Claviceps nigricans and *Claviceps grohii*: Their Alkaloids and Phylogenetic Placement

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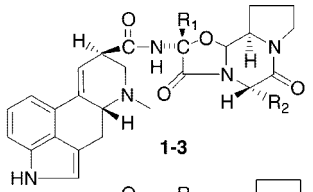
Received February 21, 2008

Claviceps purpurea, *C. grohii*, *C. zizaniae*, *C. cyperi*, and *C. nigricans* are closely related ergot fungi and form a monophyletic clade inside the genus *Claviceps*. Analysis of alkaloid content in *C. nigricans* sclerotia using UPLC detected ergocristine (**1**), ergosine (**2**), α-ergocryptine (**3**), and ergocristam (**4**). Alkaloids **1**, **3**, and **4** were found in the sclerotia of *C. grohii*. The content of **4** in the mixture of alkaloids from *C. nigricans* and *C. grohii* (over 8% and over 20%, respectively) was unusually high. Submerged shaken cultures of *C. nigricans* produced no alkaloids, whereas *C. grohii* culture formed small amounts (15 mg L^{-1}) of extracellular clavines and **1**. In the previously used HPLC method the ergocristam degradation product could have been obscured by the ergosine peak. Therefore sclerotia of a *C. purpurea* habitat-specific population G2 with the dominant production of **1** and **2** have been reanalyzed, but no **4** was detected. The phylogeny of the *C. purpurea*-related species group is discussed with regard to alkaloid-specific nonribosomal peptide synthetase duplication leading to the production of two main ergopeptines instead of a single product.

Previous studies[1,2] have shown that *Claviceps purpurea* (Fr.) Tul. (Clavicipitaceae), *C. grohii* Groves, *C. zizaniae* (Fyles) Pantidou, and *C. cyperi* Loveless are closely related and form a monophyletic clade inside the genus *Claviceps*. In addition, *C. zizaniae* and *C. cyperi* share approximately 90 bp long homologous inserts in the middle of the ITS1 spacer that are not present in *C. purpurea* and *C. grohii*.

Ergopeptine alkaloids, including **1**−**4**, have been found in three species of this group. No information exists about alkaloids produced by *C. grohii* and *C. nigricans* Tul. Ergocryptine (**3**) has been isolated from mycelium and fermentation broth of submerged cultures of *C. zizaniae* CCM 8240[3] and was also found to be the dominant alkaloid in *C. cyperi*.[4]



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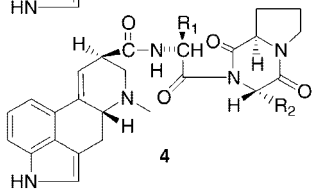

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Number	R_1	R_2
1	$CH(CH_3)_2$	$CH_2C_6H_5$
2	CH_3	$CH_2CH(CH_3)_2$
3	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$
4	$CH(CH_3)_2$	$CH_2C_6H_5$

Habitat-specific populations (races) *C. purpurea* differ in the type of alkaloids produced.[5−7] The sclerotia of population G1 contained various combinations of ergot alkaloids; all G2 produced ergosine (**2**) and ergocristine (**1**), often with small amounts of **3**, and all G3 isolates belonged to a chemotype producing **1** and **3**.

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Compared to *Claviceps* species diversity in the tropics and subtropics, few species occur in the moderate regions. *C. purpurea*, which is the most widespread, colonizes mainly pooid grasses throughout the Northern Hemisphere.[5,6] *C. zizaniae* occurs on *Zizania* spp. in North America, and *C. grohii* Groves occurs on *Carex* spp. in Canada and the northern United States.[8] *C. nigricans* has been described on both *Eleocharis* and *Scirpus* spp. in North America, Europe, and Japan.[9] There are records of *C. nigricans* from Australia[10] and recently from New Zealand,[11] which are probably imports from the Northern Hemisphere. The only species of this group that originates from the Southern Hemisphere is *C. cyperi* from South Africa.[2,12]

In this paper, alkaloid analyses of sclerotia of *C. nigricans* and *C. grohii* are presented. Except for one small drawing of *C. grohii* conidia,[13] no picture of either *C. grohii* or *C. nigricans* conidia has been published; therefore conidia shape and dimensions from the analyzed specimens and cultures were recorded. The relationship between alkaloids produced and the phylogenetic placement of the respective species inside the group is discussed.

Origins of specimens and isolates are given in Table 1. Sclerotia of *C. grohii* were obtained from the U.S. National Fungus Collections (BPI). Sporulating cultures were obtained from sclerotia of *C. nigricans*. The conidia were cylindrical in shape (length 6.6 < 9.1 < 12.5, SD 1.0 μm, width 2.9 < 3.9 < 4.5, SD 0.3 μm) (Figure 1), and the few conidia found on the surface of sclerotia were identical. The conidia from *C. nigricans* cultures resembled those found on sclerotia and conformed to the species description. The shape and size of the conidia were similar to those of the G2 and G3 groups of *C. purpurea*.

The culture of *C. grohii* did not sporulate. Conidia of the specimen BPI 633040 were found on younger sclerotia. The sphacelial part contained oblong to arcuate conidia (Figure 2); conidial dimensions were 5.0 < 7.8 < 12.0 μm (SD 1.31) and 1.6 < 2.12 < 3.0 (SD 0.32). The original description of *C. grohii* did not contain information about conidial size and shape. Langdon[9] found conidia of a slightly arcuate shape (10−16 × 3−5 μm) on a specimen IMI25952, which according to its date and origin may be related to BPI 633038. Our measurements of the specimen found smaller conidia than those observed by Langdon.[9]

Phylogenetic analysis of rDNA has shown that *C. cyperi*, *C. nigricans*, and *C. zizaniae* are grouped together, whereas *C. grohii* appeared inside the *C. purpurea* clade as ancestral to *C. purpurea* habitat-specific populations G2 (wet and/or shady) and G3 (salt marsh) (Figure 3). The ITS1 spacer of *C. nigricans* contained

10.1021/np8001173 CCC: $40.75
Published on Web 05/08/2008

Table 1. *Claviceps* Specimens and Isolates

Claviceps species	herbarium/ collection	host	location	year	rDNA sequence
C. grohii[a]	BPI 633038	*Carex brunnescens*	Canada, Quebec, Beauceville	1943	
C. grohii	BPI 633040	*C. canescens*	Canada, British Columbia, Tow Hill	1957	
C. grohii	BPI 633041	*C. phyllomanica*	Canada, British Columbia, Masset	1964	
C. grohii	CBS 124.47	*Carex* sp.	Canada, British Columbia, Milner	1943	AJ133395
C. nigricans	CCC804	*Eleocharis palustris*	CZ, Bohemian Highlands, Březina	2004	AM039814
C. nigricans		*E. palustris*	Hungary, Györ district, Pér	1998	
C. purpurea G2	CCC162	*Poa annua*	CZ, Moravia, Skalička	1993	
C. purpurea G2	CCC431-3	*Phalaris arundinacea*	Germany, Bavaria, Philippsreut	1998	
C. purpurea G2	CCC434	*Dactylis* sp.	Germany, Bavaria, Philippsreut	1998	AJ311950
C. purpurea G2	CCC480	*Molinia coerulea*	CZ, Moravia, Zubří	1998	EU344983
C. purpurea G3	CCC511	*Spartina anglica*	UK, Skeffling, Humber estuary	1998	AJ311951
C. purpurea G1	CCC207	*Triticum aestivum*	USA, Kansas	1995	EU344981
C. purpurea G1	CCC771	*Secale cereale*	CZ, Bezdědice	2003	EU344982

[a] Specimen was identified by J. W. Groves and originated from a location mentioned in the species description.[38]

another homologue of the cca 90 bp long insert detected previously in *C. zizaniae* and *C. cyperi*.

Alkaloid analyses of sclerotia of *C. nigricans* detected **1**, **2**, and **4**; α-ergocryptine (**3**) was found in only one sample. Alkaloids **1** and **4** were found in *C. grohii*, and in one sample **3** was also detected (Table 2). Attempts to produce alkaloids in submerged shaken cultures of *C. nigricans* failed. A culture of *C. grohii* produced only 15 mg L^{-1} of extracellular alkaloids (elymoclavine 38.9%, agroclavine 35.5%, festuclavine 6.3%, and **1** 19.3%) after 28 days of cultivation. No **1** or other ergopeptine was detected in the mycelium, only elymoclavine (41.3%), agroclavine (52.9%), and festuclavine (5.8%). Predominant clavine production in a submerged culture of a *Claviceps* species that is capable of peptide production *in planta* was first observed in cultures of *C. purpurea*, where only agroclavine and elymoclavine were formed.[14] *C. grohii* cultures behaved similarly. However, it is possible that the ability to produce peptide alkaloids in culture was lost due to the extended time in culture in the CBS collection (at least since 1947).

In the previously used HPLC method, the presence of the ergocristam degradation product could have been obscured by the ergosine peak. Therefore, analysis of sclerotia belonging to a *C. purpurea* G2 group characterized by a predominant production of **1** and **2**[5] has been repeated using the new extraction and UPLC method.[34] The result was identical with previous analyses, and no ergocristam (**4**) was detected (Table 2).

Ergocristam (**4**) and related lactams have been detected previously as minor components in *C. purpurea* ergotoxine-producing isolates;[15–18] however, the amounts found in *C. nigricans* and especially in *C. grohii* were unusually high. The mechanism leading to lactam formation is a competitive reaction leading to a reversal of configuration on an optically active carbon of L-proline to the D-configuration in the course of biosynthesis, which precludes the subsequent closing of the ergopeptine cyclol ring.[19]

Two major ergopeptine alkaloids were observed in *C. grohii* and *C. nigricans*. Similarly, two major alkaloids are produced by all populations of *C. purpurea*.[5] In contrast, only ergocryptine (**3**) was found in the cultures of *C. zizaniae*.[3] The situation with *C. cyperi* is rather unclear; ergocryptine has been reported as the dominant component; however, in a teff and maize silage contaminated with ergotized *Cyperus esculentus* a HPLC peak corresponding to that of ergocornine was found but not confirmed by mass spectroscopic analysis.[4] So far, ergocornine has been found only in population G1 of *C. purpurea,*[5] whereas ergocristine and ergocryptine seem to be more widespread, possibly ancestral, among species of the *C. purpurea* group. Among the tropical and subtropical *Claviceps* species, dihydroergosine is the only ergopeptine that has been found (in *Claviceps africana*).[20,21]

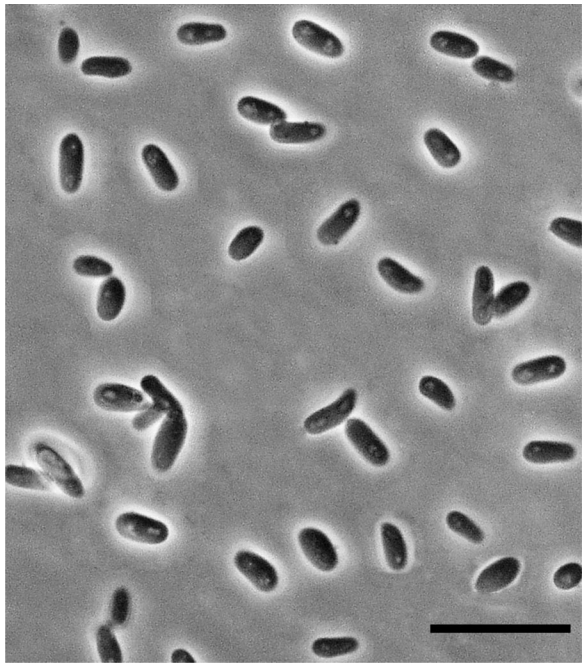

Figure 1. Conidia of *Claviceps nigricans*. Bar represents 20 μm.

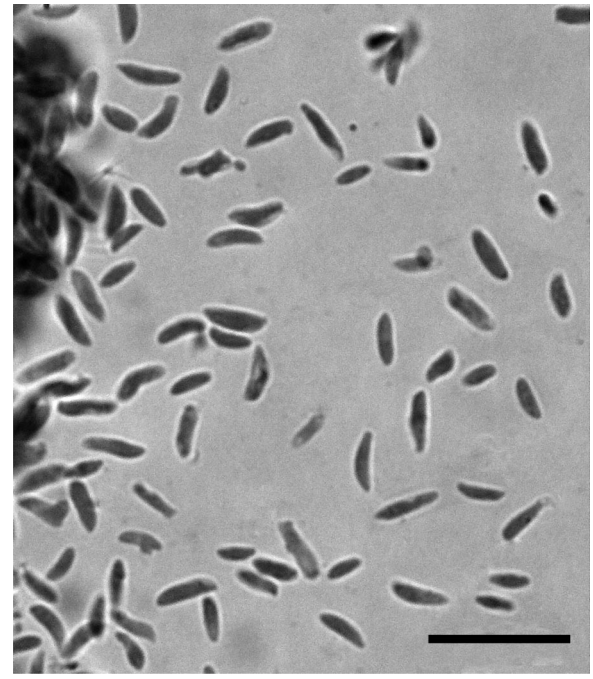

Figure 2. Conidia of *Claviceps grohii*. Bar represents 20 μm.

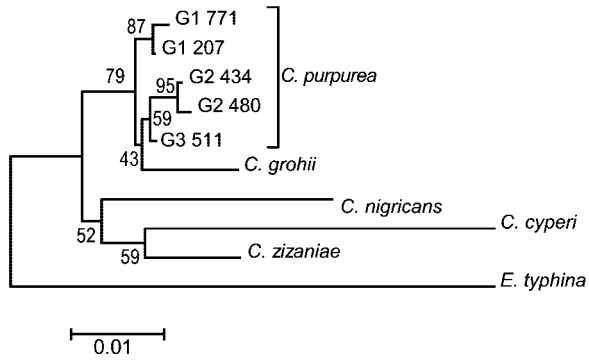

Figure 3. The evolutionary history was inferred using the neighbor-joining method.[35] The optimal tree drawn to scale with the sum of branch length of 0.16521129 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.[36] The evolutionary distances were computed using the maximum composite likelihood method[37] and expressed as base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). There were a total of 1225 positions in the final data set with 38 phylogenetically informative sites.

Table 2. Alkaloid Composition (% of total alkaloids) of *Claviceps* spp. Sclerotial Specimens

specimen	**1**	**4**	**2**	**3**
C. nigricans CZ	67.7	8.3	3.7	20.3
C. nigricans HU	86.5	8.9	4.6	
C. grohii BPI 633041	52.3	21.0		26.7
C. grohii BPI 633038	74.9	25.1		
C. purpurea CCC162	43.6		52.9	3.5
C. purpurea CCC434	42.2		56.6	1.2

Other clavicipitaceous ergopeptine producers also produce a single predominant alkaloid. Endophytes of the genus *Epichloë/Neotyphodium*, which are closest to the genus *Claviceps*, produce only ergovaline. In an endophytic *Balansia obtecta*[22] and in a yet undescribed epibiotic fungus colonizing leaf surfaces of *Ipomoea asarifolia* plants, a single ergopeptine product, ergobalansine, also has been detected.[23,24]

The presence of two dominant ergopeptine alkaloids can be explained on the basis of studies of the alkaloid biosynthesis gene clusters in clavicipitaceous fungi. The recently sequenced gene cluster of *Epichloë,*[25] a sister genus to *Claviceps*, contains only one non-ribosomal peptide synthetase (NRPS). However, two NRPS genes residing at the 3′ end of the cluster have been found in *C. purpurea*, probably as a result of a duplication in the course of evolution. Due to the differences at binding pockets for amino acid recognition, each NRPS produces a different alkaloid.[26,27] The fact that, in addition to the two main ergopeptines, a variety of minor or trace alkaloids is formed may be explained by "leaky" substrate specificity of the active sites shown *in vitro*.[28] From the phylogram (Figure 3) it seems that the duplication of NRPS may have occurred soon after separation of the *C. purpurea* clade from the ancestors of tropical and subtropical *Claviceps* species and that a single copy may be present only in *C. zizaniae*.

Experimental Section

General Experimental Procedures. Conidia from the specimens and cultures were mounted in 1% cotton blue/lactic acid, photographed, and measured using an Olympus BX51 microscope equipped with a CAMEDIA digital camera and QuickPHOTO Camera 2.2 image-processing software. The statistical treatment of spore size data was done using Kyplot 2.0 beta 15 available at http://www.pricelesswarehome.org/woundedmoon/win32/kyplot.html.[29]

Isolates and Their Cultivation. *C. nigricans* was isolated from mature sclerotia collected in Březina, CZ; the Hungarian sample was no longer viable. Sclerotia were surface-sterilized for 5−10 min in 1.3% sodium hypochlorite and rinsed twice for 5 min in distilled H_2O. Sterilized sclerotia were placed on T2 agar plates.[30] Isolates were maintained on T2 agar slants at 4 °C and subcultured every 6 months. A culture of *C. grohii* CBS 127.47 was obtained from Centraalbureau voor Schimmelcultures, Utrecht. Submerged cultivation of *C. nigricans* and *C. grohii* was carried out using sucrose-asparagine media TI (inoculation) and T2 (alkaloid production).[30] Cultivation was performed in 250 mL Erlenmeyer flasks with 60 mL of the medium. Each flask of inoculation culture was inoculated by mycelium scraped from the whole 4-week-old T2 agar slant. The cultivation proceeded at 24 °C and 250 rpm. The production cultures were harvested after 28 days, and alkaloids in the cultivation liquid were analyzed colorimetrically.[30]

Isolation and Analyses of DNA. Genomic DNA was prepared from 1-week-old mycelium grown on T2 plates covered with cellophane using UltraClean microbial DNA isolation kit (Mo-Bio Laboratories, Solana Beach, CA). rDNA regions containing ITS1-5.8S-ITS2 and part of D1D2 were amplified and sequenced using primer pair ITS5/NL4.[31] Reaction mixtures and thermocycler parameters were as in Pažoutová et al.[5] Custom sequencing was performed at Macrogen Inc. (Seoul, Korea). DNA sequences were aligned using BioEdit,[32] and the phylogenetic tree was constructed using MEGA 4.0.[33] The sequence of *C. cyperi* (AY387491) was obtained from GenBank.

Alkaloid Analysis. Alkaloids from powdered sclerotia were extracted with MeOH in H_2O (80%) containing 0.1% NH_4OH (24% aqueous solution) for 2 h at laboratory temperature. Under these conditions complete degradation of ergocristam was observed,[34] yielding the methyl ester of *N*-(D-lysergyl)-L-valine (54%) and *N*-[*N*-(D-lysergyl)-L-valyl]-L-phenylalanyl-D-prolyl methyl ester (46%). The content of ergocristam was recalculated from the concentrations of the degradation product. Individual alkaloids were determined by the UPLC method as decribed earlier, and their identity was confirmed by MS.[34] Alkaloids from submerged cultures were extracted as follows: the culture (100 mL) was centrifuged for 10 min at 8000 rpm (10 °C), and the supernatant was adjusted to pH 9.0 with NH_4OH and extracted twice with 100 mL of $CHCl_3$. Combined extracts were evaporated to dryness, and the residue was dissolved in 100 μL of MeOH (5 μL injected into UPLC). The separated mycelium was washed with distilled H_2O; 3 g of mycelium was extracted with 50 mL of an acetone/$CHCl_3$ mixture (1:1 v/v). The extract was evaporated to dryness and the residue dissolved in 100 μL of MeOH (5 μL injected into UPLC). Alkaloid production of submerged cultures was assayed colorimetrically using Van Urk's reagent[30] with ergotamine and elymoclavine as standards.

Acknowledgment. The investigation reported here was partly supported by Institutional Research Concept AV0Z50200510, Sorghum, Millet and Other Grains CRSP (USAID), and Centre LC07017 of the Ministry of Education, Youth, and Sports of the Czech Republic.

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NP8001173