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Pleomorphic conidiation in *Claviceps*

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Types of asexual sporulation in 17 *Claviceps* species and the closely related *Corallocytostroma ornicopreoides* were revised in relation to the phylogeny of clavicipitaceous fungi. We observed: (1) enteroblastic conidiation from branched phialidic conidiophores typical of the genus (anamorph *Sphacelia*) in all species including *Corallocytostroma*; (2) widespread and often sequential formation of terminal holoblastic secondary conidia on tapering hyphae arising from sphacelial macroconidia; and (3) in addition to sphacelial conidiation, sympodial holoblastic conidiation of the *Ephelis*-type in cultures of *C. zizaniae* and in both the culture and sphacelial tissue of *C. citrina*. Secondary conidiation was not found in *C. purpurea*, *C. citrina* and *C. sorghicola*. During sphacelial fructification, most species produced macroconidia and microconidia. Only macroconidia formed *in planta* underwent secondary conidiation whereas microconidia did not germinate at all. In *C. phalaridis*, the formation of holoblastic 2–3 celled appendaged conidia was observed, similar to that of *Aciculosporium* and *Neoclaviceps*. In dendrograms based on ITS1-5.8S rDNA-ITS2 sequences, genera and species with appendaged conidia grouped on a highly supported clade with ancestral *Corallocytostroma*. The clade was placed inside a group of tropical species of *Claviceps*, without any relationship to *Balansiae*.

INTRODUCTION

In the family *Clavicipitaceae*, several conidiation types and the corresponding anamorphs are known. *Ephelis* anamorphs produce allantoid to acicular or filiform macroconidia, which are holoblastic, sympodial and often form whorls consisting of 3–8 spores (Rykard & Luttrell 1984, White 1997). Ephelidial anamorphs occur in teleomorphic genera *Balansia*, *Myriogenospora* (White 1997) and *Heteroepichloë* (Tanaka *et al.* 2002).

Anamorphs of *Claviceps* (i.e. *Sphacelia*), *Epichloë* (i.e. *Neotyphodium*) *Dussiella*, and *Cordyceps* have phialides, producing endoblastic conidia (White 1997). Endophytic *Epichloë*/*Neotyphodium* species have phialides arranged in sporodochia *in planta* that form small conidial heads, whereas conidia formed in culture or on germ tubes emerging from ascospores occur mostly singly. These conidia are capable of repeated microcyclic conidiation (Bacon & Hinton 1991).

Atkinsonella produces either ephelidial macroconidia in groups of 3–5, or *Acremonium*-like oval microconidia (Morgan-Jones & White 1992).

In *Claviceps*, phialidic formation of enteroblastic conidia that remain glued first in heads and later merging in an oozing honeydew mass has been observed both *in planta* and in culture (Luttrell 1980, Rykard *et al.* 1984) and is considered the main sporulation type. Also, the formation of secondary conidia is described in *C. africana* and *C. sorghi* (Frederickson, Mantle & de Milliano 1989, Pažoutová & Bogo 2001).

However, there are some less known species of *Claviceps* and allied genera, which differ in their association with host plant and where yet different conidiation was observed. The Australian endemic *Cepsiclava phalaridis* (syn. *Claviceps phalaridis*) forms a stable endophytic association with its host plants (Walker 1957, 1970, 2004) which are infected by ascospores making contact with the coleoptile. Sclerotia occur in every floret of the colonized plant regardless of their sex, rendering it sterile. Walker (1957) described oblong to cylindrical enteroblastic dry conidia 7.5–14 × 2–3 μm on the surface of *C. phalaridis* sclerotia and also filiform holoblastic bicellular conidia (20–25 μm) with dichotomously branched antler-like appendages at the distal end of the apical cell; these latter spores are capable of microcyclic conidiation through proliferation of the basal cell.

Almost identical appendaged spores have been observed in cultures of *Aciculosporium take*, an endophyte (Nozu & Yamamoto 1972) causing a witches'

Table 1. Species of *Clavicipitaceae* studied.

Species	Host	Herbarium collection[a]	Origin of samples	GenBank accession no.
Honeydews and/or conidiomata				
Claviceps africana	*Sorghum bicolor*	*ca* 200 samples	Australia; Brazil; Bolivia; India; Mexico; South Africa; Thailand; Zimbabwe; Zambia	AJ011783
C. citrina	*Distichlis spicata*	PRM 842 966	Texcoco, Mexico	AJ133393
C. cynodontis	*Cynodon dactylon*	CCC 616	Matopos, Zimbabwe	AJ557074
C. fusiformis	*Pennisetum americanum*	CCC 525	Matopos, Zimbabwe	AJ133392
C. gigantea	*Zea mays*		Toluca Valley, Mexico	AJ133394
C. maximensis (formerly as PM)	*Panicum maximum*	CCC 398 CCC 639	Matopos, Zimbabwe; Chaco, Paraguay	AJ133396
C. paspali	*Paspalum* sp.	CCC 129	Montgomery, Ala., USA	AJ133398
C. purpurea	*Pooideae*	*ca* 100 samples	Czech Rep.; Germany; France	AJ133401
C. pusilla	*Dichanthium aristatum*	BRIP 26571	Tolga, Qld., Australia	AJ277544
C. pusilla	*Heteropogon contortus*	PRM	Matopos, Zimbabwe	
C. sorghi	*Sorghum bicolor* *Heteropogon triticeus*	CCC 623 CCC 630	Gulbarga, India	AJ306621
C. sulcata	*Urochloa brizantha*	CCC 399	Passo Fundo, Brazil	AJ133403
C. ornicopreoides	*Dichanthium* sp.	BRIP 39184	Kunumurra, WA, Australia	AJ557075
Cultures				
C. grohii	*Carex* sp.	CBS 124.47	Canada	AJ133395
C. phalaridis	*Phalaris tuberosa*	ex DAR 69619	NSW, Australia	AJ133399
C. sorghicola	*Sorghum bicolor*		Japan	AJ133397
C. viridis	*Oplismenus compositus*	CBS 125.63	India	AJ133404
C. zizaniae	*Zizania aquatica*	CCM 8231	Canada	AJ133405

[a] CCC, Czech Collection of Clavicipitales at the Institute of Microbiology CAS, Prague; PRM, Herbarium of the National Museum in Prague; and CCM, Czech Collection of Microorganisms, Brno.

broom disease of bamboos in Japan and Taiwan (Tsuda *et al*. 1997). From conidiomata at diseased leaf sheath tips, conidia ooze out in a suspension (Shinohara 1965, Tsuda *et al*. 1997, Oguchi 2001); the conidia are filiform with swollen ends and 1–2 septa. After transfer onto agar media, these conidia produce another ('cultural') type of spore with appendages at their distal end, very similar to those of *C. phalaridis*. Appendaged conidia undergo microcyclic sporulation in that they germinate from the basal end and form the same structure in a mirror-like manner, again with apical appendages (Tsuda *et al*. 1997). In shake cultures, this is repeated as the twin conidia break apart, but on agar media no further proliferation occurs.

Another rather enigmatic clavicipitalean genus, *Corallocytostroma*, forms conidiomata surrounding stems or tillers, inhibiting the formation of inflorescences (Yu & Zhang 1980, Shivas, Mitchell & Jubb 1997); the conidiomata later harden into sclerotia. Species of the genus are found in China (on rice) and Australia (on *Astrebla* and *Dichanthium*). In Australian *C. ornicopreoides*, masses of conidia are produced that ooze out of the loculi in the stromata. The conidiophores are densely packed inside the loculi resembling those of *Claviceps*. Sclerotia overwinter at the base of grass tussocks and are able to produce either stipitate perithecial heads or, after the onset of rain, eruptions appear with loculi identical to the ones from conidiomata. The production of new conidiomata on dormant structures have not been observed in other *Clavicipitaceae*.

In the present study, mechanisms and types of asexual sporulation in various *Claviceps* species and closely related genera are reassessed in relation to the phylogeny of clavicipitaceous fungi.

MATERIAL AND METHODS

Fungal material

Honeydew drops and conidiogenous layer of young sphacelia were collected from naturally infected plant material (Table 1). Also, the honeydew of yet undescribed *Claviceps* sp. SG (on *Setaria geniculata* from Brazil; Pažoutová 2001) was included. 10–14 d old cultures of *C. fusiformis*, *C. purpurea*, and *C. zizaniae* on T2 (sucrose-asparagine) agar, *C. citrina* on beer wort agar and *C. phalaridis* on Czapek-Dox Yeast Autolysate agar (CYA; Pitt 1980) were observed. The isolates were the same as those used by Pažoutová (2001). Conidiomata of *C. ornicopreoides* (BRIP 39184) were obtained by courtesy of Roger Shivas (Queensland Plant Pathology Herbarium). *C. phalaridis* was isolated from sclerotial specimen DAR 69619 obtained from John Walker.

Media and cultivation

Plates of T2 agar (sucrose-asparagine-salts; Pažoutová *et al*. 1998), beer wort agar (beer wort diluted to specific

gravity[1] 1.015, 1000 ml; agar, 20 g), and CYA (Pitt 1979) supplemented with trace elements (0.001 % $ZnSO_4 \cdot 7H_2O$ and 0.0005 % $CuSO_4 \cdot 5H_2O$); were used.

Microcyclic ability and the germination of conidia was observed in drops of honeydew or loopfuls of conidia from cultures streaked onto freshly poured T2 agar plates.

Microscopy

Spores from honeydew, teased pieces of sphacelia, or samples from cultures were stained by 1 % cotton blue in lactic acid. To produce durable slides, the suspension was mixed with polyvinylalcohol and shortly heated over a flame before covering with a coverslip. Microcyclic conidiation was observed directly on plate cultures or after transfer of a piece of agar bearing a sporulating conidial layer to a drop of the stain; mature secondary conidia were loosely attached to conidiophores and multiple conidiating structures could have been destroyed.

DNA preparation and analysis

Dry conidiomata of *Corallocytostroma ornicopreoides* were scrubbed from contaminations and pieces of the inner tissue were cut out and crushed in liquid nitrogen. The DNEasy Plant Mini Kit (Qiagen, Hilden) was used for DNA preparation. rDNA amplification was achieved using primer pairs ITS3/ITS4 and ITS1/ITS2, as the whole ITS1-5.8S-ITS2 fragment did not amplify well with ITS5/ITS4 primer pair (White *et al*. 1990). The mixture (25 µl) contained 50 ng of genomic DNA, 20 pmol of each primer, 0.2 mM dNTP's (dNTP Mastermix, Invitek, Berlin) and 1 U of DynaZyme with the respective buffer (Finnzymes, Oy). DNA from the other species was obtained and analyzed as described in Pažoutová *et al*. (2000).

The reaction mixtures were subjected to 32 cycles in a Mastercycler Gradient (Eppendorf, Hamburg) under the following temperature regime: 95 °C/3 min, 55 °/30 s, 72 °/1 min (1×), 95 °/30 s, 55 °/30 s, 72 °/1 min (30×), and 95 °/30 s, 55 °/30 s, 72 °/10 min (1×). Amplified fragments were purified by the JetQuick PCR Purification kit (Genomed, Bad Oeynhausen) and directly sequenced at Microsynth (Balgach, Switzerland). Sequences were deposited in the EMBL Nucleotide Sequence Database. Alignment of sequences was obtained using a hidden Markov-based algorithm, SAM (Hughey & Krogh 1996; http://bioweb.pasteur.fr) at the Institut Pasteur (Paris). Of the species with appendaged conidia, the *Neoclaviceps monostipa* sequence was obtained courtesy of Raymond Sullivan (Sullivan *et al*. 2001) and the sequence of *Aciculosporium take* (AB066292) was retrieved from GenBank. As representatives of various genera of *Balansiae*, database sequences of *Epichloë amarillans* (L07141), *Atkinsonella hypoxylon* (U57405), *Balansia strangulans* (U57403), *Parepichloë cinerea* (AB065425), *Heteroepichloë sasae* (AB065430) and *Myriogenospora atramentosa* (U57407) were included. *Myrothecium atroviride* (AJ302002), belonging to *Bionectriaceae*, was used as an outgroup. Quartet puzzling tree was obtained using software TREE-PUZZLE 5.0 (Schmidt *et al*. 2002). A maximum parsimony tree was computed using SEPAL Version 1.2 (Salisbury 2000) with Bremer decay values (Bremer 1988) and jackknife supports for the clades (Farris *et al*. 1996).

RESULTS

Phialidic conidiation

The conidiogenous layer of the *Claviceps* sphacelial stage has been compared to palisade parenchyma (Fig. 1). This appearance is due to the densely packed phialides. Conidiophores grow out of a mass of thick parallel hyphae that form young sphacelia in all *Claviceps* species observed (Pažoutová *et al*. 2002). If the layer is teased apart, branched conidiophores with bottle-shaped conidiogenous cells are visible (Fig. 2).

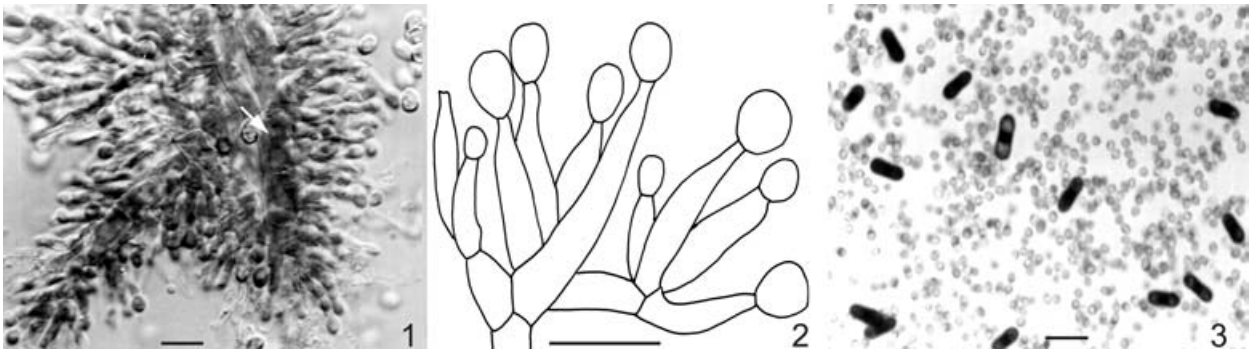
A palisade layer of conidiophores was also observed in the loculi inside the conidiomata of *Corallocytostroma ornicopreoides* (Shivas *et al*. 1997). Microscopy of BRIP39184 confirmed that the layer consisted of phialidic branched conidiophores identical to those typical of *Claviceps* (Fig. 2).

In all studied honeydews of *Claviceps* species, except *C. citrina* and *C. purpurea*, two types of phialidic conidia were observed: macroconidia of different shapes and sizes, that are typical for the species given (Loveless 1964), and oval to globose uniform microconidia (3–5 × 2–4 µm), indistinguishable between species; their ratio is variable. Whereas macroconidia are stained dark blue with cotton or aniline blue, microconidia stain poorly or not at all (Fig. 3). When honeydew is plated on T2 agar medium or water agar, macroconidia undergo secondary conidiation in 16–18 h while microconidia show no signs of germination; their biological purpose is unclear.

Conidiophores bearing macro- or microconidia do not differ and often occur in close proximity even in pure cultures. For *C. fusiformis* (incorrectly as *C. purpurea*) this was shown by Pažoutová *et al*. (1977).

In *C. citrina*, only small oval to elliptic phialidic conidia were found on the surface of younger sclerotia (Pažoutová *et al*. 1998). These spores stained weakly with cotton blue, and germination was not observed, suggesting that the spores are microconidia. Macroconidia were not found in cultures and raw plant material, which does not imply that they do not exist. For example, in some specimens of *C. pusilla* honeydew or sphacelia, the typical triangular macroconidia were

[1] Specific gravity (SG) is used in the brewing industry. A SG of 1.050 indicates that the substance is 5 % heavier than an equal volume of water.



Figs 1–3. Phialidic conidiation in *Claviceps*. **Fig. 1.** Layer of phialides resembling palisade parenchyme (*C. purpurea*) Arrow – thick basal cells from which conidiophores emerge. Bar = 10 μm. **Fig. 2.** Branched conidiophore typical of *Claviceps* (*C. purpurea*). Bar = 10 μm. **Fig. 3.** Difference in cotton blue staining between macroconidia and microconidia (*C. africana*). Macroconidia are intensively stained, whereas microconidia remain pale blue or hyaline. Bar = 20 μm.

absent and the species identity was confirmed only after a few macroconidia were found in pure cultures isolated from the same specimen.

C. purpurea produced conidia of variable lengths and shapes. The size of oval conidia typical for population G1 (Pažoutová *et al*. 2000) corresponded to that of microconidia (4–6 × 2–3 μm), but these conidia stained by cotton blue and germinated. Population G3 (from *Spartina* stands in salt marshes) formed cylindrical conidia to 13 μm, but in addition to these spores, no rounded small conidia not staining with cotton blue were observed. *C. purpurea* does not appear to form microconidia at all.

Our culture of *C. grohii* did not sporulate. Groves (1943) did not record the conidial size and shape in *C. grohii*; Langdon (1952) reported slightly arcuate conidia 10–16 × 3–5 μm, but made no mention of microconidia. However, Langdon (1952) sometimes mistook microconidia for yeast contamination of the honeydew (e.g. in *C. pusilla*), so that the existence of microconidia in *C. grohii* might have passed unrecognized.

In colonies of *C. zizaniae* on T2 medium, typical phialidic conidiophores were found bearing conidia of various elongated shapes (obclavate, oval, reniform) and sizes $(5 < 11.2 < 20) \times (1.5–2)$ μm, S.D. length 3.07. The size range was considerable, but rather gradual, and two distinct groups were not observed. Rarely, elongated shapeless spores (20–30 μm long) strongly staining with cotton blue and similar to those observed by Pantidou (1959) (Fig. 15) were found, but their origin was unknown; we cannot exclude the possibility that these cells might have been hyphal fragments.

Transfers of cultures often lead to quick loss of conidiation ability. However, isolating and maintaining pure strains on medium T2 enabled conidiation to continue for several years (depending on the isolate). The shape of macroconidia in the cultures during the transfers was less defined than in the honeydew. Conidia maintaining the shape found in honeydew were interspersed with conidia tending to be generally elongated or obclavate but all were enteroblastic in origin. Massive microconidiation was observed in one isolate of *C. gigantea* out of 35 and one isolate of *C. africana* (from *Hyparrhenia tamba*) out of six.

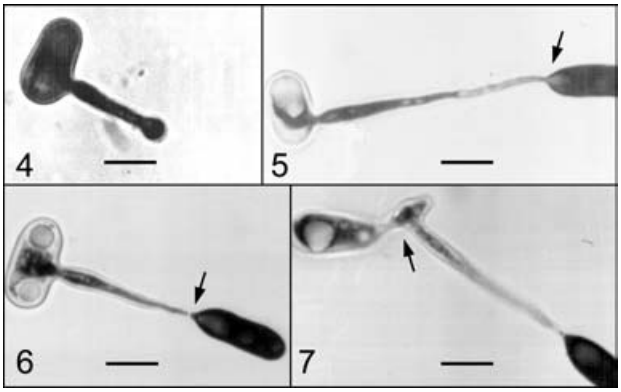
Holoblastic secondary conidiation

Macroconidia from honeydew underwent the formation of secondary terminal holoblastic conidia after plating onto water agar or medium T2. Secondary conidia were oval to pyriform and approximately the same size as the original macroconidium. *In planta* formed conidia of *Claviceps purpurea* and *C. citrina* did not undergo this process.

Secondary conidiation is documented here in *C. africana* as an example, but the course was similar in all species capable of this process. After 8–12 h depending on the freshness of the honeydew, the first germ tube always grew out perpendicularly to the substrate. During its growth, a small rounded conidium started to form on the tip (Fig. 4). As the secondary conidium increased in size, its cytoplasm started to stain dark with cotton blue; the macroconidium and germ-tube cytoplasm stained light blue. In the macroconidia germinating directly in the honeydew drop *in planta*, most of the maternal cytoplasm was transferred to the secondary conidium (Fig. 5) so that after detachment of the secondary conidium only a 'ghost' of the primary conidium remained. In the last step of conidiogenesis, a septum was formed just under the tip of the germ tube (Fig. 6). In 16–20 h the process was completed. Detached secondary conidia retain the tip as hilum.

On medium T2, most of the germinating macroconidia retained their cytoplasm and were able to produce more germ tubes. The branching of germ tubes was also observed (Figs 7–8). The proximal part of the germ tube swelled, and the branching hypha always grew out perpendicularly to the original one.

Secondary conidiation was sequential process. At the distal end of a secondary conidium, another germ tube emerged and another secondary conidium was formed on its tip in the same way. Chains containing the original macroconidium and 2–3 secondary conidia



Figs 4–7. Secondary holoblastic conidiation in *Claviceps africana*. **Fig. 4.** Germination of a macroconidium with formation of holoblastic secondary conidium starting. **Fig. 5.** Transfer of cytoplasm (arrow) from macroconidium to secondary conidium. **Fig. 6.** Formation of septum (arrow) during secondary conidium formation. **Fig. 7.** Proximal right-angle branching (arrow) of conidiophore bearing secondary conidium. Bar = 10 μm.

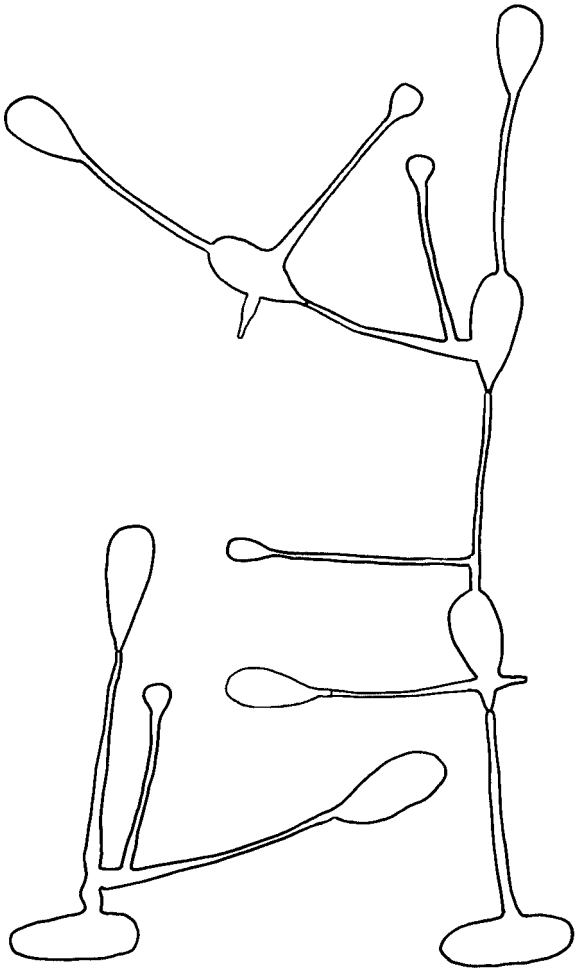


Fig. 8. Formation of secondary conidia chains in *Claviceps africana* as the first stage of colony formation. Bar = 20 μm.

were also observed *in planta*. Plated macroconidia were able to produce longer and branched chains (Fig. 8). All branching occurred strictly at right angles to the original hypha or conidium surface. The first colony formed from germinating macroconidia consisted of branching conidiophores/germ tubes, but after only 2 weeks true vegetative hyphal outgrowths appeared.

In planta formed macroconidia of *C. gigantea* are exceptional in that they complete only a single microcycle and exhibit no branching, no further microcycle of secondary conidia, or vegetative growth. Colony formation was only achieved when explants of lavender-coloured tissue of the young sclerotium were plated, but never from conidia or sphacelial tissue.

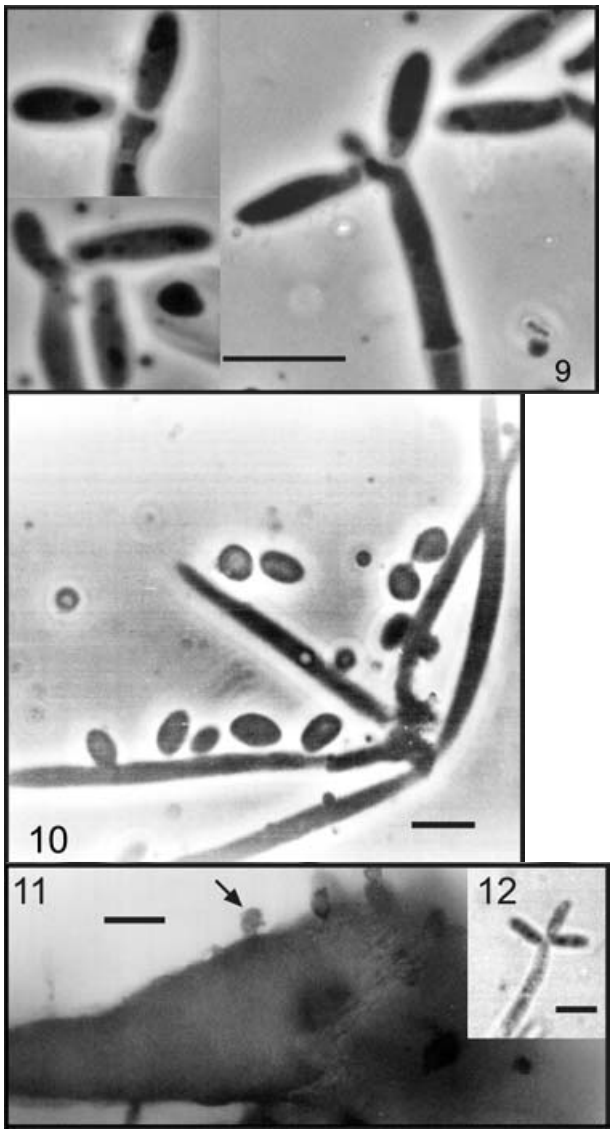
In *C. citrina* that seems to produce only microconidia, no microcyclic conidiogenesis was observed. Neither was *C. purpurea* capable of microcyclic conidiation. After plating fresh honeydew, conidia gave rise to a vegetative mycelium which started to conidiate only after 7–10 d, in the usual phialidic way. The germ tubes grew out in parallel with the substrate surface, not perpendicularly to it as in species forming secondary conidia.

Holoblastic sympodial conidiation of the ephelidial type

In some cultures of *Claviceps zizaniae* on T2 medium, holoblastic and sympodial conidiogenous cells arose terminally from hyphal conidiophores (Figs 9–12) and even predominated over the usual phialidic conidiation. Oblong aseptate conidia, $(7 < 9.6 < 17.5) \times (2.5–3.8)$ μm; (S.D. length 3.01) were borne apically in succession. Their size and shape resembled that of phialidic conidia produced in the culture, except that they were mostly truncated at the former proximal end, whereas phialidic conidia were rounded at both ends. Usually 2–3, but often only one holoblastic conidium per conidiogenous cell was found. Unfortunately, we were not able to study fresh younger sclerotia of *C. zizaniae* to search for the *in planta* conidiation type.

In the remnants of sphacelial tissue from not fully matured *C. citrina* sclerotia, acicular non-septated spores resembling setae $(17.5 < 61 < 75) \times (5 < 6.6 < 8)$ μm; (S.D. length 13.2) were observed, in addition to small phialidic conidia. In the sporogenous mycelial layer, acicular spores occurred in whorls or verticils of 3–9, interspersed with phialidic conidiophores. More detailed observation of the verticil-like structures revealed, that the conidia formation resembled the ephelidial one (Fig. 10).

In cultures of *C. citrina*, ephelidial sporulation was observed on beer wort plates, but not on T2 medium. Colonies were funiculose, consisting of thick synnemata bearing ephelidial conidiophores (Fig. 11). The conidiophores resembled those in *C. zizaniae* cultures (Fig. 12). Conidia, as well as hyphae of the synnemata, were glued together by an exudate. Ephelidial conidia from this culture were substantially smaller than the ones formed *in planta* and rounded at the ends instead



Figs 9–12. Sympodial holoblastic conidiation in *Claviceps*. **Fig. 9.** Conidiation of the ephelidial type in *C. zizaniae*. Bar = 10 μm. **Fig. 10.** Whorl of acicular conidia from the sphacelia of *Claviceps citrina*. Note also the small rounded phialidic conidia. Bar = 20 μm. **Fig. 11.** Conidiation in *C. citrina* culture on beer wort agar. Synnema with protruding conidiophores (arrow). Bar = 50 μm. **Fig. 12.** Ephelidial conidiophore of *C. citrina*. Bar = 10 μm.

of acicular $(<5.6<7.7<12.2)\times(1.7–2.4)$ μm; (S.D. length 1.59).

Holoblastic appendaged spores

From the *Claviceps phalaridis* sclerotia we cultured several isolates that differed in the sporulation intensity. The formation of 2–3 celled appendaged conidia was observed on medium T2. Some of the isolates grew yeastlike and the colonies consisted of appendaged spores only, some isolates produced partially mycelial colonies, where appendaged conidia appeared as lateral branches on sparse hyphae and microcyclic conidiation yielded star-like formations of conidia (Fig. 13). All the

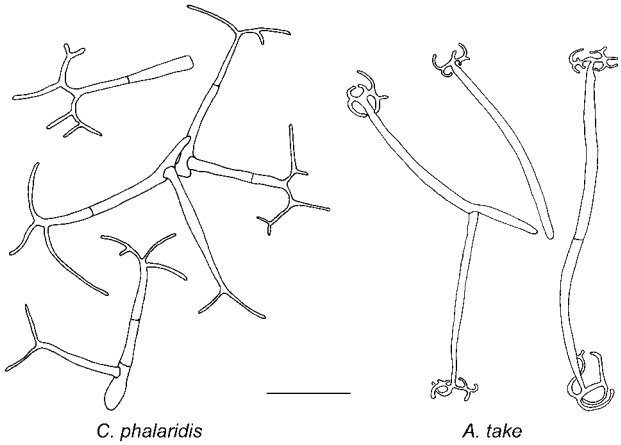


Fig. 13. Appendaged holoblastic conidia of *Cepsiclava phalaridis* and of *Aciculosporium take* (the latter after Tsuda *et al.* 1997). Bar = 20 μm.

processes we observed corresponded to those described by Walker (2004). The germination and formation of new conidia proceeded only at the proximal end. The appendages have had no role in the proliferation and were formed as the last part of the conidium.

Phylogeny

The quartet puzzling tree and the maximum parsimony tree with jackknifing (Fig. 14) separated *Claviceps* species into the *C. purpurea* group, a group of tropical species, and *C. citrina*, whose relationship to the remaining groups was unresolved.

No changes in the *C. purpurea* clade as compared to the tree in Pažoutová (2001) occurred by adding new taxa to the analysis. In *C. purpurea*, holoblastic conidiation of the microcyclic type and phialidic microconidiation are apparently lost. We did not find distinctly separated size groups among phialidic conidia in the cultures of *C. zizaniae* and nothing is known either from the literature or from our own observations about the existence of these two characters in *C. grohii*. However, we did observe both these characters in *C. paspali*, ancestral to the whole clade as well as in the tropical species *C. fusiformis* and *C. sulcata*.

Claviceps and related tropical fungi were divided into three clades. The first one contained *Corallocytostroma ornicopreoides*, *Aciculosporium take*, *Cepsiclava phalaridis* and *Neoclaviceps monostipa*, the latter three sharing identical deletions in both ITS1 and ITS2. The second clade contained *Claviceps pusilla* with a long ITS1, *C. cynodontis* and *C. maximensis* together with anamorphic *Claviceps* sp. SG, all three with another type of deletion in the ITS1 spacer but no deletion in the ITS2. The third clade was unresolved. In all species of the second and third clades, except for *C. viridis* and *C. sorghicola*, phialidic macro- and microconidia as well as holoblastic secondary conidia were found.

The clade containing *Corallocytostroma ornicopreoides* and the three species with appendaged conidia was placed amongst the tropical species of *Claviceps*,

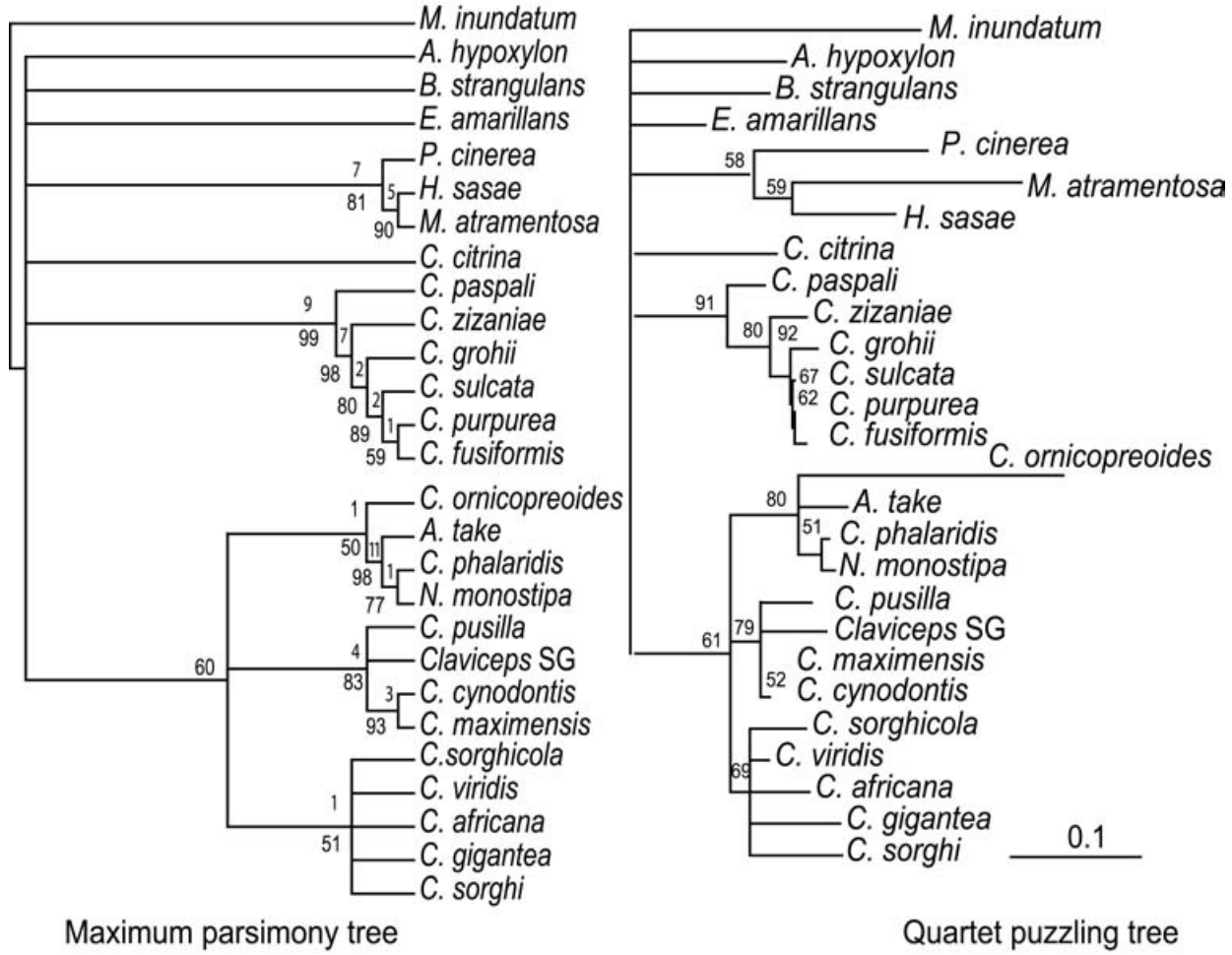


Fig. 14. Phylogenetic trees of selected *Clavicipitaceae*. (*a*) Quartet puzzling tree: Because of unequal rate of nucleotide substitution among the positions, the matrix of maximum likelihood distances was computed using model of Tamura & Nei (1993). Gamma distributed rate with six Gamma rate categories were used as model of rate heterogeneity. Parameter alpha (0.26, S.E. 0.02) was estimated from the data set. Computation was done without clock assumption. (*b*) Maximum parsimony tree: Bremer decay indices and percentages from 500 jackknife replications are given over and under the clades, respectively. Jackknife parsimony was computed using deletion 36%, 1000 replicas and splits >50%. The shortest tree length was 841 steps. The alignment contained 563 nucleotide sites from which 279 (49.6%) were constant.

instead of being related to *Balansiae* or other genus included in the analysis. In the parsimony tree, the grouping of *C. ornicopreoides* with the clade of the species with appendaged conidia has lower support than in the quartet puzzling tree.

DISCUSSION

In *Claviceps diadema* and *C. flavella*, Möller (1901) described a conidiation apparently resembling the holoblastic formation of secondary conidia (Fig. 15). Both these species feature primitive morphological markers viz., undifferentiated sclerotia (resembling those of *Balansia*) and the formation of perithecial heads *in planta*. *C. diadema* was first observed on *Panicum* sp. (Itajahý, Santa Catarina, Brazil) and originally described as *Balansia diadema* because its sclerotia consisted of a soft hyphal mass overgrowing and enclosing flower parts or even several florets.

According to Möller (1901), ascospores of *C. diadema* germinated in a liquid medium, giving rise to branching mycelium that after 3 d produced pointy conidiophores (in the liquid medium as well as on aerial hyphae) on which conidia (7–9 μm in length) tapering to the proximal end appeared. In addition, germination occurred repeatedly in conidia still attached to conidiophores giving rise to chains (Fig. 15); no formation of conidial heads was observed. Möller apparently did not observe conidia of the phialidic type in any of the above species. The morphology of these conidia leaves little doubt that they are of the same type as the secondary holoblastic conidia of various *Claviceps* species.

Conidia occurring singly on conidiophore were found also in *C. flavella* (referred to by Möller as *C. balansioides*); microcyclic conidiation was also observed. Note the right-angle branching at the proximal ends of *C. flavella* conidiophores (Fig. 15) resembling the one depicted in *C. africana* (Fig. 8).

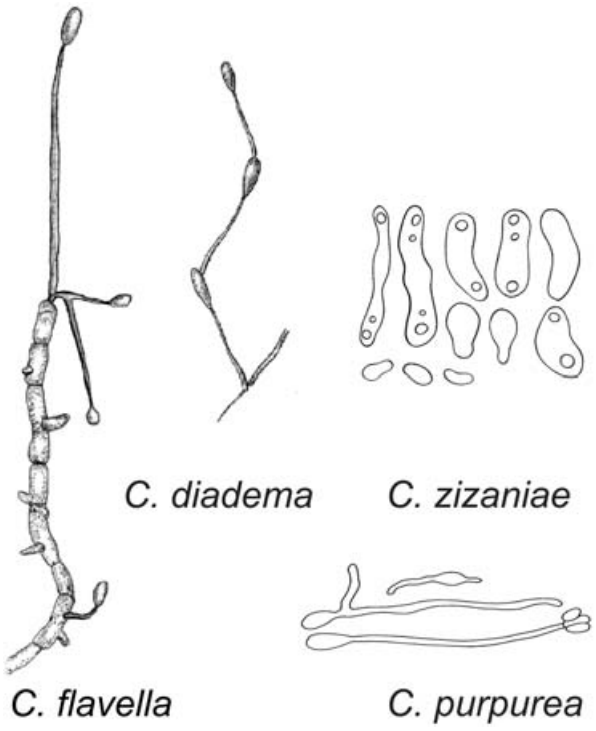


Fig. 15. Historical drawings of conidiation in some *Claviceps* species. *C. flavella* and *C. diadema* conidiation resembling holoblastic secondary conidiation redrawn after Möller (1901: figs 73–74); *C. purpurea* microcycle after de Bary (1884); and *C. zizaniae* variable conidia from Pantidou (1959).

The only previous observation of microcyclic conidiation in *C. purpurea* was mentioned in Kuhn (1858). His picture, reproduced in de Bary (1884: fig. 15), shows a small head of conidia resembling the common phialidic type, not the typical single secondary conidium tapering to the proximal end. However, we were not able to confirm this despite many observations of plated samples of fresh *C. purpurea* honeydew. In all cases, only horizontal vegetative germ tubes were found. Our previous paper about microcyclic conidiation in shake culture of a mutant strain of *C. purpurea* (Pažoutová *et al.* 1978) pertained to an isolate of *C. fusiformis* (Pažoutová & Tudzynski 1999). In *C. sorghicola*, Tsukiboshi *et al.* (1999) specifically mentioned the absence of any secondary conidiation and microconidia; our culture did not sporulate at all. In *C. viridis*, Tanda (1992) reported oval to elliptical conidia (5 μm) from honeydew, whereas in the culture, he found spores of the same shape together with few cylindrical conidia of average length 11.5 μm. Tanda (1992) considered the cylindrical spores macroconidia. No mention of the formation of secondary conidia by *C. viridis* was found. Again, our culture of *C. viridis* did not form any spores.

So far, ephelidial conidiation (holoblastic and sympodial) has not been observed in *Claviceps* and was described only in *Balansiae*. The morphology of *Claviceps* ephelidial conidiophores in culture resembled that of *Atkinsonella* (Leuchtmann & Clay 1989, Morgan-Jones & White 1989). Whereas we were able to document the formation of ephelidial conidia in *C. citrina* both in sphacelial tissue and in culture, the *in planta* behaviour of *C. zizaniae* remained unclear. The ephelidial conidia formed in culture are similar to those of *C. citrina*, but there are conflicting data about conidia formed *in planta*. Pantidou (1959) described the spores adhering to sclerotia as 'very variable in size and shape, oblong-elliptical, reniform, some close to pyriform, hyaline, with two or more oil drops' and 8–30 × 3–6 μm (Fig. 15). However, she mentioned that Wright (1942) reported 6.5–16 × 3.5–5.5 μm conidia from honeydew. Fyles (1915) observed the size of honeydew conidia suspended in water as 9–12 × 2.5–3.5 μm. Both the latter measurements are in agreement with the average size we observed in culture for both sphacelial and ephelidial conidia.

In the genus *Cordyceps*, a wide variety of anamorphs was observed, but in most of the clavicipitaceous fungi, only one type of anamorph is associated with each teleomorph. Two anamorphs, phialidic and ephelidial, were found so far only for *Atkinsonella*. *Claviceps* has two widespread anamorphs, typical enteroblastic *Sphacelia*'s, and the unnamed holoblastic anamorph related to the secondary conidiation, which in some species may be the prevailing one (Möller 1901). The occurrence of the third anamorph (holoblastic and sympodial) in the cultures of two *Claviceps* species may be regarded as a laboratory artefact, but nevertheless it shows the potential of *Claviceps* genetic information. *C. citrina*, producing *Ephelis*-like acicular spores *in vivo*, is in the current analysis placed outside the main *Claviceps* clades, which may reflect an ancestral or transitory state between *Claviceps* and *Balansiae*.

The placement of species with appendaged conidia (*Aciculosporium take*, *Cepsiclava phalaridis*, and *Neoclaviceps monostipa*) to the same clade with *Corallocytostroma ornicopreoides* and inside the tropical clade of *Claviceps* was rather surprising. Also, the phylogenetic maximum likelihood analysis of Tanaka *et al.* (2002) placed *Aciculosporium* together with *C. africana* and *C. sorghicola*.

When the morphological markers are considered, three of these species share the formation of appendaged conidia, *Corallocytostroma ornicopreoides* has phialidic conidiophores of the *Claviceps* type. Three of them form sclerotia-like resting structures, except *N. monostipa* that does not have a differentiated persistent sclerotium, only a hypothallus. *Corallocytostroma ornicopreoides*, *Cepsiclava phalaridis* and *N. monostipa* have stipitate perithecial heads of the usual *Claviceps* appearance (Walker 1957, Shivas *et al.* 1997, Sullivan *et al.* 2001), while *Aciculosporium* lost this marker and perithecia are formed immersed in the stroma that surrounds leaf sheaths (Tsuda *et al.* 1997, Oguchi 2001). The clade contains two confirmed endophytes (*C. phalaridis* and *A. take*). In the plant specimen infected with *N. monostipa*, all florets of the

panicoid host were colonized by the fungus (Jim White, pers. comm.); this is similar to the situation in grasses infected by *C. phalaridis* and may hint at endophytism. We speculate that the close similarity of rDNA sequences, endophytic life-cycle and the presence of unusual anamorphs will lead in the future to grouping of these three species into a single genus.

The situation in *Corallocytostroma ornicopreoides* is unclear. The fungus forms superficial stromata surrounding meristematic host tissues (stem nodes, inflorescences and shoot tips; Shivas *et al*. 1997), and the Chinese species was reported to cause dwarfing of the plants, reducing internodal elongation, and increasing the number of tillers (Yu & Zhang 1980) which may point at a secretion of auxin-like metabolites by endophytic mycelium, similarly to *Aciculosporium*. However, the local or systemic parasitism or the infection mechanism have not been established yet. From the phylogenetical point of view, the emergence of endophytes and non-inflorescence parasites from inside the genus *Claviceps* is not implausible. Although species of the genus *Claviceps* are primarily flower parasites, they possess the ability to other types of host colonization. After artificial inoculation into leaf sheaths, *C. purpurea* can grow and form sclerotia on stem meristems (Lewis 1956) so that there is a capacity for epiphytic and endophytic growth.

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