5.   ERGOT ALKALOIDS PRODUCED BY *CLAVICEPS CYPERI*

Abstract

The main ergopeptine alkaloid in sclerotia of *Claviceps cyperi* collected from ergotised nut sedge implicated in outbreaks of bovine ergotism in South Africa was identified by HPLC and tandem mass spectroscopy as α-ergocryptine. All sclerotial samples tested also yielded ergosine, whereas ergocornine and ergocrystine were detected at low concentrations in a freshly collected sample. Trace amounts of α-ergocryptine was produced by *C. cyperi* on solid medium, but all attempts at inducing an isolate of the species to synthesise ergopeptines in liquid culture have failed. α-Ergocryptine showed no antibiotic activity against *Cladosporium cladosporioides* and *Fusarium heterosporum*, two fungi commonly associated with *C. cyperi* honeydew.

5.1   INTRODUCTION

Various cases of bovine ergotism have recently been reported from the eastern Highveld Region in South Africa (Van der Linde & Naude 2000). Examination of the fodder implicated in the outbreaks showed that it was extensively contaminated with yellow nut sedge (*Cyperus esculentus* L.) ergotised by *Claviceps cyperi* Loveless, whereas chemical analysis indicated the presence of the ergot alkaloid α-ergocryptine, as well as traces of ergosine, ergocornine, ergocrystine and ergotamine (Naude *et al.* 2005).

Besides being important from a diagnostic/prognostic perspective, verification of the alkaloid or alkaloids produced by a *Claviceps* species is of value in confirming the identity of the species, defining chemoraces or chemotypes, and classifying intraspecific variability (Pažoutová *et al.* 2000), hence assisting in the elucidation of phylogenetic relationships and habitat specialisation. The potential pharmaceutical application of the particular alkaloid/s is also of considerable importance. α-Ergocryptine, for instance, is the source of 2-bromo-α-ergocryptine, a dopaminergic agent marketed world-wide under the trade name Parlodel by the firm Novartis. The drug is used, among others, for puerperal prevention or suppression of lactation, treatment of hyperprolactinaemia and prolactin-related menstrual and fertility disorders, and as an adjunct in the treatment of Parkinsonism (Snyman 2001).

2-Bromo-α-ergocryptine is presently produced semi-synthetically from wheat (*Triticum aestivum* L.) artificially infected with *Claviceps purpurea* (Fr.:Fr.) Tul.

(http://www.dimok.de/ergot/harvest.html). However, *C. purpurea* is not a prolific producer of α-ergocryptine (Taber & Vining 1957), and *C. cyperi* may therefore be a more suitable source. This report provides data on the ergot alkaloids present in sclerotia of *C. cyperi* and describes various attempts at inducing sphacelial isolates of the species to produce α-ergocryptine in culture.

5.2   MATERIALS AND METHODS

Sclerotia of *C. cyperi* collected between 1997 and 2000 from ergotised nut sedge at localities where bovine ergotism occurred (Table 5.1) were analysed for ergot alkaloids by Meadows Cape in Paarl, Western Cape Province, according to the HPLC method of Rottinghaus *et al.* (1993), following extraction of the sclerotia as described by Scott *et al.* (1992). Sclerotia collected in 1997, 1999, 2002 and 2003 were tested in 2003 for ergopeptines as described in 5.2.3 and 5.2.4. A preliminary experiment was also done in which *C. cyperi* PREM 56618 was grown for 8 weeks at 22 °C in the dark on Mantle's alkaloid medium (Mantle 1973) solidified with 1.5 % agar, and the cultures analysed by HPLC for α-ergocryptine at the Council for Scientific and Industrial Research in Pretoria.

Following the above, *in vitro* production of alkaloids by *C. cyperi* in liquid medium was attempted according to the procedures described below.

5.2.1   Preparation of inoculum

Pure cultures of *C. cyperi* PREM 57392, subsequently deposited as PPRI 7196, were obtained by surface-disinfecting sclerotia in 1.75 % sodium hypochlorite for 5 minutes and rinsing them three times in sterile distilled water. The sclerotia were dried on sterile tissue paper and transferred to a microscope slide cleaned with 70 % ethanol. The cortex of each sclerotium was removed with a sterile surgical blade, and the inside cut into sections which were plated on potato-dextrose agar and glucose yeast extract agar. When the colonies were about 25 mm in diameter, plugs with mycelium cut from them were used to inoculate Erlenmeyer flasks with glucose yeast extract broth. The flasks were incubated stationary in the dark for 2 weeks at 22 °C and the cultures used to inoculate the media in 5.2.2.

5.2.2 Culturing

The following liquid media were prepared (concentrations are per litre):

Mantle's alkaloid medium (Mantle 1973) (MAM):

Sucrose	150 g
L-Asparagine	15 g
KH_2PO_4	25 mg
$MgSO_4.7H_2O$	25 mg
$FeSO_4.7H_2O$	33 mg
$ZnSO_4$	27 mg

(pH adjusted to 5.5 with 1 N NaOH)

Bacon's alkaloid medium (Bacon *et al.* 1979) (BAC):

Sorbitol	100 g
Glucose	40 g
Succinic acid	10 g
KH_2PO_4	1 g
$MgSO_4.7H_2O$	0.3 g
$FeSO_4.7H_2O$	1 mg
Yeast Extract	1 g

(pH adjusted to 5.5 with 1 N NaOH)

Molasses medium (MOL):

Molasses	150 g
Yeast extract	5 g

(pH adjusted to 5.2 with 1N NaOH)

Glucose yeast extract (Fuentes *et al.* 1964) (GYE):

Glucose	10 g
Yeast Extract	10 g

(pH adjusted to 5.2 with 1N NaOH)

Each medium was dispensed into sixty-four 250 ml capacity Erlenmeyer flasks at 100 ml per

flask, and the flasks with medium were autoclaved for 15 minutes at 121 °C. When cooled, each flask was inoculated with 1 ml of the above inoculum of *C. cyperi* and sixteen flasks of each medium were incubated stationary in the dark at 15, 22, 27 and 34 °C, respectively.

5.2.3 Extraction of alkaloids

Extractions from cultures were done weekly for 8 weeks with duplicate flasks of each medium from each incubation temperature. The content of each flask was filtered through Whatman No. 1 paper. Two aliquots of 5 ml of the filtrate were each transferred to a 15 ml screw-cap vial. Drops of 0.1 N sodium hydroxide were added to increase the pH to between 9 and 10. Nine millilitres of chloroform was added to each vial and the vials agitated on a rotary shaker for 30 minutes, after which they were centrifuged for 3 minutes at 4000 rpm. The upper aqueous layer was discarded and the remaining chloroform evaporated to dryness with nitrogen in a Reacti-Vap at 50 °C.

The mycelial mass was rinsed with distilled water, divided in two portions, and each portion was transferred to a pre-weighed 15 ml screw-cap vial. The fungal material was dried for 2—3 hours at 40 °C, the vials were weighed again, and the dry mycelial mass was calculated. Five millilitres of methanol was added to each vial, the contents sonicated for 1 hour, and the tubes then centrifuged for 3 minutes at 4000 rpm. Five millilitres of the liquid in each vial was transferred to a clean vial and concentrated to dryness with nitrogen in a Reacti-Vap at 50 °C. The entire experiment was conducted three times.

5.2.4 Alkaloid analysis

Vials with dried extracts were submitted on a weekly basis for alkaloid analysis to the Veterinary Medical Diagnostic Laboratory at the University of Missouri, Columbia, USA. Ergopeptine analysis was done by the HPLC technique of Rottinghaus *et al.* (1993). Samples were extracted with alkaline chloroform, filtered and applied to Ergosil cleanup columns (Analtech, Newark, Denmark). Following elution of pigments with acetone:chloroform (4:1), alkaloids were eluted with methanol and analysed by HPLC with fluorescence detection. The presence of ergopeptines was confirmed by treating the samples with 0.2 % acetic acid and re-analysis by HPLC for the -ine isomers. The HPLC system consisted of a Perkin Elmer LC 250 pump and ISS 200 auto sampler, with detection on a Hitachi F-1200 fluorescence

detector. A Phenomenex Luna C18 column (150 mm x 4.6 mm) was used with a mobile phase of acetonitrile:water (35:65) and a 200 mg l^{-1} solution of ammonium carbonate in distilled water.

The identity of the ergopeptine alkaloids was verified by tandem mass spectroscopy (Finnigan/MAT TSQ 70 Tandem Mass Spectrometer (MS/MS)) according to Rottinghaus *et al.* (1993) at the USDA National Veterinary Services Laboratory, Ames, Iowa. A portion of each of the chloroform extracts processed through the above Ergosil cleanup columns was applied to a direct exposure probe, the solvent allowed to evaporate, and the probe inserted into the mass spectrometer. The MS/MS was operated in the negative chemical ionisation mode with methane as reagent gas and argon as collision gas. Daughter ions were collected for the α-ergocryptine parent ion m/Z 308. Typical daughter spectra were obtained with base peak m/Z 209, in accordance with Plattner *et al.* (1983) and Rottinghaus *et al.* (1993).

5.2.5   Antimycotic activity of α-ergocryptine

Colonies of the two fungal species most commonly associated with honeydew of *C. cyperi*, viz. *Fusarium heterosporum* Nees and *Cladosporium cladosporioides* (Fresen.) G.A. de Vries, were established centrally on potato-dextrose agar plates. When about 1 cm in diameter, four antibiotic assay discs saturated with chloroform solutions containing 2000, 3000, 4000 and 5000 ppm pure α-ergocryptine (Sigma, Johannesburg), respectively, were placed equidistantly around each colony on the agar. Plates were incubated at 18 °C in the dark and inspected regularly.

5.3   RESULTS

The main ergopeptine alkaloid detected in sclerotia of *C. cyperi* was α-ergocryptine, with concentrations in freshly collected samples ranging from 2500 to 5325 ppm (Table 5.1). When tested again in 2003, the α-ergocryptine content of the sclerotia on average was about 50 % less than in the initial analysis. All sclerotial samples tested in 2003 also contained ergosine at concentrations 3.4—24 times lower than that of α-ergocryptine. Small quantities of ergocornine and ergocristine were present in sclerotia collected and tested in 2003.

Cultures of PREM 56618 grown on solid MAM produced a dark brown diffusable pigment in

the agar (Figure 5.1). The concentration of α-ergocryptine in these cultures after 8 weeks was 7 ppb.

No growth of PREM 57392 occurred in any of the liquid media at 34 °C. Some growth was evident in MOL at 15 °C, but not in the other three media. Cultures in MAM and BAC at 22 and 27 °C started to produce a brown pigment in the medium after about 4 weeks. Best growth in terms of biomass produced at the latter temperatures (data not presented) occurred in GYE. No ergopeptine alkaloid could be detected in any of the media at any time at any temperature in any of the three runs of the experiment.

Colonies of *F. heterosporum* and *C. cladosporioides* grew unobstructedly over the antibiotic assay discs impregnated with α-ergocryptine (Figures 5.2, 5.3).

5.4   DISCUSSION

Environmental conditions play an important role in *Claviceps* fermentations (Mantle 1978; Arora *et al.* 1992; Wainwright 1992). With most strains, the metal ions Mg, Fe and Zn in trace amounts enhance alkaloid production. pH optima for growth are around 5.2—5.5, and temperature optima usually near 24 °C. Induction of alkaloid biosynthesis requires osmotic pressures between 10 and 20 bar, attained by high sugar concentrations which can be partially replaced by sodium chloride. The high osmotic pressure inhibits conidiogenesis, induces differentiation into sclerotium-like cells, and enhances the entrance of nutrients into the cells. As is common for most secondary metabolites, alkaloid synthesis follows rather than accompanies active growth. Maximum alkaloid yield therefore usually occurs only after prolonged incubation (12—50 days) (Abe 1948; Taber & Vining 1957; Mantle 1973). By adhering to these principles, alkaloid synthesis in culture has successfully been achieved with ergot species such as *C. purpurea* (Michener & Snell 1950; Taber & Vining 1957; Castagnoli & Mantle 1966), *Claviceps paspali* F. Stevens & J.G. Hall (Pacifici *et al.* 1962), *Claviceps gigantea* S.F. Fuentes, Isla, Ullstrup & Rodriques (Fuentes *et al.*, 1964), *Sphacelia sorghi* McRae (Mantle 1973), *Claviceps fusiformis* Loveless (Mantle 1978), *Claviceps africana* Freder., P.G. Mantle & W.A.J. de Milliano, *Claviceps sorghicola* Tsukib., Shiman. & Uematsu (Pažoutová 2001) and *Claviceps zizaniae* (Fyles) Pantidou (Kantorová *et al.* 2002).

Despite adhering to the above guidelines, and allowing for possible deviations in

environmental requirements, the present attempts at inducing *C. cyperi* to respond accordingly have failed, except for the trace amounts of α-ergocryptine produced on solid medium. The lack of success could probably be ascribed to an inherent inability of *C. cyperi* to synthesise ergopeptines in liquid culture or to the isolates used having lost the capacity to produce alkaloids. Von Bekesy (1940) showed that cultures of *C. purpurea* obtained from sclerotia with a high alkaloid content yielded the highest concentration of alkaloids. The two isolates of *C. cyperi* used in the present study, particularly PPRI 7196, were selected for that reason. Although alkaloid-producing cultures have an acute tendency to loose the ability to yield alkaloids upon repeated transfer (Mantle 1978), PPRI 7196, when inoculated into the various liquid media, had been subcultured only twice since being obtained in culture from PREM 57392 sclerotia. A more feasible explanation for the absence of ergopeptines in cultures of PPRI 7196 therefore seems to be that cultural conditions were not conducive to alkaloid production by this isolate of *C. cyperi*. Observations by Ramstad & Gjerstad (1955) indicated that mycelial tissue of *C. purpurea*, in the same way as conidia, is incapable of producing alkaloids, and that failure to produce alkaloids in culture is due to sclerotial tissue not developing under these conditions. Mantle & Tonolo (1968) similarly showed that improved *in vitro* alkaloid yields by *C. purpurea* is associated with a plectenchymatic growth form. Enlarged hyphal cells, probably sclerotial primordia, have been observed in cultures of *C. cyperi* on solid medium in Chapter 3, but it is not known if such cells were formed in liquid medium in the present study. Growth of the cultures certainly did not appear plectenchymatous. Regarding the above it is interesting to note that Pažoutová (2001) reported sclerotia and cultures of *C. zizaniae*, the *Claviceps* species phenotypically the closest related to *C. cyperi* (Chapter 6) and the only other ergot species producing α-ergocryptine as main alkaloid, to be void of alkaloids. In a subsequent study, however, Kantorová *et al.* (2002) found one wild strain of *C. zizaniae* to synthesise α-ergocryptine at concentrations of up to 1 mg g^{-1} in the same medium used by Pažoutová (2001), whereas a second strain did not produce any detectable alkaloids at all.

Contrary to the culture experiments, ergopeptine analysis of sclerotia of *C. cyperi* provided vital information regarding the chemistry of *C. cyperi*. More than 80 ergot alkaloids are known (Bock & Parberry, www.tacethno.com/info/claviceps Internet access 13.01.2005), but peptide ergot alkaloids (ergotamine, ergosine, ergocristine, ergocornine, ergostine, α-ergocryptine, ß-ergocryptine and derivatives) have thus far been detected only in *C. purpurea* (various ergopeptines), *C. zizaniae* (α-ergocryptine) and *C. africana* (dihydroergosine) (Mantle 1968; Flieger *et al.* 1997; Kantorová *et al.* 2002). *C. cyperi* is therefore the fourth *Claviceps* species

found capable of producing ergopeptines. This alkaloid profile corresponds with the alkaloid analysis of the fodder implicated in the outbreaks of bovine ergotism (Naude *et al.* 2005) and is typically associated with "summer syndrome" symptoms, e.g. hyperthermia, reduced food intake, lethargy, drop in milk production, loss of body mass, increased respiratory rate, open-mouthed breathing, seeking shade and wading into water (Ross *et al.* 1989). One of the fodder samples tested by Naude *et al.* (2005), however, contained ergotamine, which was not detected in sclerotia of *C. cyperi*. The presence of ergotamine in the above sample can probably be ascribed to contamination of the fodder by a *Lolium* sp. ergotised by *C. purpurea*, also occurring in the area.

Besides elucidating the alkaloid profile of *C. cyperi*, results of the present study verified the relatively narrow mesophilic temperature growth range of this ergot species (Chapter 3) and also showed that α-ergocryptine does not possess antimycotic activity against *F. heterosporum* and *C. cladosporioides*. The inhibition of growth of the latter two fungi by *C. cyperi* observed in dual culture in Chapter 3 must therefore have been due to a different metabolite produced by *C. cyperi* in the medium.

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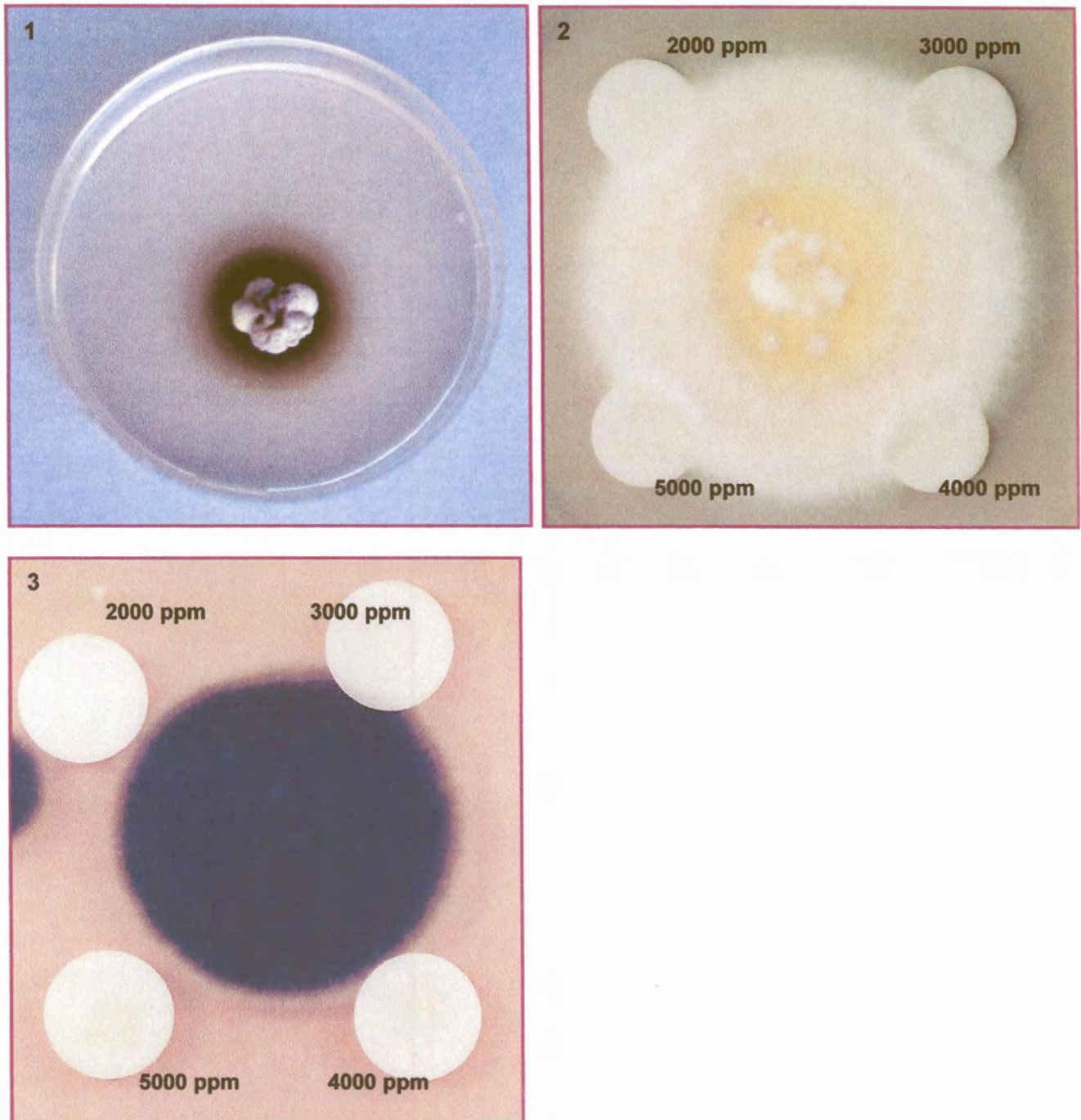
Figure 5.1 Colony of *Claviceps cyperi* growing on Mantle's alkaloid medium.
Figure 5.2 Lack of inhibition of *Fusarium heterosporum* by α-ergocryptine.
Figure 5.3 Lack of inhibition of *Cladosporium cladosporioides* by α-ergocryptine.

Table 5.1   Ergopeptine alkaloid content of sclerotia of *Claviceps cyperi* collected between 1997 and 2003 from ergotised nut sedge implicated in bovine ergotism in South Africa.

Alkaloid	Concentration (ppm)					
	1997 PREM 56618	1998 PREM 56621	1999 PREM 56623	2000 PREM 57392	2002 PREM 57394	2003 PREM 58304
α-Ergocryptine[a]	3600	3925	2500	5325	ND	ND
α-Ergocryptine[b]	2491	ND	1676	ND	1902	2687
Ergosine[b]	102	ND	97	ND	147	789
Ergocornine[b]	0	ND	0	ND	0	30
Ergocristine[b]	0	ND	0	ND	0	26

[a] Determined in year of collection.

[b] Determined in 2003

ND = Not determined

6. MOLECULAR SYSTEMATICS OF *CLAVICEPS CYPERI* AND OTHER SOUTH AFRICAN *CLAVICEPS* SPECIES

Abstract

Two South African isolates of *Claviceps cyperi*, one of *C. purpurea*, and a Canadian strain of *C. grohii* (CBS 124.47) were characterised with the aid of three primers to produce multilocus fingerprints. The internal transcribed spacers 1 and 2, the 5.8S region, as well as the β-tubulin intron 3 region were sequenced. All available sequence data for several *Claviceps* species deposited in the GenBank nucleotide database were compared and optimally aligned with the South African isolates. K-means clustering and two-dimensional discriminant analysis as well as phylogenetic relationships were determined on these data sets. *C. cyperi* and *C. zizaniae* formed a distinct cluster showing little similarity with the other *Claviceps* species which clustered in two main groups. The South African isolate of *C. purpurea* and *C. grohii* (CBS 124.47) showed high similarity with the GenBank *C. purpurea* strains and clustered with *C. sulcata*, *C. fusiformis* and *C. paspali*. *C. africana* was included in the second major cluster with *C. viridis*, *C. pusilla*, *C. sorghi*, *C. gigantea*, *C. maximensis*, *C. phalaridis*, *C. sorghicola* and *C. citrina*. It was difficult to place *C. citrina* and *C. paspali* in either of the major clusters using the phylogenetic analysis. Sequence data from the β-tubulin intron 3 region revealed a similar pattern and placed the two *C. cyperi* isolates in a distinct outgroup cluster at a large genetic distance from the other *Claviceps* species.

6.1 INTRODUCTION

Claviceps species have traditionally been identified according to morphological characteristics of the teleomorph and the anamorph, as well as the hosts from which they were recorded. However, morphological features within species tend to be variable (Pažoutová *et al.* 2000b), whereas description of a new species based solely on the host is inclusive due to the polygeneric host range of the majority of ergot fungi (Pažoutová *et al.* 2000b). Attempts to circumvent the problem have led to the establishment of varieties, special forms or races, and host-specific groups (Pažoutová *et al.* 2000b; Pažoutová 2001). Other means of distinguishing between species, such as alkaloid profiles and the ability to float on water, have also been utilised to identify chemoraces and habitat specialisation (Pažoutová *et al.* 2000b, 2002).

In recent years, much emphasis has been placed on the application of molecular methods for verifying and detecting genetic variability in *Claviceps*. Most of the studies concerned *Claviceps purpurea* (Fr.:Fr.) Tul. and relied on random amplified polymorphic DNA (RAPD) analysis utilising polymerase chain reaction (PCR) techniques (Jungehülsing & Tudzynski 1997; Pažoutová & Tudzynski 1999; Pažoutová *et al.* 2000b, 2002; Duncan *et al.* 2002). Application of RAPD, random amplified microsatellite (RAM), amplified fragment length polymorphism (AFLP) and sequence analysis of the β-tubulin gene intron 3 region and EF-1-α gene intron 4 also enabled Pažoutová *et al.* (2000a) and Tooley *et al.* (2000, 2001) to separate the *Claviceps* species associated with *Sorghum* species. Although some of the above investigations included additional ergot species as outgroups, the only comprehensive phylogenetic study was by Pažoutová (2001), who compared 16 *Claviceps* species by means of PCR amplification and alignment of 5.8S rDNA and the adjacent internal transcribed spacers (ITS) 1 and 2.

One species that has thus far not been included in any molecular study is *Claviceps cyperi* Loveless, causal agent of ergot in yellow and purple nut sedge (*Cyperus esculentus* L. and *Cyperus rotundus* L.), which has recently been implicated in several cases of bovine ergotism in South Africa (Van der Linde & Naude 2000; Naude *et al.* 2005). Observations in Chapter 2 confirmed that *C. cyperi* is morphologically distinct from *Claviceps grohii* J.W. Groves and *Claviceps nigricans* Tul., the other two ergot species recorded on *Cyperaceae*. This report describes the molecular characterisation of *C. cyperi* and its separation from other *Claviceps* species available in the GenBank sequence database with the aid of multilocus PCR fingerprinting of genomic DNA and sequence analysis of the ITS1—5.8 rDNA-ITS2 and β-tubulin gene intron 3 regions.

6.2   MATERIALS AND METHODS

6.2.1   Strains examined

Details of the *Claviceps* and outgroup strains included in the study are summarised in Table 6.1.

6.2.2 Extraction and purification of DNA

The CTAB method of Ausubel *et al.* (1989) was used for the extraction and purification of genomic DNA of the four South African isolates included in this study (Table 6.1). Purified DNA was quantified with fluorometry using Hoescht (H 33258) dye and a Hoefer Dyna Quant 200 mini-fluorometer (Hoefer, San Francisco, CA). The final DNA concentration was adjusted to between 25—50 ng μl^{-1} at which all rep-PCR and RAPD fingerprinting was performed.

6.2.3 PCR fingerprinting of genomic DNA

All reactions were performed in a total volume of 10 μl in 0.2 ml microtubes. Repetitive sequence-based (rep-PCR) typing was performed with BOXA1R and ERIC2 single primers according to Versalovic *et al.* (1994). PCR buffer for rep-PCR was changed to 10 mM Tris-HCl; 50 mM KCl; 3.5 mM $MgCl_2$; 0.1 % Triton X-100; pH 9.0 (Promega, Madison, Wisconsin). A GC-rich arbitrary primer ARP-7 was used according to Mathis & McMillan (1996). PCR products were separated in 1.5 % agarose at 80 V constant in TBE 1x buffer (89 mM Tris-base; 89 mM borate; 2 mM EDTA, pH 8.0). Amplification products were stained with ethidium bromide (1 mg ml^{-1}) for 15 minutes at 25 °C and destained for 10 minutes. DNA relative molecular mass marker (Roche Diagnostics, no VI; GmbH, Mannheim) was loaded in lane one of each gel. Gel images were captured with a CCD camera and stored as TIFF-uncompressed graphic files. Gel images were processed and a similarity matrix and maximum parsimony tree created using Bionumerics ver. 3.0 (Applied-Maths, BVBA, St.-Martens-Latem). Cluster and discriminant analysis of fingerprints were performed on the similarity matrix with Ward's clustering algorithm with Euclidean distances. (Statistica ver. 6.0; Statsoft, Tulsa, Oklahoma). K-means clustering of group means was performed based on user-specified clusters as derived from the dendrogram.

This clustering method attempts to minimise the distances within each cluster and to maximise between-cluster distances. The K-means method tests the validity of user-specified clusters based on the topology of the dendrogram. In addition to K-means,

Multivariate Analysis of Variance (MANOVA) was used to test the validity of user-delineated groups (clusters). MANOVA is a statistical method which proves that the likelihood of obtaining equally good separations and discrimination when groups are chosen at random, approaches zero. In addition, MANOVA will determine which characters are responsible for the discrimination between delineated groups. Parameter L (Wilkinson's likelihood for normal distributions) predicts the likelihood that the groups as delineated by the user, were drawn from the same population. When L is low to zero, the strains or entries most likely were drawn from different populations confirming the discrimination between user-delineated groups. Parameter p is the probability that a random subdivision into different groups would produce equally good discrimination between groups. Once again a low p value will confirm the validity of user-delineated groups.

6.2.4 Sequence analysis of the ITS1/2 and 5.8S regions (rDNA operon)

This region of the rDNA operon was chosen because sequence data of several *Claviceps* species has been submitted to the GenBank database, which makes it convenient for comparative purposes when new strains are sequenced. The complete ITS 1, 5.8 S and ITS 2 regions were amplified using primers ITS 5 (5' - GGA AGT AAA AGT CGT AAC AAG G -3') and ITS 4 (5' - TCC TCC GCT TAT TGA TAT GC-3') which are complementary to conserved sequences flanking the ITS 1 and 2 spacers. A schematic presentation of the rDNA gene cluster amplified is shown in Figure 6.1. Amplification conditions were as follows: initial denaturation 95 °C for 3 minutes; annealing at 60 °C for 1 minute; extension at 72 °C for 2 minutes; 30 cycles performed at 94 °C for 1 minute, 60 °C for 1 minute and 72 °C for 2 minutes; final extension at 72 °C for 10 minutes. The reaction mixture consisted of 10 mM Tris-HCl; 1.5 mM $MgCl_2$; 50 mM KCl; 0.1 % Triton X-100; pH 9.0; Taq polymerase 0.133 units per 10 µl reaction mixture (Promega, Madison, Wisconsin).

PCR amplified ITS1/2 and 5.8 S regions were purified using the High Pure PCR Product Purification kit (Roche Molecular Diagnostics, Johannesburg, South Africa) or Qiagen PCR Purification kit (Southern Cross Biotechnologies, Johannesburg) according to the

manufacturer's instructions. To assess the purity and concentration of the purified product, 1.0 µl was subjected to electrophoresis on a 1 % agarose gel (Promega, Madison, Wisconsin).

The purified PCR products were sequenced directly, without any additional cloning procedures, using the ABI Prism BigDyeTM Terminator Cycle Sequencing Ready Reaction kit (with AmpliTaqR DNA Polymerase, FS) (PE Applied Biosystems, Foster City, California). Each sequencing reaction was carried out in a 5 µl volume containing approximately 100 ng template DNA, 12.5 pmol primer and 2 µl ready reaction premix (supplied with the sequencing kit, containing the dye terminators, dNTP's, AmpliTaq DNA Polymerase, $MgCl_2$ and Tris-HCl buffer, pH 9.0).

Sequences were aligned in accordance with DNAMAN ver. 5.1 (Lynnon BioSoft, Quebec) using optimal alignment and a dynamic method according to Feng & Doolittle (1987) and Thompson *et al.* (1994). The following optimal alignment parameters were applied: gap open penalty (10.00), gap extension penalty (5.00), percentage delay divergent (40). Distance matrices were calculated using a) Observed Divergence in combination with the Kimura 2-parameter correction (Kimura 1980), and b) Maximum Likelihood (Hasegawa *et al.* 1985). A rooted phylogenetic tree was then constructed using the Neighbour-joining method of Saitou & Nei (1987). Bootstrap confidence values were determined using 500 permutations of the data set to establish confidence at branching points (Felsenstein 1985). Sequence data sets were submitted to GenBank. Accession numbers are listed in Table 6.1.

Distance matrix spreadsheets generated by DNAMAN software were analysed by Statistica ver. 6.0 (Statsoft, Tulsa, Oklahoma). Dendrograms depicting grouping of species were drafted using Ward's clustering algorithm with Euclidean distances. The K-means clustering procedure was used to place species in well-defined clusters or groups showing distances between clusters and the distance from the cluster centre of each strain within each cluster. Between-cluster distances were maximised and within-cluster variance minimised.

Non-metric multidimensional scaling was applied to detect meaningful hidden dimensions that reveal observed similarities and dissimilarities (distances) between strains and species. A two-dimensional scatterplot is constructed to show clusters of data points and other particular patterns of relatedness between strains and species in a two-dimensional plane. Principal component analysis is performed (standard Guttman-Lingoes) as a first step, followed by measures of goodness-of-fit or the raw stress value, which measure how well a particular configuration reproduces the observed distance matrix. Stress values are presented as D-hat, D-star, alienation and a final stress value. The lower the stress value, the better the fit of the reproduced distance matrix to the observed distance matrix. The Shepard diagram is used to test the goodness-of-fit of the data points (D-hat values) on the step-function of the diagram. The closer the fit of the data points to the step function, the better the reproduction of the distances in the input data as applied to the different dimensions. Deviations from this step-function indicate lack of fit. In order to arrive at an interpretable solution, six to nine dimensions were computed followed by comparison of dimension one with dimensions two to five. The final configuration placing the strains in distinct clusters and showing the optimal separation between strains was selected.

6.2.5 Amplification and sequencing of the β-tubulin gene intron 3 region

The β-tubulin (tub2) intron 3 region was amplified using primers complementary to conserved exonic sequences (Annis & Panaccione 1998). Forward primer BT5: 5'-GCT CTA GAC TGC TTT CTG GCA GAC C-3'; reverse primer BT3: 5'-CGT CTA GAK GTR CCC ATA CCG GCA-3'; redundancies K=G/T; R=A/G. A schematic presentation of the intron 3 region amplified is shown in Figure 6.2. Amplification conditions were as follows: initial denaturation 95 °C for 1 minute; 30 cycles of 94 °C for 15 seconds; 55 °C for 15 seconds and extension at 72 °C for 15 seconds; final extension at 72 °C for 6 minutes. PCR products were gel-purified and sequenced with an automated sequencer as described for the ITS1/2 5.8S region. Sequences were aligned and data presented as described for the ITS1/2 region spacers.

Distance matrix spreadsheets generated by DNAMAN software were analysed by Statistica ver. 6.0 (Statsoft, Tulsa, Oklahoma). Dendrograms depicting grouping of

species were drafted using Ward's clustering algorithm with Euclidean distances. The K-means clustering procedure was used to place species in well-defined clusters or groups showing distances between clusters and the distance from the cluster centre of each strain within each cluster. Between-cluster distances were maximised and within-cluster variance minimised. Multidimensional scaling was performed to produce two-dimensional scatterplots as described for the analysis of the ITS 1,2 spacers.

6.3   RESULTS

6.3.1   Multilocus PCR fingerprinting of genomic DNA

Fingerprinting with all three primers clearly distinguished between *C. cyperi*, *C. grohii* (CBS 124.47) and *C. purpurea*. The dendrogram (Figure 6.3) and similarity matrix (Table 6.2) indicated a low level of similarity between these *Claviceps* species. Using BOXA1R (Figure 6.4), ARP7 (Figure 6.5) and ERIC2 (Figure 6.6) primers, *C. grohii* (CBS 124.47) clustered closer to C. *pupurea* (53 %) and showed 44 % similarity with *Tilletia indica* Mitra, while the two *C. cyperi* isolates clustered separately from both *C. grohii* (CBS 124.47) and *C. purpurea*, showing 96 % similarity between them (Table 6.2). The maximum parsimony tree (Figure 6.7) shows the branching distances between the different strains and in particular the longer distance between *C. cyperi* and the rest of the tree. All strains showed unique patterns for each primer except the two *C. cyperi* strains, which proved the usefulness of repetitive sequence-based PCR and GC-rich arbitrary primers for grouping and distinguishing between *Claviceps* species up to strain level (Figures 6.4 — 6.6).

MANOVA discriminant analysis confirmed the validity of the dendrogram clusters (Figure 6.3, Table 6.2). The likelihood that the strains of *Claviceps* species were drawn from the same population, indicating no discrimination between the strains, was very low as indicated by parameter L. The probability that a random subdivision in groups will yield the same degree of discrimination as the user-selected groups, was negligible as indicated by $P < 0.001$ (Table 6.2).

6.3.2 Sequence analysis of ITS1/2 and 5.8 S regions

Strains used to construct a phylogram are listed in Table 6.3. Amplification of the complete ITS 1 and 2 spacers with the 5.8S gene produced only one PCR product for the isolates of *C. cyperi* (01/CC; 02/CC) of size 656 bp and 654 bp respectively. *C. grohii* (CBS 124.47) showed a product of 585 bp size, whereas a product of size 584 bp was amplified from *C. purpurea* (03/CP). Both *C. cyperi* strains possessed an extended ITS 1 spacer in comparison with *C. purpurea* 03/CP and *C. grohii* (CBS 124.47). No polymorphisms or other non-specific fragments were amplified. When *C. cyperi* (01/CC; 02/CC) and *C. purpurea* (03/CP) strains were compared to other *Claviceps* species deposited in the GenBank sequence database, a dendrogram (Figure 6.8) placed the *Claviceps* species in four major clusters. The related teleomorphic species of *Atkinsonella*, *Epichloë* and *Echinodothis* were included in a cluster (no. 4), together with *Claviceps citrina* Pažoutová, Fuĭk., Leyva-Mir & Flieger, even though the distance from the cluster centre of the latter species was larger than the distances of the other genera in this cluster (Table 6.3). *Claviceps zizaniae* (Fyles) Pantidou shows 93 % similarity in the similarity matrix with the two *C. cyperi* isolates and was consequently included in a cluster (no. 3), together with the *C. cyperi* isolates which were clearly distanced from all the other clusters according to the table of Euclidean distances (Table 6.3). *C. grohii* (CBS 124.47) and the four *C. purpurea* strains were included in a second major cluster. *Claviceps paspali* F. Stevens & J.G. Hall did not fit well in any of the four clusters (Figure 6.8, Table 6.3), because of a much larger distance from the centre of cluster 2. *Claviceps fusiformis* Loveless, *Claviceps africana* Freder., P.G. Mantle & W.A.J. de Milliano, *Claviceps viridis* Padwick & Azmatullah, *Claviceps gigantea* S.F. Fuentes, Isla, Ullstrup & Rodriquez, *Claviceps sorghi* B.G.P. Kulk., Seshadri & Hegde, *Claviceps sorghicola* Tsukib., Shiman. & T. Uematsu, *Claviceps maximensis* T. Theis, *Claviceps pusilla* Ces. and *Claviceps phalaridis* J. Walker formed the largest major cluster. In the dendrogram (Figure 6.8), *C. gigantea*, *C. sorghi* and *C. fusiformis* formed a smaller sub-cluster within this major cluster.

The two-dimensional plot (Figure 6.9) also revealed four major clusters with multidimensional scaling. Six dimensions were computed showing low stress values indicating goodness-of-fit of data points to the step function of the Shepard diagram

(Table 6.3). The first major cluster included the same nine *Claviceps* species as revealed by the dendrogram. All species in this cluster were grouped in a separate cluster (Figure 6.9). The second major cluster contained *C. purpurea*, *C. grohii* (CBS 124.47) and *C. paspali*, with *C. paspali* and one *C. purpurea* strain slightly further distanced from the cluster centre (Table 6.3). The teleomorphic species formed a less closely spaced cluster in the two-dimensional plot while *C. citrina* did not fit well in any cluster (Figure 6.9) as confirmed by the distance from the cluster centre (Table 6.3). The *C. cyperi* isolates and *C. zizaniae* formed a separate cluster well distanced from the other clusters with *C. zizaniae* further distanced from this cluster centre. The groupings generated by multidimensional scaling were similar to the clustering pattern of the dendrogram and supported the K-means partitioning of clusters.

A similar clustering pattern of four major clusters is depicted in the phylogenetic tree (Figure 6.10) which places the species in the same major clusters, except *C. paspali* which forms an outlier and occupied an intermediate position distanced from the major clusters. This positioning of *C. paspali* was more pronounced in the phylogenetic tree than in the dendrogram (Figure 6.8), the K-means table of distances (Table 6.3) and the two-dimensional plot (Figure 6.9). *C. cyperi* together with *C. zizaniae* formed a distinct separate cluster at a large distance from the other major clusters. It was apparent from the phylogenetic tree (Figure 6.10) that the related teleomorphic species, as well as *C. citrina*, were included in a single cluster, clearly separated from the other clusters as well as from *C. paspali*. All branches linking major clusters were well supported by bootstrapping.

6.3.3 Sequence analysis of the β-tubulin gene intron 3 region

Using the described amplification conditions, the β-tubulin (tub2) gene of similar size was amplified from all isolates. The isolates of *C. cyperi* 01/CC and 02/CC amplified genes of 477 bp and 473 bp size, respectively. *C. grohii* (CBS 124.47) amplified a tub2 gene of 464 bp and *C. purpurea* 03/CP a tub2 gene of 475 bp. A dendrogram presentation indicates five distinct clusters, with the two related species of *Echinodothis* and *Epichloë*, not fitting well in any of the major clusters (Figure 6.11). *C. grohii* (CBS 124.47), *C. purpurea* 03/CP and other GenBank *C. purpurea* strains formed one cluster

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(no. 4). The three *C. africana* strains formed a separate cluster (no. 3), and the *C. sorghicola* strains grouped in cluster no. 5 together with *C. fusiformis* which clustered at a larger distance from this cluster centre (Table 6.4). The two *C. paspali* strains were placed in cluster no. 2. *Epichloë* did not fit well in any cluster but linked at a larger distance with cluster no. 2. The *C. cyperi* isolates were placed in cluster no. 7, with *Echinodothis* linking at a much larger distance with this cluster (Figure 6.11). Similar to the ITS1/2 spacers sequence data, the intron 3 region also placed the *C. cyperi* isolates at the largest distance from the other species clusters as indicated by Euclidean distances (Table 6.4).

The two-dimensional plot (Figure 6.12) confirmed the clustering pattern of the dendrogram and phylogram showing the same clusters clearly distanced (Table 6.4). The *C. cyperi* isolates formed a distinct separate cluster at a large distance from the other clusters (Table 6.4). The *Echinodothis* and *Epichloë* species formed outliers and remained unclustered (Figure 6.12).

The phylogenetic tree (Figure 6.13) corresponded with the cluster pattern of the dendrogram (Figure 6.11), although the *Epichloë* and *Echinodothis* species did not fit in any cluster. As expected, the *C. cyperi* strains formed a distinct outgroup cluster (94 % sequence similarity between strains) while *C. purpurea* 03/CP and *C. grohii* (CBS 124.47) (96 % sequence similarity), clustered with other *C. purpurea* strains. The different *Claviceps* species grouped in separate clusters whereas *C. fusiformis* was only linked at a distance to the larger cluster containing *C. africana* and *C. sorghicola*. All branches linking major clusters were well supported by bootstrapping.

6.4   DISCUSSION

In this study, multilocus fingerprinting clearly differentiated between strains of *C. purpurea*, *C. grohii* (CBS 124.47) and *C. cyperi*, with the two *C. cyperi* isolates showing identical band patterns. However, due to the limited number of isolates available, variation within the species could not be established. Previous studies revealed considerable intraspecific genetic diversity within various species of *Claviceps* (Jungehülsing & Tudzynski 1997; Pažoutová & Tudzynski 1999; Pažoutová *et al.* 2000a,b, 2002; Tooley *et al.* 2000). The

above investigations indicated that multilocus fingerprinting could differentiate up to strain level, rendering it appropriate for the study of genetic characteristics of populations of ergot fungi. The lower similarity between *C. purpurea* and *C. grohii* (CBS 124.47) shown by multilocus fingerprinting in the present study, contrasted with sequence analysis of the ITS1/2 spacers and β-tubulin gene, where higher sequence similarity was established between these species. This observation is in agreement with the study by Tooley *et al.* (2000) where multilocus genotyping revealed greater genetic variation at the intraspecific level than sequence data of different genes. The random method of fragment amplification by single primers comlementary to repeated sequence motifs that are spread over the whole of the genome, should explain this intraspecific diversity.

The phylogenetic relationships indicated by the ITS1/2 spacers corresponded with those described by Pažoutová (2001), in which two distinct clades were observed, one comprising *C. citrina*, *C. phalaridis*, *C. sorghicola*, *C. sorghi*, *C. gigantea*, *C. africana*, *C. viridis* and *C. pusilla*, and the other *C. paspali*, *C. zizaniae*, *C. grohii*, *C. sulcata*, *C. fusiformis* and *C. purpurea*. In the present study, however, *C. zizaniae* showed a higher sequence similarity to the two *C. cyperi* isolates, with which it formed a distinct separate cluster based on ITS spacers sequence data. It is significant that the extended ITS1 spacer present in the *C. cyperi* isolates was also present in *C. zizaniae*. The evolutionary significance of this sequence insertion in ITS spacer 1 indicates either a common ancestor (monophyletic origin) or a paraphyletic origin of *C. zizaniae* and *C. cyperi*, though a process of convergent evolution or a speciation event could also have been involved.

Physiologically, *C. cyperi* and *C. zizaniae* share a common alkaloid profile (Kantovora *et al.* 2002; Chapter 5). Both also have hosts that prefer a moist habitat, viz. yellow nut sedge (*Cyperus esculentus* L.) and wild rice (*Zizania palustris* L. and *Zizania aquatica* L.), respectively (Loveless 1967; Pantidou 1959). Morphologically, however, the two species are quite distinct, with *C. zizaniae* producing considerably larger sclerotia than *C. cyperi*, and a differently coloured capitulum without a collar-like appendage surrounding the base (Pantidou 1959; Loveless 1967; Chapter 2). The above differences and similarities support the view of Pažoutova *et al.* (2000b, 2002), Pažoutová (2001) and Duncan *et al.* (2002) that the phylogenetic positions of *Claviceps* species tend to correspond with their alkaloid profiles and ecological specialisation rather than their morphological features and host

specificity. Further evidence regarding the unrelatedness of morphology and phylogeny in *Claviceps* can be derived from the prominent ranunculoid papilla present in *C. cyperi*, a feature it shares with *Claviceps ranunculoides* A. Möller recorded from *Setaria* sp. in Brazil (Möller 1901), that distinguishes them from all other known *Claviceps* species. No type material or GenBank strain of *C. ranunculoides* was available for inclusion in the present study. However, the species was included, together with *C. africana*, *C. fusiformis*, *C. paspali* and *C. purpurea*, in a phylogenetic analysis of the 26S large subunit rDNA of graminicolous *Clavicipitaceae* by Sullivan *et al.* (2001). According to the above analysis, *C. ranunculoides* clustered closest to *C. africana* followed by *C. fusiformis*, with *C. paspali* and *C. purpurea* forming a separate cluster. This pattern implies that *C. ranunculoides* probably would have clustered with *C. africana*, *C. fusiformis*, *C. gigantea*, *C. maximensis*, *C. phalaridis*, *C. pusilla*, *C. sorghi*, *C. sorgicola* and *C. viridis*, at a far distance from *C. cyperi*, had it been included in the present study.

Besides confirming *C. cyperi* to be a distinct species, the present investigation also provided evidence regarding the phylogeny of two "dubious" *Claviceps* species, viz. *C. citrina* and *C. paspali*. As indicated above, Pažoutová (2001) placed *C. citrina* in a cluster which included *C. africana* and *C. sorghicola*, whereas *C. paspali* grouped in a separate cluster with *C. fusiformis*, *C. grohii*, *C. purpurea*, *C. sulcata* and *C. zizaniae*. However, in a study using PCR amplification, Duncan *et al.* (2002) found *C. citrina* to cluster separately from *C. africana* and *C. sorghicola*. This grouping corresponds with the present results according to which *C. citrina* was not properly associated with any cluster, but only grouped with *Echinodothis tuberiformis* (Berk. & Ravenel) G.F. Atk., *Epichloë typhina* (Pers.) Tul. & C. Tul. and *Atkinsonella hypoxylon* (Peck) Diehl at a much larger distance. The present study also showed *C. paspali* to cluster separately from *C. fusiformis* and *C. purpurea*, which is in agreement with the sequence analysis of the β-tubulin intron 3 and EF-1 α-gene intron 4 regions reported for *Claviceps* species on sorghum by Tooley *et al.* (2001). However, in the latter study *C. fusiformis* grouped with *C. sorghicola* and *C. africana* with a bootstrap value of 82 %, possibly because resequencing of the original RAPD and ITS1 sequences reported by Pažoutova *et al.* (1998) for the strain of *C. fusiformis* referred to by Tooley *et al.* (2001) indicated that it is actually *C. purpurea* (S. Pažoutova, pers. com.). All publications based on the incorrect sequence should therefore be reconsidered.

In conclusion, although this study elucidated the phylogeny of the nut sedge ergot pathogen, extensive genetic diversity studies on South African isolates of *C. cyperi* still need to be done to establish the genetic profile of local populations of the species. The most perplexing observation was the outgroup cluster of *C. cyperi* that linked at a large distance from the other *Claviceps* species. The presence of the extended ITS1 spacer not present in any other *Claviceps* species except *C. zizaniae*, may explain this outgroup placement insufficiently. The true natural phylogenetic relationship of *C. cyperi* and *C. zizaniae* will only be resolved after a comprehensive study of many isolates from various geographic areas.

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VERSALOVIC, J., SCHNEIDER, M., DE BRUIJN, F.J. & LUPSKI, J.R. 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods in Molecular and Cellular Biology* **5**:25—40.
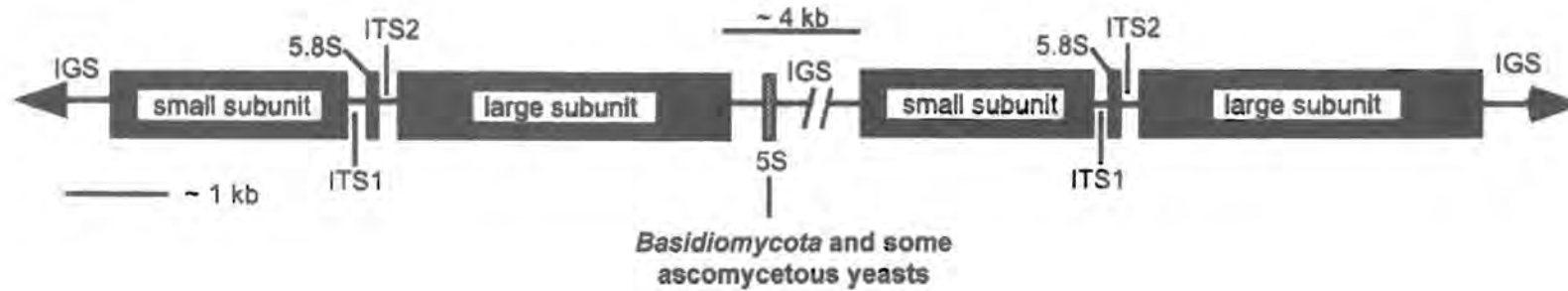
Figure 6.1   Schematic presentation of rDNA cluster of tandemly repeated ribosomal genes. Large subunit = 28 S; small subunit = 18 S; IGS = intergenic spacer; ITS = internal transcribed spacers 1 & 2; 5.8 subunit.
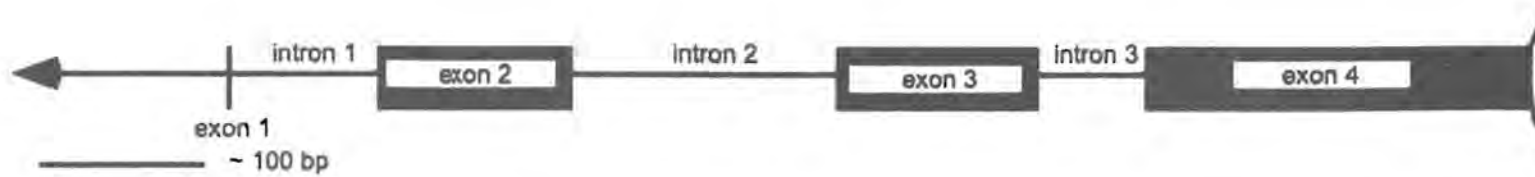
Figure 6.2   Example of an evolutionary conserved intron-rich protein-coding gene with exons 2,3,4 = conserved protein-coding sequences; introns 1-3 = variable sequences.
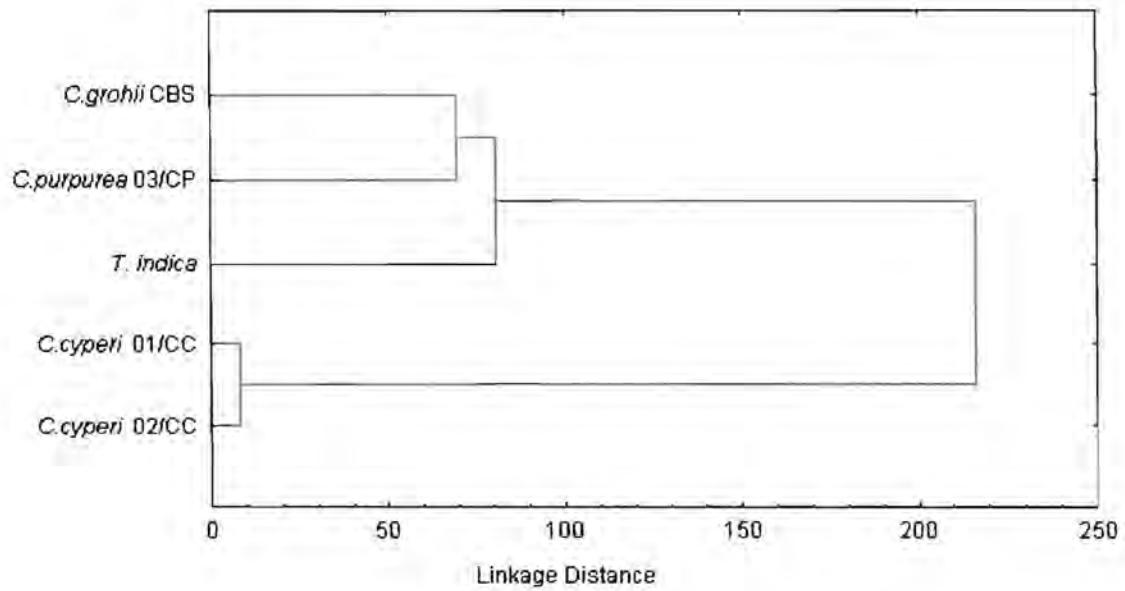
Figure 6.3   Dendrogram showing genetic differences between three *Claviceps* species and *Tilletia indica* based on multilocus fingerprinting data.

Figure 6.4   Electrophoretic band patterns using BOXA1R primer.

Lanes: M   Molecular mass marker
                1-5   *Claviceps purpurea* 03/CP
                6-7   *C. cyperi* 02/CC
                8-9   *C. cyperi* 01/CC
                10-13   *C. grohii* CBS 124.47
                14-15   *Tilletia indica* 1325

Figure 6.5   Electrophoretic band patterns using ARP-7 primer.

Lanes: M   Molecular mass marker
         1-5   *Claviceps purpurea* 03/CP
         6-7   *C. cyperi* 02/CC
         8-9   *C. cyperi* 01/CC
         10-13   *C. grohii* CBS 124.47
         14-15   *Tilletia indica* 1325

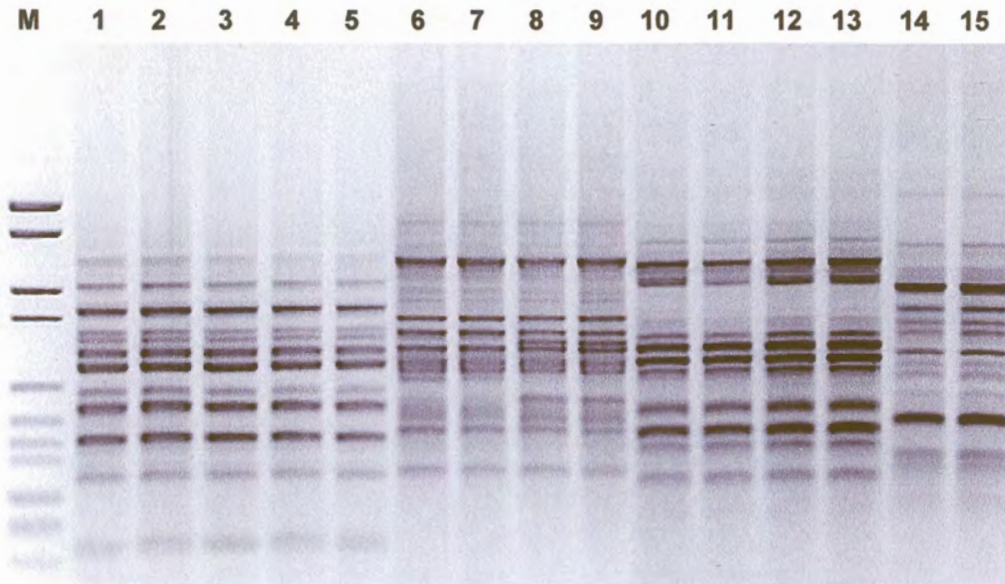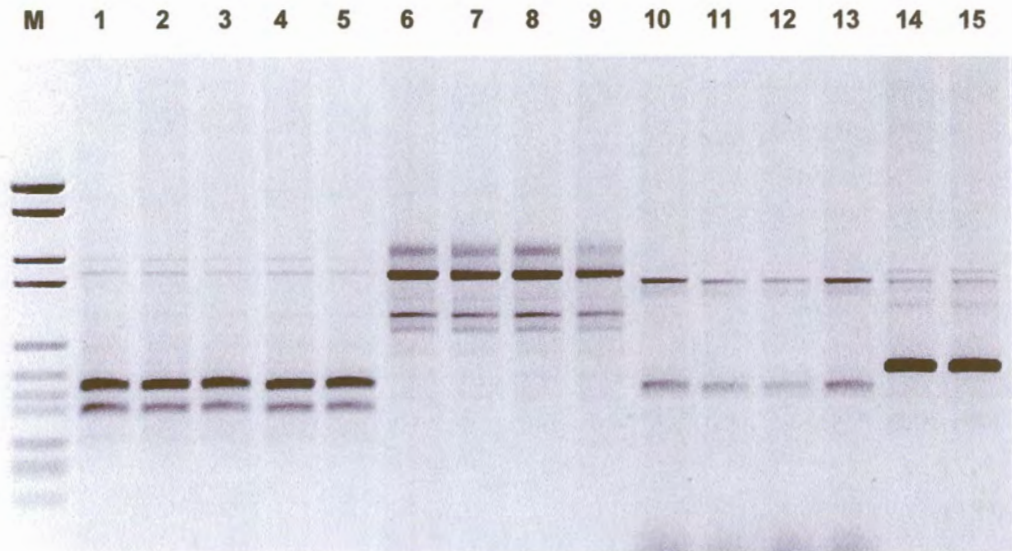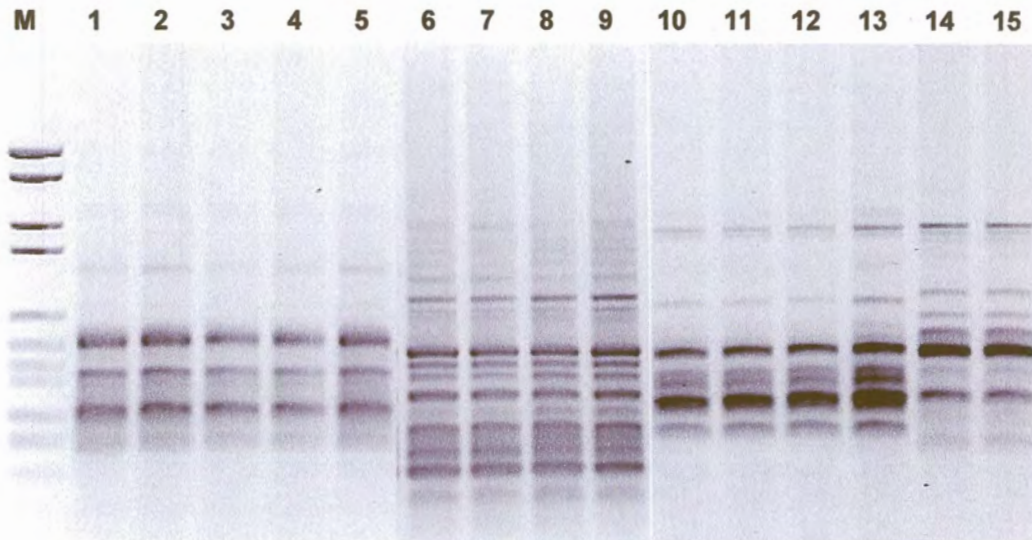Figure 6.6   Electrophoretic band patterns using ERIC2 primer.

Lanes: M   Molecular mass marker
              1-5   *Clavicpes purpurea* 03/CP
              6-7   *C. cyperi* 02/CC
              8-9   *C. cyperi* 01/CC
              10-13 *C. grohii* CBS 124.47
              14-15 *Tilletia indica* 1325

Figure 6.7   Maximum parsimony tree based on multilocus fingerprinting profiles of three *Claviceps* species and *Tilletia indica*.

Figure 6.8   Dendrogram showing genetic distances between different *Claviceps* species and related teleomorph species based on ITS1/2 spacer sequences.
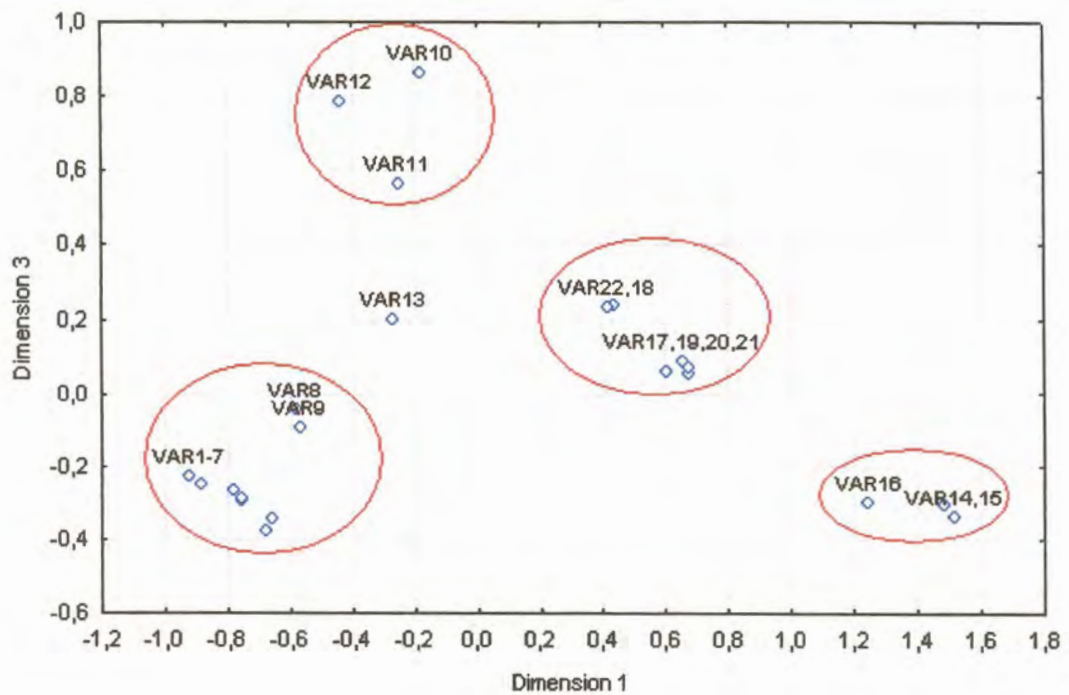
Strain code:

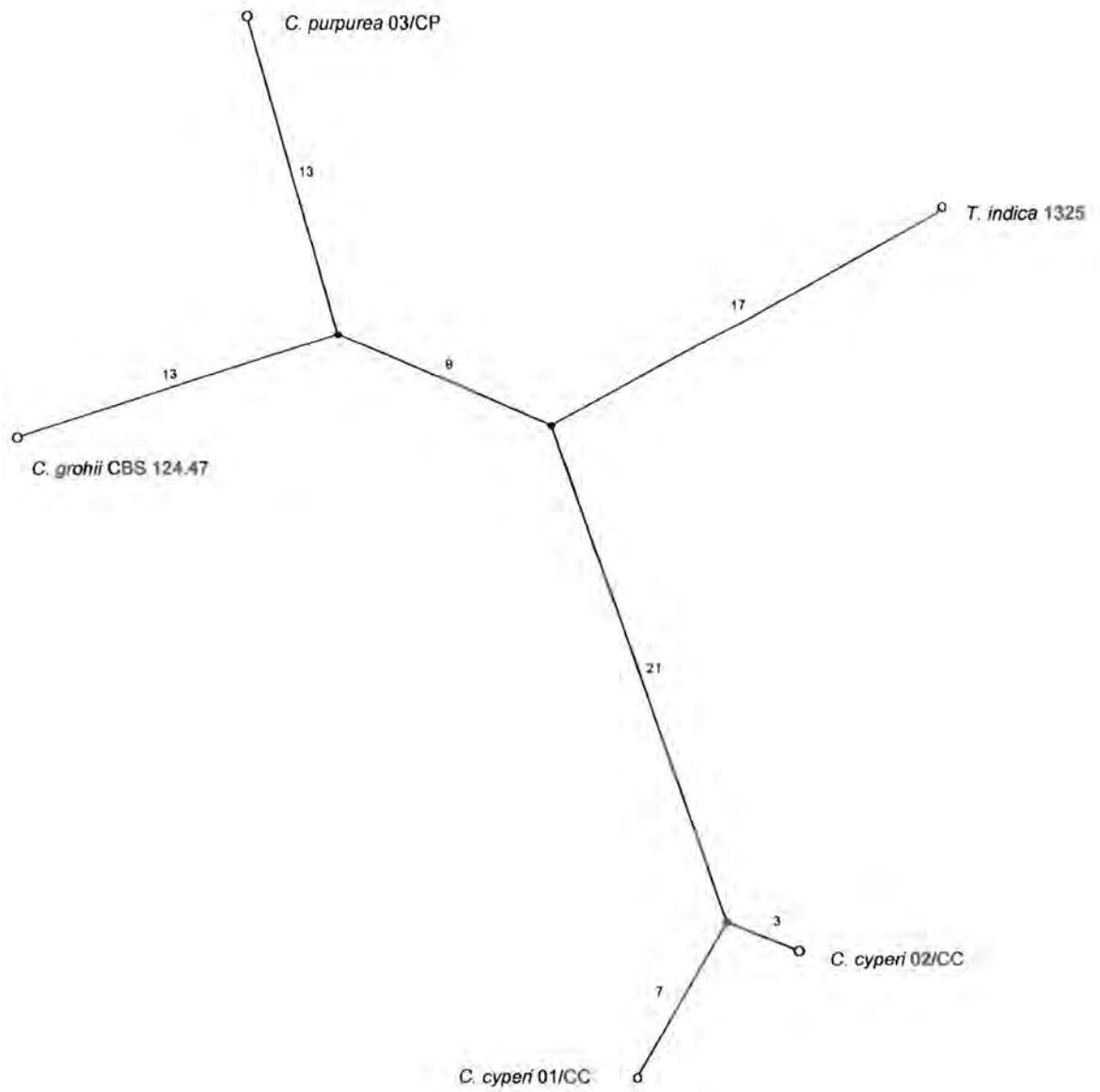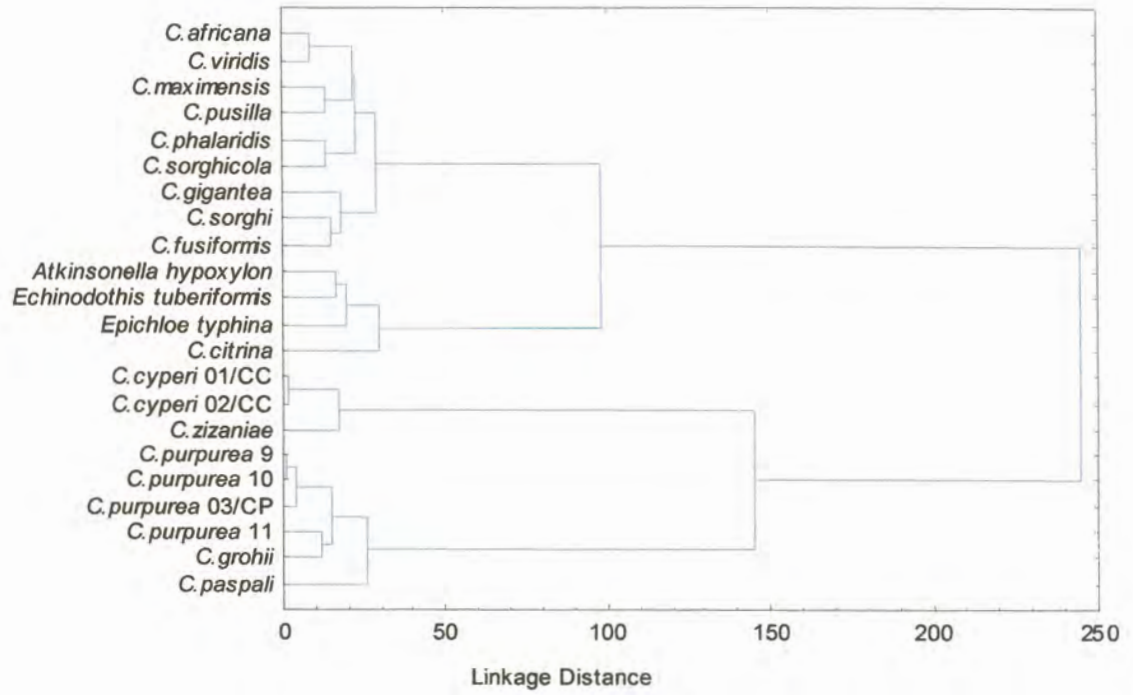|		
VAR 1	*C. africana*	NE1; Cafr
VAR 2	*C. viridis*	CBS 125.63
VAR 3	*C. gigantea*	
VAR 4	*C. sorghi*	
VAR 5	*C. fusiformis*	SD 85
VAR 6	*C. maximensis*	PM
VAR 7	*C. pusilla*	BRIP 26571
VAR 8	*C. phalaridis*	DAR 69619
VAR 9	*C. sorghicola*	
VAR 10	*Atkinsoniella hypoxylon*	CBS 125.63
VAR 11	*Echinodothis tuberiformis*	
VAR 12	*Epichloë typhina*	
VAR 13	*C. citrina*	
VAR 14	*C. cyperi*	01/CC
VAR 15	*C. cyperi*	02/CC
VAR 16	*C. zizaniae*	CCM 8231
VAR 17	*C. purpurea* 9	134 AU
VAR 18	*C. purpurea* 11	
VAR 19	*C. purpurea*	03/CP
VAR 20	*C. purpurea* 10	
VAR 21	*C. grohii*	CBS 124.47
VAR 22	*C. paspali*	MAL-1

Figure 6.9   Two-dimensional scatterplot showing genetic differences between *Claviceps* species and related teleomorphic species based on ITS1/2 spacer sequence data.

Figure 6.10   Phylogenetic tree showing phylogenetic relationships between *Claviceps* species and related teleomorphic species based on ITS1/2 spacers sequence data.
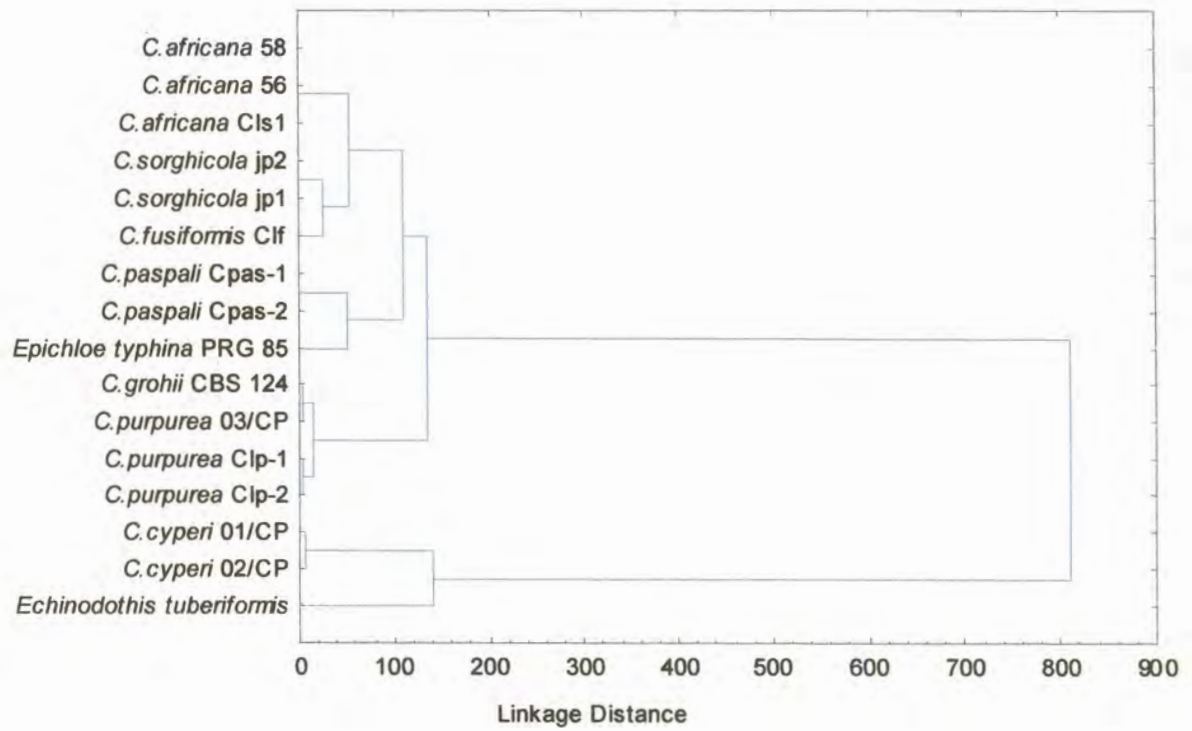
Figure 6.11   Dendrogram showing genetic distances between different *Claviceps* species and related teleomorphic species based on β-tubulin gene intron 3 region sequences.
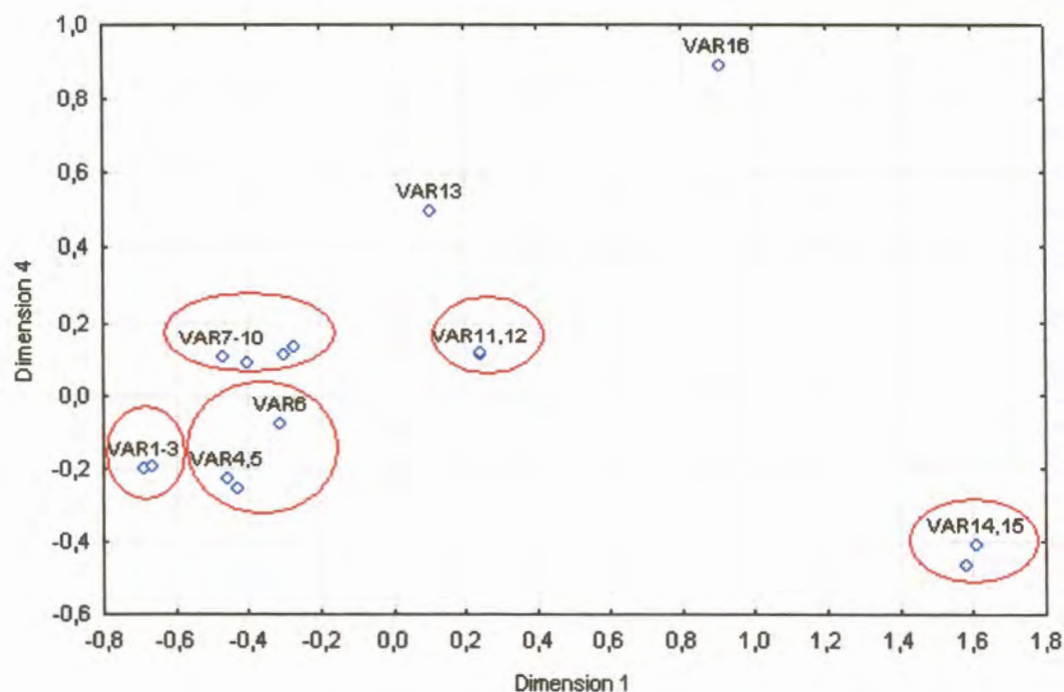
Strain code:

VAR1	C. africana	Cls-1
VAR2	C. africana	Cla-56
VAR3	C. africana	Cla-58
VAR4	C. sorghicola	Cjap-1
VAR5	C. sorghicola	Cjap-2
VAR6	C. fusiformis	Clf -1
VAR7	C. grohii	CBS 124.47
VAR8	C. purpurea	03/CP
VAR9	C. purpurea	Clp-1
VAR10	C. purpurea	Clp-2
VAR11	C. paspali	Cpas-1
VAR12	C. paspali	Cpas-2
VAR13	Epichloë typhina	PRG; 85
VAR14	C. cyperi	01/CC
VAR15	C. cyperi	02/CC
VAR16	Echinodothis tuberiformis	B 351

Figure 6.12   Two-dimensional scatterplot showing genetic distances between different *Claviceps* species and related teleomorphic species based on β-tubulin gene intron 3 sequences.
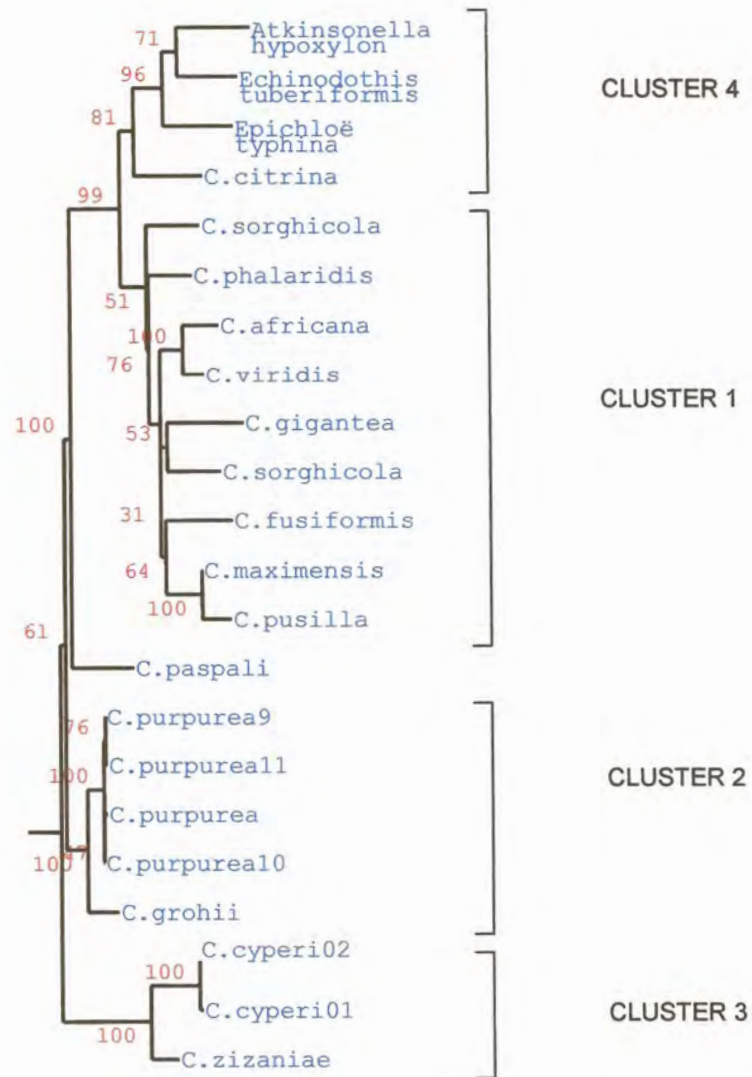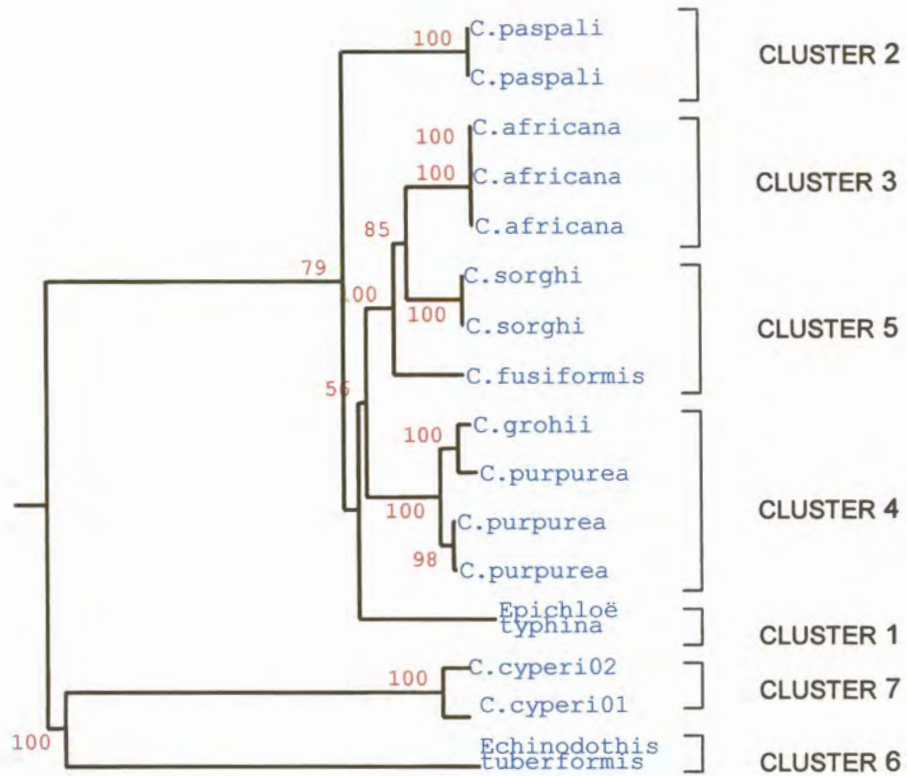
Figure 6.13   Phylogenetic tree showing phylogenetic relationships between *Claviceps* species and related teleomorphic species based on β-tubulin gene intron 3 region sequences.

Table 6.1   Strains of *Claviceps* species and related teleomorphic genera included in this study.

Species	Strain code	Date isolated	Locality	Host	Genbank accession no. for ITS1/2 spacers
C. cyperi	01/CC	2000	Greylingstad, SA	*Cyperus esculentus*	AY387492
C. cyperi	02/CC	2003	Greylingstad, SA	*Cyperus esculentus*	AY387491
C. purpurea	03/CP	2002	Onderstepoort, SA	*Festuca eliator*	AY387490
Tilletia indica	1325	2001			
C. fusiformis	SD 85			*Pennnisetum* sp.	AF133392
C. viridis	CBS 125.63	1963	India	*Oplismenus compositus*	AJ133404
C. grohii	CBS 124.47	1947	Canada	*Carex* sp.	AJ133395
C. zizaniae	CCM 8231	1996	Canada	*Zizania aquatica*	AJ133405
C. phalaridis	DAR 69619	1996	New South Wales, Austr	*Phalaris tuberosa*	AJ133399
C. pusilla	BRIP 26571	1996	Queensland, Australia	*Dicanthium aristatum*	AJ277544
C. sorghi		2000	India	*Sorghum bicolor*	AJ242869
C. sorghicola		1996	Japan;Kanto	*Sorghum bicolor*	AJ133397
C. paspali	MAL-1	1999	USA, Alabama	*Paspalum* sp.	AJ133398
C. purpurea	10	1996	Georgia, USA	*Dactylis glomerata*	U57669
C. purpurea	9 134 AU	1999	Victoria, Australia	*Phalaris tuberosa*	AJ133400
C. purpurea	11	1999		Strain Pepty 695/S	AJ133401
C. sulacata		1999	Brazil	*Brachiaria* sp.	AJ133403
C. citrina		1999	Mexico	*Distichlis spicata*	AJ133393
C. africana	NE1; Cafr	2000	Nebraska, USA	*Sorghum*	AF281176
C. viridis	CBS 125.63	1999	India	*Oplismenus compositus*	AJ133404
C. gigantea		1999	Mexico	*Zea mays*	AJ133394
C. maximensis	PM	1999	Paraguay	*Panicum maximum*	AJ133396
Atkinsonella hypoxylon		1996	Georgia, USA	*Danthonia spicata*	U57405
Echinodothis tuberiformis		1996	Alabama, USA	*Arundinaria tecta*	U57667
Epichloë typhina		2003			AB105952

Species	Strain code	Date isolated	Locality	Host	Genbank accession no. for β-tubulin intron 3
C. cyperi	01/CP	2000	Greylingstad, SA	Cyperus esculentus	AY497005
C. cyperi	02/CP	2003	Greylingstad, SA	Cyperus esculentus	AY497775
C. grohii	CBS 124.47	1947	Canada	Carex sp.	AY438671
C. purpurea	Clp-1		Montana, USA	Hordeum vulgare	AF263567
C. purpurea	Clp-2		East Germany	Secale cereale	AF263568
C. purpurea	03/CP	2002	South Africa	Festuca eliator	AY438670
C. fusiformis	Clf-1		Africa	Pennisetum typhoideum	AF263569
C. paspali	Cpas-1	1998	North Carolina	Paspalum sp.	AF263605
C. paspali	Cpas-2	1997	Georgia	Paspalum sp.	AF263606
C. africana	Cls-1	1997	India	Sorghum bicolor	AF263596
C. africana	Cla-56	1998	Potchefstroom, SA	Sorghum bicolor	AF263591
C. africana	Cla-58	1998	Potchefstroom, SA	Sorghum bicolor	AF263592
C. sorghicola	Cjap-1	1989	Tochigi, Japan	Sorghum bicolor	AF263600
C. sorghicola	Cjap-2	1996	Tochigi, Japan	Sorghum bicolor	AF263601
Epichloë typhina	PRG; 85	1990		Lolium perenne	X52616
Echinodothis tuberiformis	B 351	1997			L78268

Table 6.2   Dendrogram and K-means clustering of South African isolates of *Claviceps purpurea*, *C. grohii*, *C. cyperi* and *Tilletia indica* based on multilocus fingerprinting.

Euclidean distances between clusters
Distances below diagonal

	No. 1	No. 2
No. 1	0	
No. 2	46.13407135	0

Distance from respective cluster centre *

	Variable	Cluster	Distance *
C. grohii CBS 124.47	1	2	15.5083758
C. purpurea 03/CP	2	2	15.5083758
T. indica 1325	3		
C.cyperi 01/CC	4	1	1.815852417
C.cyperi 02/CC	5	1	1.815852417

MANOVA discriminant analysis for BOX A1R; ERIC2 and ARP-7 multilocus primers

DISCR_01	EIGV= 62.3%	L= 0.0001	P<=0.001%
DISCR_02	EIGV= 37.7%	L= 0.0154	P<=0.001%

Similarity matrix

	C. grohii CBS	*C. purpurea*	*T. indica*	*C. c 01/CC*	*C. c 02/CC*
C.grohii CBS 124.47	100	52.65	43.61	41.56	46.18
C.purpurea 03/CP	52.65	100	52.2	34.09	32.16
T.indica	43.61	52.2	100	20.92	22.85
C.cyperi 01/CC	41.56	34.09	20.92	100	95.69
C.cyperi 02/CC	46.18	32.16	22.85	95.69	100

Table 6.3   Dendrogram and K-means clustering of different *Claviceps* species and related teleomorphic species showing genetic distances based on ITS1/2 spacers sequence data.

Euclidean distances between clusters
Distances below diagonal

	No. 1	No. 2	No. 3	No. 4
No. 1	0			
No. 2	8.539524078	0		
No. 3	12.09759808	9.195717812	0	
No. 4	6.131327629	8.274378777	10.75427914	0

Distance from respective cluster centre *

Variable		Cluster	Distance *
C. africana	1	1	2.142656396
C. viridis	2	1	1.702083295
C. gigantea	3	1	3.271944819
C. sorghi	4	1	2.24644875
C. fusiformis	5	1	2.646069444
C. maximensis	6	1	2.716711594
C. pusilla	7	1	2.418075173
C. phalaridis	8	1	2.817050658
C. sorghicola	9	1	2.530274602
Atkinsonella hypoxylon	10	4	2.568753456
Echinodothis tuberiformis	11	4	2.691838464
Epichloë typhina	12	4	2.391574938
C. citrina	13	4	3.727855068
C. cyperi 01/CC	14	3	0.836599659
C. cyperi 02/CC	15	3	1.022795733
C. zizaniae	16	3	1.837172289
C. purpurea 9	17	2	1.250676585
C. purpurea 11	18	2	1.678548555
C. purpurea 03/CP	19	2	1.014416287
C. purpurea 10	20	2	1.17838273
C. grohii	21	2	1.403063027
C. paspali	22	2	3.144202618

Multidimensional scaling: final configuration

No. of dimensions = 6
Measure of goodness-of-fit: Stress values

D-star:Raw stress	D-hat:Raw stress	Alienation	Stress
0.023605	0.0088243	0.0069836	0.0042699

Table 6.4   Dendrogram and K-means clustering of different *Claviceps* species and related teleomorphic species showing genetic distances based on β-tubulin gene intron 3 region sequence data.

Euclidean distances between clusters
Distances below diagonal

	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7
No. 1	0						
No. 2	9.537975311	0					
No. 3	10.74645329	11.3850803	0				
No. 4	9.716282845	11.5703497	10.1820211	0			
No. 5	9.56938076	9.92785358	5.14885426	8.502935	0		
No. 6	46.33493423	47.7928696	51.6772575	51.13251	50.81779099	0	
No. 7	48.63568497	49.4319077	53.3657837	52.53014	52.49116898	26.51585	0

Distance from respective cluster centre *

	Variable	Cluster	Distance*
C. africana Cls-1	1	3	0.06454972
C. africana Cla-56	2	3	0.06454972
C. africana Cla-58	3	3	0.12909944
C. sorghicola Cjap-1	4	5	1.53769759
C. sorghicola Cjap-2	5	5	1.52661572
C. fusiformis Clf-1	6	5	3.06055777
C. grohii CBS124.47	7	4	0.98862673
C. purpurea 03/CP	8	4	1.37541187
C. purpurea Clp-1	9	4	1.16479947
C. purpurea Clp-2	10	4	1.01621617
C. paspali Cpas-1	11	2	0.073951
C. paspali Cpas-2	12	2	0.073951
Epichloe typhina	13	1	0
C. cyperi 01/CC	14	7	0.77237459
C. cyperi 02/CC	15	7	0.77237459
Echinodothis tuberiformis	16	6	0

Multidimensional scaling: final configuration

No. of dimensions = 6
Measure of goodness-of-fit: Stress values

D-star:Raw stress	D-hat:Raw stress	Alienation	Stress
0.0000033	0.000001	0.00001139	0.00000624

7. GENERAL DISCUSSION

The previous chapters have provided descriptions of the symptoms of ergot on yellow nut sedge (*Cyperus esculentus* L.) and of live specimens of the causal organism, *Claviceps cyperi* Loveless. Evidence was also presented regarding the phylogeny and alkaloid profile of the pathogen, the morphology, taxonomy, physiology and mode of infection of its anamorph, and some aspects pertaining to the epidemiology and ecology of the disease. Although many aspects have been elucidated in the study, some questions remained unanswered or unclear, the most notable being the taxonomic position of the pathogen and the origin, distribution and management of the disease.

Phylogenetically, *C. cyperi* proved to be a distinct species, but closely related to *Claviceps zizaniae* (Fyles) Pantidou, with which it also shared a common alkaloid profile. However, the presence of the extended ITS regions unique to these two species observed in the present study may explain their outgroup placement insufficiently. Sequence data derived from ITS regions should be interpreted with caution because not all copies of these regions are identical in the same strain. Several strains of different species within a genus need to be screened to ensure that only copy type is amplified and sequenced. Multiple intron-rich protein coding genes should be sequenced as well, and compared with existing sequence data in GenBank. These protein-coding genes evolve faster than the ITS regions and are able to discriminate between closely related phylogenetic cryptic species within a morphological species (Taylor *et al.* 2000). O'Donnell & Cigelnik (1997) have demonstrated the potential of intron-rich protein-coding genes as species markers for *Fusarium*, and probably other fungal genera as well. According to Geiser *et al.* (1998) and Carbone & Kohn (2001), introns flanked by the more conserved exons evolve at a higher rate than ITS regions, hence increasing the resolving power of these genes to reveal variation at intraspecific level. Determination of the genealogies of these genes within the genus *Claviceps* may also prove rewarding with regard to the overall phylogeny of the genus and determining a common ancestor or ancestors for different species. The ongoing process of speciation within the genus *Claviceps* in association with different hosts, as well as the geographic origin of different species may also be established using appropriate species markers and the concept of Genealogical Concordance Phylogenetic Species Recognition.

The apparent holoblastic conidiogenesis observed in the spacelial state of *C. cyperi* in this study was a matter of concern as it implied a revision of the genus *Sphacelia*. Very recently, however, Pažoutová *et al.* (2004) showed that conidiation in *Claviceps* is pleomorphic, with conidiogenesis in *C. zizaniae* and *Claviceps citrina* Pažoutová, Fucík., Leyva-Mir & Flieger being ephelidial (holoblastic and sympodial), typical of the genus *Ephelis*. Although the existence of holoblastic conidiation in *Claviceps* would have resolved the taxonomy of *C. cyperi*, and placed its anamorph in *Ephelis*, this could not be done as the conidia of *Ephelis* species are produced sympodially and often form whorls consisting of 3 to 8 spores (Rykard *et al.* 1984; White 1997), whereas *C. cyperi* produces conidia singly and certainly not in sympodial succession. The issue is further compounded by the claim of Pažoutová *et al.* (2004) that both macro- and microconidia have been observed in all studied phialidic *Claviceps* species except *C. citrina* and *C. purpurea* (Fr.:Fr.) Tul., though microconidia could not be discerned in honeydew or cultures of *C. cyperi*. The confusion surrounding the taxonomic status of the nut sedge ergot pathogen therefore remains.

From an ecological perspective the most perplexing question obviously is the apparent confinement of *C. cyperi* to South Africa and its disappearance for more than 50 years since first recorded from in and around Pretoria during World War II until the incidents of ergotism associated with the intake of ergotised nut sedge in 1996/1997 at Greylingstad, Memel and Vrede. Despite concerted efforts the past eight years, the disease could not be detected in the Pretoria/Kempton Park area. It should nevertheless be noted that Greylingstad, Memel and Vrede do not feature in Doidge (1950) and the possibility therefore exist that these sites have never been visited in previous disease surveys. There is, however, a reference in Doidge (1950) to a *Cerebella* species on *Cyperus* from Mount Edgecombe in KwaZulu-Natal. Examination of the specimen, which was collected in 1945, indicated that the host was *Cyperus rotundus* L. and indeed colonised by *Cerebella*. Although no sign of ergot could be found, the presence of *Cerebella* is a sure indication of previous infection with a species of *Claviceps* (Langdon 1942; Loveless 1964). It is thus possible that *C. cyperi* has been present in other areas of South Africa, but remained unnoticed due to the inconspicuousness of the symptoms and the fact that it was not involved in any reported incident of ergotism. The recent sporadic eruptions can probably be ascribed to a combination of the following: (i) changes in tillage practices, (ii)

conducive climate, (iii) invasion by, or an increase in populations of spotted maize beetle (*Astylus atromaculatus* Blanchard) and the unidentified thrips species associated with the disease, and (iv) the conversion to forced crib-feeding by the farmers concerned.

Deep ploughing is an effective means of eliminating or confining ergot sclerotia (Bandyopadhyay *et al.* 1998; Bhuiyan *et al.* 2002). This practice has, since about 1980, increasingly been replaced by no-tillage or alternatively by shallow ripping and discing in the affected areas, and could therefore have resulted in a gradual build-up of inoculum of *C. cyperi*. Tillage is also an essential component in nut sedge control (Hauser 1962; Glaze 1987), and the reduction in mechanical cultivation necessitated the use of herbicides, which not only does not provide total control, but possibly could have predisposed the weed to ergot infection (Altman & Campbell 1977). The first outbreak of ergotism in 1996 was preceded by an exceptionally cold winter followed by high rainfall in spring and early summer, a climatological pattern which incidentally also prevailed during the latter part of World War II (SA Weather Services). Cold weather followed by rain and high humidity is conducive to the germination of ergot sclerotia (Brentzel 1947; Eleutherius & Meyers 1974). The ensuing inoculum propably was disseminated by insects such as thrips and spotted maize beetle of which the numbers increased because indiscriminate spraying of crops destined for fodder with insecticides obviously is undesirable.

The main reason for the outbreaks of ergotism undoubtedly is the practice of forced crib-feeding. When allowed to graze ad-lib it is highly unlikely that cattle would consume nut sedge plants, particularly when they are ergotised and the honeydew colonised by a trichotecene-producing fungus such as *Fusarium heterosporum* Nees, as in the present study. Crib-feeding is in any case not an effective farming practice and leads to various metabolic and reproductive disorders, most notably acidosis. Unfortunately dairy farmers in the new South Africa are compelled to resort to this practice for financial and security reasons such as AgriBEE, accompanied by deregulation of the agricultural sector, abolition of subsidies, land restitution, dwindling research funding and capacity, and the ever-increasing incidence of stock theft, and maiming farm attacks.

Fortunately, it is not the end of the road for the farmers affected. World sales of Parlodel, the dopaminergic agent of which the active ingredient 2-bromo-α-ergocryptine, is derived

from α-ergocryptine, the main alkaloid produced by *C. cyperi*, amounts to approximately $143 million (± R 860 million) per annum. This represents an annual usage of 247 kg of 2-bromo-α-ergocryptine, containing about 222 kg of α-ergocryptine. Yellow nut sedge produces on average 600 000 inflorescences per hectare (Hill *et al.* 1963). When ergotised, the mean number of sclerotia per inflorescence is 10 (Chapter 4), each between 8—15 x 1.5 mm in size (Chapter 2), with a mean volume of 6.5 μl and mean α-ergocryptine content of 3600 mg l^{-1} (Chapter 5). This implies that one hectare of ergotised nut sedge yields about 140 g of the alkaloid to the value of R 436 842. Even when accepted that the producer's price of sclerotia would probably be only 10 % of that of the final product, it still means a profit of more than R 40 000 per hectare after harvesting. To supply the world demand for Parlodel would require about 1 600 ha of ergotised nut sedge. Synthetic production of α-ergocryptine with species such as *C. zizaniae* (and *C. cyperi* if it can be induced to produce the alkaloid in culture) could add R 11 to R 70 g^{-1} to the production cost. The cost of producing α-ergocryptine from wheat ergotised by *C. purpurea*, the present means of commercial production, is not known but is likely to be relatively high due to the low α-ergocryptine yield of *C. purpurea* and the added expenditure of growing, infecting and maintaining the wheat.

As indicated in the first paragraph, facets of the nut sedge ergot complex that still need to be clarified include the molecular systematics of *C. cyperi* and the taxonomic position of the anamorph, as well as the distribution and dissemination of the disease. Negotiations are presently underway to obtain material of the ergotised specimens of *Cyperus latifolius* Poir. and *Cyperus rigidifolius* Steud. maintained at the International Mycological Institute for inclusion in molecular analysis with new and existing isolates of *C. cyperi*. The above two *Cyperus* species also occur in South Africa (Gordon-Gray 1995) and are morphologically closely related to *C. esculentus* and *C. rotundus*. It should therefore be worthwhile to conduct a survey of these and other related and common unrelated *Cyperus* species in South Africa for ergot infection. Lastly, a collaborative study is planned with a specialist entomologist to elucidate the involvement of the thrips species and spotted maize beetle, the latter which incidentally also produces a toxin that can be lethal when large numbers of the beetle are ensilaged in fodder fed to cattle (Drinkwater 1997), in the epidemiology of nut sedge ergot and pathology of ergotism.

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