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An analysis of ergot alkaloids in the Clavicipitaceae (Hypocreales, Ascomycota) and ecological implications

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Abstract
Fungi of family Clavicipitaceae (Hypocreales; Ascomycetes) were evaluated according to their ecological habits (free-living, insect parasite, epibiotic or endophytic) and trophic mode (saprotrophic/insect necrotroph; scale insect necrotroph/plant biotroph; or plant biotroph). In order to infer evolutionary relationships phylogenetic analysis on large subunit rDNA sequence data were used on taxa selected to represent a range of ecological habits and trophic modes in the family. The capacities of the fungi to produce ergot alkaloids (or ergoline structures) in culture were evaluated. In this study we found that production of ergot alkaloids was a feature of Clavicipitaceae that were biotrophs on plants. Ergoline alkaloid-producing plant biotrophs were distributed in two clades of the Clavicipitaceae that included plant biotrophic forms. This is the first study that links ergot alkaloid production in the Clavicipitaceae with plant biotrophy. To explain this linkage we offer the hypothesis that the abundance of nutrients in living plants may permit the plant biotrophic forms to produce ergot alkaloids for defensive purposes. The exposure of these fungi on the surfaces of plants increases their vulnerability to herbivory; production of ergot alkaloids reduces that vulnerability.

Keywords: Ergot alkaloids, defensive mutualism, endophytes, epiphytes, fungi, molecular systematics

1. Introduction

The family Clavicipitaceae (Hypocreales, Ascomycota) is composed of saprotrophic and symbiotic species associated with insects and fungi (*Cordyceps* spp.) or grasses, rushes, or sedges (*Balansia* spp., *Epichloë* spp., *Claviceps* spp.) (Bacon and White, 2000). Symbiotic interactions in the Clavicipitaceae range in a continuum from antagonism to mutualism (Clay, 1990; Schardl et al., 2004). The plant biotrophic forms within this family can be characterized based on the nature of associations with their hosts, being epibiotic during part or the entire life cycle or strictly endophytic with hyphae growing intercellularly in the aboveground plant parts.

In the grass symbiotic plant biotrophs, *Epichloë* and *Balansia*, the symbiotic association with many grasses is often recognized as a 'defensive mutualism' (Clay, 1988). Host protection from mammalian and insect herbivory is largely attributable to the production of various biologically active compounds, mainly alkaloids, which accumulate within infected host tissues (Bush et al., 1997; Porter, 1994). The fungal biotroph benefits from access to nutrients provided by the host plant and dissemination through seed. There are additional documented benefits for the plant host including drought tolerance, increased field persistence and resistance to nematodes and fungal pathogens (Clay, 1990; Schardl and Phillips, 1997; Bacon et al., 1997; Malinowski and Belesky, 2000; Clarke et al., 2006). In addition, this fungal group has received attention due to economic losses produced in agricultural systems when cattle graze endophyte-infected grasses. Endophyte-infected grasses contain ergot alkaloids and other secondary metabolites that are the cause of reduced performance of grazing animals due to toxic syndromes (Hoveland, 1993; Glenn and Bacon, 1977; Siegel and Bush, 1996).

A significant number of clavicipitaceous species are scale insect parasites. Genera *Hyperdermium*, *Dussiella*, *Hypocrella*, and *Ascopolyporus*, are parasitic on soft scale insects (homopterans, Cocooidea). In general these species are distributed in the tropics and associated with plants of no economic value receiving less attention than the grass

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endophytes (Petch, 1921; Sullivan et al., 2000). The relatively large stromata size in some species (e.g. *Hyperdermium pulvinatum*, *Dussiella tuberiformis*, *Ascopolyporus philodendrum*, *Hypocrella macrostoma*) have been attributed to a particular nutritional mode where the fungus first parasitizes and totally consumes the scale insect and then continues to acquire nutrients from the plant through the hole left behind by the insect stylet (Sullivan et al., 2000; Koroch et al., 2004; Bischoff and White, 2005; Chaverri et al., 2005a, b; Hywel-Jones and Samuels, 1998).

Frequently clavicipitaceous species found in soil are known only in their anamorphic states. Several hyphomycetous soil genera such as (e.g. *Chaunopycnis*, *Paecilomyces*, *Lecanicillium*, *Beauveria*) complete their life cycles as soil saprotrophs or insect parasites (Hodge, 2003). Their capability to survive as soil saprotrophs augment their potential as agents of biological control. Insect pathogenic species of genus *Cordyceps* and *Hypocrella* produce numerous secondary compounds with biological activity with possible applications in medicine and insect control (Isaka et al., 2003). However, little is known regarding ergot alkaloid production in clavicipitaceous fungi that are not grass endophytes (Glenn and Bacon, 1997). In this study we assessed cultural production of ergot alkaloids or alkaloids with the ergoline structure in several clavicipitaceous species to evaluate how ergot alkaloid production relates to ecological habit and phylogeny in the family Clavicipitaceae. Alkaloids with the ergoline structure were confirmed by subjecting compounds in the alkaloid fraction after extraction (acid/base separation system) to MS and UV analysis.

2. Materials and Methods

Phylogenetic analysis

Twenty-seven LSU rDNA Clavicipitaceae sequences were selected from GenBank to represent a range of ecological habits. GenBank accession numbers are listed in Table 1. Members of the family Nectriaceae were used as outgroup taxa. Sequences were manually aligned using the secondary structure of *Saccharomyces cerevisiae* (U53879) from the comparative RNA Web site (CRW) database (Cannone et al., 2002). The matrix was annotated as described by Kjer (1995). The matrix containing 831 characters was analyzed using the program PAUP v4.0 (Swofford, 2002) by maximum likelihood and Bayesian analysis. ModelTest v.3.06 (Posada and Crandall, 1998) was used to select the best-fit model of sequence evolution determined by Akaike information criterion (Akaike, 1974).

Analysis of ecological habit

The fungi were analyzed with respect to hosts (e.g. insect, scale-insect, plant), trophic mode (e.g. saprotroph, insect necrotroph, plant biotroph), and if on a plant, the degree of plant colonization (restricted or systemic; endophyte, epibiont) (Table 2).

Culture conditions and extraction of ergot alkaloids

Twelve isolates were cultured; the isolates are listed in Table 1. For ergot alkaloid production cultures we modified the procedure of Bacon (1988). Fungi were cultured in 250 ml Erlenmeyer flasks containing 100 ml of M102 liquid medium and grown for 2 weeks in a shaker at 100 rpm and then transferred to liquid medium M104T. Flask were maintained in shaker at 100 rpm for 2 weeks and then left as stationary phase in dark for 8 weeks. Extractions were performed by adding an equal volume of chloroform:methanol (8:2) to the culture (media+mycelia). Samples were stirred over a period of 1 hour and the aqueous fraction discarded. The extraction was repeated three times. The extracts were evaporated to dryness in a rotavapor. The residues were dissolved in methanol and filtered using 0.45 µm Nylon (CostarR) filters held in a 2.0 ml polypropylene tube and centrifuged at 5000 rpm for 45 sec before analysis.

Samples were analyzed by reverse phase HPLC coupled to a photodiode array detector to record the spectra from 210 to 600 nm at 1.5 nm step and by mass spectrometer (LC-MS).

Samples were analyzed by high performance liquid chromatography coupled with mass spectrometry (HPLC-MS). The HPLC system (Shimadzu Co., 10VP Series, Columbia, MD, USA) employed a C_{18} MCM column (4.6 X 150 mm; 5 µm; MC Medical, Inc, Tokyo, Japan). Twenty micro liters were injected into the column, and a gradient elution was used for separations. Solvent A consisted of 10% MeOH in H_2O adjusted to pH 3.5 with formic acid. Solvent B consisted of 20% H_2O (pH 3.5), 20% MeOH, and 60% acetonitrile. At a flow rate of 0.3 ml min^{-1}, the following gradient was used: 0 min, 100% A; 10 min, 20% A; 20 min, 40% A; 40 min, 0% A; held at 0% A for 15 min. Five minutes of equilibration at 100% A was performed before and after each injection. Effluent from the column was introduced into a single-quadrupole mass spectrometer (API 150 MCA, PE Sciex, Concord, Ontario, CA) equipped with electrospray ionization (ESI).

The following parameters were used: capillary voltage at 5,500 V, nebulizer gas (N_2) at 10 (arbitrary units) focusing potential at 300 V, entrance potential at 10 V, declustering potential at 75, drying gas (N_2) heated to 320°C and introduced at a flow rate of 8000 cm^{-3} min/l. Data were acquired in both positive and negative ionization modes, with full scan acquisition being performed from m/z 50–1,200. Data were processed by Analyst software 1.4.1 (Applied Biosystems, Applera Co., Foster City, CA, USA). The standards of ergocristine, ergocornine, ergonovine,

Table 1. Taxa, cultures and GenBank accession numbers of fungi used in this study.

Species	Culture collection source*	GenBank accession no.
Ascopolyporus polychrous Möller	–	AY886547
Balansia henningsiana (Möller) Diehl	–	U57678
Balansia nigricans (Speg.) White, Drake & Martin	RUTPP-BN1	U68119
Balansia strangulans (Mont.) Diehl	CBS334.96	U57679
Beauveria bassiana (Bals.-Criv.) Vuill.	–	AF245300
Chaunopycnis alba Gams	ATCC-MYA201787	AF245296
Chaunopycnis pustulata Bills, Polishook & White	–	AF389190
Chaunopycnis sp.	–	AF245297
Claviceps fusiformis Loveless	–	U17402
Claviceps paspali Stevens & Hall	–	U47826
Claviceps purpurea (Fr.) Tul.	RUTPP-CP1	U57085
Cordyceps militaris (L.) Link	–	AF043135
Cordyceps spegazzinii Torres, White & Bischoff	ATCC-MYA3684	DQ196435
Cordyceps subsessilis Petch	–	AF373285
Dussiella tuberiformis (Berk. & Ravenel) Atk.	ATCC-MYA2810	U57083
Epichloë festucae Leuchtm., Schardl & Siegel	RUTPP-EF1	AF385214
Epichloë typhina (Pers.:Fr.) Tul.	–	U17396
Hyperdermium bertonii (Speg.) White, Sullivan, Bills & Hywel-Jones	ATCC-MYA68	AF242354
Hyperdermium pulvinatum White, Sullivan, Bills & Hywel-Jones	ATCC-MYA-69	AF242353
Hypocrella phyllogena (Mont.) Speg.	RUTPP-4iso8	AY518372
Lecanicillium lecanii (Zimm.) Zare & Gams	–	U17414
Leuconectria clusiae (Samuels & Rogerson) Rossman, Samuels & Lowen	–	U17412
Nectria radicicola (Gerlach & L. Nilsson) Mantiri & Samuels	–	U17415
Neotyphodium coenophialum (Morgan-Jones & W. Gams) Glenn, Bacon & Hanlin	–	U57681
Paecilomyces lilacinus (Thom) Samson	RUTPP-GB5460	AF172339
Tolypocladium inflatum Gams	–	AB103381
Torrubiella luteorostrata Zimm.	–	AF327388
Torrubiella superficialis Kobayashi & Shimizu	RUTPP-SU70	–

*Cultures from ATCC (American Type Culture Collection); CBS (Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre); RUTPP (Rutgers Mycological Herbarium; Strain stored at –80°C; +Sclerotia, herbarium specimen).

agroclavine (Sigma, St. Louis, MO) and extract of *Claviceps purpurea* sclerotia were included in the analyses.

3. Results

Phylogenetic analysis

The evolution model selected was General Time Reversible with proportion of invariable sites (I) and gamma distribution (G). The parameters include base frequencies A=0.2488, C=0.2225, G=0.3321, T=0.1966; rate matrix [A-C]=1.0, [A-G]=2.1682, [A-T]=1.0, [C-G]=1.0, [C-T]=10.2163; [G-T]=1.0; I=0.5455 and G=0.4054 and this model was incorporated into PAUPv.4.0. The most likely tree (-ln 3006.913) is shown in Fig. 2. Bayesian inference was used to estimate branch support (posterior probability) under likelihood using Mr Bayes 3.0 (Huelsenbeck, 2000). Bayesian analysis was run three times with four mcmc (Markov Chain Monte Carlo) chains for 1,000,000 generations, sampling every 100 generations. The 30,000 trees resulting from the three runs were pooled and 28,500 were imported into PAUP to construct a majority rule consensus tree after discarding the asymptotic trees (burn in).

In our phylogenetic analysis members of the Clavicipitaceae were separated into three clades (A, B, C) (Fig. 2). Clade A (90% posterior probability) accommodates all the strict plant biotrophs included in this analysis and scale-insect necrotrophs *Hypocrella phyllogena*, *Dussiella tuberiformis* and *Torrubiella luteorostrata*. Clade B (89% posterior probability) accommodates insect necrotrophs *Cordyceps subsessilis* and its anamorph *Tolypocladium inflatum*, soil saprotrophs *Chaunopycnis* spp. and *Paecilomyces lilacinus*. Clade C (100% posterior probability) was well supported as a distinct group including insect necrotrophs (*Beauveria bassiana, Cordyceps* spp. and *Lecanicillium lecanii*) and scale-insect necrotrophs (*Hyperdermium bertonii*, *H. pulvinatum* and *Ascopolyporus polychrous*).

Analysis of ecological habit

The Clavicipitaceae may be separated into three groups

Table 2. Comparisons of ecological habit among genera of Clavicipitaceae.

Trophic mode	Taxa	Substrate/host groups	Habit
Insect necrotroph (In)/Soil saprotroph (Ss)	*Chaunopycnis*	Soil detritus/insects	Free-living/insect parasitic
	Cordyceps	Soil detritus/insects	
	Lecanicillum	Soil detritus/insects	
	Paecilomyces	Soil detritus/nematodes	
	Tolypocladium	Soil detritus/insects	
Insect necrotroph (In)/Plant biotroph (Pb)	*Ascopolyporus*	Scale insects/tropical plants	Non-systemic/epibiont
	Dussiella	Scale insect/bamboo	
	Hyperdermium	Scale insect/dicots	
	Hypocrella	Scale insect/dicots	
	Torrubiella	Scale insect/dicots	
Plant biotroph (Pb)	*Balansia*	Warm-season grasses	Systemic/endophyte
	Epichloë	Cool-season grasses	Systemic/endophyte
	Claviceps	Grasses and sedges	Non-systemic/epibiont

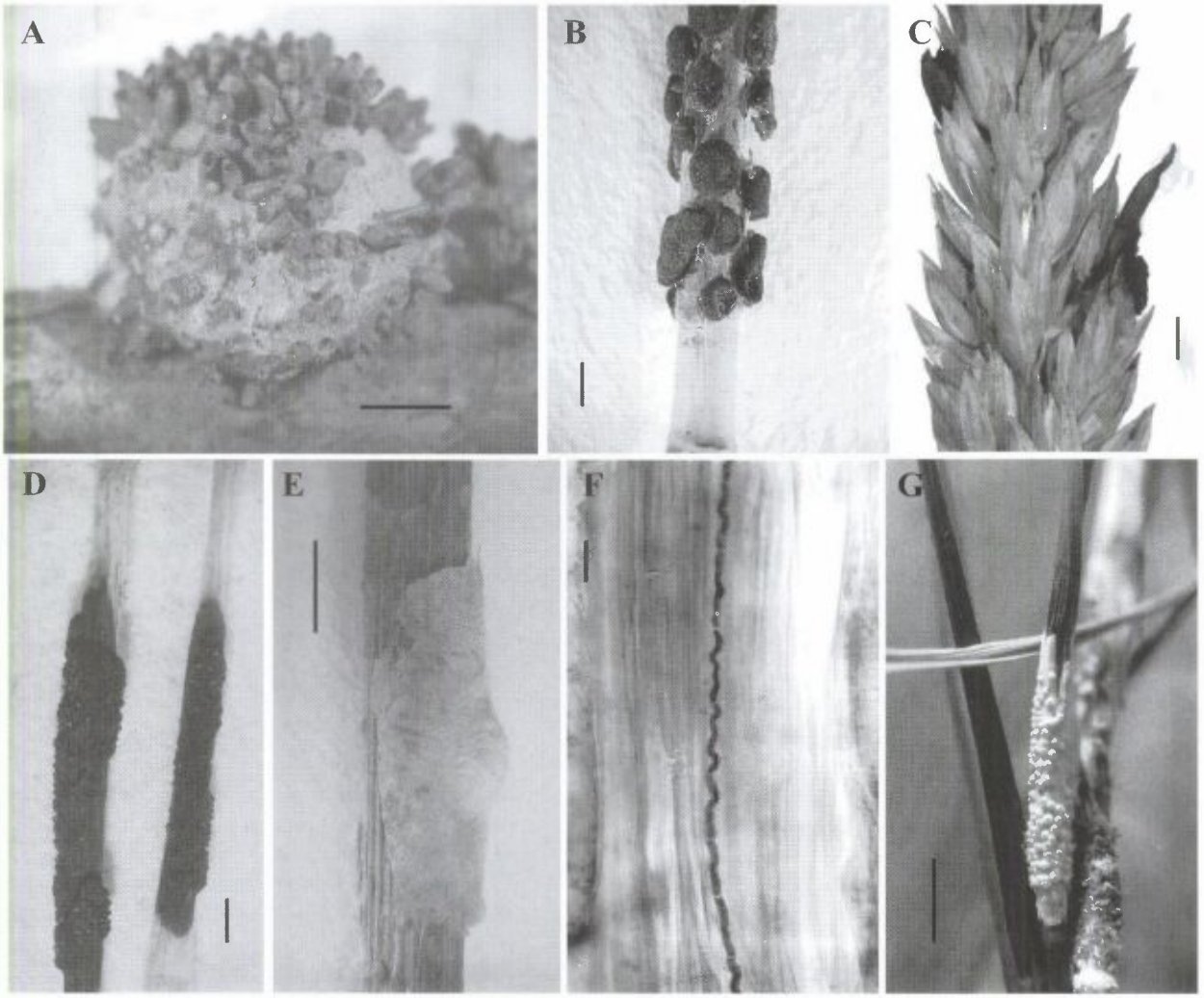

Figure 1. Clavicipitaceae. A. Stromata of *Dussiella tuberiformis* on plant culms (Bar = 1 cm). B. Perithecial stroma of *Balansia discoidea* (Bar = 3 mm). C. Sclerotia of *Claviceps purpurea* on grass inflorescence (Bar = 2 mm). D. Stromata of *Balansia nigricans* (Bar = 5 mm). E. Stromata of *Hyperdermium bertonii* on plant stem (Bar = 1 cm). F. *Neotyphodium* sp. endophytic mycelium growing intercellularly in plant (Bar = 7 μm). G. *Epichloë* sp. stroma on culm of a grass plant (Bar = 2 cm).

based on ecological habits, including 1) soil saprotrophs, 2) insect necrotrophs/plant biotrophs, and 3) plant biotrophs (Table 2). The first group is comprised of soil inhabitants represented by *Chaunopycnis alba*, *Cordyceps subsessilis*, *Cordyceps militaris*, *Tolypocladium inflatum*, *Paecilomyces lilacinus* and *Lecanicillium lecanii*. Soil inhabitant species such as *Ch. alba*, tend to be cosmopolitan and possess a high saprobic capacity being able to live on many different substrates found in soil (M.S. Torres and J.F. White, unpublished data). *Lecanicillium* (anamorph of *Torrubiella*) and *Paecilomyces* (anamorph of *Cordyceps*) (Zare and Gams, 2001) are two genera of common insect necrotrophs frequently isolated from soils. A second group of species, insect necrotrophs/plant biotrophs, infects immature stages of scale insects, degrades their bodies and continue to live as epibionts on the surface of plants (Sullivan et al., 2000; Koroch et al., 2004; Bischoff and White, 2005; Chaverri et al., 2005b). These species include *Hyperdermium* spp. (Fig. 1E), *A. polychrous*, *H. phyllogena* and *D. tuberiformis* (Fig. 1A). A third group is comprised of 'plant biotrophs' including grass symbiont species of genera *Epichloë* (Fig. 1G), *Neotyphodium* (Fig. 1F), *Balansia* (Fig. 1B, D), and *Claviceps* (Fig. 1C).

Ergot alkaloid analysis

Several approaches were used to asses the presence of ergot alkaloids in the fungal extracts, including comparisons with standards, UV spectra characteristic of ergot alkaloids with max absorption at 316-318 nm and a minimum at 268 nm (Smedsgaard, 1997; Nielsen and Smedsgaard, 2003) and MS ion fragment pattern (Lehner et al., 2004; 2005).

Ergot alkaloid identification in the fungal extracts through standard comparison was possible for *C. purpurea* (Table 3). For the rest of the species we used LC-MS fragment patterns of *m/z* 208, 223, 268 (Table 3) as indicators of the ergoline structure (Lehner et al., 2004; 2005). Further studies using MS and NMR or comparable methods would be needed to further characterize the ergot alkaloids.

In this study ergot alkaloids were detected in *Balansia nigricans*, *B. strangulans*, *Claviceps purpurea*, *Dussiella tuberiformis*, *Epichloë festucae*, *Hyperdermium pulvinatum* and *Hypocrella phyllogena* (Table 3).

4. Discussion

Phylogenetic placement of trophic groups in the Clavicipitaceae

Clavicipitaceae has been clearly recognized as a member of the Hypocreales (Rehner and Samuels, 1994; Spatafora and Blackwell, 1993). A recent study (Spatafora et al., 2007) using a multigene phylogenetic analysis separated clavicipitaceous species into three main groups or clades. In the same study, based on ancestral character state reconstruction the authors proposed that the plant biotroph-grass symbionts originated from an animal/insect pathogen likely close to the scale insect pathogenic species (e.g., genera *Ascopolyporus, Dussiella, Hypocrella*, etc...) through a dynamic process of interkingdom host jumping.

Our phylogenetic analysis based on LSU rDNA is congruent with results of previously published analyses based on a single gene (Bischoff and White, 2005) and multigene analyses (Chaverri et al., 2005a; Spatafora et al., 2007). In our analysis the soil saprotrophic *Paecilomyces lilacinus* and *Chaunopycnis* spp. are limited to clade B; however both clades B and C also contain species that infect insects and may grow saprophytically in soil (Pateman and Kinghorn, 1976). The scale insect necrotroph/plant biotrophs (including in clade A, *Dussiella tuberiformis* and *Torrubiella luteorostrata*; and in clade C, *Hyperdermium* spp. and *Ascopolyporus polychrous*) are scattered in clades A and C (Fig. 2). These scale insect infecting species may be intermediary stages in adaptation to complete plant biotrophy (Koroch et al., 2004; Bischoff and White, 2004; Chaverri et al., 2005b). Consistent with this hypothesis is placement of *Torrubiella* and *Dussiella* as deeply-rooted sister groups to the rest of clade A. In our analyses *Hypocrella* is more derived but still basal to genus *Claviceps* with 97% posterior probability support (Fig. 2).

Ergot alkaloids in the family Clavicipitaceae

Ergot alkaloids are widely known to be produced by ergots (*Claviceps* spp.), plant biotrophic species that infect developing ovaries of grasses and replace them with structures known as sclerotia. Phylogenetically distant species in the genera *Aspergillus* and *Penicillium* (Eurotiales) are also reported to produce ergot alkaloids that are accumulated in resting structures or in conidia (Panaccione, 2005). A few other reports indicate production of ergot alkaloids (clavines and ergopeptides) in taxa related to the Clavicipitaceae (see Rehacek and Sadjl, 1990).

In the family Clavicipitaceae, the abundance of ergot alkaloids in grass endophyte associations has been interpreted as a unique characteristic of this group (Bacon et al., 1979; Glenn and Bacon, 1997; Siegel and Bush, 1996). Endophyte-infected grasses contain a variety of biologically active secondary metabolites that are not found in non-infected plants. Ergot alkaloids are known toxins to mammals having multiple effects on the neurological, cardiovascular, reproductive, and immune systems (Panaccione, 2005; Schardl et al., 2006). Ergot alkaloids are also frequently considered the primary mechanism for antiherbivore activity (Panaccione, 2005; Panaccione et al., 2006; Schardl et al., 2006; Gloer, 1995; Bush et al., 1997).

Table 3. Ergot alkaloid detection in species of Clavicipitaceae.

Taxa	Ergot alkaloid type[1]	$[M+H]^{+P}$ and Fragmentation in Electrospray-MS	References
Balansia henningsiana	Clavines, ergonovine	326.3, 223.2, 208.1, 180.0, 239.3, 223.1, 208.1	Bacon et al., 1979
Balansia nigricans	Unknown ergot alkaloids (2)	356.1, 336.4, 326.4, 223.1, 205.9, 363.0, 344.9, 269.2, 223.1, 208.3	This study
Balansia strangulans	Clavines, ergopeptides, unknown ergot alkaloids (4)	529.4, 387.3, 365.2, 268, 223.2, 208.2, 497.4, 362.2, 300.1, 223, 208, 180, 385.3, 363.1, 320.2, 239.3, 223.1, 208.1, 345.2, 318.3, 268, 223.2, 208.2	Bacon et al., 1979 This study
Claviceps fusiformis (sclerotia)	Clavines	326.3, 223.2, 208.1, 180.0	Rehacek and Sadjl, 1990
Claviceps paspali (sclerotia)	Lysergic acid derivatives	269.3, 223.2, 208.2	Rehacek and Sadjl, 1990
Claviceps purpurea (sclerotia)[*]	Clavines	326.3, 223.2, 208.1, 180.0	Rehacek and Sadjl, 1990
	Ergotamine	582.2, 268.2, 223.2	This study
	Ergocornine	562.4, 268.2, 223.2	This study
	Ergocrystine	610.2, 268.2, 223.2	This study
	Ergocryptine	576.4, 268.2, 223.2	This study
	Unknown ergot alkaloids (2)	613.9, 325.2, 223.2, 208.1, 592.2, 268.4, 223.2, 208.1	This study
Dussiella tuberiformis	Unknown ergot alkaloids (2)	420.3, 376.3, 223.2, 208.1, 465.1, 420.3, 376.3, 223.2, 208.1	This study
Epichloë festucae	Unknown ergot alkaloids (1**)	223.2, 208.1	This study
Hyperdermium bertonii	Unknown ergot alkaloids (1**)	344.4, 326.4, 312.4, 253.9, 223.2, 208.0	This study
Hyperdermium pulvinatum	Unknown ergot alkaloids (3)	312.4, 294.5, 223.2, 208.0, 347.1, 312.4, 294.5, 223.2, 208.0, 448.3, 426.3, 337.2, 295.2, 223.2, 208.0	This study
Hypocrella phyllogena	Unknown ergot alkaloids (1**)	223.2, 208.0	This study

[*]Commercial standards were used to identify alkaloids ergotamine, ergocornine, ergocryptine, and ergocrystine in *Claviceps purpurea*. [1]Numbers in brackets indicate number of ergot alkaloids detected by LC-MS. [**]Molecular weight not defined.

Both epiphytic and endophytic species in genera *Balansia* and *Epichloë* produce ergot alkaloids (Bacon et al., 1979; Glenn and Bacon, 1997; Schardl et al., 2006).

In a previous survey of ergot alkaloids in insect and fungal parasitic species, *Cordyceps* spp. (*C. militaris*, *C. ophioglossoides*, and *C. sphecocephala*) were negative for ergot alkaloid production; however *Dussiella* was identified as a producer of ergot alkaloids (Glenn and Bacon, 1997). In our analysis of the Clavicipitaceae we demonstrated that *Hypocrella* spp. and *Hyperdermium* spp. are also ergot alkaloid producers and observed a tendency for production of ergot alkaloids where a plant is part of the life cycle (Table 3). Although we did not screen *Torrubiella luteorostrata* and *Ascopolyporus polychrous*, the collective data suggest that the presence of ergot alkaloids is associated with plant biotrophy in the Clavicipitaceae (see Fig. 2). Our results at the minimum suggest that ergot alkaloid production is a physiological capacity that is more extensive in the family than previously assumed. We hypothesize that access to a continuous supply of carbon and nitrogen in plant nutrients may have enabled the biotrophic species to divert energy from vegetative growth to alkaloid production for defense. The plant biotrophic forms including scale insect parasites are also highly vulnerable to herbivory by a variety of herbivores since they are immobilized on the surfaces of plants and may accumulate sugars and other nutrients from plants (Torres et al., 2007a). Production of ergot alkaloids would reduce their vulnerability to herbivory. It is possible that soil saprotrophic and insect necrotrophic species also may produce ergot alkaloids but perhaps at lower concentrations than the plant biotrophic forms.

Additional research using a variety of culturing conditions will be needed to determine if soil saprotrophic and insect necrotrophic forms may also be induced to produce ergot alkaloids. Because the plant biotrophic forms occur in multiple clades of Clavicipitaceae, we propose that enhanced ergot alkaloid production by plant-associated species may be more a phenomenon of 'enhanced expression' of defensive genes rather than 'newly-evolved' genetic capacities.

'Self defense' vs 'host defense'

Ergot alkaloids in grass endophytes are clearly associated with host defense (Clay and Schardl, 2002; Clay, 1988). However, it is not clear that this is the case in insect necrotroph/plant biotrophs or the epibiotic biotrophic species, such as *Claviceps*. In these species mycelium is restricted on plants either on the scale insect or in the plant

ovary. In these restricted epibionts, there is no evidence, either ecologically or chemically, that the alkaloids diffuse into plant tissues or that plants are defended in any way. In the restricted epibionts (*Hyperdermium* spp., *Hypocrella* sp., *Claviceps* spp., etc...) ergot alkaloids could act as a self defense mechanism to deter herbivores of the fungus and prevent colonization by other organisms, without any apparent benefit for the plant host. A similar defense strategy was proposed by Gloer (1995) for indole diterpinoid function in sclerotia of *Aspergillus flavus*, where the presence of alkaloids resulted in deterrence of feeding by dried fruit beetles (*Carpophilus hemipterus*). It is reasonable to hypothesize that fungal defensive metabolites developed the host defensive function after endophytism evolved (Bischoff and White, 2005; Torres et al., 2007b).

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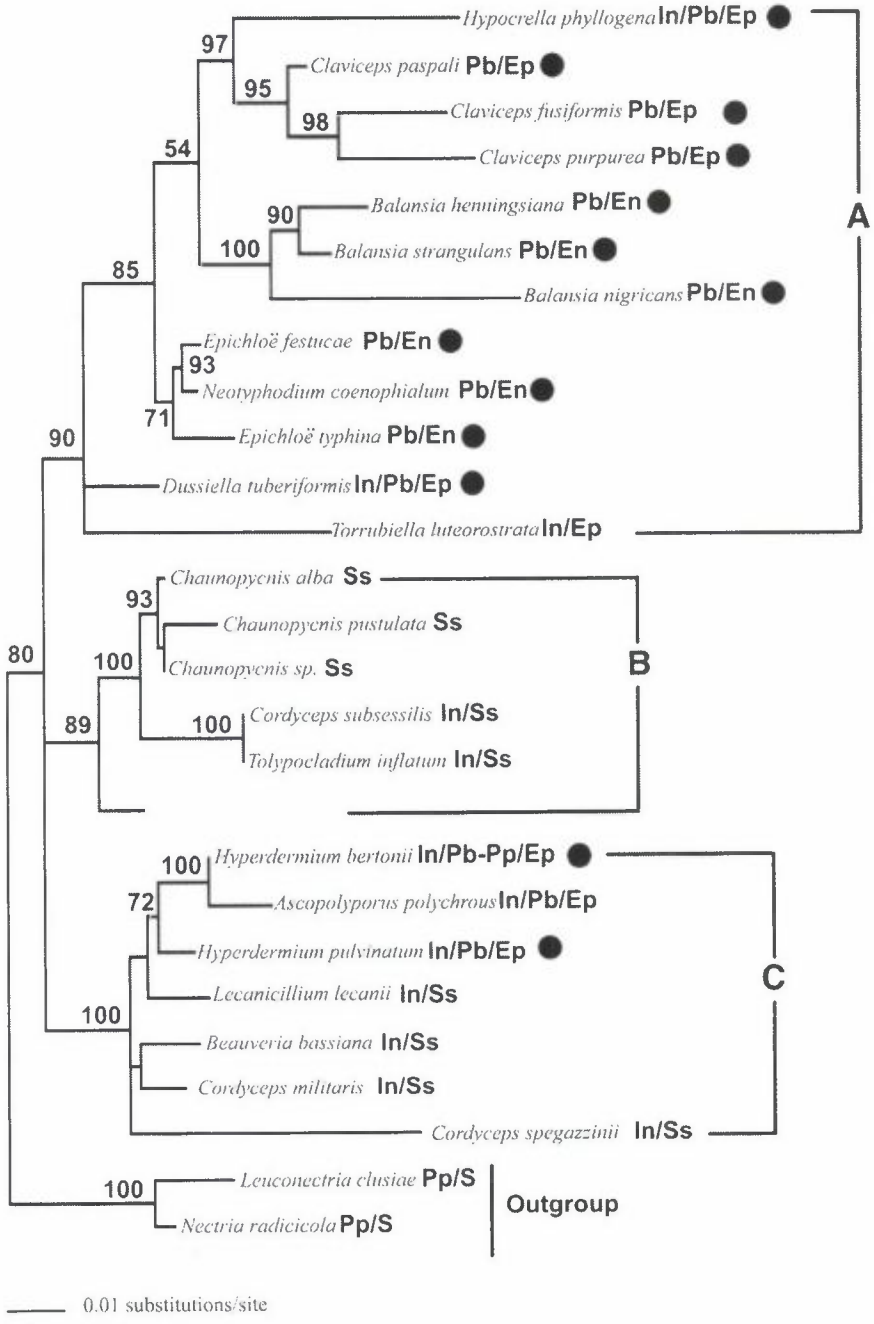

Figure 2. The most likely tree (-ln 3006.913) as determined by Paup using GTR+I+G model of evolution. The numbers on the branches indicate the posterior probability as a percentage for the node they proceed (only ≥50% shown). (● = ergot alkaloids). In = insect necrotroph, Pb = plant biotroph, Ep = epibiont, En = endophyte, S = saprotroph, Ss = soil saprotroph, Pp = plant parasite.

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