3. THE TAXONOMY AND PHYLOGENY OF *CLAVICEPS*

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3.1. THE TAXONOMIC POSITION OF *CLAVICEPS*

The evolution and to some extent the taxonomy of the parasitic fungi belonging to the genus *Claviceps* are linked to the evolution of their host plants (grasses, rushes, and sedges). Although the recent Internet version of the Index of Fungi (http://nt.ars_grin.gov/indxfun/frmlndF.htm) lists 38 recorded species of *Claviceps,* at least 7 species are missing and *C. oryzae-sativae* and *C. virens* were removed from *Claviceps,* so that there are 43 known species.

The genus *Claviceps* is in the family Clavicipitaceae which was initially placed in the order Hypocreales. During the 50's, however, doubts about this based on comparative study of conidiogenous stroma development resulted in its transfer into the Xylariales (Luttrell, 1951) and then to the Clavicipitales, an order close to Hypocreales and erected specifically to accommodate clavicipitaceous fungi (Gäumann, 1952). More recently, application of DNA analysis has been used to test the relationships between different members of Hypocreales and has confirmed the initial placement of the monophyletic Clavicipitaceae as family belonging to the order Hypocreales. Molecular phylogenies suggest that the genus *Claviceps* was the first group derived from a common ancestor line, then *Epichloe/Neotyphodium* followed by *Atkinsonella* and with the last clade containing species of *Balansia* and *Myriogenospora* (Spatafora and Blackwell, 1993; Rehner and Samuels, 1994; Glenn *et al.,* 1996). This contradicts the formerly held hypothesis that *Balansia* contains the most primitive clavicipitoids.

3.2. TAXONOMIC MARKERS

The taxonomic criteria used to deliminate *Claviceps* species are: the color, size and shape of sclerotia, the color of stipes and capitula, the présence or absence of loose hyphae on the stroma, the size and shape of perithecia, asci, ascospores and conidia (Langdon, 1942).

3.2.1. Sclerotium

Sclerotium size is largely dependent on host. For example, *C. purpurea* sclerotia produced in florets of *Poa annua* or *Phalaris tuberosa* are about 1–2 mm long,

those formed in florets of *Secale cereale* are up to 50 mm. Similarly, there is a threefold difference also between the *C. gigantea* sclerotia formed in *Zea mays* and *Z. mexicana*. Sclerotia contain lipid reserves that are consumed during germination (Mitchell and Cooke, 1968). In the wet tropics, where hosts may flower for most of the year, the selective pressure for sclerotium production is so low for some ergot species that they produce only few sclerotia erratically or even not at all (*Sphacelia* spp.). For example, although some African mature sclerotia of *C. africana* were successfully germinated (Frederickson *et al.,* 1991), this ergot is worldwide found in the sphacelial state. Other ergots, such as *Sphacelia tricholaenae* are known only for its anamorph.

Langdon (1954) described three basal types of sclerotia:

1. primitive, irregularly globose, where the mycelium emerges from the infected ovary and envelops parts of the spikelet(s) into pseudosclerotia resembling those of balansioid genera. Species producing these primitive sclerotial forms, *C. diadema* and *C. flavella,* occur only on panicoid hosts with C3 type of photosynthesis in tropical forest regions
2. subglobose to elongated, usually light-colored, (*C. paspali, C. queenslandica, C. hirtella* and *C. orthocladae*), occurring on Panicoids
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 *C. purpurea* which exhibits intercalary growth in the proliferative zone distal to the sclerotial foot (site of contact with the plant vascular system). This category, however, should be subdivided, because elongated sclerotia differ greatly in the resistance to drought, frost and long storage as well as in dormancy and germination requirements.

3.2.2. Stroma

Stroma (also clava) consists of stipe and capitulum. The coloration of clavae is either straw to yellow or in shades of brown-purple to black-purple, with the exception of *C. viridis* which is green. The "yellow" clavae are mostly encountered in species from panicoid hosts. The young capitulum is smooth, the ostioles of the perithecia appear as darker pores. During the maturation the ostioles enlarge and become papillate, very prominently in some species *(C. ranunculoides, C. fusiformis)*. This, however, appears to be due more to the shrinkage of the tissue of the outer capitulum cortex than to the emergence of perithecia beyond it although the end result would be the same.

The size and shape of perithecia is dependent on their degree of maturation. Young perithecia are small and oblong to oval, whereas fully matured ones are often ellipsoid to pyriform. Once the filamentous asci which form inside perithecia are mature, they protrude through the ostiole prior to discharging their ascospores. Loveless (1964) considered the length of asci an unreliable

criterion, because asci at different stage of maturity and therefore different length may occur in a single perithecium and the stage of maturity is difficult to determine. The length of filiform ascospores should be measured after their discharge from asci.

3.2.3. Conidia

The size and shape of conidia is less dependent on environmental factors than are ascospores and are valuable traits for species determination. Loveless (1964) mapped the range in size and shape of conidia in fresh honeydew and from dried sclerotial material collected from Rhodesian grasses. Thirteen conidial types were defined, six of them belonged to known species *C. paspali, C. digitariae, C. sulcata, C. maximensis, C. pusilla* and *C. cynodontis,* seventh group with large falcate or fusiform conidia occurring on *Cenchrus ciliaris* and *Pennisetum typhoideum* was probably *C. fusiformis,* described three years later by the same author (Loveless, 1967). Conidial characters together with considerations of host range are generally sufficient to determine if a sphacelial ergot is the anamorph of an already named species or is representative of a species new to science. For e.g., the tenth conidial group described from *Sorghum caffrorum* resembled anamorph of *C. africana* described much later by Frederickson *et al.* (1991).

A study of conidia of English *C. purpurea* collections revealed the similarity in conidial shape among the samples from certain host groups suggesting the existence of *C. purpurea* host races (Loveless, 1971).

3.2.4. Chemotaxonomic Markers

The qualitative analysis of alkaloid content as a possible marker was made in only seven species from the 43 described, these being *C. purpurea* (peptide alkaloids: ergotamine, ergocornine, ergocristine), *C. fusiformis* (agro-, elymoand chanoclavine), *C. paspali* (lysergic acid amides), *C. gigantea* (festuclavine) (for detailed review see Flieger *et al.,* 1997). Mantle (1968) detected the peptide alkaloid dihydroergosine as the main component in *C. africana* and traces of agroclavine in *C. sorghi*. Tanaka and Sugawa (1952) and Yamaguchi *et al.* (1959) found the peptide alkaloids, ergometrine and agroclavine in sclerotia of *C. imperatae*. Porter *et al.* (1974) described the occurrence of ergometrine related alkaloids in ergotized *Cynodon dactylon,* but did not identify the *Claviceps* species which was probably *Claviceps cynodontis*.

In other species, evidence of alkaloid production was obtained colorimetrically using vanUrk's reagent which gives blue coloration with ergoline compounds. Prior to describing new species, Tanda assayed sclerotial extracts for alkaloids as well as testing their toxicity to mice. Only in *C. bothriochloae,* some positive colorimetric reaction was found (Tanda, 1991), whereas in *C. microspora* and *C. yanagawaensis,* the weight loss and other signs of mice toxicity were not apparently connected with alkaloid content (Tanda, 1981) and may therefore

have been caused by other toxic metabolites. Taber and Vining (1960) detected alkaloid production colorimetrically in shake cultures of *C. maximensis* and the ergot isolated from wild rice, that could possibly be *C. zizaniae*. As yet unidentified alkaloids have been found in the shaken cultures *of C. grohii* and *C. sulcata* (Pazoutová *et al.,* unpublished).

An indirect proof of alkaloid production by *C. cinerea* was the occurrence of abortions in cattle in North Mexico grazing *Hilaria mutica,* colonized by this ergot (Zenteno-Zevada, 1958).

Walker (personal communication) found no alkaloids in *C. phalaridis* sclerotia. Similarly, HPLC analysis at the Inst. Microbiology CAS, Prague failed to detect alkaloids at the ppm level. Preliminary results confirmed the présence of a group of unknown metabolites. No alkaloids were also detected in the sclerotia of new species of ergot *C. citrina* from *Distichlis spicata* (Pazoutová *et al.,* in press).

Another attempt to find chemotaxonomic markers for distinguishing *Claviceps* species was presented by Mower and Hancock (1975). The sugar composition of the thick liquid honeydew secreted during the sphacelial stage to protect and disseminate conidia, was determined for nine known species and possibly five undescribed species collected on specified grasses and rushes.

The Claviceps and Sphacelia species differed in the amount and representation of 23 mono-, di- and oligosaccharides with two basic types being identified; those with prevalent glucose and fructose (*C. purpurea, C. fusiformis, C. nigricans, C. grohii* and *Sphacelia* isolates from *Pennisetum, Setaria* and *Juncus*) and those with arabinitol and mannitol *(C. gigantea, C. cinerea, C. tripsaci)*. An intermediary group containing both sugars and sugar alcohols included *C. paspali, C. uleana, C. zizaniae* and a *Sphacelia* from *Andropogon tener*.

3.3. THE CHARACTERIZATION OF SOME CLAVICEPS SPECIES

3.3.1. *C. paspali*

According to Hitchcock (1950) and Langdon (1952), this species originates from South America (Uruguay or Argentina) and was introduced to USA about 1850. In the years 1927 to 1937 it was reported from Australia and New Zealand, 1947–1948 from the Mediterranean region (where it followed the introduction of *Paspalum distichum* in 1929) and now is found worldwide where *Paspalum* species grow. Infection is followed 4–5 days later with the production of abundant honeydew containing primary and secondary (microcycle) conidia. Subglobose light brown sclerotia encompass the remnants of disintegrated ovary (Luttrell, 1977). Cold and moist storage is required for sclerotium germination, the germination ability is lost after dry storage and exposure to temperatures under 0°C (Cunfer and Marshall, 1977). Similar species, however, endemic to Australia and detected there before the introduction of *C. paspali* is *C. queenslandica* (Langdon, 1952).

3.3.2. *C. phalaridis*

Walker (1957) discovered this Australian endemic ergot, that persists as systemic endophyte similarly to *Epichloe/Neotyphodium* and forms sclerotia in florets of the diseased plants, rendering them sterile. Intercellular hyphae are present in tillers, stems, leaf sheaths and blades. It was found on pooid grasses including *Lolium rigidum* and species of *Phalaris, Vulpia, Dactylis,* as well as on arundinoid grasses in the genus *Danthonia* (Walker, 1970). A white fungal mass envelopes young anthers and the ovary. Infected florets are later incorporated in the mature subglobose sclerotium. The sclerotia were able to germinate after 5-month storage either dry at room temperature or humid at 6°C (Walker, 1970). Ascospore formation in this species differs from that of either *C. purpurea* and *E. typhina* (Decker, 1980). Clay (1988) considered this species as possible intermediate between Balansiae and *Claviceps,* but our current investigations of DNA sequences do not support this view (Pazoutová, unpublished).

3.3.3. *C. viridis*

C. virdis was found on *Oplismenus compositus* in India (Padwick and Azmatullah, 1943; Thomas *et al.,* 1945) and on O. *undulatifolius* recently in Japan (Tanda, 1992), in forest regions with monsoon rains. Its sclerotia are cylindrical, blackish with a tinge of green and require a dormancy period prior germination (Tanda, 1992).

3.3.4. *C. gigantea*

C. gigantea is found on maize *(Zea mays)* in the high valleys of Central Mexico (Fuentes *et al.,* 1964). Its occurrence seems to be limited by the annual mean temperature of 13–15°C (Fučikovský and Moreno, 1971). The formation of sclerotia (Fuentes *et al.,* 1964) is quite different from that in *C. purpurea* and *C. paspali* as described by Luttrell (1977, 1980 respectively). It starts as a soft and hollow structure that produces many conidia suspended in honeydew. The resting structure develops later in the internal cavity as firm pseudoparenchymatous tissue surrounded by thin dark rind. Maturation proceeds from the interface with the host progressing distally. The surface of the mature sclerotium is covered with dried remnants of the sphacelia. With respect to climatic conditions, the sclerotia survive short frost periods and the dry winter.

3.3.5. *C. africana*

C. africana colonizes sorghum *(Sorghum bicolor)* and is endemic in eastern and southern Africa, especially in Kenya, Zimbabwe and South Africa. However, *C. africana* is spreading to Southeast Asia, Japan (Bandyopadhyay, 1992) South America (Reis *et al.,* 1996) and Australia (Ryley *et al.,* 1996), presenting danger

for the male sterile A-lines of sorghum and seed production. In 1997, it reached Texas, USA. Infection ability is enhanced by the extensive secondary conidiation on the surface of excreted honeydew that presents source of windborne propagules (Frederickson *et al.,* 1993). *C. africana* sclerotia were able to germinate after 1 year of dry storage at ambient temperature (15–30°C) (Frederickson *et al.,* 1991). The most often encountered alternative host is *S. halepense* (Johnson grass). A common feature of *C. africana* and *C. gigantea* is the production of dihydrogenated ergoline alkaloids.

3.3.6. *Claviceps pusilla*

C. pusilla is a widespread ergot of the Mediterranean region, Africa, India, Australia and probably China, colonizing seventeen andropogonoid Old World species (among them *Bothriochloa, Dichanthium, Capillipedium, Cymbopogon, Heteropogon, Vetiveria* and *Themeda*) (Langdon, 1954; Loveless, 1964). Its sclerotia are probably of the advanced type, and a characteristic marker is the triangular shape of its conidia.

3.3.7. *Claviceps cynodontis*

C. cynodontis is one of the few ergots colonizing chloridoid grasses (Langdon, 1954; Loveless, 1965). It grows in the florets of *Cynodon dactylon* (Bermuda grass) and it is distributed from southern Europe to Africa, India, Burma and Philippines. It often occurs in sphacelial state without developing sclerotia.

3.3.8. *Claviceps sulcata*

This species was originally found in Rhodesia (Zimbabwe) on a *Brachiaria* species (Langdon, 1952), an important forage grass in warm regions. Since then, it has been found in other areas of southern Africa, but recently, the sphacelial stage was reported as widespread from Brazil (Fernandes *et al.,* 1995). It is characterized by large conidia and prominent broad furrows running along each side of the sclerotium.

3.3.9. *Claviceps fusiformis*

C. fusiformis is widespread in semi-arid regions of Africa and India. It occurs on pearl millet *(Pennisetum americanum)* which was domesticated in Sudanian region in Africa and introduced to India (for review see de Wet, 1992). Distribution of *C. fusiformis* extends from the Transvaal to Equatorial Africa (Loveless, 1967) and widely within India. Despite its typical fusiform elongated conidia, Thirumalachar (1945) concluded that it was *C. microcephala* (syn. *C. purpurea*) and this misidentification persisted until the 70's (Sundaram *et al.,* 1972). Rapid spread and high infection rates are enhanced by the formation of secondary conidia and its sclerotia are drought-resistant (Thakur *et al.,* 1984).

Loveless (1964) found the conidia of *C. fusiformis* as the falcate type on the species of *Cenchrus ciliaris, P. typhoideum* and *P. maximum*. Ramakrishnan (1952) successfully cross-inoculated *C. fusiformis* from six species of *Pennisetum* to species of *Cenchrus* and *Urochloa*.

3.3.10. *Claviceps purpurea*

C. purpurea occurs in all temperate regions and has undoubtedly the widest host range of any *Claviceps* species. As a result, its name has been suspected of being a blanket term for several distinct taxa. Morphology descriptions of the species reflect this variability. Sprague (1950) described *C. purpurea* as having purple-black sclerotia and flesh-colored capitula, Dickson (1956) characterized its stromatal heads as pale-fawn, while Tanda (1979a) reported pale purple to blackish brown sclerotia and light orange to pale red capitula.

Sclerotium morphology is influenced by the size of host florets and climatic differences (for e.g., the sclerotial size ranges from 1–50 mm). However, for precisely these reasons it happens that almost any ergot with elongated dark brown to black sclerotia and purplish capitulum is classified as *C. purpurea* whereas it could represent yet another undescribed species. A good example of such misdetermination is the above mentioned designation of *C. fusiformis* from Indian *Pennisetum typhoideum* as *C. microcephala* which persisted for many years. *C. grohii* (Groves, 1943) was described as a new species mostly because of its colonization of *Carex,* which was considered too great an extension of its host range for *C. purpurea*. One other discriminative marker distinguishing the two species was the insect damage of sclerotia in *C. grohii* collections when *C. purpurea* sclerotia remained untouched. Later it was found (Langdon, 1952) that conidia of *C. grohii* differ in the shape from those of *C. purpurea*. However, *C. purpurea* was recently isolated from *Carex* and its identity confirmed by RAPD (Jungehülsing, 1995). Thus, both these species occur on *Carex hosts*.

It has been noted, that isolates of *C. purpurea* from certain hosts infect other grass species under laboratory conditions with varying results. The successful infection is highly dependent on the length of the period of anthesis and the inoculation technique used. Stäger (1903, 1905, 1908, 1922, cf. Campbell, 1957) used three methods: spraying the heads with a conidial suspension, prying the glumes apart and spraying the florets and dipping grass heads in conidial suspension. These methods succeeded unless the florets were closed or waxy or haired glumes protected them.

Stäger found three races of *C. purpurea* in Europe that should infect only certain groups of hosts and later, together with other authors seven races was identified:

P1, *Bromus sterilis, Festuca elatior, Hordeum,* four species of *Poa, Secale, Triticum*
P2, *Brachypodium sylvaticum*
P3, *Bromus erectus, Lolium* sp.

P4, *Bromus erectus, Festuca arundinacea, Lolium perenne* (Mastenbroek and Oort, 1941)
P5, *Lolium* spp., *Secale* (Baldacci and Forlani, 1948)
P6, *Aira, Molinia, Nardus, Phragmites* (Arundinoideae)
P7, *Poa annua* only (also considered *C. microcephala*)

Other authors, however, came to different conclusions when they transferred ergot from *Lolium* sp. to rye (Békésy, 1956) or to wheat (Bretag and Merriman, 1981). Kybal and Brejcha (1956) succeeded in infecting rye with ergot from *Phragmites* and *Molinia,* whereas Campbell (1957) found no host preferences at all in Canadian isolates.

Campbell removed the glume tips at anthesis and sprayed the heads with conidial suspension but when infecting *Hordeum,* it was necessary to inoculate the heads just emerging from the leaf sheath. Inoculation at anthesis was not successful. Inoculations were repeated until the first signs of sphacelial development appeared.

In the first part of his experiments, Campbell took the conidial suspensions from ergots collected on 38 host species in 19 genera from three subfamilies (as defined by Watson and Dalwitz, 1996):

Pooideae
Agropyron, Agrostis, Arrhenatherum, Avena, Bromus, Calamagrostis, Dactylis, Elymus, Festuca, Glyceria, Hordeum, Lolium, Phleum, Poa, Secale, Triticum
Arundinoideae
Stipa
Chloridoideae
Calamovilfa, Spartina

and inoculated rye, wheat and barley. Every isolate infected each cereal with the exception of one isolate from *Glyceria borealis*. Other isolates from *G. borealis,* however, were infectious.

In a reverse procedure, conidial inoculum of rye ergot to was applied to plants in the following subfamilies and genera:

Pooideae:
Agropyron, Agrostis, Alopecurus, Bromus, Calamagrostis, Dactylis, Elymus, Festuca, Koeleria, Phalaris, Phleum, Poa, Polypogon, Sitanion Sphenopholis
Arundinoideae
Stipa, Danthonia
Chloridoidae:
Eragrostis, Sporobolus
Panicoideae
Setaria

Again, all plants became infected both in the field and in the greenhouse. These experiments confirmed that with the use of a suitably aggressive inoculation technique, *C. purpurea* is capable of colonizing a wide range of not only pooid, but also chloridoid and some panicoid hosts. Natural occurrences of *C. purpurea* on the species of Setaria and Pennisetum were reported from South Africa (Doidge, 1950). Brewer and Loveless (1977) took honeydew from *Pennisetum macrourum* and inoculated wheat getting a mean of 1.6 sclerotia per head of wheat compared with 3.9 sclerotia when they transferred honeydew from wheat to wheat.

However, the question of host races and specificity of *C. purpurea* continues. Loveless (1971) found that the host-specific differences in conidial size and shape in English collections were retained even in laboratory cultures and corresponded to some extent to the Stäger's groups. Following numerous cross-inoculation experiments, Tanda allocated Japanese isolates of *C. purpurea* from different pooid and arundinoid hosts to four varieties: *C. purpurea* var. *purpurea,* var. *alopecuri,* var. *phalaridis* and var. *sasae* (summarized in Tanda, 1979a, b). The latter variety occurs on a bambusoid host and could be probably species different from *C. purpurea*.

It seems that the races of *C. purpurea* are associated with regions and most probably they cannot be world-wide generalized. The host ranges of the Stägerian groups are not the same as the ones listed for Tanda's varieties, and even other European authors came to different conclusions. Also in North America, *C. purpurea* seems to have other preferences.

C. purpurea sclerotia contain peptide alkaloids in varying ratios. Kobel and Sanglier (1978) after screening of alkaloid content in sclerotia collected for years in Europe and North America on rye and wild grasses found 10 chemoraces:

Combination	*% of samples*
Ergotamine	13.1
Ergocristine	7.3
Ergosine	2.2
Ergotamine + ergosine	7.3
Ergocornine + ergocryptine	22.6
Ergocornine	1.5
Ergocristine + ergotamine	4.4
Ergocristine + ergosine	20.4
Ergocryptine α	1.5
Ergocryptine $\alpha + \beta$	1.5

The authors observed that the strains containing the combination ergocristine-ergocornine-ergokryptine were unstable and dissociated in the culture into ergocristine strains and ergocornine+ergocryptine strains.

Gröger (1979) found 6 chemoraces among strains capable of alkaloid production in laboratory culture. These strains were isolated from sclerotia found on rye, triticales or fescue:

Ergotamine
Ergocristine
Ergosine
Ergocornine+ergosine (1:1)
Ergotamine+ergocryptine (1:1)
Ergocornine+ergocryptine (3:2)

In our laboratory, the measurements of alkaloid content in natural sclerotia collected on wild grasses and cereals in Europe and in USA were made. We observed that the European strains tend to belong either to ergotamine or to ergotoxine group, whereas the American strains contained various combinations of ergotamine with ergotoxines, covering almost the whole spectrum.

3.4. CLAVICEPS INTRASPECIFIC VARIABILITY ASSESSED BY DNA ANALYSIS

3.4.1. Variable *C. purpurea*

Jungehülsing (1995) addressed the problem of intraspecific variability of *C. purpurea* using the methods of molecular genetics (RAPD analysis and electrophoretic karyotyping). Her RAPD results were probably the most variable of any RAPD's found among filamentous fungi studied so far, although one or several species-specific bands was observed with each primer. The isolates originated mainly from Germany and England, the host being species of *Agropyron, Agrostis, Dactylis, Elymus, Festuca, Holcus, Lolium, Molinia, Phleum, Secale,* and non-grass host Carex. The analysis of data pooled from nine primers showed that the *Secale* isolates from various parts of Germany were related and that isolates from the same locality were the most similar. Bootstrap analysis confirmed a marked tendency towards relatedness of the isolates from the same region followed by relatedness according to host preference.

Jungehülsing also found possible reason for the extreme variability even among the isolates from the same host species and locality (see also Tudzynski, this volume). The chromosome number and size in *C. purpurea* isolates was found to be variable. Despite this, crosses were possible between parents with different sized chromosomes and translocations resulted in progeny of different karyotypes.

RAPD fingerprinting of different *Claviceps* species has also been done in the Institute of Microbiology CAS. When testing various *C. purpurea* isolates with the primer RP2 (AAGGATCAGA) (Lehmann *et al.*, 1992), we obtained similar results as Jungehülsing (1995) confirming that the strains collected in the same region are more related than the geographically distant isolates (Figure 1).

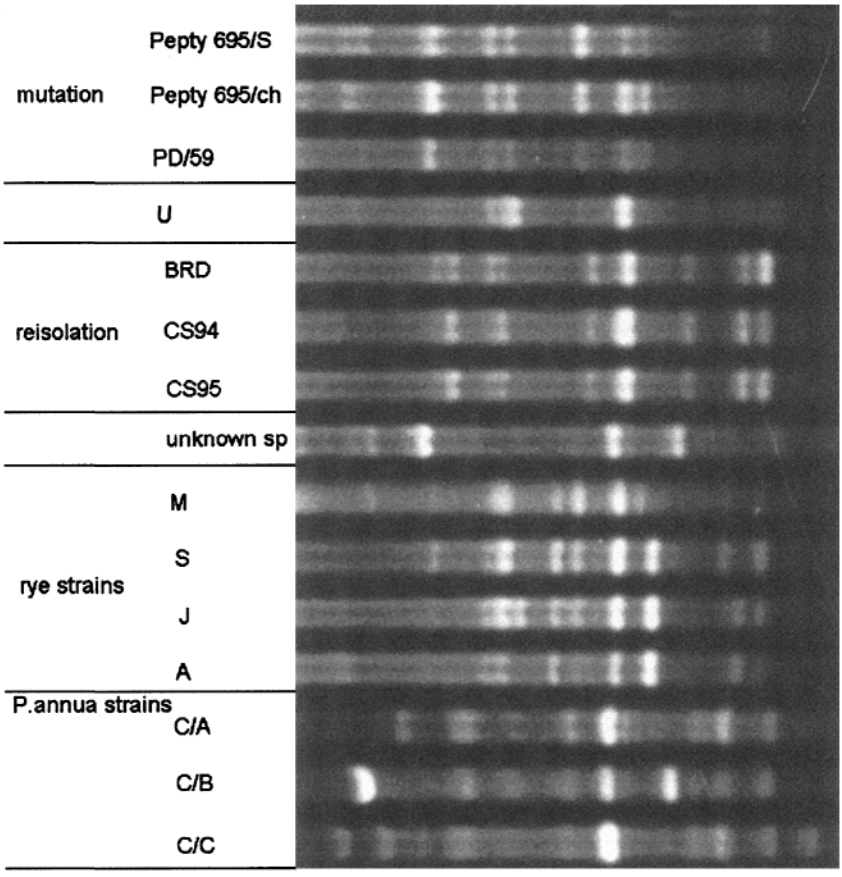

Figure 1 RAPD patterns of *C. purpurea* strains obtained with the primer RP2. The strain Pepty 695/S and its two mutants, strain U isolated from *Lolium multiflorum* in South Africa, strain BRD used for field infection and two strains CS94 and CS95 reisolated from the resulting sclerotia in two subsequent years, independent isolates M, S, J, A from the spontaneously infected rye field and independent isolates from spontaneously infected *Poa annua* field are shown. The species specific band is marked by black arrow

Variability among natural isolates from the same locality differed. Single sclerotium isolates from the same rye field (strains M, S, J, A) showed fewer differences than the isolates from a seed producing field of *Poa annua* (strains C/A, C/B, C/D). This variability was due to the présence of a population of distinct strains on the given locality and not to variations in the progeny of a single strain, because the banding pattern of the strain BRD used to inoculate one cultivar of *Poa pratensis* and reisolated from the field sclerotia in two subsequent years (CS 94, CS 95) remained unchanged. Also the mutation-and-screening procedure did not change the RAPD patterns, shown here for the

ergotoxine producing strain Pepty 695/S, its clavineproducing blocked mutant Pepty 695/ch isolated by Schumann *et al.* (1982) and another isolate PD59 isolated in our laboratory and differing slightly in morphology from the parent Pepty 695/S strain.

Another experiment assessed the degree of variability among isolates from sclerotia collected from the same flower head, on different flower heads of the same plant and from the different plants of the same locality. Although the colony morphology of the isolates was almost identical, we found that even the isolates from the same flower head of *Lolium* sp. exhibited different RAPD patterns (data not shown).

In contrast to the RAPD's, the sequence of the ribosomal region containing 5.8S rDNA and spacers ITS1 and ITS2 is less variable. We sequenced the 556 bp fragments obtained from Middle-European cereal isolate *C. purpurea* P695/S (Schumann *et al.,* 1982) and from *C. purpurea* isolated in our laboratory from sclerotia from *Phalaris* sp. collected in Australia. The sequences were compared to two American *C. purpurea* sequences. U57669 (GenBank) from isolate GAM 12885 collected on *Dactylis glomerata* near Athens, Georgia, USA (Glenn and Bacon, 1996; unpublished) and the sequence of the isolate 109 from *Festuca arundinacea,* Lexington, Kentucky (Schardl *et al.,* 1991).

The European and Australian isolates differed only in a single base out of 556 localized in ITS2. The American isolates GAM 12885 and 109 differed from the European Pepty 695/S each in 5 and 6bp respectively, all in ITS1 spacer, but not the identical ones (Figure 2), whereas the difference between them was in 10 positions of ITS1.

This result seems to support the hypothesis that *C. purpurea* is a relatively recent introduction to Australia together with cereals from Europe. The

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Pepty 695/S ATCATTACCG AGTTTACAAC TCCCAAACCC ACTGTGAACT TATACCC-AA AACGTTGCCT
Australia   .......... .......... .......... .......... .......... ..........
GAM 12885   .......... .......... .......... .T........ .......... ..........
109         .......... .......... .......... .......... .......G.. ..........

Pepty 695/S CGGCGGGCAC AGCGGTACCC GAGCCCCC-G CAAGGGAG-C AGAGGCGCC- CGCCCGCCAG
Australia   .......... .......... .......... .......... .......... ..........
GAM 12885   ......A... .......... .......... .......... .A.......C ..T.......
109         .........- .......... ........C. ........G. .........G ..........

Pepty 695/S GGGACCAAAA CTCTTCTGTA TACCCATAGC GGCATGTCTG AGTGGATTTA AAAACAAAT
Australia   .......... .......... .......... .......... .......... .........
GAM 12885   .......... .......... .......... .......... .......... .........
109         .......... .......... .......... .......... .......... C........
```

Figure 2 The differences in ITS1 spacer sequence among *C. purpurea* isolates from different regions. P695/S—European isolate; Australia—Australian isolate from *Phalaris* sp.; GAM 12885—*D. glomerata,* Georgia, USA; 109—*F. arundinacea,* Kentucky, USA

differences between European and American isolates could be attributed to the présence of indigenous strains of *C. purpurea* that evolved independently of the European populations.

3.4.2. Other Claviceps Species

We made RAPD's of other Claviceps species with the primer 206 (TCAACAATGTCGGCCTCCGT) (Figure 3). Isolates of *C. paspali* and *C. fusiformis,* although originating from distant regions, had almost the same banding patterns. The patterns of three Mexican *C. gigantea* isolates were identical (only one representative shown) as well as the patterns of two *C. africana* strains from Bolivia and Australia. Probably, *C. africana* is spreading too quickly to develop genome differences among distant isolates. The patterns with the primer 206 were markedly species-specific and that could enable the identification of sphacelial stages once the teleomorph RAPD was determined. We used the RAPD pattern as the first criterion for excluding the possibility of *D. spicata* ergot *(C. citrina)* being *C. purpurea*.

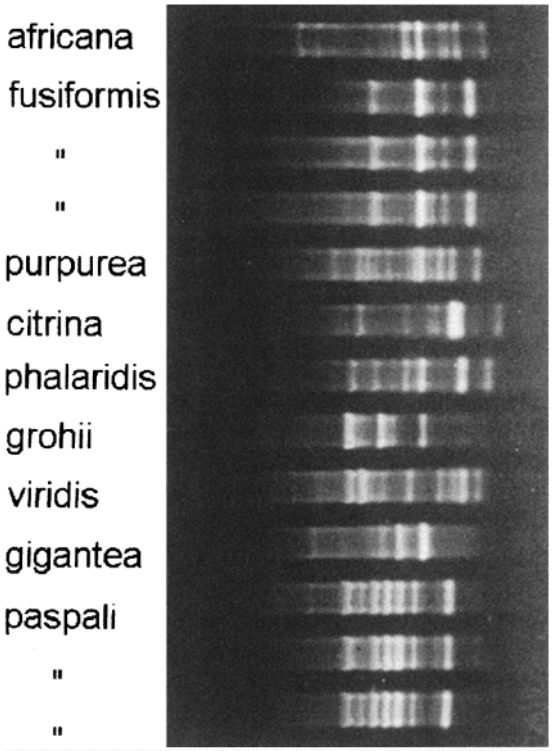

Figure 3 RAPD patterns of different *Claviceps* species with the primer 206. Note the almost identical patterns of *C. paspali* and *C. fusiformis* isolates

3.5. THE CLAVICEPS PHYLOGENY

Its main course is connected with the evolution of grasses and the global climatic changes, in the side branches the incidence of endemic species is correlated with ecological niches.

3.5.1. Grass Evolution

The evolution of grasses is summarized in Brown and Smith (1972) and Jones (1991). The grasses probably appeared during the Jurassic period (Mesozoic), in the wet tropics. The Centothecoideae, Bambusoideae, and Arundinoideae with C3 type of photosynthesis are supposed to be the most primitive. Their descendants, the subfamilies Chloridoideae and Panicoideae developed C4 photosynthetic pathways and thus acquired competitive advantage in warmer regions.

Chloridoideae are adapted to stressful arid or saline habitats (sand dunes, coastal marshes, semi-deserts). Their widespread distribution with the radiation center in South Africa suggests that they acquired the C4 photosynthesis and expanded before the breakup of the Pangea (Middle Cretaceous).

One section of the Panicoideae (the tribe Paniceae) acquired C4 photosynthesis shortly before South America was separated from the rest of Gondwana in the Late Cretaceous. In the then isolated South America, Paniceae became dominant. C3 photosynthesis was retained in apparently more primitive shade loving species (*Oplismenus, Icnanthus,* and some species of *Panicum*) whereas C4 species colonized open habitats in the tropical and subtropical zones (e.g. *Paspalum, Cenchrus, Brachiaria, Pennisetum*).

In southern Asia, the Andropogoneae *(Sorghum, Zea, Saccharum, Bothriochloa, Imperata)* developed from some C4 ancestor and rapidly occupied savanna habitats and dry open woodlands. It is supposed that this occurred some 25–30% Mya (million years ago). Andropogonoid species form 30–40% of all grass species in India and Africa. The transfer of Andropogoneae to America probably occurred via southern Europe, before their separation in the Tertiary period while they probably reached Australia via island chains.

The subfamily Pooidae retained C3 photosynthesis, as the C4 type does not provide any advantage in cooler climates. It expanded mainly in the northern hemisphere. The southern hemisphere does not support its climatic needs except New Zealand, southern Australia, and South America. As distinct from other subfamilies, Pooidae possess large chromosomes.

The grass phylogenies derived from plastid as well as nuclear DNA sequences show two main groups. In the PACC group (including Panicoideae, Arundinoideae, Chloridoideae, and Centothecoideae) chloridoids and arundinoids are sister groups to the ancestor of the panicoids, which correlates well with the theory based on the geographical distribution of grass subfamilies. The group BOP contains Bambusa-Oryza and Pooids (Mathews and Sharrock, 1996).

3.5.2. Ergot Distribution

Knowledge of the geographic occurrence of *Claviceps* species is influenced by the présence of the grass hosts and also by the human factor. With the exception of Brazil and West India at the end of the 19th century (Möller, 1901; Hennings, 1899), the information concerning the occurrence of tropical forest ergots is scarce. The best information about geographical and host distribution is available for the ergots that colonize cereals and pasture grasses.

The distribution of ergot species throughout the world has several interesting features. First, there is a striking difference in the number of ergot species colonizing the chloridoid, pooid, and panicoid subfamilies. The only chloridoid-specializing species known so far are *C. cynodontis (Cynodon) C. yanagawaensis (Zoysia)* and *C. cinerea (Hilaria)* and we add new species *C. citrina* found in Central Mexico on *Distichlis spicata*. Also the occurrence of *C. purpurea* on recent *Spartina* hybrid in England was documented (Gray *et al.*, 1990).

The pooid ergot is represented only by *C. purpurea* with Laurasian distribution and the unusual endophytic *C. phalaridis* endemic to Australia. Both species colonize also arundinoid species like *Danthonia* and have wide host ranges, although *C. purpurea* is considered advanced species and *C. phalaridis* a rather primitive one. There is also *C. litoralis* occurring on the northern Japanese islands and Sakhalin on *Elymus* and *Hordeum* (Kawatani, 1944), but some mycologists doubt its species status and consider it a variety of *C. purpurea*.

Some morphologic similarity to *C. purpurea* is seen in *C. grohii, C. cyperi* and *C. nigricans* that colonize sedges in the North temperate regions.

In the genus *Claviceps,* the panicoid species with monogeneric *(C. paspali, C. viridis, C. gigantea)* to polygeneric host ranges *(C. fusiformis, C. pusilla)* predominate. The most primitive species *C. orthocladae, C. flavella* and *C. diadema* (undifferentiated sclerotium encompassing the flower parts, germinating directly on the host) are found on *Orthoclada* (Centothecae) and panicoids in South America tropics, whereas none are found in wet tropical and subtropical forests of Africa and South Asia. Also none of the South American ergots was found in the other regions of the world except *C. paspali* which has been spread by human influence. On the other hand, several species are common in Africa and India and some also in Australia. No colonization of arundinoid grasses has been documented for panicoid ergots.

It seems, that species of *Claviceps* should be considered predominantly parasites of panicoids. The colonization of chloridoid and pooid hosts is somewhat obscured by the vast occurrence of *C. purpurea,* but in fact it is as marginal as the occurrence on cyperaceous hosts. The distribution of *Claviceps* species suggests, that the genus origin probably dates from the period of the expansion of panicoids from South America in the Late Cretaceous. The speciation then continued independently on all continents of the former

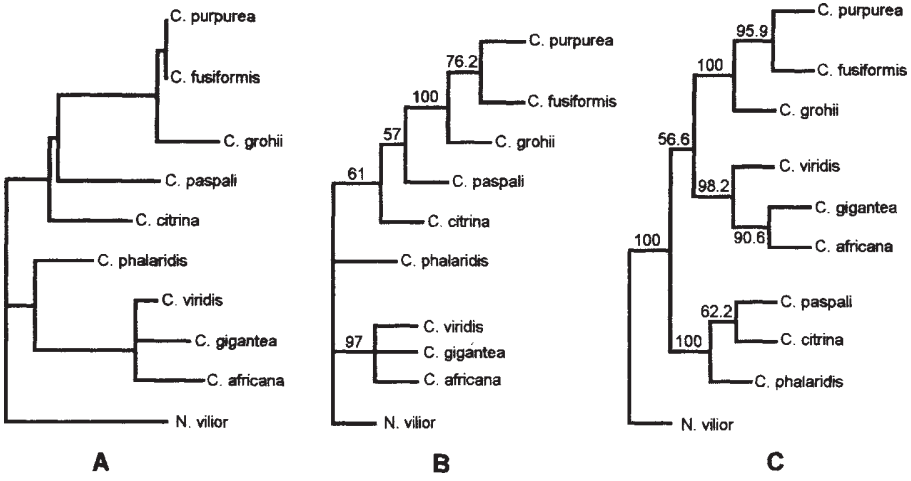

Figure 4 Phylogenetic relationships among *Claviceps* species based on rDNA ITS1 region.
A—maximum likelihood tree;
B—neighbor-joining tree (Jukes-Cantor distance matrix and 500x bootstrap);
C—maximum parsimony tree (500x bootstrap);
Strains used for sequencing:
C. purpurea Pepty 695/S; *C. fusiformis* SD-58; *C. grohii* strain 124.47 (CBS, Barn); *C. paspali*—our conidial isolate (*P. dilatatum,* Alabama); *C. citrina* sp. nova, our isolate (*D. spicata,* Central Mexico); *C. phalaridis*—our isolate (*Phalaris,* sample DAR 69619 Australia); *C. viridis* strain 125.63 (CBS, Barn); *C. gigantea*—our isolate (*Zea mays,* Toluca Valley, Mexico); *C. africana*—our isolate (*Sorghum,* Bolivia)

Gondwana. The exchange among African and Indian species was enabled probably at the same time as the spreading of andropogonoid grasses.

3.5.3. Claviceps Phylogenetic Tree

In our laboratory, we made phylogenetic analysis of the nine *Claviceps* species based on the comparison of internal transcribed spacers ITS1. *Nectria vilior* (Glenn *et al.,* 1996) was used as an outgroup species. The trees (Figure 2) acquired with different algorithms contained in PHYLIP (Felsenstein, 1995) differed, but the segregation of two distinct groups was always supported over 95% of bootstraps:

1. *C. purpurea, C. fusiformis, C. grohii*
2. *C. viridis, C. gigantea, C. africana*

The remaining species *C. phalaridis, C. citrina and C. paspali* were separated by the maximum parsimony as the third group (100%) containing more, "primitive" species. Also, these species share a marked deletion of 38 bp.

On the other side, maximum likelihood tree (out of 211 tested) placed Australian *C. phalaridis* basal to the group 2, whereas American *C. paspali* and *C. citrina* were closer to the ancestors of group 1. The branches with zero length were collapsed to polytomies. The neighbor-joining tree resembles the ML tree (with the exception of *C. phalaridis*) and their log likelihoods were almost identical (ML=-990.245; NJ =-990.256). The NJ tree branches supported by less than 50% bootstraps were reduced to polytomies.

The advanced species of the group 1, with drought and cold resistant sclerotia may be related to the group of ergots with prevailing glucose and fructose as described by Mower and Hancock (1975). The high similarity of ITS1 DNA of these species otherwise so different in their habitats and host specificity is rather puzzling, but the 95.6% identity in 960 bp fragment of 28S rDNA of *C. purpurea* and *C. fusiformis* was also observed by Rehner and Samuels (1995). The evolution course in this branch is unclear, but we very tentatively speculate about the Laurasian origin from more primitive ancestors related to *C. paspali* and *C. citrina* and relatively recent transfer to Africa.

The second group, encompassing two andropogonoid ergots together with *C. viridis* from rather primitive C3 panicoid from Asian monsoon regions, clearly correlates with the radiation origin of andropogonoids in Southern Asia. It also shows that the andropogonoids took their ergots with them on their way to Africa and North America and were not colonized by the local ergot species. The ergots with the prevalent mannitol and arabinitol in their honeydew (Mower and Hancock, 1975) should be expected in this branch. Together with related *C. phalaridis* it represents probably the genuine ergots that developed in the Old World warm regions.

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