88C	*C. africana*	*S. bicolor*	2000	1
SE90D[D]	*C. africana*	*S. bicolor*	2000	1
SE94C	*C. africana*	*S. bicolor*	2000	1
T10763[D]	*C. africana*	n.a.	1996	1
T10765[D]	*C. africana*	n.a.	1996	1
T10779	*C. africana*	n.a.	1996	1
GRS0[D]	*C. africana*	*S. bicolor*	1999	1
63503	*C. africana*	Forage sorghum	1999	
		Australia—central Queensland		
CQ2C	*C. africana*	*Sorghum* sp.	2000	1
CQ6B[D]	*C. africana*	*S. almum*	2000	1
CQ6C	*C. africana*	*S. almum*	2000	1
CQ6D	*C. africana*	*S. almum*	2000	1
CQ7	*C. africana*	*S. bicolor*	1999	2
CQ8[D]	*C. africana*	*S. bicolor*	1999	1
CQ9	*C. africana*	*S. bicolor*	1999	1
CQ10[D]	*C. africana*	*S. almum*	1999	2
CQ11	*C. africana*	*S. almum*	1999	1
CQ12	*C. africana*	*S. almum*	1999	2
CQ13	*C. africana*	*S. almum*	1999	1
CQ14B[D]	*C. africana*	*S. bicolor*	1999	1
CQ15[D]	*C. africana*	*S. bicolor*	1999	2
CQ16	*C. africana*	*Sorghum* sp.	1999	2
CQ17	*C. africana*	*Sorghum* sp.	1999	1
CQ19[D]	*C. africana*	*Sorghum* sp.	1999	2
CQ20[D]	*C. africana*	Forage sorghum	1999	2
CQ21	*C. africana*	*Sorghum* sp.	1999	1
CQ22	*C. africana*	*Sorghum* sp.	1999	1
		Australia—north Queensland		
NQ60	*C. africana*	*Sorghum* sp.	2000	1
NQ62	*C. africana*	*Sorghum* sp.	2000	1
NQ70[D]	*C. africana*	*Sorghum* sp.	2000	1
NQ71[D]	*C. africana*	*Sorghum* sp.	2000	2
NQ74A	*C. africana*	*Sorghum* sp.	2000	1
NQ74B	*C. africana*	*Sorghum* sp.	2000	1
NQ75	*C. africana*	*Sorghum* sp.	2000	1
NQ85	*C. africana*	*Sorghum* sp.	2000	1
		Australia—Northern Territory		
NT27	*C. africana*	Forage sorghum	1999	1
NT28[D]	*C. africana*	n.a.	1999	1
NT30[D]	*C. africana*	Forage sorghum	1999	1
NT32[D]	*C. africana*	n.a.	1999	2
		Australia—New South Wales		
NSW33A[D]	*C. africana*	*S. bicolor*	2000	1
NSW33B	*C. africana*	*S. bicolor*	2000	1
NSW96[D]	*C. africana*	*S. bicolor*	2000	1
NSW97	*C. africana*	*S. bicolor*	2000	1

n.a., Information not available. [A]Supplied by P. Tooley. [B]Supplied by R. Bandyopadhyay. [C]Supplied by S. Pazoutová. [D]Used for morphological identification.

Radiolabelled amplified DNA fingerprint analysis (RAF)

Ten arbitrary primers of 10 base pairs each were used in RAF (J. Waldron, M. Graham, and B. Carroll, pers. comm.). The reaction mixture of 10 µL contained 1 µL of 10× DAF-buffer (10 mM Tris–HCl, pH 8.0; 10 mM KCl; 5 mM $MgCl_2$), 20 µM of dNTPs, 1.5 U Ampli-Taq DNA Polymerase Stoffel Fragment (PE Biosystems), 0.25 µL α-^{33}P-dATP, 5 µM primer, and 30–50 ng genomic DNA. DNA amplification was performed in a thermal cycler (MJ Research PTC 100) programmed at 94°C/7min, 30 cycles of 94°C/30s, 57°C/1 min, 56°C/1 min, 55°C/1 min, 54°C/1 min, 53°C/1 min, and one time 72°C/5 min. After completion, an equal volume of 98% (w/v) formamide; 10 mM EDTA, pH 8.0; 0.05% (w/v) bromophenol blue; and 0.05% (w/v) xylene cyanol was added to the reaction mixtures and the reaction products were denatured at 95°C for 3 min and immediately put on ice. The amplification products were analysed by electrophoresis on 4% denaturing polyacrylamide gels on a Biorad sequencing gel system (50 by 21 by 0.4 cm). The gels were dried on a gel dryer (Biorad Model 583) at 80°C for 1 h and exposed to X-ray film (Kodak Biomax MR-2) at room temperature overnight.

Strong bands consistently present in duplicate samples of each isolate were scored as present or absent. A distance matrix was calculated using the Jaccard's coefficient and a dendrogram was generated from cluster analysis of the similarity values by unweighted pair-group method with arithmetic average (UPGMA) using the MVSP program (Multi Variate Statistical Package, Version 3.11h, Kovach Computing Service, Pentraeth, Wales, UK).

Molecular identification

Two Australian isolates, SE88C from RAF Cluster 1 and CQ10 from RAF Cluster 2, were chosen to sequence the internal transcribed spacer (ITS) 1 region and part of the 5.8S rDNA using the primers ITS1 and ITS2 (White *et al.* 1990). Twenty-five µL of reaction mixture contained 5 µL of 5× reaction buffer (67 mM Tris–HCl, pH 8.8; 16 mM $[NH_4]_2SO_4$; 0.45% Trition X-100; 0.2 mg/L of gelatin; 0.2 mM dNTPs, Fisher Biotech), 1.5 mM $MgCl_2$, 0.2 µM of each primer, 2.75 U DNA Taq polymerase (Fisher Biotec), and 30–50 ng genomic DNA. DNA amplification was performed in a thermal cycler (MJ Research PTC 100) as described by Pazoutová *et al.* (2000). PCR products were purified with a QiaQUICK PCR Purification Kit (QIAGEN, Germany). The ITS 1 region was sequenced from both strands using the Applied Biosystem's BigDye Terminator RR Mix. Automated DNA sequencing was performed by the Australian Genomics Research Facility (AGRF), Brisbane. The sequences were aligned using the program Sequencher Version 3.O (Applied Biosystems, Australia) and compared with the sequences of 3 *C. africana* isolates from Australia (AJ011783), Bolivia (AJ011590), and India (AJ011784) as described by Pazoutová *et al.* (2000).

Results

Morphological identification

For all 33 isolates inoculated in the glasshouse, first symptoms usually became visible after 5–6 days as white fungal stroma emerging through glumes of sorghum florets. Between Days 6 and 7, clear honeydew started to exude from the stroma. Soon after production the surface of honeydew gradually turned white with a powdery appearance. Over time, honeydew turned dull with a light to dark brown coloration. The oval or spherical sphacelium had a white to grey appearance and protruded slightly beyond the glumes.

The honeydew collected after inoculation with homogenised mycelium in the glasshouse contained 3 types of conidia: round microconidia, elliptical to oblong macroconidia, and pear-shaped secondary conidia (Fig. 1). Microconidia were generally 3–4 µm in diameter. The size of macroconidia varied between isolates. Table 2 shows the average length and width of macroconidia in 3 *C. africana* isolates to demonstrate the range of conidia dimensions found in the 33 isolates. Only few secondary conidia were found in the samples and these were generally 8.5–17.5 µm long and 3–7 µm wide.

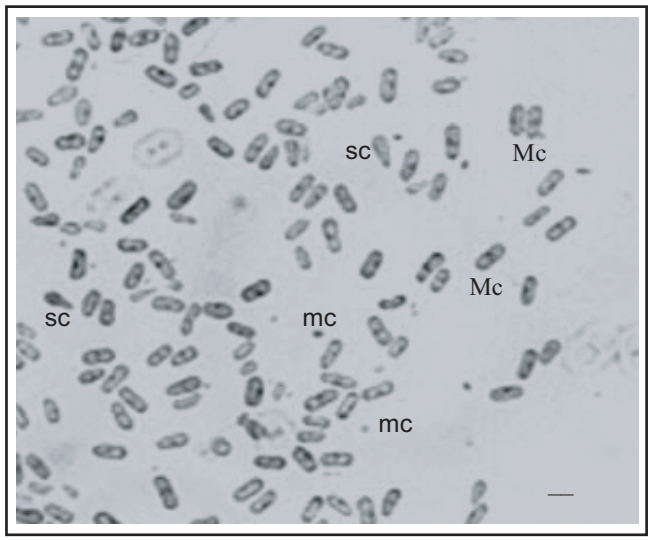

Fig. 1. Spore types found in honeydew of 30 isolates of *Claviceps africana*; mc, round microconidia; Mc, oblong macroconidia; sc, pear-shaped secondary conidia (bar represents 12 µm).

RAF analysis

RAF analysis resulted in characteristic banding patterns for the different *Claviceps* species (Fig. 2). Reproducible amplification products in the range of 100–550 bp were scored and a dendrogram was generated for the 110 *Claviceps* spp. isolates (Fig. 3). All Australian isolates collected from *Sorghum* spp. were at least 80% similar to the overseas isolates of *C. africana*. None of the putative *C. africana* isolates from Australia showed the same degree of similarity to any of the other *Claviceps* species used in this study. *Claviceps pusilla* showed the least similarity to *C. africana* (<5%), followed by *C. sorghicola* (20%), and *C. sorghi* and *Claviceps* sp. (both approximately 30%).

Table 2. Average length and width (µm) of 100 macroconidia in the honeydew of three selected *Claviceps africana* isolates from Australia

Isolate	Mean length ± s.d.	Mean width ± s.d.
SE58A	9.84 ± 1.54	4.1 ± 0.44
NQ71	11.9 ± 1.41	4.72 ± 0.96
NSW33A	14.03 ± 1.21	5.26 ± 0.97

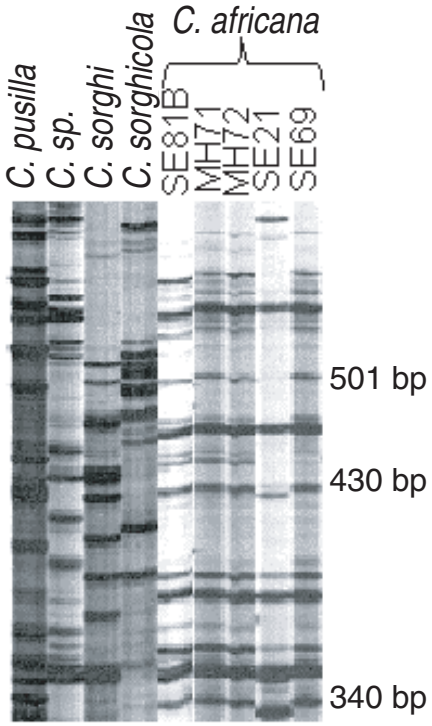

Fig. 2. Radiolabelled amplified fragment (RAF) fingerprint for 5 different *Claviceps* species generated with Primer OPV7.

Analysis of intra-specific diversity among the 106 putative *C. africana* isolates revealed DNA polymorphism for all 10 primers used. Polymorphism obtained with the primer OPL2 for a subset of isolates is shown in Fig. 4*a*. The putative *C. africana* isolates could be grouped into 2 clusters (Fig. 3). Cluster 1 contains 93 isolates from Australia, India, Japan, Africa, and the Americas. The Australian isolates in this cluster are 85–100% similar and these originate from grain and forage sorghum, *S. almum*, *S. halepense*, and unidentified *Sorghum* spp. This cluster contains several subgroups of isolates with identical RAF fingerprints. The largest, with 27 isolates, contains isolates from geographically distant regions in Australia as well as 3 isolates from India. Fig. 4*b* is an example of a RAF gel generated with primer OPH13 showing fragments of approximately 150 bp in size shared by the Australian and Indian isolates but not by the American and African isolates. Similarly, the American isolates show fragments not shared by the Indian or Australian isolates (Fig. 4*c*). There are other fragments shared by the American and African isolates, but not by the Australian or the Indian isolates. Cluster 2 contains 13 Australian isolates collected in central, north, and south-east Queensland from native *Sorghum* spp. and grain sorghum.

We have detected more than one RAF fingerprint among isolates collected from the same panicle, e.g. isolates SE73, SE73A, and SE73C, which show different RAF fingerprints.

Molecular identification

Alignment of nucleotide sequences of the ITS1 region of the 2 Australian isolates with 3 previously described *C. africana* isolates showed no differences between the 5 isolates (data not shown). This indicates that the Australian isolates belong to *C. africana*.

Discussion

Symptom development, spore morphology, and sphacelial characters of the Australian isolates of the ergot pathogen correspond to the description of *C. africana* by Frederickson *et al.* (1991). The ITS 1 sequence of 2 Australian isolates from the 2 RAF clusters is identical to the sequences of 3 *C. africana* isolates from Australia and overseas reported by Pazoutová *et al.* (2000). Additionally, all Australian sorghum ergot isolates and isolates of *C. africana* from overseas showed a high degree of similarity in our RAF analyses. Therefore, it can be concluded that *C. africana* is the only species causing sorghum ergot in Australia.

Using a larger number of isolates we have detected more genetic variation in the Australian population than the generally low level of intra-specific variation reported in previous studies (Pazoutová *et al.* 2000; Tooley *et al.* 2000). The 85 Australian isolates could be grouped into 2 clusters and none of the 21 overseas isolates examined in this study was similar to the 13 isolates in Cluster 2. Pazoutová *et al.* (2000) did not detect any difference between 5 Australian *C. africana* isolates from south-east Queensland using RAPD analysis. Two of these isolates (10763 and 10765) were used in this study; both were in Cluster 1 and they showed minor differences in their RAF fingerprints (Fig. 3). This points to a higher resolution of genetic variation using the RAF technique compared with RAPD. However, the overall diversity among the Australian isolates is generally low, with more than half of the isolates showing at least 95% similarity. *Claviceps africana* predominately reproduces through asexual means and the role of sclerotia in the life cycle of this fungus is still unclear (Frederickson *et al.* 1991; Bandyopadhyay *et al.* 1998). Consequently, low variability between isolates of this pathogen is to be expected. This is in contrast to the sexually reproducing *C. purpurea* where a single RAPD primer distinguished more than 20 genotypes among 29 isolates (Jungehülsing and Tudzynski 1997).

Despite the overall similarity among isolates, the small but extensive variations between isolates are unexpected given that sorghum ergot has only been in Australia for the past 5 years. RAF analysis has revealed a number of putative genotypes among isolates from the same region, the same field, or even the same panicle. This is consistent with previous observations of differences in spore morphology between honeydew samples from the same panicle (Ryley and Henzell 1999). Although we have shown here that the variation in conidial morphology is within the range

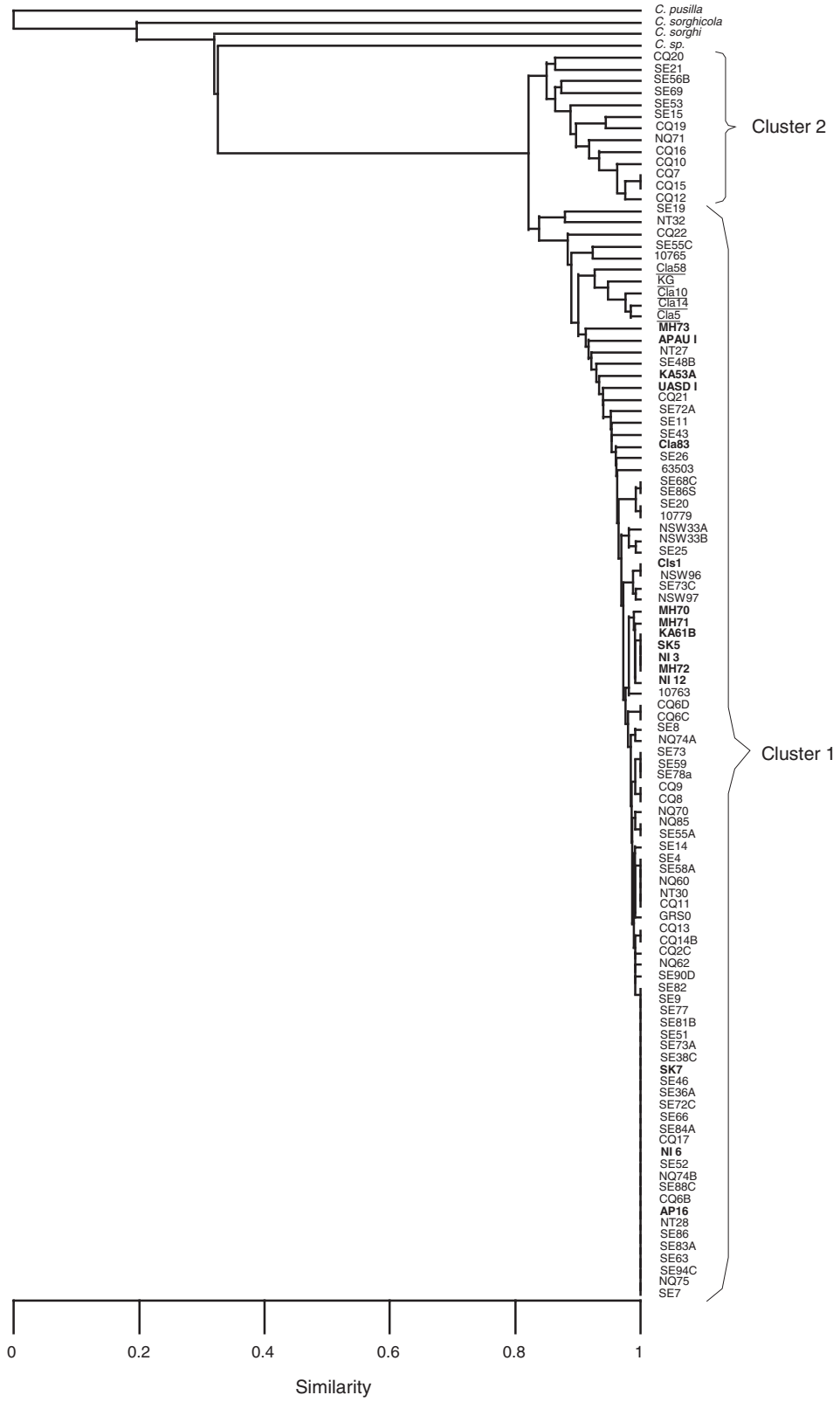

Fig. 3. Genetic relationship of 110 *Claviceps* spp. from Australia, India, Japan, South Africa, Puerto Rico, and the USA (Table 1), based on radiolabelled amplified fragment analysis (RAF). American/African isolates are underlined; Indian/Japanese isolates are in bold.

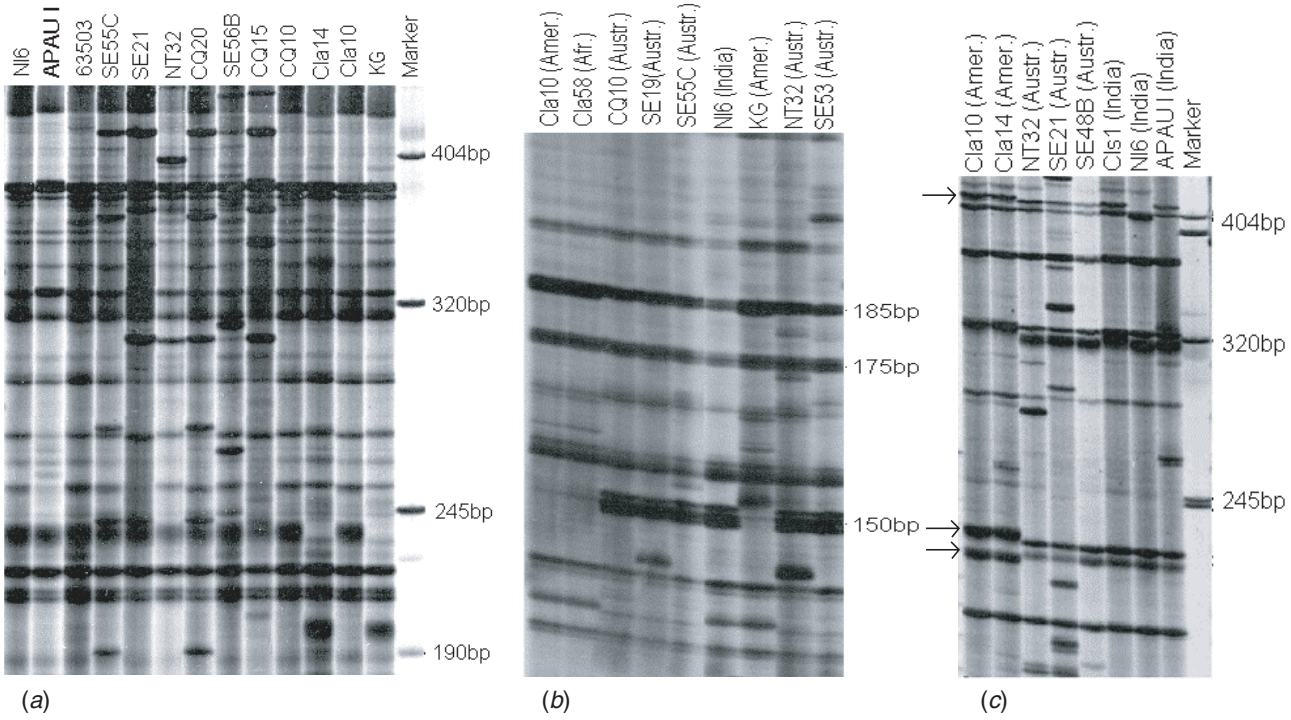

Fig. 4. (*a*) An example of a radiolabelled amplified fragments (RAF) polyacrylamide gel showing a number of polymorphisms for 13 *Claviceps africana* isolates with Primer OPL2. (*b*) RAF gel showing an example of a polymorphic fragment at 150 bp distinguishing Australian (Austr.) and Indian (India) isolates from African (Afr.) and American (Amer.) isolates using Primer OPH13. (*c*) RAF gel showing examples of polymorphic fragments only found in American isolates with Primer OPX3 (arrows).

described for *C. africana* (Frederickson *et al.* 1991), this variation is still extensive. Tooley *et al.* (2000) reported similar findings in their assessment of the American population of *C. africana*. The origin of this variation is unclear. Bandyopadhyay *et al.* (1998) suggested that the pathogen could have been introduced before 1996 but failed to cause severe epidemics due to unfavourable weather. During a series of dry seasons in the late 1980s and early 1990s in south-east and central Queensland the pathogen may have survived on grain and native sorghum and generated this level of variation. Seventeen of the 25 described species of *Sorghum* are native to Australia and at least 9 of these can be a collateral host to *C. africana* (D. Herde, pers. comm.).

In this study, the Indian *C. africana* isolates showed a high degree of similarity to the Australian isolates. Australian and Indian isolates shared several fragments not found in the American or African isolates (Fig. 4*b*) and fragments shared by the American and African isolates were not present in the Australian or Indian isolates. This concurs with earlier findings of Asia as the source of incursion for Australia and Africa as the original source of isolates causing the 1997 epidemics in the mainland USA and Puerto Rico (Pazoutová *et al.* 2000; Tooley *et al.* 2000). However, only a limited number of overseas isolates was used in this study and further work should include a more extensive range of isolates from other countries.

Three Indian isolates had identical RAF fingerprint to 24 isolates that originated from geographically separated regions of Australia, including the Northern Territory, approximately 3000 km from the site of the first reported outbreak of sorghum ergot (Ryley *et al.* 1996). This subgroup may represent one of the founder clonal lineages from India initially colonising Australia.

Cluster 2 represents the most diverse group of isolates with a number of unique putative genotypes not found in other isolates from Australia or overseas. Whether this variation has developed within Australia following the original introduction or from new introductions from another exotic source is uncertain at this stage.

It seems unlikely that contaminated seed lots were the cause of introduction to Australia, since quarantine regulations require all legally imported sorghum seed to be grown for one generation in a containment glasshouse. The role of airborne secondary conidia as the principal dispersal agent over local and moderate distances is well established (Frederickson *et al.* 1989, 1993). Bandyopadhyay (1999) attributes the rapid spread of *C. africana* within Brazil in 1995 and Australia in 1996 to long-distance dispersal of secondary conidia. Although there is still uncertainty about

the role of secondary conidia in the intercontinental spread of *C. africana*, long-distance aerial dispersal of conidia is the likely explanation to the widespread distribution of identical genotypes within Australia.

In conclusion, this study has established an inventory of Australian strains of *C. africana*, which can be used for further monitoring of Australian sorghum ergot populations to detect the appearance of new genotypes. Although Asia appears to be the major source of introduction, it is still not clear how and when the pathogen was introduced. The level of genetic diversity in the Australian *C. africana* population is higher than expected and the origin of this diversity is speculative. More targetted studies of *C. africana* are needed to gain a better understanding of the mechanisms that are likely to generate variation in this pathogen.

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