The Evolutionary Strategy of *Claviceps*

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1. INTRODUCTION

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

Until now, about 45 teleomorph species of *Claviceps* have been described, but presumably many species may exist only in anamorphic (sphacelial) stage and therefore go unnoticed. Although *C. purpurea* is type species for the genus, it is in many aspects untypical, because most *Claviceps* species originate from tropical regions, colonize panicoid grasses, produce macroconidia and microconidia in their sphacelial stage and are able of microcyclic conidiation from macroconidia. Species on panicoid hosts with monogeneric to polygeneric host ranges predominate.
2. PHYLOGENETIC TREE

We compared sequences of ITS1-5.8S-ITS2 rDNA region for 19 species of *Claviceps*, Database sequences of *Myrothecium atroviride* (AJ302002) (outgroup from Bionectriaceae), *Epichloe amarillans* (L07141), *Atkinsonella hypoxylon* (U57405) and *Myriogenospora atramentosa* (U57407) were included to root the tree among other related genera. To the *Claviceps* species included in Pažoutová (2001), *Neoclaviceps monostipa* sequence was added (Sullivan et al. 2001) (sequence obtained courtesy of R. Sullivan) as well as four unpublished ones - *C. cynodontis* (Loveless 1965), anamorphic *Claviceps* spp. Erag (from *Eragrostis* sp. Zimbabwe), Hyp (from *Hyparrhenia rufa*, Zimbabwe) and UroPas (on *Urochloa* and *Paspalum*, Mexico). North American species *C. zizaniae*, already proved to belong to *C. purpurea* group was omitted from this data set because of long insertion and rearrangements in its ITS1 region.

Parsimonious and the quartet puzzling tree with maximum likelihood branch lengths were computed (Fig 1, 2). While other related genera were placed outside *Claviceps* clade, *N. monostipa* was firmly positioned inside the clade as sister species to *Claviceps phalaridis*. When the sequences were aligned, we found that certain *Claviceps* species have short ITS1, and the species with longer ITS1 filled that gap with entirely different sequences, probably as a result of independent events. Short ITS1 appears predominantly in species on more ancestral positions as is *C. paspali*, *C. citrina*, *C. cynodontis*, *Claviceps* spp. SG and PM. Pair *C. phalaridis* and *N. monostipa* had also shorter ITS1. From the differences in the neighboring sequences it may be result of repeated deletion, as it is probably the case of *C. sorghicola*.

The quartet tree separated *Claviceps* species into *C. purpurea* group, group of tropical ergots and *C. citrina*, whose relationship to the remaining groups was unresolved. On parsimonious tree, *C. citrina* was ancestral to the group of all tropical *Claviceps* species. No changes in the *C. purpurea* clade as compared to the tree in Pažoutová (2001) occurred by adding new taxa to the analysis.

*C. purpurea* has Paleartic distribution and colonizes pooids, arundinoids and even panicoids. *C. paspali* originates from South America. *C. grohii* is North American species from *Carex*, whereas *C. sulcata* (Brachiaria and *Urochloa*) is an African species and *C. fusiformis* (Pennisetum and *Cenchrus*) occurs in Africa and India (Loveless 1967)*1. *C. purpurea* and as far as it is known also *C. grohii* lack microconidia and secondary conidiation. Despite

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* According to corrected sequence, *C. fusiformis* belongs in the clade of tropical ergots. Isolates formerly identified as *C. sulcata* isolates belong to *Claviceps maximensis*, same as *Claviceps* sp. PM. The *C. purpurea* clade now contains *C. paspali*, *C. zizaniae* and *C. grohii* only.

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different distributions, the similarity of rDNA sequences in this group is striking - at least 97% identity amongst *C. purpurea* and *C. grohii* was found..

Ergot species of tropical regions were divided in three clades. The first one contained African *Claviceps* sp. Hyp, *C. pusilla*, *C. cynodontis* (both widespread in Old World tropics) and two South American anamorphic species

**FIGURE 1** Maximum parsimony tree. Percentages from 500 bootstrap replications and Bremer decay indices are given over and under the clades, respectively. 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, dnaphars and consense from the package PHYLIP 3.573c. Dnapars was run with the “jumble” option changing 100x the species input order.

Erratum 1:
*C. sulcata* and *Claviceps* sp. PM isolates are now identified as *C. maximensis*. Correct phylogenetic placement is close to *C. cynodontis*.

Erratum 2:
*C. fusiformis* belongs now close to *Claviceps* sp. Erag. My apologies for sequencing error.
Claviceps sp. SG (from *Setaria geniculata*) and PM (from *P. maximum*). Conidial morphology of the latter two isolates was not sufficiently related to described teleomorphic species to allow for their unequivocal association. 

*C. pusilla* is easily recognizable due to triangular macroconidia. Its host spectrum covers about twenty andropogonoid genera (for e.g., *Bothriochloa, Cymbopogon, Capillipedium, Dichanthium, Heteropogon, Hyparrhenia, Vetiveria* and *Themeda*) (Langdon 1954, Loveless 1964a, b). Sequences of Hyp and *C. pusilla* were 97.6% identical. *C. cynodontis* has elongated to reniform macroconidia and it occurred in Paleotropics. Its host plant, chloridoid grass *Cynodon dactylon* is believed to originate in Turkey and Pakistan and from there it was introduced to all tropical and subtropical regions of the world.

The second clade contained *C. phalaridis* (pooid parasite, endemic in Australia) and *N. monostipa* (panicoid parasite from Costa Rica). Surprisingly,
their rDNA sequences were 98.2% identical, although there are considerable morphological differences between these species.

Third clade was mostly unresolved. *C. africana*, and Asian species *C. sorghicola* and *C. sorghi* are all parasites of *Sorghum*. *C. viridis* is occurring on *Oplismenus* (Paniceae) in India (Padwick and Azmatullah 1943, Thomas et al. 1945) and in Japan (Tanda, 1992). *C. gigantea* is specialized on the genus *Zea*. It represents so far the only parasite of andropogonoids that had to evolve in America from an ancestor introduced during expansion of andropogonoid grasses. Sphacelial stage of *Claviceps* sp. UroPas was found by the author in Mexico (2000) as parasite of *Urochloa* and *Paspalum*, *Claviceps* sp. Erag from *Eragrostis* sp. was collected by Dr D. Frederickson in Zimbabwe (2001). Both these species cannot be assigned to any described teleomorph. Until now, it was assumed that the only species occurring on *Paspalum* is *C. paspali*. No teleomorphic species was recorded on *Eragrostis*.

### 3. HOST DISTRIBUTIONS

The distribution of ergot species among tribes of host grasses is unequal. There is considerable difference in the number of ergot species colonizing different subfamilies. The only chloridoid - specializing species known so far are *C. cynodontis* (*Cynodon*) *C. yanagawaensis* (*Zoysia*, Japan) *C. cinerea* (*Hilaria*, Mexico, and southern USA) and *C. citrina* (*Distichlis spicata*, Mexico) (Pažoutová et al. 1998). This may be caused by the fact, that chloridoid grasses often inhabit very dry habitats where *Claviceps* spp. are not able to survive. *C. cinerea* and *C. citrina* sclerotia develop in ascostromata 20-25 days after being placed on wet sand without requiring months of dormancy (Griffiths 1901, Pažoutová et al. 1998). This enables them to produce ascospores quickly after rain period sets in.

However, recently arundinoid genera (*Phragmites*, *Molinia* and *Danthonia*) were transferred to subfamily Chloridoideae (Grass Phylogeny Working Group, 2000) and on these genera, *C. purpurea* and *C. phalaridis* occur.

The only parasites of pooid grasses are *C. purpurea* in North temperate regions and endophytic *C. phalaridis* endemic to Australia. As *C. purpurea* and *C. phalaridis* are quite distant species and despite that share the wide host range, it is probable that there is no barrier between genera of Pooideae that would prevent *Claviceps* species able to colonize one of them from spreading to all other genera, i.e., the wide host range may be caused by host metabolism and not by the parasite multiple adaptation.

*C. grohii*, *C. cyperi* and *C. nigricans* colonize sedges and rushes in the North temperate regions.

Most species of the genus *Claviceps* are found on panicoid hosts. Their host specificity ranges from monogeneric (*C. paspali*, *C. viridis*, *C.
gigantea) to polygeneric (C. fusiformis, C. pusilla). C. orthocladae, C. flavella and C. diadema, all with primitive undifferentiated sclerotium encompassing the flower parts and often germinating directly on the host, are found on Orthoclada (Panicoideae, tribe Centotheceae) and Panicum species in South America tropics. No such species were found in wet tropical and subtropical forests of Africa and South Asia.

Phylogeny of Claviceps does not mirror phylogeny of grasses. As the number of Claviceps sequences increases, there appears certain tendency of the genus to fall apart into groups of closely related species, which may colonize quite unrelated host taxa (for e.g., C. cynodontis from chloridoid grass related to Claviceps spp. PM and SG from Paniceae, or C. purpurea, C. sulcata, C. fusiformis and C. grohii. One explanation for this may be the introduction of the group ancestor into new area or ecological niche and subsequent colonization of the available host species with specialized populations from which later species arise. This may explain why C. viridis specialized on Oplismenus, a C-3 panicoid grass with broad leaves (all primitive markers), is close relative of Claviceps spp. colonizing andropogonoid grasses which are the most advanced panicoid tribe. Similar strategy is now probably operating in C. purpurea and will be discussed later.

4. BIOGEOGRAPHY

The best mapped distribution is that of Claviceps spp. occurring on cereals and pasture grasses, especially in Africa. Only few species, C. diadema, C. flavella and C. orthocladae, were observed in forest regions of tropics and subtropics. These were collected in 19th century in South America (Brazil, Guadeloupe, Cuba) and according to the original descriptions they possess primitive sclerotial characters or characters intermediate between Claviceps and Balansiae (Möller 1901, Hennings 1899, Diehl 1950). No similar species was described from any other continent. Unfortunately, no recent collections of these fungi were made that would enable more detailed studies.

Original distributions of Claviceps species in certain regions were especially well documented. Möller (1901) described Ascomycetes and Zygomycetes in the Brazilian state Santa Catarina, in the region around Blumenau. He spent almost two years in 1891-1893 (he even founded a small local mycological society) and his book of 1901 is an interesting crossover of detailed observations with a kind of diary. He did not record the occurrence of any Claviceps species that would have resembled species from Paleotropics, later described and/or revised by Langdon (1952) and Loveless (1964a b,1965). Langdon, in his PhD Thesis (1952), described Australian Claviceps species which consisted of endemic species and Paleotropical ones. Moreover, he revised available herbarium specimens of most species known so far and corrected their descriptions. It is a pity that this work was never published as a
monograph. Loveless (1964-1985) spent about twenty years collecting and describing savannah *Claviceps* species in southern Africa, continuing the work of Doidge (1950). Except *C. maximensis* which he believed to be introduced from Africa to South America with guinea grass, no common species between Neotropics and Paleotropics were found. As these detailed works precede the explosion of global seed and germplasm exchange, we may assume, that Neotropical and Paleotropical *Claviceps* species were separated and introductions of new species occurred probably only with host expansions.

In temperate regions of both hemispheres, *C. purpurea* is prevailing. Due to seed transfer by settlers it is not possible to find out if the original distribution was Northern Hemisphere only. There’s no other grass-colonizing *Claviceps* species, related species are from sedges and rushes only, or in case of *C. zizaniae*, an oryzoid host.

Original distribution of *Claviceps* species was affected during the last century by fodder grass seed and grain transfers. Examples are spreading of *P. maximum*, *C. dactylon* (Loveless 1964a, 1965), *Brachiaria brizantha*, *Paspalum* spp. and sorghum. *C. paspali* spread from South America to USA about 1850, in the years 1927 to 1937 it reached Australia and New Zealand, 1947-1948 the Mediterranean region, following the introduction of *Paspalum distichum* in 1929. (Hitchcock and Chase, 1950; Langdon, 1952). *C. sulcata* (*Brachiaria* spp.) was introduced from Africa to Brazil in 1995 (Fernandes et al., 1995). *C. africana* managed to affect sorghum growing regions worldwide during 20 years (Bandyopadhyay et al 1998, Paž outová et al., 2000), *C. fusiformis* (pearl millets in Africa and India) was recently found in Mexico on buffalo grass (*Cenchrus*) (San Martín et al. 1997). On the other hand, *C. gigantea* was never introduced outside Central America. *C. cynodontis* was originally distributed in the Paleotropics only. However, the author collected ergotized sample of *Cynodon* in Mexico (2000). Its conidial morphology and RAPD patterns were as similar to the ones of isolate from Zimbabwe as in two populations of the same species. Porter et al. (1974) described production of ergometrine and clavines in the cultures of the fungus isolated from ergotized *C. dactylon* (Mississippi) but no species identification was given. “Bermuda grass tremors” were reported in the USA since 50’s. The origin of *Claviceps sp. UroPas* is uncertain. Although it was found in Mexico, its relatedness to Paleotropical species is remarkable.

5. ORIGIN

Langdon (1954) placed the origin of the genus *Claviceps* in South American part of former Gondwana. The first *Claviceps* species probably arose on the predecessors of panicoid grasses in the warm and humid climate of the South America region of former Gondwana in the Upper Cretaceous. Our tree

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*It was an unusual outbreak of *C. maximensis* on *Urochloa* (*Brachiaria*) *brizantha* (S. Pažoutová, unpublished).*
supports this hypothesis as the species on ancestral are predominantly from South and Central America. Moreover, the radiation centre of panicoid grasses is in that region and *Claviceps* species with primitive undifferentiated sclerotia were recorded in that area. 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 panicoid grasses in early Tertiary probably gave rise to *Claviceps* species of the tropical clade. Further event influencing *Claviceps* evolution could have been the radiation of andropogonoid grasses from southern Asia to Africa, southern Europe and Central America (Jones, 1991) reflected on phylogenetic tree in close relatedness of the Mexican maize parasite *C. gigantea* to Paleotropical andropogonoid parasites. Tropical *Claviceps* species are well adapted to semi-arid conditions, but cold resistance enabling them to spread northwards is limited.

The ancestors of species close to *C. purpurea* (relatives of *C. paspali* or *C. citrina*) might have migrated from South America 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 but also with semi-arid conditions. Sequence relatedness among the extant species of this clade occurring in the colder climatic regions and semi-arid Africa suggests that the species diverged relatively recently.

Hypothesis of an early divergence between *C. purpurea* group and tropical species is supported by the low or absent homology between DMAT synthase genes of both *Claviceps* clades. DMAT synthase is the first specific enzyme of alkaloid biosynthesis. *This gene appears to be more variable than the ITS rDNA region.* Tudzynski et al. (1999) found 68% sequence similarity between the deduced sequence of the DMAT synthase protein of *C. purpurea* (CPD1) and its homologue DMAW from *C. fusiformis* (Tsai et al., 1995), whereas ITS rDNA sequences of *C. purpurea* and *C. fusiformis* are 98.7% identical and Rehner and Samuels (1995) observed 95.6% identity in a 960 bp fragment of 28S rDNA of *C. purpurea* and *C. fusiformis*.

Pažoutová (2001) hybridized digested genomic DNA of various *Claviceps* species with 0.8kb fragment of *cpd1* gene. *C. purpurea*, *C. fusiformis*, *C. sulcata* (=*C. maximensis*), *C. zizaniae* and *C. grohii* gave strong reactions whereas more ancestral *C. paspali* with different ITS1 structure gave a much weaker hybridization signal. Among tropical species, weak (*C. africana*, *C. gigantea* and *C. pusilla*) or nonexistent DMAT signals in the DNA were observed which might suggest the differences in secondary metabolism of both clades. *C. africana* (Mantle, 1968) and *C. gigantea* (Olš ovská 1999) produce alkaloids derived from the dihydroergoline skeleton, whereas in the *C. purpurea* group ergoline alkaloids were found. Moreover, there is evidence that the species from the tropical 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.
6. MORPHOLOGICAL CHARACTERS

The taxonomic criteria used to delimitate *Claviceps* species are: the color, size and shape of sclerotia, the color of ascostromata (stipe and capitulum), the presence or absence of loose hyphae on the stroma, the size and shape of perithecia, asci, ascospores (Langdon 1942). From these markers, only sclerotium formation and type of asexual fructification bear phylogenetical importance.

Among both main clades, species with yellow, red-brown or vinaceous to violet ascostromata are scattered without any tendency. On the *C. purpurea* clade, ancestral *C. paspali* belongs to so called “yellow ergots”, so does *C. sulcata*, whereas other species have red to dark red shades of coloration.

Perithecial dimensions, presence of septa in ascospores and their length were also distributed between the species regardless to their position on phylogenetic tree. From 21 species where the presence or absence of ascospore septa was mentioned in the description, 15 species had nonseptate, 5 had three- or multiseptate ascospores and in *C. paspali*, 1 or no septum was recorded. Stipes of ascostromata are phototropic so their length is to some extent influenced by the amount and direction of light available during germination. Size and shape of macroconidia is quite variable, mostly elongated between 10-15 x 3-5 μm. There is only a few species easily recognizable in sphacelial/conidial stage of development which are: *C. pusilla* (triangular), Hyp (truncated), *C. rhynchelytri* (reniform) and *C. fusiformis* (fusoid). (Loveless 1964b).

6.1. Sclerotium

Sclerotium size and to some extent shape is largely dependent on the space available inside the host floral cavity. For example, *C. purpurea* sclerotia produced in florets of *Poa annua* are about 1-2 mm long, those formed in florets of *Secale cereale* are up to 50 mm. Although sclerotia from wheat are more rounded than these from rye or common reed, they always protrude from the glumes so their shape differs from that of the healthy seed.

In sorghum ergot, *Claviceps africana*, the sclerotia were thought to be rounded, 3-5 mm in diameter with reddish brown spots, until another population of this fungus specialized on *Hyparrhenia* spp. was discovered (D. Frederickson and S. Paž outová unpublished), whose sclerotia are cylindrical and brown to black, 2-4 mm in length and 0.5-1 mm wide. Here, the sclerotium is shaped only by floral cavity and even the color is influenced by the pigments supplied by host. Sclerotia formed on sorghum cultivars with dark seeds are more pigmented than those of yellow cultivars. These two examples document that morphology of sclerotium is not sufficient for species identification on different hosts.
Function of sclerotium is that of a dormant or resting structure. Its formation differs in \textit{Claviceps} species. The simplest way is probably the proliferation of thick-walled cells accumulating lipids over the sphacelia with cortex made up of layers of dead cells as demonstrated in \textit{C. paspali} (Luttrell, 1977). In \textit{C. sorghicola} (Tsukiboshi et al. 1999) and \textit{C. citrina} (Paž outová unpublished), maturing sclerotium remains partially covered by layer of conidiating sphacelial mycelium. In \textit{C. gigantea}, sphacelia differentiates as hollow structure and sclerotium arises inside this cavity as compact pale lavender-colored tissue covered with thin pigmented rind (Fuentes et al. 1964). In \textit{C. purpurea}, the most organized sclerotium formation was observed (Luttrell 1980). Sclerotal and sphacelial differentiation are separated and sclerotal apically directed intercalary growth starts in proliferative zone distal to the sclerotal foot (site of contact with the plant vascular system).

Langdon (1954) described three types of sclerotia based on their development and resistance against climatic factors:

1. 1 - primitive, (balansioioid), irregularly globose, where the mycelium emerges from the infected ovary and envelops parts of the spikelet(s) into pseudosclerotia or hypothallus resembling those of balansioioid genera. Species producing these primitive sclerotal forms, \textit{C. diadema} and \textit{C. flavella}, occur in tropical regions
2. 2 - subglobose to elongated, usually light-colored, (\textit{C. paspali}, \textit{C. queenslandica}, \textit{C. hirtella}), may contain remnants of floral parts
3. 3 - elongated sclerotia, ovoid to cylindric in shape, dark coloured. On the distal tip of this sclerotium type, there is usually a cap formed by the remnants of sphacelial tissue. Species forming this type are found on members of all gramineous subfamilies. Their most advanced representative is \textit{C. purpurea}.

6.2. Asexual Fructification

For the genus \textit{Claviceps}, enteroblastic conidiation is typical. Phialides borne on short branched sporophores exhibit colarette and the conidia forming conidial heads remain attached by honeydew-like exudate. Branched sporophores (Fig. 3a) are in planta densely clustered on the surface of the young stroma resembling palisade pseudoparenchym. In vitro, conidiation occurs on short sporophores, occasionally branched, arising vertically from hyphae growing on substrate surface. While macroconidia have different and often characteristic shapes, microconidia of all species forming them are rounded to
Asexual fructification in *Claviceps*. (a) Typical branched sporophores with protruding conidia. Conidia seen on the sporophores are invariantly immature as the mature conidia are easily detached in aqueous mounting media. (b) Universal shape of *Claviceps* microconidia. (c) - Microcycle conidiation with pearlike and oval secondary conidia. (d) Ephelidial fructification of *C. citrina*. Conidia (3-7) are clustered on a whorl-like sporophore. Bar – 10 μm

Oval, about 4-6 μm in diameter (Fig. 3b). Micro- and macroconidia may occur on neighboring sporophores (Pažoutová et al., 1977).

Most of the species form also single secondary conidia on germ tubes emerging from macroconidia (microcycle) either in honeydew drops or when plated on nutrient media (Fig. 2c). Their shape is oval or pearlike, always with spiky proximal end, 10-15 μm in length, corresponding roughly to the macroconidium size. Microcycle is mostly finished in the course of 24 h after plating. Macroconidia of most species start colony growth in five days after secondary conidiation. Formation of secondary conidia is good test of macroconidial viability. *C. gigantea* is exceptional in that its macroconidia die after completing microcycle and colony growth is only achieved when explants of lavender tissue of the young sclerotium are plated.

In the descriptions from the end of 19th and beginning of 20th century, conidial size and mode of formation was often not sufficiently recorded or even completely omitted. The authors were more focused on sexual structures and sclerotial characters and even the host plant was not properly identified. Möller’s (1901) descriptions were an exception, because he not only described collected specimens but observed conidiation *in vitro* and documented it by excellent drawings. Diehl (1950) and Langdon (1952) revised descriptions of herbarium specimens where *Claviceps* or *Balansia* affiliation was unclear, using conidiation type as criterion.
7. CLAVICIPITOID SPECIES WITH INTERMEDIARY CHARACTERS

There is a number of species with either primitive characters or characters intermediary between Balansiae and Claviceps. Some of the descriptions originate from 19th century and are not easily available, therefore I will give them in details (Fig. 3).

7.1. *Claviceps flavella*

The type for this species was collected 1869 in Cuba, and deposited in herbarium Kew as related to *Cordyceps*. The host was not stated, the fungus being found among leaves. Petch (1933) noted that the sample contained “one sclerotium bearing five stalks and one and half detached heads”. Langdon (1952) described Kew type sclerotium as brown, and enveloping the glumes, stipes and capitula were yellow, ostioles protruding.

![Claviceps flavella and Claviceps diadema](image)

*Figure 4*  *Claviceps flavella*: (a) conidiophores on basal hypha; (b) sexual fructification; (c) perithecial head, arrow indicates collar. *Claviceps diadema*: (d) perithecial head; (e) chain of conidia and germination hyphae; (f) sclerotium germinating *in planta*.

Patouillard (1899) described specimen collected by A. Duss in Guadeloupe as *C. pallida*. No identification of host was given. Samples were found on gramineous seeds, lying on the ground. Color of the head was whitish amber with prominently protruding ostioles, stipe whitish, partially translucent.
Perithecia were oval, 300x200 μm, ascospores nonseptated, up to 200 μm. Patouillard noted, that the fungus does not produce true sclerotium, but grows on the “blackened seed tissue enveloped in glumes”.

Petch (1933) described other Duss specimens deposited in Herb. Berlin at that time. Here, mostly one clava was found on each sclerotium with stalk about 1 cm. Apex of the stalk was surrounded by a collar about 0.25 mm. Sclerotia were seated on or surrounding spikelet, with glume tips visible. Heads were dark brown, stalks pale red brown, subtranslucent, sclerotia dark reddish brown with cortex red-brown in section.

Möller (1901) gave detailed description of *Claviceps balansioides* from Brazil. The infection of host plant (*Echinochloa*) starts in the upper fertile floret, which is later with the lower sterile one and/or the whole spikelet overgrown with hyphae. On the surface of glumes, layer of mycelium appears forming single elongated conidia of the sphacelial type 9-12 x 5 μm, without occurrence of conidial heads (Fig. 3a). Sporophores are thinner than normal hyphae. Later, mycelial mass fills the space available between the glumes in conical form, partially enclosing floral parts. The conidiating layer is replaced by dark blue-black rind. Irregular shape of sclerotium corresponds to the spikelet parts included, size depends on the amount of nutrition provided by the spikelet (Fig. 3b). The sclerotium was able to survive detached for several months. Only after that period, the germination into pale yellow perithecial heads occured. The stipes were unusually long, up to 8 cm, ending with collar (Fig. 3c).

*C. balansioides* was considered (Petch 1933) to be the same as type *C. flavella* (Berk. & Curt). Diehl (1950) transferred to this species also *Claviceps pallida* (Pat.) from Guadaloupe. Sclerotial shape and structure for all the specimens is similar, however, Möller and Patouillard described it as black, whereas Petch observed reddish-brown coloration. Stipes and capitula on the Kew, Möller’s and Patouillard’s fungus were of yellow shades, whereas the ones on Petch’s samples were reddish-brown which Petch ascribes to storing.

### 7.2. *Claviceps diadema*

Möller (1901) first described it as belonging to *Balansia* because of its loosely woven sclerotium, although the asexual fructification was more of sphacelial type (Fig. 3e). Therefore Diehl (1950) transferred this species into genus *Claviceps*. Möller observed that one or two neighboring *Panicum* spikelets were overgrown by hyphal mass. Structure of glumes was not destroyed and still recognizable. Although this stroma was not hard as for e.g., *C. purpurea* one, it developed dark yellow rind. Formation of anastomoses between the hyphae was frequently observed. The formation of 5-6 perithecial yellow heads with 2-4 mm stipes and very slightly protruding ostioles (Fig. 3d) occurred in planta so that the germinating sclerotium resembled little crown (hence the name) (Fig. 3f).
diadema formed conidia that were 7-9 μm long, oval, tapering to lower end and Möller noted that they usually divide in two cell prior germination. However, the germination occurred also in conidia still attached to conidiophore. Chains of conidia and germination hyphae were observed, but no formation of conidial heads glued together occurred. On the picture (Fig. 3b), they resemble more secondary conidia of other Claviceps species (Fig. 2c). Anastomoses occurred frequently between hyphae.

7.3. **Claviceps orthocladae**

*Clavices orthocladae* (P. Henn.) Diehl (*Orthoclada*, Brazil) first described as *B. pallida* var. *orthocladae* (Hennings 1900), also develops yellowish villous stipes and pale yellow capitula while its sclerotium is still on the host plant. Hennings (1904) merged this specimen with Möller’s *B. diadema* from *Panicum* and another Ule’s specimen (No. 1100) also from *Panicum* (Rio de Janeiro, Palmeiras) to new taxon *Balansiella orthocladae*. However, Diehl (1950) found in the original specimen sphacelial fructification only and placed this species into the genus *Claviceps*, unfortunately without revealing any details about conidial shape, size and formation.

7.3.. **Balansia pallida**

*Balansia pallida* (collected by Ule, 1885, Sao Francisco, Sta Catarina, Brazil, *Luziola peruviana*) (Winter 1887) resembles *C. diadema* and Hennings (1899) transferred it to the genus *Claviceps*. However, Winter observed that young stromata were covered with fructification layer producing long (44-62 μm) curved conidia resembling ephelidial spores which was the reason for Diehl (1950) returning the species to *Balansia* again. Diehl reexamined the specimen and found hypothallus (0.5-2 mm diameter) seated within florets of the host and partially enclosing palea and lemma, with yellow surface and whitish interior. Yellow layer consisted of ephelidial fructifications. Ascostromata were yellow, with stipes 1-3 mm long, and heads 0.5-2 mm in diameter.

The more recent records *Claviceps* species with “intermediary” characteristics are *C. phalaridis*, related *Neoclaviceps monostipa*, and according to yet unpublished observations, *C. citrina*.

7.4. **Neoclaviceps monostipa**

*Neoclaviceps monostipa* was found on panicoid grass in Costa Rica. It does not have true sclerotium, branching hyphae permeate the ovule tissues forming hypothallus. No host-parasite interface is established. From hypothallus, single reddish brown stipe (up to 3 mm) with capitulum emerges. Sequence of ITS1-5.8SrDNA-ITS2 region of *N. monostipa* was 98.2% identical to that of *C. phalaridis* Walker that is Australian endemite. In cultures, *N. monostipa*
produces conidia (36-72 x 1.2-1.6 μm) which undergo microcyclic conidiation partially resembling ephelidial fructification (Sullivan et al. 2001).

7.5. **Claviceps phalaridis**

*Claviceps phalaridis* persists as systemic endophyte (Walker 1957) in tillers, stems, leaf sheaths and blades similarly to *Epichloe/Neotyphodium* and forms sclerotia in florets of the diseased plants, rendering them sterile. It occurs on pooid grasses as well as on grasses of the chloridoid genus *Danthonia* (Walker 1970). Sclerotium differentiation begins as white fungal mass encompassing anthers and ovary. Infected florets are later incorporated in the mature sclerotium. As distinct from *N. monostipa*, sclerotia of *C. phalaridis* are true resting structures with rind, however, in wet weather, they are capable of germinating *in planta*. Ascostromata are of dark vinaceous color. Sphacelial fructification is almost nonexistent, few oblong to cylindrical conidia (7.5-14 x 2-3 μm) were found on sclerotial surface. Probably the same spores are formed during ascospore germination (Uecker 1980). Each cell of the septate ascospore may give rise to one conidium borne on short stalk. In addition to that, other type of conidia, their shape resembling those of *N. monostipa*, was observed (J. Walker, personal communication).

7.5. **Claviceps citrina**

*Claviceps citrina* was isolated and characterized in our laboratory from the sclerotia on chloridoid grass *Distichlis spicata* (Mexico). Its sclerotia are true ones, readily germinating when placed in humid chamber in yellow ascostromata with stipes 20-25 mm. In our previous publication (Pažoutová et al. 1998) we described only rounded to oval sphacelial conidia 4-6 μm in diameter on the surface of mature sclerotia collected in 1996. However, in the sample of younger sclerotia obtained in 1999, their proximal surface was partially covered with white mycelial layer where, besides branched conidiophores of sphacelial type (Fig. 2a) producing oval conidia, long “setae” protruded. Recent examination revealed that these long spores are ephelidial conidia (47.5< 59.3<70 μm. x 1.5-2 μm) (Fig. 2d). On some sclerotia ephelidial spores prevailed, on other the sphacelial ones. Among the remaining sclerotia from 1996 we found also two younger specimens containing both spore types. *C. citrina* appeared in our phylogenetical analysis as the most ancestral species.
Recent rDNA sequence analyses (Spatafora and Blackwell 1993, Glenn et al. 1996, Kulda et al. 1997, Sullivan et al. 2001) confirmed monophyly of Clavicipitales and their relatedness to Hypocreales/Bionectriaceae. *Cordyceps* appeared in ancestral position to plant parasitic Clavicipitaceae. The clades depicting groupings of genera of plant-parasitic Clavicipitaceae have low statistical support. If *C. citrina* sequence is compared with Gen Bank database using Blast algorithm, the most related species are (in order of decreasing similarity) *C. phalaridis*, *Myriogenospora atramentosa*, *Balansia strangulans*, *E. amarillans* and various other *Epichloe* spp. Next *Claviceps* species appears as 11th. On our trees, the clade separating *Claviceps* from other genera has low or no statistical support, only the clades separating groups of related species are supported.

Not only sequences of Clavicipitaceae, but also morphological characters gave unequivocal information. Anamorphs with holoblastic (ephelidial) conidiation and enteroblastic (sphacelial, typhodial) conidiation are encountered as well as species and genera with synanamorphs of both types.

If we consider some *Cordyceps* characters (enteroblastic phialidic conidiation, stipitate hermipspherical ascostroma, formation of hard resting stroma, then all these are found also in the genus *Claviceps*. Specific characters relating to plant parasitism are colonization of florets (more or less specialized) with a potential for colonization of meristems (as demonstrated by *C. purpurea* inoculation experiments). Production of slime glueing together conidial heads is increased to such extent that honeydew with suspended conidia drops outside the florets. Among *Claviceps* species on ancestral positions, characters typical for other clavicipitoid genera are found, like ephelidial conidia and endophytism.

Plant parasitic clavicipitoid genera might have arisen from variable *Claviceps*-like ancestor/s as no other genus has extant species sharing so many characters with other related genera as *Claviceps*. Such ancestor might have had stipitate ascostromata, loose sclerotium of *C. flavella* type encompassing flower parts without establishing fungus/host interface on vascular bundle. Some of these early species might have occurred as occasional endophytes. The existence of synanamorphs of phialidic and ephelidial type as in *C. citrina* is probable, *Ephelis* anamorph being derived character.

**9. POPULATIONS AND SPECIATION MECHANISMS IN CLAVICEPS**

**9.1. Claviceps purpurea**

*Claviceps purpurea* is an ergot fungus with a wide host range including the entire subfamily Pooideae and also genera belonging to chloridoids and
panicoids (Loveless 1971, Brewer and Loveless 1977). Its distribution is basically Holarctic, but it has been recorded in Arctic regions (Linder 1948) and also occurs in southern temperate and subtropical regions.

Most attempts at establishing host-specific populations or sub-species taxa like varieties, special forms or races was made on C. purpurea, probably because of its wide host range and also greater accessibility of different isolates and collections. It can be argued now, that the range is not the character of C. purpurea, but that of pooid grasses as C. phalaridis is also able to colonize various pooid hosts, even the genera introduced to Australia.

Morphology of C. purpurea is variable. Sclerotial length ranges from 2 to 50 mm and the color of the stromata varies over a wide scale of red shades. Conidial size and shape also are polymorphic, ranging from oval spores 5 μm in length to cylindric or elongated and up to 13 μm in length (Loveless 1971, Sprague 1950, Tanda 1979). The sclerotia contain peptide alkaloids that belong to three basic groups - ergotamines (with alanine as the first amino acid entering the cyclopeptide moiety), ergotoxines (with valine), and rarely found ergoxines (with 2-aminoisobutyric acid) (Walzel et al. 1997).

In the herbaria, C. purpurea specimens can often be found under the names of C. wilsonii (used for ergot from Glyceria fluitans, Barger 1931), C. microcephala (samples from Poa annua), or C. sesleriae. There were misidentifications of C. fusiformis as C. microcephala (Thirumalachar, 1945) persisting until the 70’s (Sundaram et al. 1972).

A historical overview of races and varieties of C. purpurea introduced by different authors is given in Barger (1931), Loveless (1971) and Pažoutová and Parbery (1998). Especially Stäger ( 1903, 1908) defined several races which were subsequently modified and rearranged, however one of them went almost unnoticed. Stäger (1922) observed that sclerotia formed on grasses from wet habitats could float on water, but that sclerotia from Secale, Lolium, Brachypodium sylvaticum, Sesleria coerulea, Arrhenatherum elatius, Agropyron (now Elytrigia) repens, Alopecurus myosuroides and other land grasses sank in water. On Dactylis glomerata, Calamagrostis epigeios as well as some Holcus and Poa spp., sclerotia of both types were found. He named that race f. sp. Phalaridis arundinaceae natans. Defining this taxon based on habitat was against the host-based system of his other races, so unfortunately this line of research was discontinued.

Loveless (1971) found that the conidia of isolates from grasses from wet/shady habitats were longer (6.5-8.5 μm) than those from isolates found on land grasses (5-6 μm) and the spores from laboratory cultures showed more variation than the ones from natural host. Grouping of specimens according to conidial size and host corresponded partially to Stäger’s groups.

Kobel and Sanglier (1978) found 10 chemoraces in sclerotia collected in Europe and North America, the most usual combinations being
ergocornine/ergocryptine (22.6% of samples), ergocristine/ergosine, (20.4%), and ergotamine (13.1%). Composition of alkaloid mixture produced is hereditary and independent on host grass (Kybal and Brejcha 1955).

However, no attempt was made as to compare the alkaloid races with host-based grouping except for Czech study of natural occurrence of chemoraces (Kybal et al. 1957) together with characterization of host and location. Seventeen sclerotial specimens (out of 32) were of ergocristine/ergosine type, found on *Phalaroides arundinacea*, *Calamagrostis* spp., *Holcus* spp., *Molinia* spp., *Festuca rubra*, *Festuca gigantea*, *D. glomerata* and *Phragmites communis* growing in wet or forest locations. Thirteen specimens occurring on *Hordelymus europaeus*, *Elytrigia repens*, *F. pratensis*, *H. lanatus* and *S. cereale* growing on meadows contained mixture of ergosine, ergocornine and ergocristine. Only two specimens produced small amounts of ergotamine in addition to ergocornine and ergocryptine and both originated from *Lolium* growing along the roads.

Another group of *C. purpurea* isolates was found on *Spartina* spp. populating salt marshes of Atlantic shore in the Americas. This group was characterized (as analyzed by thin-layer chromatography) by predominant production of ergocryptine, ergocryptinine and lysergylvalylmethylester (Eleuterius and Meyers 1974). *Spartina* stands at British Isles have been colonized by *C. purpurea* only after 1960 (Hubbard 1970, Raybould et al. 1998). These isolates have the longest conidia (8.4 μm) from all the British samples studied (Loveless 1971).

Jungehülsing and Tudzynski (1997) established two main groups using RAPD typing: one consisted mainly of the English isolates from *Molinia*, *Holcus* and *Dactylis*, the other group contained the isolates from land grasses.

Our study (Pažoutová et al 2000) established the population structure of *C. purpurea* and characterized the groups and isolates by host or habitat preferences, phenotypic traits used in previous studies (conidial morphology, alkaloid type, properties of sclerotia) as well as by DNA analysis (using RAPD and EcoRI restriction site polymorphism in the 5.8S rDNA). Thus, the ambiguous, even contradictory groupings found by previous researchers and based on only one or two characters were incorporated into one system. Three groups were identified:

- G1 from fields and open meadows
- G2 from shady or wet habitats
- G3 from *Spartina* salt marshes

The sclerotia of G1 contained various ergotamines and ergotoxines, its conidia were 5-8 μm long. G2 produced ergosine and ergocristine with small amounts of ergocryptine, conidia were 7-10 μm long. G3 produced ergocristine and ergocryptine and conidial length was 10-12 μm. Sclerotia of the G2 and G3
isolates floated on water. In the 5.8S rDNA, an EcoRI site was found in G1 and G3 but not in G2.

Typical hosts of G1 were *S. cereale*, *Lolium* spp., *E. repens*, *F. pratensis*, *Helictotrichon pubescens* and *Bromus* spp.

Isolates of G2 were more commonly recovered from *Calamagrostis*, *Holcus*, *Molinia*, *Phalaroides*, and *Phragmites* growing at pond and river banks, ditches, forests, mountain woods.

*Alopecurus pratensis*, *Ammophila arenaria*, *Arrhenatherum elatior*, *Dactylis* sp., *Festuca ovina*, *F. rubra*, *Phleum* sp., and *Poa pratensis* could be naturally colonized by isolates of both G1 and G2. The habitat seems to be more important, as the common occurrence of different groups in the same locality is rare.

The third group, G3 was found on *Spartina alterniflora* (introduced from North America) and *S. anglica* stands in coastal salt marshes in Wales, Essex and Yorkshire and at four locations in Southampton region. RAPD profiles of American and British isolates from these grasses were uniform (Pažoutová et al., submitted) suggesting transfer of G3 group from American Atlantic coast to Europe. Mass ergot infection on *S. anglica* and *S. alterniflora* by *C. purpurea* with unusually long conidia appeared first in 60’s (Loveless 1971, Rayboould 1998).

To some degree, host preferences were observed. Our preliminary inoculation experiments using spraying of florets with conidial suspension showed that host species typical for the given habitat (like *Phragmites* or *Phalaroides*) are only weakly colonized by *C. purpurea* isolates from different habitat (those belonging to G1 or G3). Host genera common to both the G1 and G2 (especially *P. pratensis* and *Dactylis* spp.) are grasses which may be encountered in either habitat. These preferences result from adaptation to available hosts.

*C. purpurea* isolates were analyzed using RAPD and AFLP (Pažoutová et al. 2000; Pažoutová et al. 2002). The differences between populations were considerable, as both methods detected only 1-3 bands shared between populations. Together with comparison of rDNA (ITS1-5.8S-ITS2 region) all three methods suggest that G1 population is ancestral to G3 and G2 which are more homogenous.

Possible cause for increased intraspecific variation in *C. purpurea* might be chromosomal rearrangements. Hüsgen et al. (1999) observed variations in the chromosome number and size in a set of *C. purpurea* isolates. We have typed some of their isolates that were part of the set analyzed by Jungehülsing and Tudzynski (1997) to our G1 or G2 groups. Different karyotypes, and ploidy were found across the groups. Two triploids were found, both from G2 group, and the most of the isolates were diploids or aneuploids. Only 4 out of 23 isolates were haploid.
G2 and G3 group also share some phenotypical similarities like elongated or cylindrical conidia, floating sclerotia and ergocristine as one of major alkaloids. Therefore it may be inferred that a group of isolates with floating sclerotia arose from ancestors of extant group G1 which later diverged into G3 and G2 (the latter lost in addition a conserved Eco RI site in 5.8S rDNA). Indeed, we found such an isolate with RAPD and alkaloid composition (ergosine, ergocornine, ergocryptine) typical for G, however, its sclerotia floated and it was found on water grass *Glyceria fluitans*.

Interestingly, similar habitat association was recently discovered in an insect pathogen *Metarrhizium anisopliae*, where forest and field populations were found differing in UV and cold tolerance and no host specificity was found (Bidochka et al. 2001).

### 9.2. *Claviceps africana*

*C. africana* spreads mainly via secondary conidia. Formation of secondary conidia from primary macroconidia is widespread especially among tropical *Claviceps* species, however, we observed that this process occurs either inside honeydew drop or *in vitro* when primary macroconidia are plated on agar medium. In *C. africana*, secondary conidiation occurs on macroconidia close to the surface of honeydew drop (Frederickson et al. 1989). Resulting microconidia form dry layer outside the drop and are spread by wind (Frederickson et al. 1993). This strategy differs from usual spreading through insect vectors attracted by sweet honeydew and enables long distance jumps.

The importance of ascostroma formation in population variability is unclear. Most sphacelia formed in sorghum fields does not mature in sclerotia. Germination of sclerotia in vitro or observed in the field was erratic (Frederickson et al. 1991).

Recent spreading of *C. africana* from Africa to sorghum growing regions worldwide prompted studies about its internal variation and population structure (Pažoutová et al. 2000a, Tooley et al. 2000). RAPD and AFLP were used and both methods revealed only very small differences between isolates confirming homogeneity of sorghum populations. American isolates were almost identical and three isolates of the same type were also found in South Africa, suggesting the origin of the invasion clone in the Americas (West group) first recorded in Brazil, 1995 (Reis 1996).

In India, sorghum ergot was caused by endemic *C. sorghi*. Although conidia of this species are indistinguishable from those of *C. africana*, no windborne conidia are formed, its sclerotia protrude from glumes and are easier to germinate. Early observations during 1914 to 1917, 1946 (Ramakrishnan 1948) and during the early 1960s (Singh 1964), mention mostly large elongated sclerotia found on sorghum. Both long and short sclerotia were found in the late 1970s (Sangitrao and Bade 1979) to mid 1980s which might suggest start of *C. africana*...
infections. Since the late 1980s, the typical long and protruding sclerotia of *C. sorghi* have not been observed.

DNA-typing studies confirmed *C. africana* replacing *C. sorghi* (Pažoutová et al. 2000a, Tooley et al. 2000). The population was different from that invading the Americas. Despite all quarantine precautions it reached Australia in 1996 (Ryley et al. 1996). Inside that population (East), Australian isolates were distinguishable from Indian ones by acquisition of single RAPD band difference which appeared also in an isolate from Thailand. This suggests infection path to Australia through Southeast Asia islands.

RAPD markers of East and West group behaved like bar codes – the isolates lacked or shared them all. However, in African isolates, RAPD markers appear like independent characters and occur in combinations. Still, sorghum population is very homogenous.

Recently, a *Claviceps* sp. on *Hyparrhenia rufa* was found in southern Africa (Frederickson and Pažoutová unpublished) whose conidia were very similar to *C. africana* and its sclerotia were of the same shape as seeds of the host plant. RAPD patterns showed that it is indeed another population of *C. africana*, but more different than any of the sorghum isolates, which may be the beginning of speciation based on host adaptation. Although *Hyparrhenia* plants were neighbors of sorghum field, no RAPD pattern similar to that of *Hyparrhenia* population was found among sorghum isolates.

### 9.3. *Claviceps gigantea*

In 1996, we collected in Central Mexico about thirty isolates of *C. gigantea* (Fuentes et al. 1964) from five locations in the region of Toluca (*Zea mays*) and from a location near Amecameca (*Z. mays* and *Z. mexicana*) (Paž outová and Fuèíkovský, unpublished). Sclerotia of *C. gigantea* remain in the field and those harvested with corn are easily discarded due to their size so that no transfer with seeds occurs. As the sphaecelia is completely wrapped in husks, no honeydew with conidia is available for insects to spread. Therefore differences between populations isolated in the small valleys were expected. However, although the locations were isolated from each other by mountain ridges, neither RAPD with 50 primers nor AFLP with three primer pairs detected any reproducible differences, not even between the Toluca Valley and Amecameca isolates. *C. gigantea* did not spread from Central America to maize-growing regions worldwide because it has specific temperature and humidity requirements that are met especially in high valleys of central Mexico (Fuèíkovský and Moreno, 1971).

The role of sexuality in speciation process is unclear. *C. purpurea* was proved to be homothallic which should (similarly to asexual reproduction) support separation of the population in clonal lineages. Small variability of *C. gigantea* and *C. africana* shows that this is not occurring. On the other hand, no
isolates that might be hybrids of ecoraces were found in *C. purpurea* so far, maybe due to effective separation of the populations which rarely occur on the same locality.

From these three analyses it may be hypothesized that *Claviceps* species infraspecific variation remains rather low in stable environment, as it was observed in *C. gigantea* adapted to mountain climate and in habitat-specialized ecoraces G2 and G3 of *C. purpurea*. *C. africana* shows small but detectable variability of sorghum isolates, however, RAPD pattern of *Hyparrhenia* isolate differs considerably with all primers used. It seems that strong external impulses like change of host or transfer into another environment trigger population variability and lead to speciation, aimed at the ecological niches available. Recent transfers of ergot fungi are precisely the kind of events that stimulated their evolution so far, so the adaptation of introduced species to new host genera and further spreading is to be expected.

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