The phylogeny and evolution of the genus *Claviceps*

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Phylogenetic trees of 16 *Claviceps* species were constructed based on alignments of 5.8S rDNA and the adjacent ITS1 and ITS2 spacers. Two highly supported clades were found: (1) *C. paspali*, *C. zizaniae*, *C. grohii*, *C. sulcata*, *C. fusiformis*, and *C. purpurea*; and (2) *C. citrina*, *C. phalaridis*, two unidentified *Claviceps* spp. (isolates PM and SG), *C. sorghicola*, *C. gigantea*, *C. sorghi*, *C. africana*, *C. viridis*, and *C. pusilla*. No relationship was found between the species placement and its morphological markers. The probe from *C. purpurea* gene *cpd1* for dimethylallyl tryptophan synthase, the first enzyme of alkaloid biosynthesis, was hybridized to *Pst I* digested genomes of the above species under non-stringent conditions. Hybridizing DNA was present in all species of clade 1, although the signal of the *C. paspali* gene was weaker. In clade 2, only *C. africana*, *C. gigantea*, and *C. pusilla* gave weak positive signals. Colorimetric detection found small amounts of alkaloids in cultures of *Claviceps* sp. SG and PM but despite that, no *cpd1* hybridizing bands were found. The occurrence of two major clades of *Claviceps* and their biogeography suggests, that the genus originates from South America and that the evolution of its species was influenced by comigration with their hosts and with the global climatic changes that influenced spreading of grass subfamilies.

INTRODUCTION

The genus *Claviceps* belongs to the family *Clavicipitaceae*, tribe *Clavicipeae*. The family is placed in the order *Hypocreales*, but in the 1950s doubts concerning the morphology of the conidiogenous stromata led Luttrell (1951) to transfer it into *Xylariales* and Gäumann (1952) to erect the order *Clavicipitales*. Recently, molecular data confirmed *Clavicipitaceae* as part of the 'hypocrealean complex' either as a family (Spatafora & Blackwell 1993, Rehner & Samuels 1995) or as a closely related but distinct order (White 1997a).

Plant-associated *Clavicipitaceae* (White 1997b) form a monophyletic group with a paraphyletic *Cordyceps* (Spatafora & Blackwell 1993, Rehner & Samuels 1995, Glenn *et al*. 1996). In the analysis of Kuldau *et al*. (1997), *Balansia*, *Myriogenospora* and *Atkinsonella*, all with holoblastic ephelidial microconidia, formed a clade, whereas *Claviceps* and *Epichloë/Neotyphodium* with enteroblastic conidia only, appeared either in a more ancestral position or as a sister clade. Epiphytic *Echinodothis* also with enteroblastic conidia appeared as a monotypic clade. These results contradict the hypotheses formerly held about *Balansiae* being ancestral to *Claviceps* (Gäumann 1964, Oddo & Tonolo 1967).

Members of the genus *Claviceps* are specialized parasites of grasses and sedges that specifically infect florets. The host reproductive organs are replaced with a sclerotium. However, it had been shown that after artificial inoculation, *C. purpurea* can grow and form sclerotia on stem meristems (Lewis 1956, and references therein) so that there is a capacity for epiphytic and endophytic growth. *C. phalaridis*, an Australian endemic, colonizes whole plants of pooid hosts in a way similar to *Epichloë*. However, it forms sclerotia in all florets of the infected plant, rendering it sterile (Walker 1957, 1970).

Until now, about 45 species of *Claviceps* have been described. Their distribution throughout the world has several interesting features. First, there is a striking difference in the number of species colonizing the chloridoid, pooid, and panicoid subfamilies of the *Poaceae* (Table 1). Most species have been found on panicoid hosts, whereas only a few have been collected on grasses of other subfamilies.

In the genus *Claviceps*, the species on panicoid hosts with monogeneric to polygeneric host ranges predominate. The species also differ in the mode of sclerotium formation and its

Table 1. Distribution of ergot species among the subfamilies of *Poaceae*.

Subfamily		No. of species
Cyperaceae (non-grass)		3
Arundinoideae		1 (+2)
Pooideae		3
Bambusoideae		1 (+1)
Centothecoideae		1
Chloridoideae		4 (+1)
Panicoideae	*Paniceae*	19
	Andropogoneae	11

Numbers in brackets represent species that are also found on hosts from other subfamilies.

properties. In South American tropics, *C. flavella* (redescribed in Langdon 1952) and *C. diadema* (Möller 1901) are found on panicoid grasses. Their sclerotia are undifferentiated mycelial mass encompassing the flower parts of one or several florets and they germinate directly on the host. This type of sclerotium is presumed to be a primitive character (Langdon 1954). However, no such species have been found in tropical and subtropical forests of Africa and South Asia. With the exception of Brazil and the West Indies (Möller 1901), no records of *Claviceps* species resembling *C. flavella* or *C. diadema* exist from other parts of the world.

None of the *Claviceps* species from the American tropics and subtropics has been found in the other warm regions of the world except *C. paspali* which has been spread by humans. On the other hand, several species (e.g., *C. fusiformis*, *C. pusilla*, *C. cynodontis*) are common in Old World tropics and subtropics. According to Langdon's (1954) hypothesis based on biogeography and the type of sclerotia, *Claviceps* is of tropical origin and the species with advanced sclerotia resistant to xeric conditions and temperature variations may have evolved as their hosts spread from the diminishing tropical forests to less favourable areas.

Recently, several *Claviceps* species appeared at new localities due to the transfer with seeds. African species *C. sulcata* (Fernandes, Fernandes & Bezerra 1995) and *C. africana* (Reis, Mantle & Hassan 1996) entered Brazil and *C. fusiformis* was described in Mexico (San Martin *et al*. 1997). Such events may also lead to the extinction of endemic species colonizing the same hosts and seriously disturb the original biogeography of *Claviceps* species. In India, the African sorghum parasite *C. africana* has almost replaced the endemic *C. sorghi* (Pažoutová *et al*. 2000).

The aim of this study was to learn more about the evolutionary relationships among *Claviceps* species from different parts of world by means of rDNA sequence analysis. Comparison of these results with morphological characters, host specificity and biogeography of the species analysed is discussed with respect to the possible coevolution of the genus *Claviceps* with grasses.

MATERIAL AND METHODS

Isolates

C. fusiformis SD58 (ATCC 26019) and *C. purpurea* Pepty 695/S were obtained from D. Gröger (Institute of Plant Biochemistry, Halle/Saale), *C. viridis* (125.63 ex *Oplismenus compositus*, India, 1963, CBS, Utrecht), *C. grohii* 124.47 ex *Carex* sp., Canada, 1947, CBS, Utrecht) were obtained from these collections. *C. zizaniae* CCM 8231 was collected in Canada, 1996, and isolated by L. Marvanová (Czech Collection of Microorganisms, Brno). *C. purpurea* T5 from rye was kindly supplied by P. Tudzynski (Westfällische Wilhelms Universität, Münster). *C. phalaridis* was isolated from the sample of sclerotia (DAR 69619) collected in January 1996 and kindly donated by J. Walker (Baulkham Hills, NSW, Australia). *C. pusilla* was isolated from herbarium sample BRIP 26571 (Plant Pathology Herbarium, Indooroopilly) collected from *Dichanthium aristatum* in 1996. The *C. sorghi* sequence was determined from DNA extracted from sclerotia formed in England in 1987 on male sterile sorghum line 2219A inoculated by an isolate originating from Akola, Maharashtra, India and identical to that described in Frederickson, Mantle & de Milliano (1991). Sclerotia of *C. sorghicola* were similarly derived in England in 1996 from male sterile sorghum inoculated with conidia of the pathogen provided by T. Tsukiboshi. The remaining species were isolated at the Institute of Microbiology from the sclerotium or at the *in planta* sphacelial stage.

Isolation and cultivation of the mycelial cultures

Sclerotia or sphacelia were surface-sterilized 2–15 min (according to size) in 1.3% sodium hypochlorite (suitably diluted Clorox), then washed 1–2 min in 95% EtOH and rinsed three times in distilled water. Sterilized sclerotia were placed on T2 agar plates ($g\,l^{-1}$: sucrose 100; L-asparagine 10; yeast extract 0.1; KH_2PO_4 0.25; $MgSO_4 . 7H_2O$ 0.25; $FeSO_4 . 7H_2O$ 0.02; $ZnSO_4 . 7H_2O$ 0.015; KCl 0.12; $Ca(NO_3)_2 . 4H_2O$ 1; agar 20; pH 5.2) (Spalla 1973) supplemented with 100 $\mu g\,ml^{-1}$ of ampicillin or 20 $\mu g\,ml^{-1}$ of tetracycline. The isolates were maintained on T2 or PDA agar slants at 4 °C and subcultured every 3–6 months. The method for growing submerged cultures and colorimetric alkaloid detection with vanUrk's reagent were described previously (Pažoutová *et al*. 1981).

Preparation of genomic DNA

DNA was prepared from colonies grown on cellophane overlaid on T2 or PDA agar plates (Pažoutová *et al*. 1998). The DNA samples (300 µl) containing excess polysaccharides (*Claviceps* sp. SG and PM, *C. africana* and *C. sulcata*) were further purified by addition of 150 µl of Nucleon PhytoPure DNA extraction resin (Amersham Life Science) and 400 µl of chloroform cooled to −20 °, shaken 10 min at room temperature and centrifuged at 13000 rpm for 10 min. DNA from water phase was precipitated with 0.7 vol isopropanol/0.3M sodium acetate. For the preparation of DNA from sclerotia, the volumes were scaled down 10 ×.

Nonradioactive hybridizations

Fragments of genomic DNA (10 µg) digested with *PstI* were separated on 0.8% agarose gel (450 Vh) and blotted to Nylon + (Roche Molecular Biochemicals, Mannheim, Germany). The probe was an 0.8 kb long *Sal I* fragment of the gene for dimethylallyl tryptophan synthase from isolate T5 of *C. purpurea* (Tudzynski *et al*. 1999) cloned into pBluescript/SK and labelled by alkali-labile digoxigenin-11-2′-deoxy-uridine-5′-triphosphate (DIG-dUTP) by PCR using T3 and T7 universal primers. The probe was kindly donated by P. Tudzynski (Westfälische Wilhelms-Universität, Münster). Membranes were hybridized with the probe (approx. 30 $ng\,ml^{-1}$) at 55 ° overnight and washed for 2 × 15 min at 55° in 5 × SSC/0.1% SDS. DIG-dUTP probe labelling, hybridisation and colour detection was according to manufacturer's protocol (Roche Molecular Biochemicals, Mannheim).

ITS-DNA region sequencing

The genomic region containing ITS1, 5.8S rDNA and ITS2 was amplified by PCR using ITS1 and ITS4 primers (White *et al*. 1990). The mixture (25 µl) contained 50 ng of DNA, 20 pmol of each primer, 0.2 mM dNTP's and 1 U of DynaZyme with appropriate buffer (Finnzymes, Oy, Finland). The reaction mixtures were subjected to 32 PCR cycles in a GeneE thermal cycler (Techne, Cambridge, UK) under the following temperature regime: 95 ° for 3 min, 55 °/30 s, 72 °/1 min (1 ×), 95 °/30 s, 55 °/30 s, 72 °/1 min (30 ×) and 95 °/30 s, 55 °/30 s, 72 °/10 min (1 ×). Amplified fragments were purified by Wizard DNA Clean-Up System (Promega, Madison, WI) and subjected to automatic AmpliTaq polymerase cycle sequencing process using ITS1, ITS2, ITS3 and ITS4 primers with dye terminator labelling and an ABI 373A sequencer (Perkin Elmer) in the sequencing facility of the Institute of Biotechnology (Graz). The sequences were deposited in EMBL Nucleotide Sequence Database (*C. fusiformis* AJ133392, *C. citrina* AJ133393, *C. gigantea* AJ133394, *C. grohii* AJ133395, *Claviceps* sp. PM AJ133396, *Claviceps* sp. SG AJ133402, *C. sorghicola* AJ133397, *C. paspali* AJ133398, *C. phalaridis* AJ133399, *C. purpurea* Pepty 695/S AJ133401, *C. sulcata* AJ133402, *C. viridis* AJ133404, *C. zizaniae* AJ133405, *C. africana* isolate T 10 765 AJ011783, *C. pusilla* AJ277544, and *C. sorghi* AJ242869).

Alignment analysis

Sequences were aligned by Sequence Alignment and Modelling System (SAM) using the hidden Markov model (Krogh *et al*. 1994, Hughey & Krogh 1996) with the sequence of *Epichloë amarillans* (L07141) as an outgroup (Tsai *et al*. 1994). The software is available at the server of Computational Biology group, Computer Science and Engineering University of California, Santa Cruz (http://www.cse.ucsc.edu/research/compbio/sam.html). The alignment was edited manually using BioEdit version 4.7.1. created by Tom Hall (Department of Microbiology, North Carolina State University, Raleigh). Indels were recorded as missing information except the ones informative for parsimony. Distance analysis was performed using PUZZLE 4.0.2 (Strimmer & von Haeseler 1996). Because of unequal rate of nucleotide substitution among the positions, the matrix of maximum likelihood distances was computed using model of Tamura & Nei (1993) with gamma distance corrections for substitution rate heterogeneity. Parsimony and iterative strongest evidence analyses were done using program SEPAL (Strongest Evidence and Parsimony AnaLyzer Ver.1.1 from B. A. Salisbury, Yale University), bootstrap values were obtained using seqboot, dnapars and consense from the package PHYLIP 3.573c (Felsenstein 1989). Dnapars was run with the 'jumble' option changing 100 × the species input order. Bootstrapped (500 ×) dataset was also run with 'jumble 100 × ' option.

RESULTS

Characterization of Claviceps *species*

The distribution, morphological features and alkaloid production of species are given in Table 2. In the species that were disseminated by humans together with grass and cereal seeds, the original geographical distribution is given when documented in the literature. *C. africana*, *C. gigantea* and *C. pusilla* were cultivated in shaken cultures and after 2 weeks the production of 10–20 mg l^{-1} of alkaloids was found. The species isolated from sphacelia are characterized only by conidial morphology.

Claviceps sp. ex Setaria geniculata

The SG isolate was isolated by the author from the sphacelial stage on *S. geniculata* collected by E. M. Reis (Universita de Passo Fundo, Fac. de Agronomia) in Passo Fundo, Brazil (Fig. 1a). Elongated and arcuate macroconidia predominated in the honeydew, and their dimensions were $(13 < 15.5 < 20) \times (4 < 5 < 6.5)$ µm. Microconidia found in sphacelia were oval, homogenous in size, on average 5 × 2.5 µm. Microcyclic conidiation producing microconidia was observed *in planta* in original specimens. The colorimetrical test detected small amounts of unidentified alkaloids (< 10 mg l^{-1}) produced in shaken flask culture. *C. ranunculoides* (Möller 1901) occurs on *Setaria* in Brazil. However, the conidial size of this species is substantially smaller (7–8 × 3–4 µm) than in the SG isolate.

Claviceps sp. ex Panicum maximum

PM was isolated by the author from the sphacelial stage on a plant collected by A. Glatzle (Estación Experimental, Asunción) in Chaco, Paraguay. The only taxonomic markers available were the identity of the host plant and the size and shape of conidia. The spores from honeydew (Fig. 1b) were $(13 < 16.5 < 20) \times (4.5 < 5 < 6)$ µm, and their shape was oblong, oval, and rarely reniform. Microcyclic conidiation giving rise to rounded spores max. 5.5 µm diam was observed in the infected plant specimen. The isolate produced trace amount (< 10 mg l^{-1}) of unidentified alkaloids in shaken flask culture. Ergot from *P. maximum*, *C. maximensis* Theis has been described twice; once by Theis (1952) from Puerto Rico and then by Loveless (1964) from African collections. Loveless considered *P. maximum* to be of African origin and that its transfer to South America was with infested guinea grass seeds. However, the conidial descriptions are not completely identical and without teleomorph analysis the identification of the PM isolate is impossible.

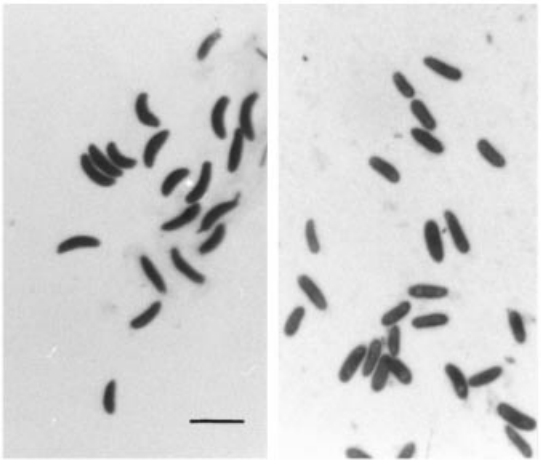
Phylogenetic analysis

Choosing the alignment and tree-building methods

The preliminary alignments of ergot species exhibited great variability in the ITS1 region, therefore the structure of this region was modelled by RNAStructure Ver. 2.52 (Mathews *et al*. 1997). Most of the variability was due to the possible stem-loop structure No. 2, beginning with CCTC motif around 55–60th nucleotide (Fig. 2). In some of the *Claviceps* species this structure was short, spanning only 30 bp. The *Claviceps* species in the later diverging lineages had apparent insertions in this structure (about 60 nucleotides), with differing sequences. Moreover, *C. zizaniae* exhibited another approx. 70 bp insertion that appears to be a modified duplication of the whole structure. The *Claviceps* sequences are shown in Fig. 3 as schematic restriction maps.

Table 2. The morphological markers, alkaloid production and the origin of analyzed *Claviceps* species.

Species	Host range (genera)	Host taxonomy	Main alkaloids	Capitulum	Stipe	Conidia size (μm)	Locality
C. gigantea	*Zea*	*Panicoideae/ Andropogonodae*	festuclavine dihydrolysergamides	pink to reddish brown	pink	8–27 × 4.5–6	Central Mexico
C. sorghi	*Sorghum*	*Panicoideae/ Andropogonodae*	caffeine	dark buff with white frill	bronze to terracotta	8–19 × 4–5	India, Burma (?)
C. pusilla	*Andropogoninae*	*Panicoideae/ Andropogonodae*	detected	dark straw	pale straw	10–15.5 × 5–7.5 often triangular	South Europe, Paleotropic, Australia
C. viridis	*Oplismenus*	*Panicoideae/ Panicodae*	in the culture not detected	green to yellowish brown	olivaceous	3–7 × 1.5–3	India, Japan
C. africana	*Sorghum*	*Panicoideae/ Andropogonodae*	festuclavine dihydroergosine	light buff, then dark purple	purple	9–17 × 5.8	Africa, now worldwide
C. sorghicola	*Sorghum*	*Panicoideae/ Andropogonodae*	tricyclic clavines caffeine	bronze to brown	dark brown	5–11.5 × 2.5–4	Japan
Claviceps sp. SG	*Setaria geniculata*	*Panicoideae/ Panicodae*	detected			13–20 × 4–6.5	Brazil
Claviceps sp. PM	*Panicum maximum*	*Panicoideae/ Panicodae*	detected			(13–20) × (4.5–6)	Paraguay
C. phalaridis	*Phalaris, Vulpia, Lolium, Dactylis, Danthonia*	*Pooidae Arundinoidae*	not detected	dark purple to black	pale purple to lilac	7.5–14 × 2–4	South Australia
C. citrina	*Distichlis*	*Chloridoidae*	not detected	lemon yellow	pale yellow	3.5–7 × 2.5–3	Central Mexico
C. paspali	*Paspalum*	*Panicoideae/ Panicodae*	lysergamides, clavines	dull yellow	yellow	3.5–15 × 2.5–7.5	South America
C. zizaniae	*Zizania*	*Oryzoideae*		tawny to russet	tawny, light lilac when young	8–30 × 3–6	Canada
C. grohii	*Carex*	*Cyperaceae: Caricinae*	probably clavines	buff-pink to orange-vinaceous	blackish violet	10–16 × 3–5	Canada
C. sulcata	*Brachiaria,*	*Panicoideae/ Panicoidae*	detected	very pale yellow (?)	?	9–22 × 3–5.5	South Africa
C. fusiformis	*Cenchrus, Urochloa, Panicum, Pennisetum, Setaria*	*Panicoideae/ Panicoidae*	clavines	greyish purple	lavender when young then cream	12–26 × 2.5–6	Africa, India
C. purpurea	many genera	*Pooidae, Arundinoidae, Chloroidoidae,* (rarely *Panicoideae*)	peptide ergoline alkaloids, clavines	varying from cream to purple	pale lilac to purplish	5–14 × 2–4	Paleoarctic



A B

Fig. 1. Conidia of two unidentified *Claviceps* strains. A, *Claviceps* sp. SG; B, *Claviceps* sp. PM. Stained with 1% cotton blue. Bar = 20 μm.

The SAM algorithm uses a hidden Markov model that estimates the possibilities of nucleotide change which are then used as penalties in the alignment cost (Krogh *et al*. 1994). From all algorithms tested, SAM alignment of the critical part of ITS1 region was most in accord with the hypothesized structure. In each of the species for which a short No. 2 structure was likely (*C. paspali*, *C. citrina*, PM, SG, *C. phalaridis* and partially *C. sorghicola*) gaps were recoded as missing information and the second insertion of *C. zizaniae* was omitted from the alignment. In the alignment with 559 sites, there were 111 sites informative for parsimony and 182 variable sites. The sequence of *Epichloë amarillans* was used as an outgroup because *Epichloë* appears as a sister clade to *Claviceps* in the analysis of Kuldau *et al*. (1997).

Restriction maps of the rDNA region

Restriction maps of the ITS region are shown in Fig. 3. Note the placement of gaps and the specific restriction sites *Sca I* located in 5.8S rDNA and *Sac II* in the ITS2 spacer.

Phylogenetic trees

The quartet puzzling tree with maximum likelihood branch lengths is shown in Fig. 4. The following parameters were found: transition/transversion parameter (estimated from data set): 0.74 (S.E. 0.08), Y/R transition parameter: 1.88 (S.E. 0.31), expected transition/transversion ratio: 0.73, expected pyrimidine transition/purine transition ratio: 2.04. Gamma

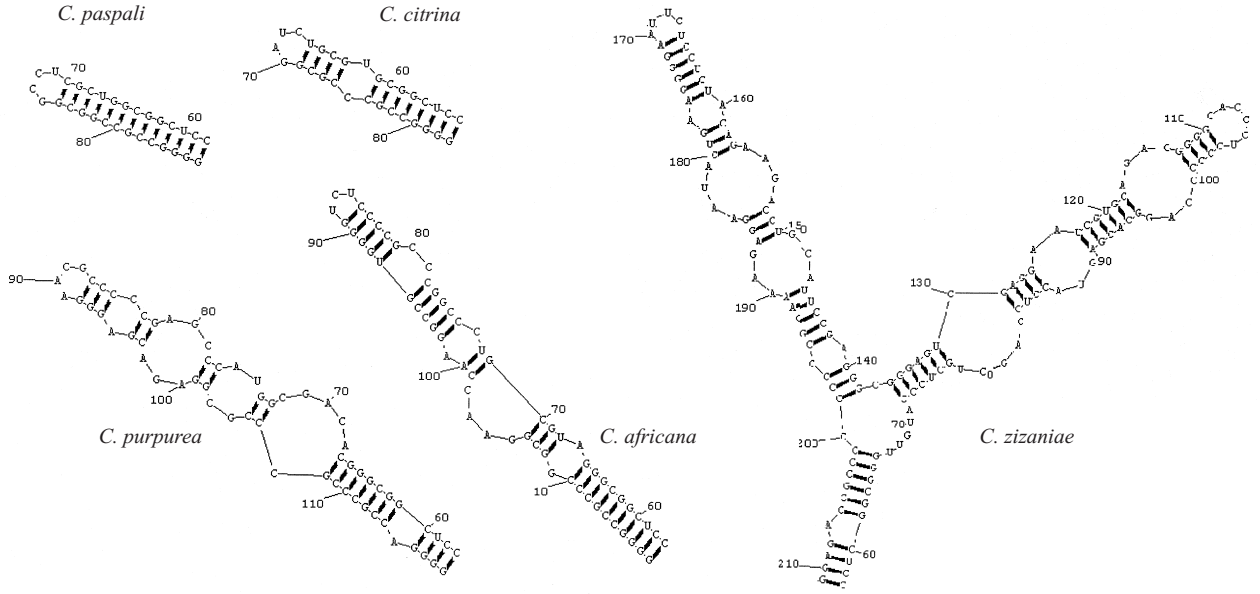


Fig. 2. Suggested structures of the variable part of the ITS1 region.

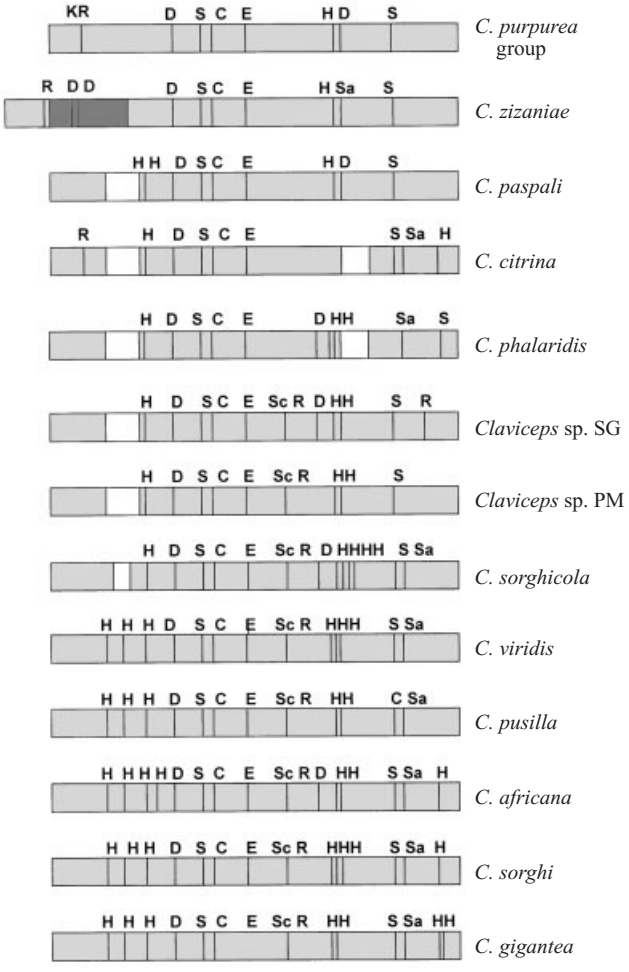


Fig. 3. Schematic restriction map of the ITS-rDNA region of different *Claviceps* species. Insertion in ITS1 of *C. zizaniae* is shown in dark grey, deletions are in white.

Abbreviations: C – *Cla I*; D – *Dde I*; E – *Eco RI*; H – *Hpa I*; K – *Kpn I*; R – *Rsa I*; S – *Sau3A*; Sa – *Sac II*; Sc – *Sca I*; *C. purpurea* group: *C. purpurea, C. fusiformis, C. sulcata, C. grohii*.

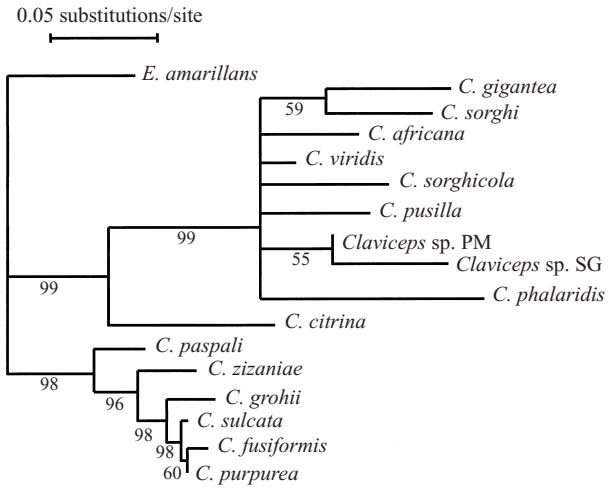


Fig. 4. Quartet puzzling tree with maximum likelihood branch lengths. *E. amarillans* was defined as the outgroup.

distribution parameter alpha (estimated from data set) was 0.27 (S.E. 0.03) assuming eight gamma rate categories. Quartet puzzling was used to choose from the possible tree topologies and to simultaneously infer support values for internal branches. Number of puzzling steps was 1000, with 2380 analysed quartets and 176 unresolved quartets (= 7.4%). The log L was −2553.44.

Maximum parsimony analysis produced two trees with 341 steps switching the positions of *C. phalaridis* and the clade containing *Claviceps* sp. SG and PM. The tree identical with the strict consensus tree resulting from bootstrap analysis is shown in Fig. 5. The clades supported by less than 50% of bootstraps were collapsed to polytomies. The same topology was observed in trees obtained by the maximum parsimony method and iterative strongest evidence (Salisbury 1999) methods implemented in the SEPAL program.

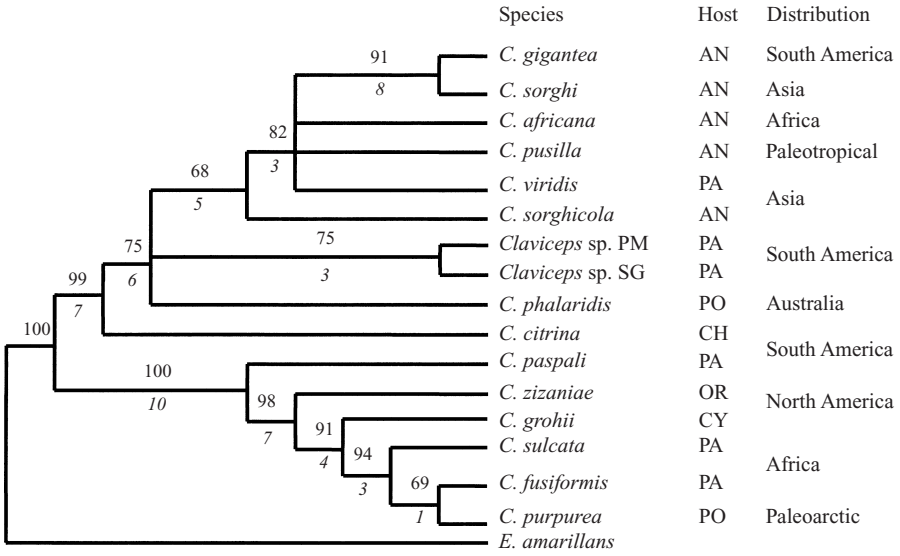


Fig. 5. Strict consensus cladogram resulting from maximum parsimony and iterative strongest evidence analysis. The numbers above the branches are bootstrap supports. Under the branches, decay values from iterative strongest evidence analysis are given. Clades with bootstrap support under 50% were collapsed to polytomies. *E. amarillans* was defined as the outgroup.
Abbreviations: PO – *Pooidae*; OR – *Oryzoidae*; CH – *Chloridoidae*; PA – *Panicoidae*; AN – *Andropogonae*; CY – *Cyperaceae*.

The quartet puzzling tree with maximum likelihood branch lengths as well as the parsimony tree show two highly supported distinct clades:

(1) *C. paspali*, *C. zizaniae*, *C. grohii*, *C. sulcata*, *C. fusiformis*, and *C. purpurea*.
(2) *C. citrina*, *C. phalaridis*, *Claviceps spp.* SG and PM, *C. sorghicola*, *C. sorghi*, *C. gigantea*, *C. africana*, *C. viridis*, and *C. pusilla*.

The first clade contains in ancestral position *C. paspali* (the only one with short ITS1 spacer), followed by species adapted to cold winters, but also two African species from semi-arid regions. The second clade has *C. citrina* on the ancestral position, followed by polytomy of *C. phalaridis*, clade of two sister South American species, *C. sorghicola* (all with short ITS1 spacers) and the clade formed by species with long ITS1 spacer. Within the second clade more polytomies were observed, especially in the maximum likelihood tree. In the parsimony tree, the clade containing species with longer ITS1 that colonize mainly andropogonoid hosts was supported by 82% bootstrap value. The clades containing *Claviceps* spp. PM and SG as well as *C. sorghi* and *C. gigantea* were supported in both maximum likelihood and parsimony trees.

As the analysis was based on only one third of known *Claviceps* species, the relationships are far from absolute. However, two main conclusions are evident: the existence of two main clades and the ancestral positions of species with short ITS1 that are of South American or Australian origin.

DMAT synthase gene in various Claviceps *species and the alkaloid production*

Hybridization of the DMAT (dimethylallytryptophan) synthase gene probe from *C. purpurea* T5 designed *cpd1* (sequence AJ011963; Tudzynski *et al.* 1999) was conducted to compare the rDNA data with some information about the homology of a structural gene (Fig. 6). As nothing is known about alkaloid production of several species used in this study, results of the hybridization should detect at least the potential for it.

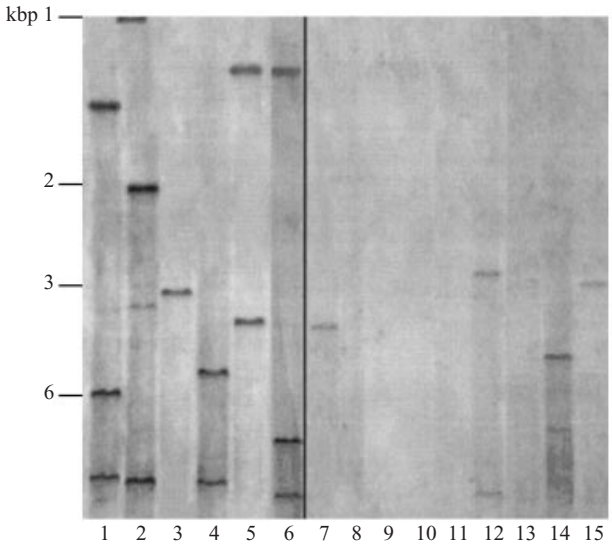


Fig. 6. Hybridization of DMAT synthase probe with genomic DNA of *Claviceps* species. Lanes: 1, *C. purpurea* 256; 2, *C. purpurea* T5; 3, *C. fusiformis*; 4, *C. sulcata*; 5, *C. grohii*; 6, *C. zizaniae*; 7, *C. paspali*; 8, *C. citrina*; 9, *Claviceps* sp. SG; 10, *Claviceps sp.* PM; 11, *C. phalaridis*; 12, *C. gigantea*; 13, *C. viridis*; 14, *C. africana* T10 765; and 15, *C. pusilla*. Samples 1–6 were developed 90 min, the remaining ones overnight.

Hybridization revealed that a gene that is homologous to the *C. purpurea* probe is present in all closely related species of the first clade. The signal intensity in these samples (Fig. 6, lanes 1–6) was clearly visible after 90 min development time, whereas the remaining part of the membrane containing DNA belonging to *C. paspali* and species of clade 2 had to be incubated overnight to visualize a signal. However, weak

positive bands were found only in *C. paspali*, *C. gigantea*, *C. africana*, and *C. pusilla*.

According to the colourimetric detection, *Claviceps* sp. SG and PM both product small amounts of alkaloids in culture, but despite that, no hybridizing bands were found. Also, in *C. viridis*, *C. phalaridis* and *C. citrina* DNA no hybridizing bands were obtained. No alkaloid production was reported for sclerotia of *C. viridis* and in our laboratory, no alkaloids were found in either the sclerotia of *C. phalaridis* and *C. citrina* or in the submerged culture of *C. viridis*. However, no alkaloids of *C. zizaniae* were found either in the sclerotia or culture despite the indication that it possessed the gene coding for DMAT synthase.

DISCUSSION

The morphological features summarized in Table 2, as stroma colour or conidial size and shape, do not track with the position of the species in the phylogenetic tree. No separation of species with yellow stromata from species with dark (purple, brown, vinaceous) stromata into different clades was observed. Host specificity and/or the correlation between the evolution of grasses and that of *Claviceps* species was also analyzed. *Claviceps* differs from all other genera of Clavicipitaceae in that it colonizes hosts from all taxa of *Poaceae*, whereas the remaining genera are mostly genus-specific. The clades of *Claviceps* do not suggest that they have directly coevolved with their grass hosts, although the species in more ancestral positions tend to colonize *Paniceae* and *Chlorideae*. Especially in the first clade, the species colonizing panicoid, oryzoid and pooid grasses are found, suggesting more complex pattern of evolution. In the second clade, species colonizing andropogonoid hosts are in later diverging clades, however, their closest relative *C. viridis* is specialized on *Oplismenus*, which is C-3 panicoid grass.

In clade 1, species with a Paleoarctic distribution that have cold resistant sclerotia are united with species typical of the African savanna. The similarity of rDNA sequences in this group is striking – at least 97% identity among *C. purpurea* and *C. grohii* was found which may suggest relatively recent divergence.

In clade 2, three species colonizing the same host genus in different localities are included – *C. africana*, *C. sorghi* and *C. sorghicola*. Also, parasites of andropogonoid grasses including the maize parasite *C. gigantea* from Central America and sorghum parasites from Africa and Asia are closely related. These relationships suggest two mechanisms, the spreading of *Claviceps* species with hosts during their radiation and the subsequent colonization of other often unrelated grass genera present in the new locality.

The third criterion, the biogeographic distribution of *Claviceps* species, was compared to the spreading of grasses during their evolution. There are three hypotheses concerning the origin of grasses; one suggests an origin in humid tropical forests with increasing evolutionary advancement correlated with the adaptation to aridity (Whyte 1974), a second is based on an origin in the open forest followed by a migration into xerophytic zones (Bews 1929), and the third suggests an origin in mesophytic and xerophytic zones with subsequent mixing of types (Aubreville 1962, Thomas 1966). *Claviceps* phylogeny is most consistent with the first hypothesis, because the primitive sclerotia consisting of rather loosely interwoven hyphae without well-defined rind (Möller, 1901) did not offer a possibility of survival in xerophytic zones. The absence of species with apparently primitive sclerotia in Africa is probably due to the fact that the Gondwanan tropical forests there did not survive the climate changes (Raven & Axelrod 1974), especially the aridisation in the Oligocene and fluctuations in the late Pliocene-Pleistocene. These fluctuations led to changes in vegetation types and expansion-contraction cycles of savanna habitats (Coughenour 1985) which may have stimulated the development of *Claviceps* species with more resistant sclerotia. In South America, climatic extremes did not occur in rain forest areas (Stebbins 1981) and the rainfall was more evenly distributed, whereas in African rain forest there was a 1–2 month period with precipitation under 100 mm (Richards 1973).

Based on these data, the following hypothesis about *Claviceps* evolution may be formulated:

The first *Claviceps* species probably appeared on the predecessors of panicoid grasses in the warm and humid climate of the South American region of former Gondwana in the Upper Cretaceous. This assumption is based on the radiation centre of panicoid grasses in that region, the South American records of *Claviceps* species with primitive undifferentiated sclerotia and the ancestral position of South American species on the rDNA phylogeny tree. Preservation of these lineages was probably facilitated by the isolation of South America from the end of Cretaceous until the end of Tertiary (Stebbins 1981).

The first expansion of *Claviceps* species gave rise to the species of the second clade and occurred most probably with the spreading of panicoid grasses in early Tertiary. Further events that influenced the evolution of species of this clade was the spreading of andropogonoid grasses from their radiation centre in southern Asia to Africa, southern Europe and Central America (Jones 1991). The close relatedness of the Mexican maize parasite *C. gigantea* to parasites of andropogonoids from Paleotropics supports this hypothesis. Members of the second clade appear to be well adapted to semi-arid conditions, but their cold resistance is limited.

The geographical distribution of ergot species from the *C. purpurea*–related group suggests another evolutionary path as follows. The ancestors of this clade might have migrated from South to North America after the formation of the Panama land bridge and then to Europe and Africa. Only these species developed the ability to deal with cold winters. Taking into consideration the possibility of long distance dispersals, this could have happened some 6–7 Myr ago. The extant species of the first clade occurring in the colder climatic regions and semi-arid Africa evolved relatively recently, filling the ecological niches available which required adaptation to survive low temperatures and/or longer periods of dryness.

An additional support to the hypothesis of an early divergence between clades 1 and 2 is the absence or low homology between DMAT synthase genes of both *Claviceps* clades. This gene appears to be even more variable than the ITS-rDNA region. Tudzynski *et al.* (1999) found that sequence

similarity between the deduced sequence of the DMAT synthase protein of *C. purpurea* (CPD1) and its homologue DMAW cloned by Tsai *et al.* (1995) from the isolate considered as *C. purpurea* but identified later (Pažoutová & Tudzynski 1999) as *C. fusiformis* is only 68%. However, the ITS-rDNA sequences of *C. purpurea* and *C. fusiformis* generated in this study are 98.7% identical and Rehner & Samuels (1995) observed 95.6% identity in a 960 bp fragment of the 28S rDNA of *C. purpurea* and *C. fusiformis*. As expected, *C. paspali* with a different ITS1 structure gave a much weaker hybridization signal. Weak (*C. africana*, *C. gigantea* and *C. pusilla*) or nonexistent DMAT signals in the DNA of the species from the second clade might suggest the differences in secondary metabolism of both clades. *C. africana* (Mantle 1968) and *C. gigantea* (Olšovská 1999) produce alkaloids derived from the dihydroergoline skeleton, whereas in the first clade, ergoline alkaloids were found. Moreover, there is evidence that the species from the second clade may produce non-ergoline alkaloids. Bogo & Mantle (2000) found caffeine in *C. sorghi* and *C. sorghicola* which have not been shown to produce significant amounts of alkaloids of the conventional ergoline type.

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REFERENCES

Aubreville, A. (1962) Savanization tropicale et glatiations quaternaires. *Adansonia* **2**: 16–84.

Bews, J. W. (1929) *The Worlds Grasses: their differentiation, distribution, economics and geology*. Longmans Green, New York.

Bogo, A. & Mantle, P. G. (2000) Caffeine: also a fungal metabolite. *Phytochemistry* **54**: in press.

Coughenour, M. B. (1985) Graminoid responses to grazing by large herbivores: Adaptations, exaptations and interacting processes. *Annals of the Missouri Botanical Garden* **72**: 852–863.

Felsenstein, J. (1989) PHYLIP – Phylogeny Inference Package (Version 3.2). *Cladistics* 5: 164–166.

Fernandes, C. L., Fernandes, A. T. F. & Bezerra, J. L. (1995) 'Mela': Uma nova doença em sementes de *Brachiaria* spp. no Brasil. *Fitopatologia brasileira* **20**: 501–503. [In Portuguese.]

Frederickson, D. E., Mantle, P. G. & de Milliano, W. A. J. (1991) *Claviceps africana* sp. nov., the distinctive ergot pathogen of sorghum in Africa. *Mycological Research* **95**: 1101–1107.

Gäumann, E. A. (1952) *The Fungi*. Hafner Publishing, New York.

Gäumann, E. A. (1964) *Die Pilze*. Birkhauser Verlag, Basel.

Glenn, A. E., Bacon, C. W., Price, R. & Hanlin, R. T. (1996) Molecular phylogeny of *Acremonium* and its taxonomic implications. *Mycologia* **88**: 369–383.

Hughey, R. & Krogh, A. (1996) Hidden Markov models for sequence analysis: extension and analysis of the basic method. *Computer Applications in the Biosciences* **12**: 95–107.

Jones, C. A. (1991) *C4 Grasses and Cereals: growth, development and stress response*. John Wiley, New York.

Krogh, A., Brown, M., Mian, I. S., Sjolander, K. & Haussler, D. (1994) Hidden Markov models in computational biology: applications to protein modeling. *Journal of Molecular Biology* **235**: 1501–1531.

Kuldau, G. A., Liu, J.-S., White, J. F., Siegel, M. R. & Schardl, C. L. (1997) Molecular systematics of *Clavicipitaceae* supporting monophyly of genus *Epichloe* and form genus *Ephelis*. *Mycologia* **89**: 434–441.

Langdon, R. F. N. (1952) *Studies on ergot*. PhD thesis, University of Queensland.

Langdon, R. F. N. (1954) The origin and differentiation of *Claviceps* species. *University of Queensland Papers* (*Botany*) **3**: 61–68.

Lewis, R. R. (1956) Development of conidia and sclerotia of the ergot fungus on inoculated rye seedlings. *Phytopathology* **46**: 295–296.

Loveless, A. R. (1964) Studies of Rhodesian ergots 1. *Claviceps maximensis* Theis and *Claviceps pusilla* Cesati. *Kirkia* **4**: 35–40.

Luttrell, E. S. (1951) Taxonomy of pyrenomycetes. *University of Missouri Studies* **24**: 1–120.

Mantle, P. G. (1968) Studies on *Sphacelia sorghi* McRae, an ergot of *Sorghum vulgare* Pers. *Annals of Applied Biology* **62**: 443–468.

Mathews, D. H., Andre, T. C., Kim, J., Turner, D. H. & Zuker, M. (1997) An updated recursive algorithm for RNA secondary structure prediction with improved free energy parameters. In *Molecular Modeling of Nucleic Acids* (N. B. Leontis & J. Santa Lucia, eds): 246–257. [ACS Symposium Series No. 682.] American Chemical Society, Washington DC.

Möller, A. (1901) Phycomyceten und Askomyceten von Brasil. In *Botanisches Mitteilungen aus Tropen* (A. F. W. Schimper, ed.): 1–319. Jena.

Oddo, N. & Tonolo, A. (1967) Origin and formation of perithecia of *Claviceps paspali* Stev. et Hall. *Annali dell' Istituto Superiore di Sanità* **3**: 16–26.

Olšovská, J. (1999) *Isolation and identification of ergot alkaloids and their application as chiral selectors in HPLC*. PhD thesis, Faculty of Sciences, Charles University, Prague. [In Czech.]

Pažoutová, S. & Tudzynski, P. (1999) *Claviceps* sp. PRL 1980 (ATCC 26245), 59 and Pepty 695/ch – 1: their true story. *Mycological Research* **103**: 1044–1048.

Pažoutová, S., Bandyopadhyay, R., Frederickson, D. E., Mantle, P. G. & Frederiksen, R. A. (2000) Relations among sorghum ergot isolates from the Americas, Africa, India, and Australia. *Plant Disease* **84**: 437–442.

Pažoutová, S., Fučíkovský, L., Leyva-Mir, S. G. & Flieger, M. (1998) *Claviceps citrina* sp. nov.; a parasite of the halophytic grass *Distichlis spicata* from Mexico. *Mycological Research* **102**: 850–854.

Pažoutová, S., Taisinger, J., Flieger, M., Sajdl, P., Bass, A. & Řeháček, Z. (1981) The relationship between intensity of oxidative metabolism and predominance of agroclavine or elymoclavine in submerged *C. purpurea* cultures. *Journal of Natural Products* (*Lloydia*) **44**: 225–235.

Raven, P. H. & Axelrod, D. I. (1974) Angiosperm biogeography and past continental movements. *Annals of the Missouri Botanical Garden* **61**: 539–673.

Rehner, S. A. & Samuels, G. J. (1995) Molecular systematics of the Hypocreales, a teleomorph gene phylogeny and the status of their anamorphs. *Canadian Journal of Botany* **73** (Suppl 1): S816–S823.

Reis, E. M., Mantle, P. G. & Hassan, H. A. G. (1996) First report in the Americas of sorghum ergot disease, caused by a pathogen diagnosed as *Claviceps africana*. *Plant Disease* **80**: 463.

Richards, P. W. (1973) Africa the 'odd man out'. In *Tropical Forest Ecosystems in Africa and South America: a comparative review* (B. J. Meggers, E. S. Ayensu & W. D. Duckworth, eds): 21–26. Smithsonian Institution Press, Washington, DC.

Salisbury, B. A. (1999) Strongest evidence: maximum apparent phylogenetic signal as a new cladistic optimality criterion. *Cladistics* **48**: 137–149.

San Martin, F., Lavín, P., García, A. & García, G. (1997) Estados anamórficos de *Claviceps africana* y *Claviceps fusiformis* (*Ascomycetes*, *Clavicipitaceae*) asociados a diferentes pastos en Tamaulipas, México. *Revista Mexicana de Micología* **13**: 52–57.

Spalla, C. (1973) Genetic problems of production of ergot alkaloids in saprophytic and parasitic conditions. In *Genetics of Industrial Microorganisms* (Z. Vaněk, Z. Hošťálek & J. Cudlín, eds): 393–403. Elsevier, Amsterdam.

Spatafora, J. W. & Blackwell, M. (1993) Molecular systematics of unitunicate perithecial ascomycetes: the *Clavicipitales – Hypocreales* connection. *Mycologia* **85**, 912–922.

Stebbins, G. L. (1981) Coevolution of grasses and herbivores. *Annals of the Missouri Botanical Garden* **68**: 75–86.

Strimmer, K. & von Haeseler, A. (1996) Quartet puzzling: a quartet maximum likelihood method for reconstructing tree topologies. *Molecular and Biological Evolution* **13**: 964–969.

Tamura, K. & Nei, M. (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular and Biological Evolution* **10**: 512–526.

Theis, T. (1952) An undescribed species of ergot on *P. maximum* Jacq. var. common guinea. *Mycologia* **44**: 789–794.

Thomas, P. T. (1966) Herbage breeding at the crossroads. In *Proceedings of the 9th International Grassland Congress*: 211–214. Departamento de Producao Animal da Secretaria da Agriculture do Estado Sao Paulo.

Tsai, H.-F., Liu, J.-S., Staben, C., Christensen, M. J., Latch, G. C., Siegel, M. R. & Schardl, C. L. (1994) Evolutionary diversification of fungal endophytes of tall fescue grass by hybridisation with *Epichloë* species. *Proceedings of the National Academy of Sciences, USA* **91**: 2542–2546.

Tsai, H.-F., Wang, H., Gebler, J. C., Poulter, D. & Schardl, C. L. (1995) The *C. purpurea* gene encoding dimethyallyl tryptophan synthase, the committed step for ergot alkaloid biosynthesis. *Biochemical and Biophysical Research Communications* **216**: 119–125.

Tudzynski, P., Hölter, K., Correia, T., Arntz, C., Grammel, N. & Keller, U. (1999) Evidence for an ergot alkaloid cluster in *C. purpurea*. *Molecular and General Genetics* **261**: 133–141.

Walker, J. (1957) A new species of *Claviceps* on *Phalaris tuberosa* L. *Proceedings of the Linnean Society of New South Wales* **82**: 322–327.

Walker, J. (1970) Systemic fungal parasite of *Phalaris tuberosa* in Australia. *Search* **1**: 81–83.

White, F. W. (1997a) Perithecial ontogeny in the fungal genus *Epichloë*: an examination of the clavicipitalean centrum. *American Journal of Botany* **84**: 170–176.

White, F. W. (1997b) Systematics of the graminicolous Clavicipitaceae. Application of morphological and molecular approaches. In *Neotyphodium/Grass Interactions* (C. Bacon & N. Hill, eds): 109–124. Plenum Press, New York.

White, T. J., Bruns, T., Lee, S. & Taylor, J. W. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: a guide to methods and applications* (M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White, eds): 315–322. Academic Press, San Diego.

Whyte, R. O. (1974) *Tropical Grazing Lands: communities and constituent species.* W. Junk, The Hague.

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