INDOLE-ALKALOID PROFILES IN NORWEGIAN *CLAVICEPS PURPUREA* POPULATIONS - RELATIONSHIPS BETWEEN CHEMORACES, GENETIC GROUPS, AND ECOLOGY

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Preface

This thesis is part of the master's degree biology (study program toxicology) at the University of Oslo (UiO), and is a collaboration between MERG (Microbial Evolution Research Group) at UiO and the Norwegian Veterinary Institute (NVI). By my wish for the subject to be something in the direction of natural toxic compounds, Trude brought together a team of co-supervisors with competence in various areas; mycology, natural toxic compounds, chemistry, phylogeny and ecology, statistics, and good old botany. Everything a master student of biosciences could wish for.

The laboratory work was carried out at NVI, supervised by Trude Vrålstad and Silvio Uhlig.

Acknowledgements (takksigelser)

To år har gått, og det fortere enn jeg trodde var mulig. I løpet av disse to årene har jeg fått veiledning, hjelp og støtte av mange forskjellige mennesker som jeg ikke kunne ha vært foruten.

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Trudes kunnskaper om *Claviceps purpurea,* både molekylær biologisk og mykologisk, har vært inspirerende. Også inspirerende har vært Silvios kunnskaper om HPLC-MS og indole-alkaloidene, samt en upåklagelig musikksmak som har gjort arbeidsdagen morsommere.

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Takk til Tom, statistikk kunnskapene dine har hjulpet meg enormt mye. Jeg var veldig bestemt på å inkludere statistikk så mye som mulig. Håper jeg ikke har mast for mye, men ikke bare kan R være litt som en treåring i trassalder, men også er R interessant og givende, når man får ut mer enn bare ERROR, verdt å merke seg… sånt som rosa elefanter.

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Til slutt må jeg gi en takk til studie venner, som har gitt meg den muligheten til å diskutere bekymringer og dele gleder med noen som går igjennom det samme, og ellers bare ta en kaffe og slappe av. Og, takk til mine foreldre som har holdt ut med en til tider deppa og irritert masterstudent, og takk til pusen min som alltid får de negative tidene til å gå fortere over med noen sandpapir-rue slikk.

Oktober, 2013

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Abstract

Claviceps purpurea is a phytopathogenic ascomycete that parasitizes various grasses, most notably rye. Instead of a grain the infected host plant produces a fungal sclerotium in the autumn, containing toxic indole-alkaloids hazardous to animals and humans. Previously, three different genetic groups (G1-G3) with divergent habitat preferences and alkaloid chemistry have been detected, often referred to as *C. purpurea* ecotypes and chemoraces.

The main aims of this study were to (1) analyse the genetic variation in *C. purpurea* in Norway, (2) characterise the indole-alkaloid profiles in relation to genetic groups, and (3) reveal the relationships between genetic groups, chemoraces and ecology.

In total, 596 sclerotia from 14 different grass species were subjected to various analyses, including a sclerotial floating test, genetic analyses from which rDNA ITS and partial beta tubulin sequences were generated, and mass peaks of indole-alkaloids that were measured using HPLC-MS. After the floating test, all sclerotia were cut in halves in order to analyse one half genetically and one half chemically.

Phylogenetic analyses and haplotype networks of genetic data supported three known (G1-G3) and one new (G4) genetic groups of *C. purpurea*, of which G1, G2 and G4 was present in Norway while G3 was absent. The new G4 group was supported also from chemical and ecological data. G4 produced sclerotia that were consistently floating, and was predominantly found in very wet habitats on *Molinia caerulea*, and occasionally also in saline habitats on *Leymus arenarius*. The G4 indole-alkaloid profile resembled that of G2, but differed with high amounts of the ergopeptam ergosedmam, a lactam congener of ergosedmine. Previously, G2 has been referred to as a chemorace based on its sole production of ergosine and ergocristine. The G2 sclerotia analysed in this study contained also mainly ergocristine, but in addition similar relative amounts of ergocryptine, and also lesser amounts of the other ergot alkaloids. A consistent presence of indole-diterpenoids in sclerotia from the G2 and G4 genetic groups was demonstrated, while these compounds with few exceptions were absent in the G1 genetic group. This study support that alkaloid chemistry is a function of the genetic groups, and that

the indole-alkaloids profiles of *C. purpurea* sclerotia can be used to assign them to a specific genetic group.

While G1 and G2 were found on numerous host plants and in different habitats, G3 and G4 seem to be more host and habitat specific. Co-occurrence of up to three genetic groups on the same host plant within the same habitat was observed. Thus, the results provided here suggest that the four groups G1-G4 in the *C. purpurea* complex represent four biological (cryptic) species rather than only ecotypes.

Sammendrag

Meldrøye (*Claviceps purpurea)* er en fytopatogenisk sekksporesopp som parasitterer ulike gressarter, særlig rug. I stedet for korn produserer den infiserte vertsplanten et sklerotium om høsten, som inneholder giftige indolalkaloider som er farlige for dyr og mennesker. Tidligere har det blitt beskrevet tre ulike genetiske grupper (G1 - G3) innenfor *C. purpurea* med divergerende habitat-preferanser og alkaloid profiler, ofte referert som *C. purpurea* økotyper og kjemoraser.

Hovedmålene med dette studiet var å (1) analysere den genetiske variasjonen som finnes i *C. purpurea* i Norge, (2) karakterisere alkaloid profiler (både ergotalklaoider og indolditerpenoider) i forhold til genetiske grupper, og (3) avdekke mulige sammenhenger mellom genetiske grupper, kjemoraser og økologi.

Total ble 596 sklerotier fra 14 forskjellige gressarter plukket ut til ulike analyser. Dette inkluderte en flytetest for sklerotiene, genetiske analyser hvor rDNA ITS-regionen og en del av beta-tubulin-genet ble DNA-sekvensert, og kjemiske analyser hvor alkaloid profilene i sklerotiene ble analysert ved hjelp av HPLC -MS. Etter flytetesten ble alle sklerotier kuttet i to for å kunne analyseres både genetisk og kjemisk.

Fylogeni og haplotype-nettverk støttet tre kjente (G1-G3) og en ny (G4) genetiske grupper av *C. purpurea*, hvorav G1, G2 og G4 var tilstede i de undersøkte norske populasjonene, mens G3 var fraværende. Den nye G4 gruppen ble også støttet av kjemiske og økologiske data. G4 produserte sklerotier som fløt konsistent, og ble fortrinnsvis funnet i svært våte habitater på

blåtopp (*Molinia caerulea*), og av og til i salte habitater på strandrug (*Leymus arenarius)*. alkaloid profilen hos G4-sklerotiene lignet G2 profilen, med skilte seg ut ved å inneholde store mengder av ergopeptamet ergosedmam, som er laktam varianten av ergosedmin. Tidligere har G2 blitt referert til som en «kjemorase» basert på innhold av ergosin og ergocristin i sklerotiene. Imidlertid inneholdt G2-sklerotiene analysert i dette studiet i stor grad ergocristin, ergocryptin, ergosin og mindre mengder av andre ergotalkaloider. Det ble demonstrert konsistent tilstedeværelse av indolditerpenoider i sklerotiene til G2 and G4 gruppene, mens disse forbindelsene med få unntak manglet i G1 gruppen. Dette studiet støtter at alkaloid profilen er en funksjon av genetisk gruppe, og at alkaloid profiler av *C. purpurea* sklerotier kan brukes for å bestemme en spesifikk genetisk gruppe.

Mens G1 og G2 ble funnet på en rekke vertsplanter og i ulike habitater, så virker G3 og G4 å være mer vert- og habitatspesifikke. Sameksistens av opptil tre genetiske grupper på same vertsplanteart i samme habitat ble observert. Disse resultatene tyder på at de fire gruppene G1-G4 i *C. purpurea*-komplekset representerer fire biologiske (kryptiske) arter.

Abbreviations

Abbreviation	Full expression
CTAB	cetyltrimethyl ammonium bromide
RNase A	Ribonuclease A
EtOH	Ethanol
Te buffer	Tris-EDTA buffer
MeOH	Methanol
EBK	Extraction control blank
EMK	Extraction environmental control
NPK	Negative PCR control
PCR	Polymerase chain reaction
RAPD	Random Amplified Polymorphic DNA
ITS	Internal transcribed spacer
tub	Betatubulin
TBR	Tree-Bisection-Regrafting
HPLC-MS	High performance liquid chromatography mass spectrometry
MeCN	Acetonitrile
ITMS	Ion trap mobility spectrometry
ESI	Electrospray ionisation interface
APCI	Atmospheric chemical ionization
PDA	Photodiode array (detector)
PCA	Principal component analysis
ANOVA	Analysis of variance

MEGA	Molecular evolutionary genetics analysis

Abbreviations of geographic locations introduced by the author

Location shortened	Location full name in Norway (location, municipality, county)
MAR	Maridalen, Oslo
TRY	Tryvann, Oslo
SOL	Solvik, Kragerø, Telemark
JAR	Orre, Klepp, Rogaland (Jæren)
FAR	Havika, Farsund, Vest-Agder
KOR	Korsvoll, Oslo

Note: Jæren (JAR) was by mistake taken as the location, when it is a general name of a stretch of beach at this location.

Abbreviations of host plants introduced by the author

Host shortened	Host full name	Norwegian name
AA	*Ammophila arenaria*	Marehalm
AO	*Anthoxanthum odoratum*	Gulaks
CA	*Calamagrostis arundinacea*	Snerprørkvein
CE	*Calamagrostis epigejos*	Bergørkvein
DC	*Deschampsia cespitosa*	Sølvbunke
DG	*Dactylis glomerata*	Hundegras
ER	*Elymus repens*	Kveke
FR	*Festuca rubra*	Rødsvingel
LA	*Leymus arenarius*	Strandrug

MC	*Molinia caerulea*	Blåtopp
PA	*Phalaris arundinacea*	Strandrør
PO	*Poa pratensis*	Engrapp
PP	*Phleum pratense*	Timotei
SP	*Schedonorus pratensis*	Engsvingel

Abbreviations of indole-alkaloids introduced by the author

<u>Indole diterpenoids shortened</u>

422 I	Indole-diterpenoid with m/z 422
422 II	Indole-diterpenoid with m/z 422
422 III	Indole-diterpenoid with m/z 422
420 I	Indole-diterpenoid with m/z 420
420 II	Indole-diterpenoid with m/z 420
406 I	Indole-diterpenoid with m/z 406
406 II	Indole-diterpenoid with m/z 406

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1. Introduction

1.1 General background

The ergot fungus *Claviceps purpurea* is a phytopathogenic ascomycete. It parasitizes several grass species, where the infection is located to the ovaries of the host plant, resulting in a sclerotium that replaces the seed grain. These contain different ergot alkaloids with severe effects on the nervous system and smooth muscles (Panaccione, 2005; Uhlig et al., 2011), but also less studied tremorgenic indole-diterpenoids (Uhlig et al., 2009) that are associated with staggers in mammals through neurological disturbances (Cawdell-Smith et al., 2010; Moyano et al., 2010). The range of host plants is extensive, including about 400 plant species in the grass family (Poaceae) including wild and cultivated pasture- and forage grasses, and common cereals (Lorenz et al., 2009; Schiff, 2006). Among these are species from the economically important subfamily Pooideae, such as rye (*Secale cereale*), oat (*Avena sativa*), barley (*Hordeum vulgare L*.) and wheat (*Triticum spp.)* (Schiff, 2006), and also a few species from Arundinoideae and Chloridoideae.

Ergot has long caused problems for human health. Ergotism, the name for the disease caused by some of the alkaloids found in ergot sclerotia, was a major concern during the Middle Ages when epidemic outbreaks occurred regularly. *Claviceps purpurea* often infects rye, and this was the most used cereal grain at that time in continental Europe (Schiff, 2006). However, even though ergotism is now rare among humans due to good agricultural practices, it is still a problem among wildlife and sometimes livestock (Botha et al., 1996; Botha et al., 2004; Løken et al., 1979). In Norway, especially elk are regularly observed with visible signs of ergotism (Handeland and Vikøren, 2005).

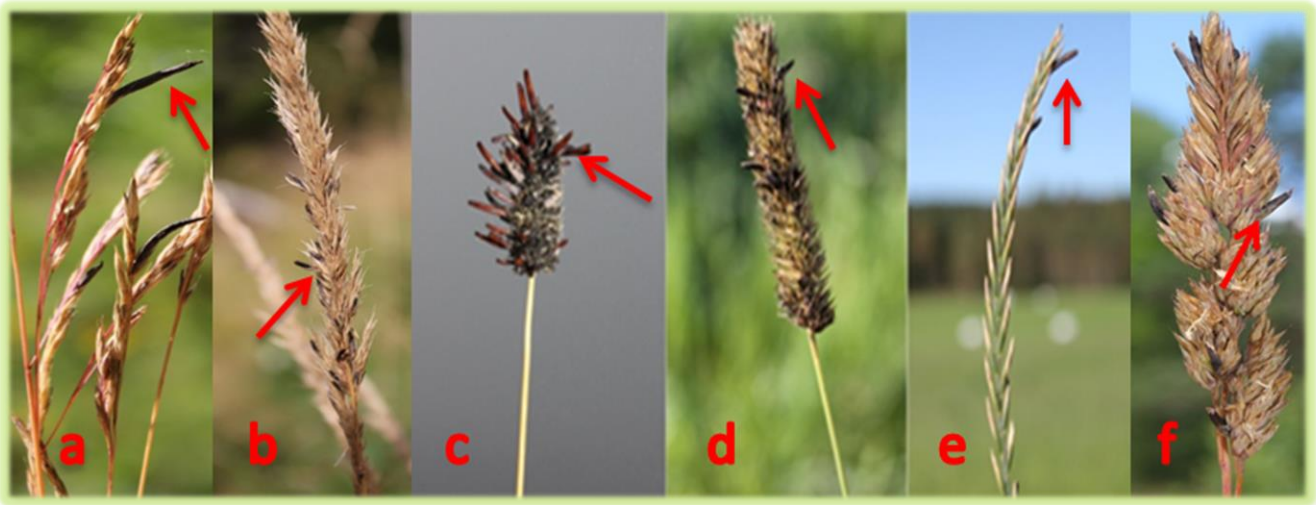


Figure 1. Various infected grass species

Claviceps purpurea infects and replaces the seed grain with a toxic sclerotium (red arrowheads) in a wide range of cereals and grasses such as a) *Festuca rubra*, b) *Calamagrostis arundinacea,* c-d) heavily and less heavily infected *Phleum pratense*, e) *Elymus repens*, and f) *Dactylis glomerata.*

Photos: Trude Vrålstad, Norwegian Veterinary Institute

The life cycle of *C. purpurea* involves stromatas with perithecia (ascocarps) that germinate from the sclerotium in the spring. These release ascospores, which may land on host plants. The process of infection is by mimicking a pollen grain thereby giving the invading spores access to the ovary. By this method of infection the plant species most vulnerable are those with open flowers, such as rye. Furthermore, as the infection progresses, the ovary is replaced, and from the newly formed sphacelia the production of honeydew begins. This sweet sap contains conidia (asexual spores) and is attractively sweet to insects, and by these vectors new host plants can be infected. Towards the end of summer, the sclerotium develops from the sphacelia, and at this stage, the alkaloids accumulate within the sclerotium. A matured hardened sclerotium will drop to the ground and survive the winter until spring, and begin the cycle again (Alexopoulos et al., 1996). It has been speculated that the grasses may benefit from the infection, e.g. the cost of losing a few seeds is outweighed by the toxicity preventing grazing by mammals and insects (Lane et al., 2000; Wäli et al., 2013).

1.2 The chemistry of *C.purpurea*

The sclerotium (plural sclerotia) consists of a hardened mycelium. Different alkaloids comprise only 0.5-2% of the total sclerotium weight, while the major contents is fat (30-40%) (Komarova and Tolkachev, 2001). The brown - purple colouring of the exterior of the sclerotia comes from anthraquinolinic acid-derived pigments (Řeháček and Sajdl, 1990).

The ergot alkaloids are likely the most important bioactive compounds produced by several *Claviceps* ssp. Such compounds are also produced within other fungal genera (e.g. *Penicillium* and *Aspergillius*) and also plant species (Convolvulaceae) (Flieger et al., 1997; Schardl et al., 2006). They can be divided into three major groups; clavines, simple lysergic acid derivatives, and peptidic ergot alkaloids (Komarova and Tolkachev, 2001; Schardl et al., 2006). These metabolites all share the ergoline ring structure (fig. 2) (Schardl et al., 2006). Today, more than 50 ergot alkaloids have been characterized from *C. purpurea* (Flieger et al., 1997).

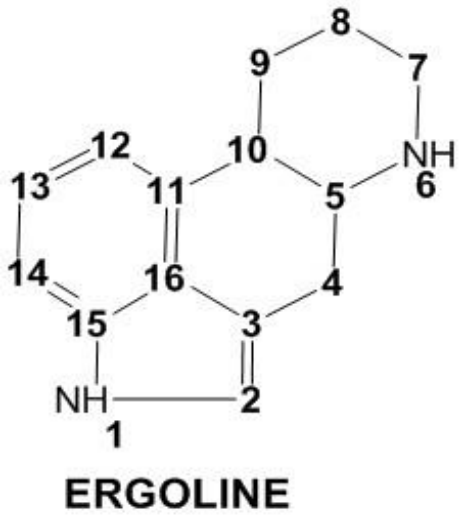


Figure 2. Ergoline ring structure

The ergoline ring structure is the commonality between all ergot alkaloids. Different ergot alkaloids are primarily modified or substituted at the C-8.

The simple lysergic acid derivatives are non-peptidic ergot alkaloids, consisting of the ergoline structure and an additional amide group at C-8 (Schiff, 2006). These alkaloids are also water-soluble (Schardl et al., 2006) (fig. 3a).

The largest group of ergot alkaloids, the ergopeptines, contain a tripeptide moiety that is esterified with the ergoline part via the amide group at C-8. In addition, unlike the lysergic acid

derivatives these are water-insoluble (Schardl et al., 2006) and are the end products of ergot alkaloid biosynthesis. Nearly all naturally occurring ergopeptines contain the amino acid L-proline. The composition of the remaining two amino acids is variable to some degree (Mukherjee and Menge, 2000) (fig. 3b).

The ergot alkaloids of pharmacological interest are the bioactive D-lysergic acid derivatives, which are the true biosynthetic products. However, they are also accompanied by the biologically inactive D-isolysergic variations, which are epimerized at C-8 (8*S*) (Eadie, 2003; Komarova and Tolkachev, 2001; Schiff, 2006) . This epimerization also occurs during storage and analysis of samples (Krska and Crews, 2008). The C-8 epimers (8*S*) are given the suffix "-inine" instead of the "-ine" used for the 8*R* derivatives of D-lysergic acid (e.g ergotamine vs. ergotaminine) (Komarova and Tolkachev, 2001).

The lactam ergot alkaloids (ergopeptams) are the non-cyclol counterparts to the ergopeptines and are the result of racemisation of L-proline during ergot alkaloid biosynthesis (Komarova and Tolkachev, 2001) (fig. 3b). Cyclisation of the amino acid adjacent to the ergoline part of the molecules does not occur in the variants containing D-proline, possibly because of steric hindrance. Lactam ergot alkaloids are relatively unstable and so hard to detect, but, theoretically there exists one lactam analogue for each ergopeptine (Floss et al., 1974; Stadler, 1982).

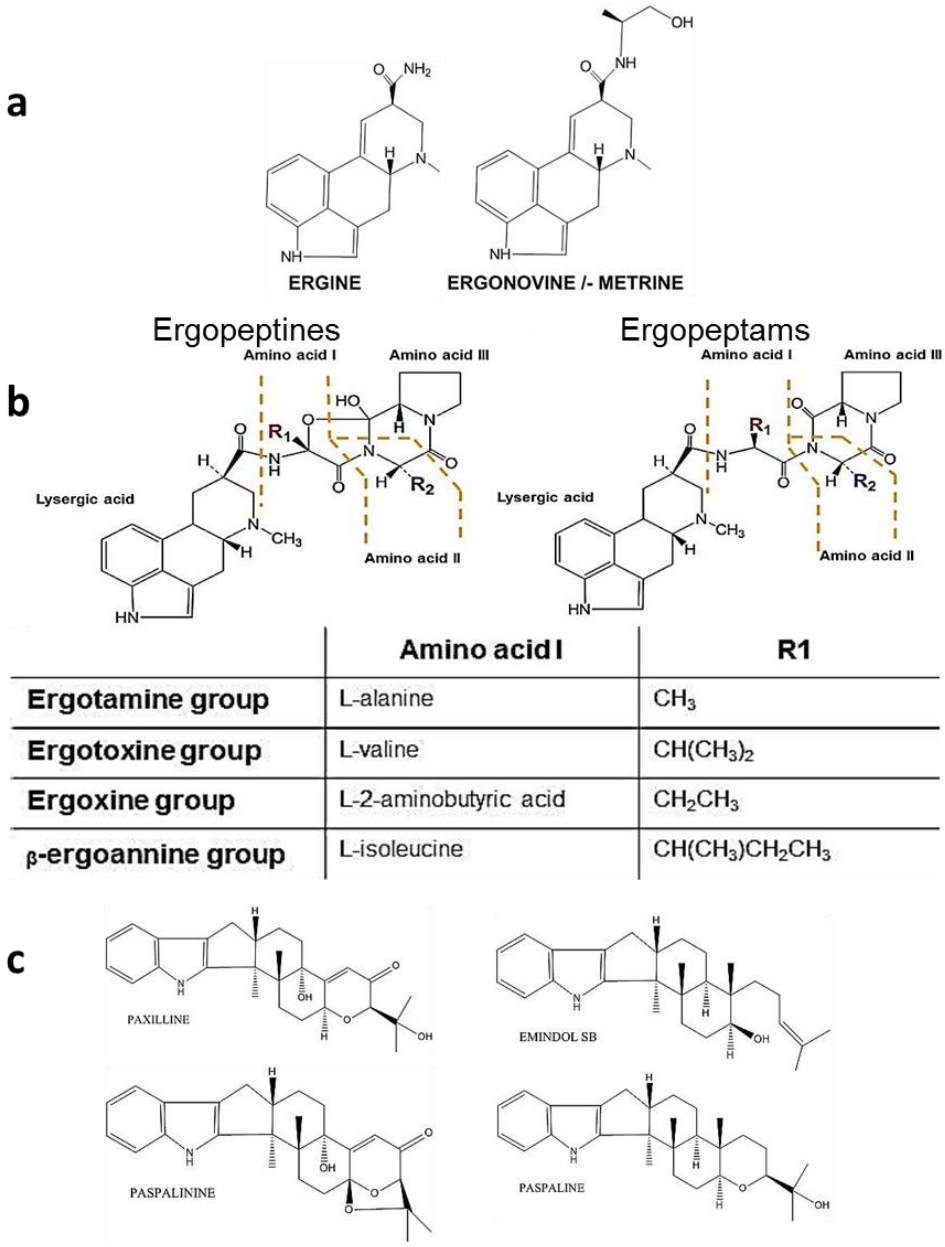


Figure 3. Structural examples for ergot alkaloids and indole-diterpenoids

The simple lysergic acid derivatives included in this study were ergine and ergonovine (syn. ergometrine), shown in (a).

The ergopeptines (b) are variable by the amino acids at R1 and R2, where R1 is used to subgroup ergopeptines into ergotamines, ergotoxines, ergoxines, and ergoannines. The structure to the left shows the general structure of ergopeptines, and the structure to the right of ergopeptams, that are lactam derivatives of the ergopeptines.

(c) Shows four examples of indole-diterpenoids related to *Claviceps* species.

The ergot alkaloids act as partial agonists and antagonists on various kinds of dopamine, serotonin and noradrenaline receptors and can produce effects both on the central nervous system and blood vessels with vasoconstrictive properties. This is possible due to the similarity between the basic molecular shape of lysergic acid and the neurotransmitters normally acting on these receptors (Pertz and Eich, 1999; Tudzynski et al., 2001) (fig. 4).

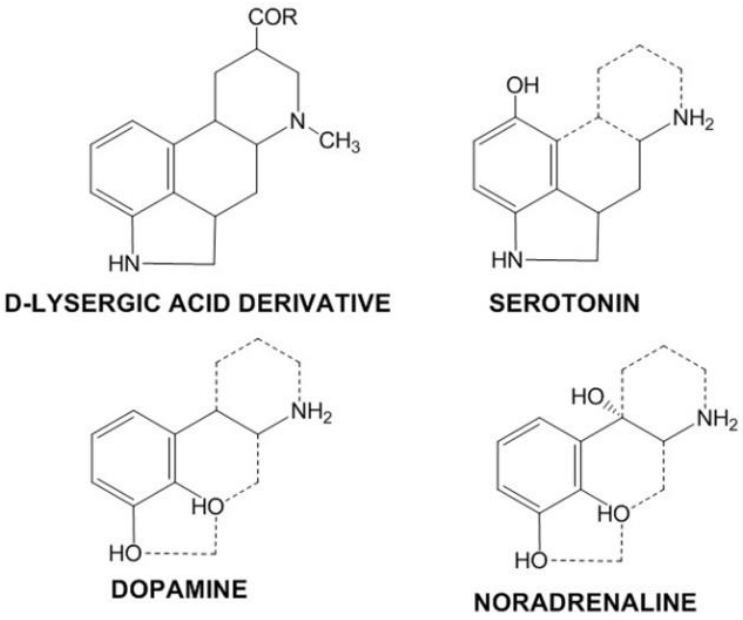


Figure 4. Neurotransmitter similarity

The lysergic acid moiety of the ergot alkaloids is similar to the neurotransmitters serotonin, dopamine and noradrenalin. This close similarity is the cause for the effects and symptoms these alkaloids can induce as vasoconstrictors and on the central nervous system.

Unpublished data (S. Uhlig pers. com) suggests that *C. purpurea* also produces indole-diterpenoids that are largely unexplored secondary metabolites of potential toxic importance. The indole-diterpenoids have a cyclic diterpene backbone and an indole group (Saikia et al., 2008) (fig 3c), and several analogues are known for the staggers/tremors they can produce in mammals (Knaus et al., 1994). Several types of indole-diterpenoids are also known to possess insecticidal activities (Lane et al., 2000; Li et al., 2002). The effects have been shown to be induced by inhibition of large conductance calcium – activated potassium channels called BK channels (Imlach et al., 2008; Knaus et al., 1994; Saikia et al., 2008).

The syndrome/disease the indole-diterpenoids give often refer to the host plant, such as "perennial ryegrass staggers" (endophytic *Neotyphodium* ssp. in ryegrass) or "paspalum staggers" (*Claviceps paspali* parasitizing *Paspalum* ssp.) (Botha et al., 1996; Cole et al., 1977; Imlach et al., 2008).

1.3 Genetic groups and chemoraces

In previous studies, three main genetic groups of *C.purpurea* (often referred to as "genotype" G1, G2 and G3) have been recognized based on RAPD (Pazoutova et al., 2000), and later confirmed by DNA sequencing of among others ITS and Beta-tubulin (Douhan et al., 2008). It has been demonstrated that these genetic groups are linked to different habitats (Pazoutova et al., 2000). G1 is typically found in dry locations such as fields / meadows, and roadsides, G2 is associated with wet and shady habitats, such as forests and forests' edges, ponds and rivers, while G3 seems to be confined to saline habitats and the grass genera *Spartina* and *Distichlis* (Fisher et al., 2005b; Pazoutova et al., 2000; Pazoutova et al., 2002b). The G3 group has so far been detected in Argentina, Germany, Ireland, the UK, and the USA on coastal salt marshes (Douhan et al., 2008; Fisher et al., 2005a; 2005b; Nehring et al., 2012; Pazoutova et al., 2002b)

The different genetic groups seem further to represent different chemoraces where each group seemingly produces different sets of alkaloids. According to Pazoutova et al (2002a; 2000), sclerotia of G2 contained equal amounts of ergosine and ergocristine and 5-15% of ergocryptine. None of the other alkaloids were found. Moreover, G3 from *S. anglica* included a mixture of ergocristine and ergocryptine. Both G2 and G3 therefore constitute stable chemoraces. However, sclerotia of the G1 group showed no specific alkaloid pattern and contained from one to seven different ergot alkaloids. Noteworthy, G1 contained ergotamines and ergotoxines (Pazoutova et al., 2002a; Pazoutova et al., 2000).

1.4 Hypotheses and research aims

In the few studies that have been conducted until now, the *C. purpurea* sclerotia show a remarkable adaptability to the different habitats. Sclerotia produced by G1 confined to dry habitats have no ability to float on water, while sclerotia produced by G2 and G3, confined to wet habitats, are able to float for a long time (Pazoutova et al., 2002a; Pazoutova et al., 2000). It has been hypothesized that only one genetic group is normally detected in a single location,

and the hosts within. Exceptions have been found, though, in locations including bordering habitats and regular introduction of new species (Pazoutova, 2007).

Previous phylogenetic analyses support the presence of the three lineages G1-G3 with little or no gene flow between the groups. It has therefore been hypothesized that habitat driven evolution are leading to separate species with unique chemistry within the *C.purpurea* complex (Douhan et al., 2008). Apparently, the host plant has no influence on alkaloid composition in *C. purpurea* sclerotia, but the quantitative amount of the various alkaloids may vary between hosts (Pazoutova et al., 2002a; Pazoutova et al., 2000). However, this topic has not been investigated thoroughly. Furthermore, the geographic coverage of previous studies covers predominantly the Americas and a small part of Western Europe.

No similar studies have been conducted for *C. purpurea* in Northern European habitats. Preliminary data from research at the Norwegian Veterinary Institute (NVI) suggests a high diversity of ergot alkaloids and indole-diterpenoids in ergot sclerotia from Norway. This thesis is the first comprehensive study of the relationship between genetic groups, habitat, host plant species and indole - alkaloid profiles in Norwegian populations of *C.purpurea*.

Study aims and questions

1. Explore genetic diversity of *C.purpurea* in Norway from selected host plants and habitats
    a. Is the genetic diversity larger than previously described?
2. Characterise the indole-alkaloid (both ergot alkaloids and indole-diterpenoids) profiles in relation to genetic groups
    a. Is the sclerotial chemistry a function of the genetic groups?
3. Reveal relationships between genetic groups, chemoraces and ecology
    a. Are there host preferences in the genetic groups?
    b. Is the indole-alkaloid composition and/or quantity dependent on the host plant?
    c. Are the genetic groups specific to habitats (e.g. dry, wet, and saline)?
    d. Can genetic groups co-occur upon a single host plant and within a single habitat?
    e. Are the adaptations to different habitats consistent within genetic groups?

2. Materials and Methods

2.1 Study areas and sampling

Claviceps purpurea sclerotia were collected during the autumn 2011 and 2012 at six locations in Southern Norway from common grass hosts (table 1). These locations represented four different habitats with various levels of humidity and salinity (table 1). If possible, the same grass host species were collected between locations and habitats, but often, different host species were represented in the different habitats. From each plot within a location, 5-15 *C. purpurea* infected grass individuals per grass species were collected and brought to the laboratory.

2.2 Floating test and material preparation

Sclerotia for one grass species per plot were collected, cleaned for debris and stored in petri dishes in room temperature. All present sclerotia up to a number of ~50 per location were dissected. However, in many cases only a few sclerotia were present thus some samples are thus rather small (table 1). In total, 596 sclerotia were included in the further analyses. The sclerotia were first submitted to a floating test (Pazoutova et al., 2000) where the sclerotia were submerged in purified H_2O for 48 hours. This was done to reveal whether sclerotia with different types of floating ability were present and whether the ability to float can be linked to genetic group and habitat type. The sclerotia were then dried and cut in half. One piece of a sclerotium was subjected to DNA analyses, and the other was stored for assembling for the alkaloid analyses.

Table 1. Sample overview

An overview of the sampling locations and what year each location was visited, with associated coordinates. Also, the habitat type and host plants at these locations are given. The number of sampled sclerotia is given in parentheses.

Season	Location	Plot	Latitude	Longitude	Habitat	Host (# sclerotia)
2011	Farsund, Vest-Agder	1	58.06984	6.72405	Saline - salt exposed beach	*Leymus arenarius* (4)
2011	Farsund, Vest-Agder	2	58.06984	6.72405	Saline - salt exposed beach	*Leymus arenarius* (5)
2011	Farsund, Vest-Agder	3	58.06984	6.72405	Saline - salt exposed beach	*Calamagrostis epigejos* (20)
2012	Farsund, Vest-Agder	4	58.06984	6.72405	Saline - salt exposed beach	*Leymus arenarius* (15)
2011	Jæren, Rogaland	1	58.72455	5.51944	Saline - salt exposed beach	*Ammophila arenaria* (13)
2011	Jæren, Rogaland	2	58.72455	5.51944	Saline - salt exposed beach	*Ammophila arenaria* (10)
2011	Korsvoll, Oslo	1	59.97072	10.74814	Very wet - constantly subermerged	*Molinia caerulea* (8)
2011	Korsvoll, Oslo	2	59.97174	10.7477	Very wet - constantly subermerged	*Molinia caerulea* (20)
2011	Korsvoll, Oslo	3	59.97301	10.74723	Very wet - constantly subermerged	*Molinia caerulea* (15)
2011	Korsvoll, Oslo	4	59.96967	10.74828	Wet - shady wet forest	*Phleum pratense* (5)
2012	Korsvoll, Oslo	5	59.96972	10.74832	Wet - shady wet forest	*Calamagrostis arundinacea* (15)
2012	Korsvoll, Oslo	5	59.96972	10.74832	Wet - shady wet forest	*Deschampsia cespitosa* (11)
2012	Korsvoll, Oslo	6	59.96887	10.74753	Wet - shady wet forest	*Elymus repens* (6)
2012	Korsvoll, Oslo	7	59.96828	10.74738	Wet - shady wet forest	*Phleum pratense* (10)
2012	Korsvoll, Oslo	8	59.96832	10.7481	Wet - shady wet forest	*Schedonorus pratesis* (14)
2011	Maridalen, Oslo	1	59.97219	10.76052	Dry - open dry field	*Phleum pratense* (12)
2011	Maridalen, Oslo	2	59.97263	10.76325	Dry - open dry field	*Phleum pratense* (16)
2011	Maridalen, Oslo	3	59.97296	10.76408	Dry - open dry field	*Dactylis glomerata* (28)
2011	Maridalen, Oslo	3	59.97296	10.76408	Dry - open dry field	*Elymus repens* (8)
2011	Maridalen, Oslo	3	59.97296	10.76408	Dry - open dry field	*Schedonorus pratesis* (13)
2012	Maridalen, Oslo	4	59.9723	10.75967	Dry - open dry field	*Calamagrostis arundinacea* (20)
2012	Maridalen, Oslo	5	59.97898	10.75447	Very wet - constantly subermerged	*Molinia caerulea* (20)
2012	Solvik, Telemark	1	58.97152	9.26459	Dry - open dry field	*Anthoxanthum odoratum* (15)
2012	Solvik, Telemark	1	58.97152	9.26459	Dry - open dry field	*Dactylis glomerata* (20)
2012	Solvik, Telemark	1	58.97152	9.26459	Dry - open dry field	*Festuca rubra* (20)
2012	Solvik, Telemark	1	58.97152	9.26459	Dry - open dry field	*Phalaris arundinacea* (15)
2012	Solvik, Telemark	1	58.97152	9.26459	Dry - open dry field	*Poa pratensis* (15)
2012	Solvik, Telemark	2	58.97147	9.26508	Very wet - constantly subermerged	*Molinia caerulea* (20)
2012	Solvik, Telemark	3	58.9682	9.25837	Dry - open dry field	*Calamagrostis arundinacea* (29)
2012	Solvik, Telemark	3	58.9682	9.25837	Dry - open dry field	*Calamagrostis epigejos* (34)
2012	Solvik, Telemark	3	58.9682	9.25837	Dry - open dry field	*Phleum pratense* (31)
2011	Tryvann, Oslo	1	59.9838	10.66953	Dry - open dry field	*Phleum pratense* (40)
2011	Tryvann, Oslo	2	59.98401	10.66767	Dry - open dry field	*Phleum pratense* (36)
2012	Tryvann, Oslo	3	59.98268	10.67023	Dry - open dry field	*Deschampsia cespitosa* (8)
2012	Tryvann, Oslo	3	59.98268	10.67023	Dry - open dry field	*Festuca rubra* (10)
2012	Tryvann, Oslo	4	59.97965	10.66966	Dry - open dry field	*Calamagrostis arundinacea* (15)
					sum sampled sclerotia	596

2.3 Molecular analyses

2.3.1 DNA extraction

DNA was extracted from one half-piece of each sclerotium, using a CTAB extraction protocol (Gardes and Bruns, 1993), including the modifications described by Vrålstad et al (2009), and with some minor modifications for this study.

Briefly, 100 µL CTAB buffer [20 g/L, Calbiochem, Darmstadt, Germany; 1.4 M NaCl, 0.1 M Tris-HCl, 20 mM Na_2 EDTA] was added to each of the eppendorf tubes containing sclerotium-halves and heated to 95°C (5 min), then crushed manually with a pistil (cleaned with household bleach, EtOH and autoclaved), and another 600 µL CTAB was added. Samples were frozen to -70 °C (for at least 10 min), then heated to 65 °C (5-10 min, until thawed), and centrifuged for 10-15 seconds. Then to the samples there were added 10 µL RNase A (10 mg/mL), and shortly vortexed before incubated at 65°C (30 min). This was followed by identical treatment with proteinase K (20 mg/mL). Chloroform (600 µL) was added, and the samples were vortexed well before being centrifuged (~16000 g for 15 min). From the upper aqueous phase containing DNA, 400 µL was transferred to new eppendorf tubes. 300 µL cold (-20 °C) isopropanol was added and samples were inverted and incubated at room temperature (15 min) to precipitate the DNA. The samples were then centrifuged (~16000 g for 15 min), which concentrated the DNA in a pellet. The supernatant was removed and discarded. The pellet was washed with 300 µL cold 70 %-EtOH (-20 °C), and shortly vortexed, before the supernatant was again removed. The pellet was left to dry in a laminar flow bench. Finally, 100 µL TE buffer was added in order to resuspend the DNA. The samples were stored in a freezer (-20 °C) until further PCR analyses.

During the DNA extraction two controls were established; extraction blank control (EBK), to control any carry-over contamination, and extraction environment control (EMK) to control contamination from the laboratory environment. EBK consisted of a tissue (and DNA) free tube that followed all steps of the extraction protocol together with the samples, while EMK consisted of a tube with 200 µL milliQ-H_2O that was left open during all work with the samples in the laboratory. EBK and EMK followed through the extraction procedure and the subsequent PCR analysis.

2.3.2 PCR and sequencing

For amplification of ITS the primers ITS1 (5'- TCCGTAGGTGAACCTGCGG- 3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990) were used.

The puReTaq Ready-To-Go PCR Beads kit (Amersham Biosciences, UK) was used for PCR amplification according to the producer's directions. To each tube 23 µL mastermix was added consisting of 17 µL milliQ-H_2O and 6 µL primers (5 µM), and 2 µL DNA template, and also here a control was established, negative PCR control (NPK), to control the purity of the milliQ-H_2O used.

PCR was performed on a DNA Engine Dyad® Peltier Thermal Cycler (PTC-0220, MJ Research, Waltham, MA, USA), the setup consisted of pre-denaturation at 95 °C (5 min), followed by 35 cycles with a denaturing step at 95 °C (30 s), then annealing at 56 °C (20 s), and synthesis at 72 °C (30 s), and lastly a final elongation at 72 °C (5 min).

From a smaller subset of the samples representing unique ITS sequences and the three different ITS genetic groups, a part of the betatubulin (*tub*) gene was sequenced. The *tub* fragment was amplified with the primers Bt-3NeoF (5`-GCTCTAGACTGCTTTCTGGCAGACC-3`) and Bt-3NeoR (5`-CGTCTAGAKGTRCCCATACCGGCA-3`) (Annis and Panaccione, 1998) using the PCR kit as described above.

The setup on the PCR machine (DYAD®) for *tub* was as follows, a pre-denaturation at 94 °C (4 min), and then 35 cycles of denaturation at 94 °C (30 s), followed by an annealing step at 66 °C (30 s), and synthesis at 72 °C (1 min), and lastly an final elongation at 72 °C (5 min).

The PCR products (5 µL) mixed with 1-2 µL loading solution (Thermo Scientific) were controlled with gel electrophoresis on a 1.5 % agarose gel and stained with GelRed Nucleic Acid Stain (Biotium, CA, USA). And pUC Mix Marker 8 ready-to-use (19-1118 bp; Fermentas; USA) was used as size marker.

5 µL PCR products were purified with 2 µL Exosap-IT (Amersham Biosciences). The purified PCR products were diluted 10 times with miliQ-H_2O (*tub* samples were not diluted due to the small

amount of DNA available even after PCR), and finally, 9 µL diluted PCR product and 1 µL of each respective primer were added to new set of plates.

The PCR products were sequenced in both directions using ABI BigDye Terminator sequence buffer, v3.1 Cycle sequencing kit and ABI PRISM® 3730 Genetic Analyzer / ABI PRISM® 3100 – Avant Genetic Analyzer (Applied Biosystems, Life Technologies, USA). The ITS sequences were generated at the ABI-lab at the Department of Biosciences, University of Oslo, while the *tub* sequences were generated at the molecular laboratory unit at the Norwegian veterinary Institute.

2.4 Alkaloid analyses

2.4.1 Reference compounds

The following ergot alkaloid reference standards were available from commercial sources or had previously been isolated and purified at the Norwegian Veterinary Institute or collaborating institutions: ergonovine, erginine, ergosine, ergotamine, α-ergocryptine, ergovaline, ergocornine, ergocristine and ergosedmine. Other ergot alkaloids and lactam ergot alkaloids were earlier studied and tentatively identified in *C. purpurea* sclerotia (Uhlig and Petersen, 2008). The indole-diterpenoid diversity in *C. purpurea* sclerotia has previously been explored, and different analogues tentatively characterised in sclerotia from *Phalaris arundinacea* (Uhlig et al., unpublished results).

2.4.2 Preparation and homogenization

The remaining halves of the sclerotia that had been DNA-sequenced and assigned to genetic groups were pooled prior to extraction and subsequent alkaloid analyses were performed using HPLC-MS. The pooled samples consisted of genetically identical sclerotia that originated from a specific plant host species of a specific plot within each location. The pooled samples typically consisted of 10-20 sclerotia halves, but in some cases less.

The pooled samples were further homogenized (Ultra-Turrax T25, Janke and Kunkel, Staufen, i. Br. Germany) with 1-4 mL acetone/water (4:1, v/v), depending on the available amount of sclerotia. The homogenates were left to sediment for at least 30 min, and 1 mL of the supernatants was filtered through a 0.22 µm Spin-X– Nylon membrane (Costar, Corning Inc, Corning, NY, USA) and transferred to chromatography vials.

2.4.3 HPLC-MS and HPLC-MS^2

The ergot alkaloid and indole-diterpenoid profiles were studied using a HPLC coupled to photodiode-array detector (PDA) and a linear ion trap mass spectrometer (ITMS). The instrument used consisted of a Finningan Surveyor MS Pump Plus and Autosampler Plus (Thermo Fisher, San Jose, CA, USA) coupled to a Surveyor PDA Plus photodiode array detector and Finnigan LTQ linear ion trap mass spectrometer (Thermo Fisher).

Ergot alkaloids

Extracts aliquots of 5 µL were injected into the HPLC-ITMS, which was equipped with an electrospray ionisation interface (ESI) operated in the positive mode. Separation of ergot alkaloids was either achieved on a 50 × 2.1 mm i.d. 3.0 µm SunFire C_{18} column (Waters, Milford, MA, USA), or a 50 × 2.1 mm i.d. 2.6 µm Kinetex C_{18}- XB column (Phenomenex, Torrance, CA, USA), using a mobile phase consisting of MeCN (A) and H_2O, containing 2 mM ammonium carbonate (B) at a flow rate of 300 µL/min. A linear gradient was applied to the column starting with 20 % A to 97 % A over 15 min. The column was then flushed with 97 % A for 2 min, before returning to standard conditions. Important parameters for the ESI interface were a capillary temperature at 300 °C, a sheath gas flow at 40 units, an auxiliary gas flow at 15 units, and a source voltage at 5 kV. The capillary voltage and the tube lens offset were tuned by infusion of a solution of 5 µg/mL of ergovaline (MeOH) prior to analysis.

Indole-diterpenoids

For the analysis of indole-diterpenoids an atmospheric pressure chemical ionization (APCI) interface was used and operated in the positive mode. Separation of indole diterpenoids was achieved using a 100 × 4.6 mm i.d. 2.6 µm Kinetex C_{18}-XB column (Phenomenex, Torrance, CA, USA), using a mobile phase consisting of 97.5 : 2.5 MeCN : H_2O (A) and H_2O (B), both containing

2 mM ammonium formate (in phase A this was dissolved in the water first) and 2 mM formic acid, with a flow rate of 700 µL/min. A linear gradient was applied to the column starting with 50 % to 100 % A over 12 min. The column was then flushed with A for 2 min, before returning to starting conditions. The parameters for the APCI interface were: a vaporizer temperature at 410 °C, a capillary temperature at 300 °C, a sheath gas flow at 55 units, an auxiliary gas flow at 20 units, and a corona discharge voltage of 6.0 kV. The capillary voltage and tube lens offset were tuned by infusion of a 5 µg/mL solution (MeOH) of paxilline.

The ITMS was run in the full-scan mode (m/z range for ergot alkaloids was 200-700 and m/z range for indole-diterpenoids was 350–800), and with data-dependent scanning (performing MS^2 of maximum three peaks with a threshold intensity of 10^4). The collision energy for data-dependent scanning was set to 25 units, the activation Q to 0.25, and the activation time to 30 ms.

Results from the HPLC-MS and HPLC-MS^2 analyses were processed using Xcalibur version 2.0.7 (Thermo Fisher Scientific).

2.5 Bioinformatics and statistical analyses

All sequence chromatograms were checked and corrected manually in BioEdit Alignment Editor v.7.1.3 (Hall, 1999).

Phylogenetic analysis were conducted using MEGA vs.5 (Tamura et al., 2011) on a combined ITS and *tub* dataset. The dataset, consisting of 34 samples where both ITS and *tub* sequences existed, was aligned in BioEdit. The 34 sequences represented unique as well as representatives of the common sequences within the recorded genetic groups, and there were a total of 924 positions in the final dataset. As I wanted to retain and also use the information given by the presence of indels (gaps) parsimony analyses were conducted. Indels were scored so that multi-position gaps only counted as one evolutionary event. The maximum parsimonious tree was obtained using the Tree-Bisection-Regrafting (TBR) algorithm [pg. 126 in ref (Nei and Kumar,

2000)] with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates), and with bootstrap test with 1000 replicates.

Minimum spanning (haplotype) networks were constructed separately for ITS and *tub* using Arlequin with default settings (Excoffier and Lischer, 2010), and manually drawn in Adobe Illustrator CS4.

Both the combined maximum parsimonious tree and haplotype networks contained reference sequences of the genetic groups downloaded from GeneBank.

All statistical analyses were conducted in R v. 3.0.2 (R Development Core Team, 2013) using the lattice (Sarkar, 2008), vegan (Oksanen et al., 2011) and RColorBrewer (Brewer et al., 2013) packages. See supplementary information for the R-script.

The major variance patterns in the alkaloid distributions were revealed by Principal Component Analysis (PCA). Rows of the indole-alkaloids by sample matrix contained relative concentrations where the sum of alkaloid peak areas was set to 100%, while the columns were not scaled but fourth root transformed due to large variations and zero-values present in the dataset. Procrustes tests showed high similarity between PCAs from untransformed, as well as square root and fourth root transformed columns, with the latter being chosen due to better readability. None of the ordination diagrams shown any arch effect, which is a well-known artefact of PCA ordinations of data sets with many zeros (Legendre and Gallagher, 2001)

To supplement the PCA analysis, box and whiskers plots (referred to as bwplots) were generated with focus on a selection of the ergot alkaloids that dominated in the G1 genetic group. The bwplots are similar to boxplots, but allow the conditioning for a third variable (i.e. host, genetic group and relative indole-alkaloid concentration). Similar plots for all indole-alkaloids by all genetic groups were also included as supplementary information (supplementary fig. 2).

One-way ANOVA was used to test any relationship between indole-alkaloids content in G1 and host plant identity. ANOVA was also used to test the variability in floating ability within G2.

3. Results

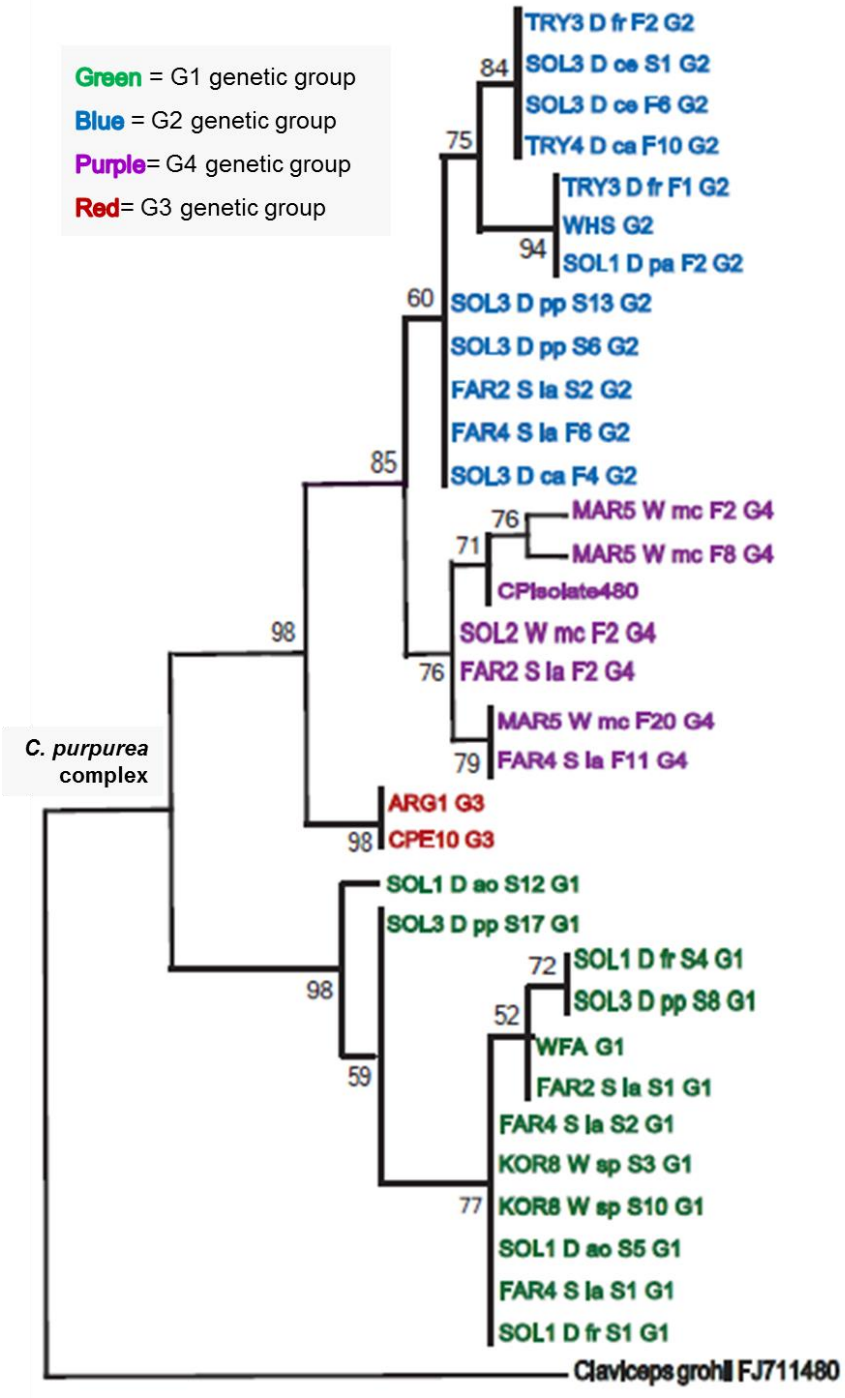
3.1 Genetic analyses

In this study 596 full-length ITS sequences were obtained from the collected sclerotia, out of which 15 unique ITS sequences were found. From a subset of 48 samples, representing the different ITS types, 40 *tub* sequences were obtained, yielding 11 unique sequences.

A phylogenetic analysis of a concatenated ITS/*tub* matrix revealed four clades representing the different main groups, G1 – G4 (fig. 5). Noteworthy, G4 has not been identified as a separate genetic group before. G1 is highly supported (98% bootstrap support) and forms a separate clade, while G2-G4 groups together in another main clade. The G3 group, represented only by two representative sequences from GeneBank, is also highly supported (98% bootstrap support). Less support was obtained for G2 and G4, which are closely related clades (fig. 5). As can be seen from the tree (fig. 5) intra-group genetic variation appeared within the four main groups. However, the chemistry data (see below) support the division of these four main groups.

Haplotype networks were constructed separately for the ITS and *tub* datasets (fig 6 a-d).

The networks depicting the distribution of genetic groups G1-G4 (fig 6 a), shows in agreement with the phylogenetic tree that the G1 group forms a separate assemblage that is separated from the G2 group by six to eight mutational steps (fig.6). Fewer mutational steps separate G2 and the G4 group, and they have one haplotype in common in the ITS network, but is well-separated in the *tub* network (fig 6a). The G3 group, represented by a GeneBank sequence, is most closely related to G2, these two being separated by four (ITS) and two (*tub*) mutational steps, respectively (fig 6a). Within each genetic group several haplotypes are found in both networks and also some reticulate patterns, possibly reflecting intra-group recombination (intra-locus) (fig. 6a). The ecological characteristics superimposed onto the networks are followed up under section 3.3.



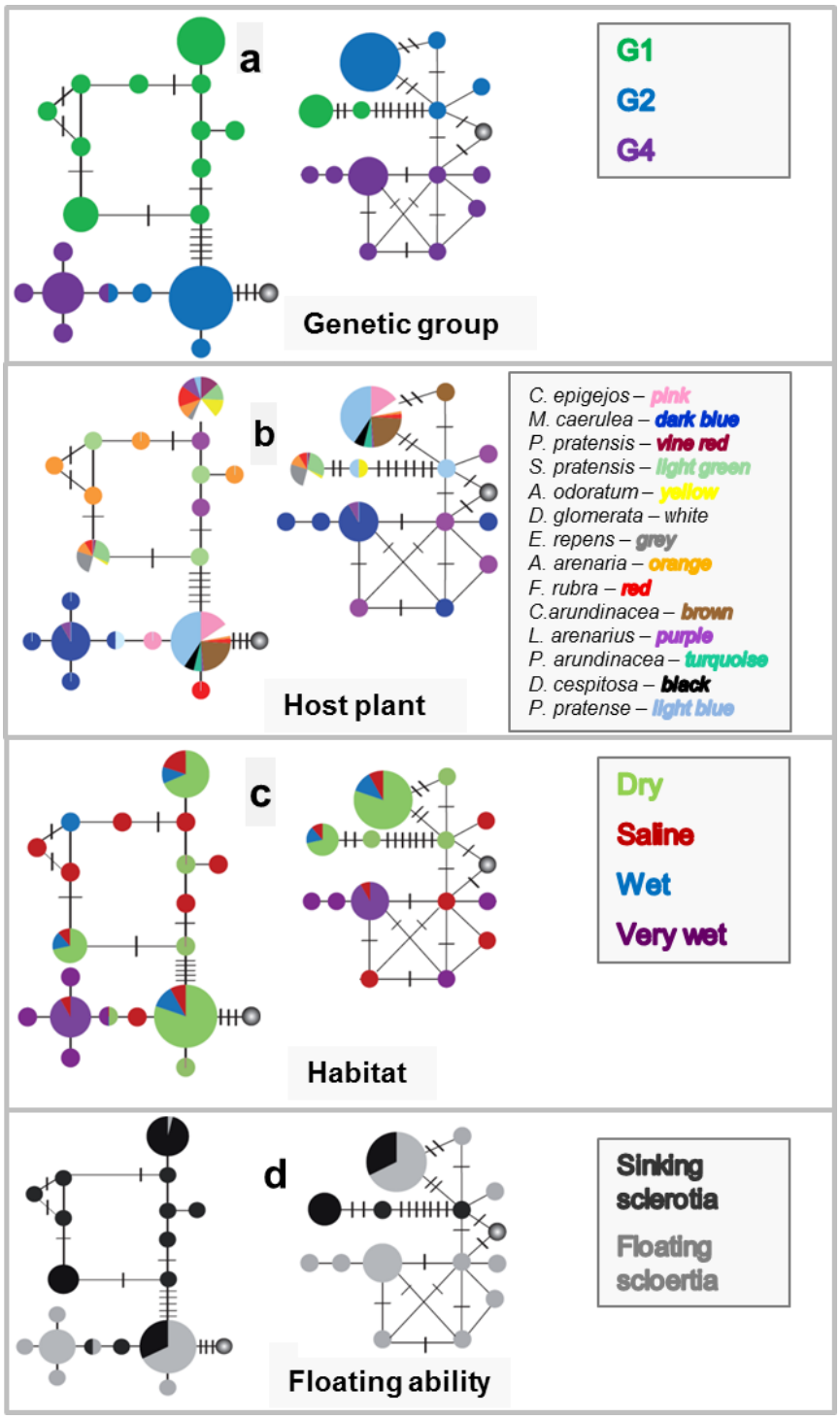
One out of two most parsimonious trees are shown (length = 59). Three clades with sequences from this study are referred to as genetic groups G1, G2 and G4. The genetic group G3 was not found in this study, but two reference sequences from GeneBank are included as references for this genetic group. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown on the branches (= bootstrap support). A reference sequence of *Claviceps grohii* from GenBank was used as outgroup. WHS, CPisolate480, ARG1, CPE10 and WFA represent reference sequences of *C. purpurea.*

Figure 5. Maximum Parsimony tree of the *C. purpurea* complex constructed from a combined ITS and *tub* matrix

Figure 6. Haplotype networks of unique ITS and *tub* sequences.

The ITS networks are placed to the left and *tub* to the right. Each circle represents one haplotype and there is one mutational step between each circle or short crossing line (representing hypothetical haplotypes). The size of the circles reflect the abundance of the haplotypes ranging from approximately N=1 to N=350. Grey shaded circle represent G3 reference sequences from GeneBank.

Different information is superimposed in the different version a-d: (a) genetic groups inferred phylogenetic analysis (cf. Fig. 5), (b) host plant, (c) habitat, and (d) floating ability.



3.2 Alkaloid profiles

Figure 8 summarises the relative amounts of ergot alkaloids (a) and indole-diterpenoids (b) in the sclerotia from G1, G2 and G4. The chromatograms from the HPLC-MS analyses (fig. 9a) demonstrate that the alkaloid profile in sclerotia of the genetic group G2 was largely dominated by ergocristine and ergocryptine. Similarly, sclerotia from the G1 group contained mostly these two ergotoxines (fig. 9a). However, the sclerotia of the G1 group contained considerably higher relative concentrations of other ergopeptines like ergotamine and ergocornine (fig. 8a). The relative concentrations of ergopeptams (ergocristam and ergosedmam) in sclerotia of the G4 group were considerably higher than in the other two (fig. 8a). Specifically, the G4 sclerotia contained ergosedmam as the principal ergot alkaloid, that has earlier been tentatively identified as lactam congener of ergosedmine, (fig. 8a) (Uhlig et al., 2011). G4 sclerotia differed further from G2 sclerotia with an evident smaller production of ergocryptine than the other two groups (fig. 8 a).

The G1 sclerotia contained only traces of indole-diterpenoids, while the indole-diterpenoid production in G2 and G4 was pronounced and mostly similar (fig 8b). The major indole-diterpenoid was a m/z 422 compound that has previously been tentaively identified as emindole SB (S. Uhlig et al, unpublished results) (fig. 8b). Furthermore, the HPLC-MS chromatograms showed the presence of two other m/z 422 compounds, of which one likely corresponds to paspaline (S. Uhlig et al, unpublished results) (fig. 9b). The remaining m/z 406 and m/z 420 indole-diterpenoid compounds are yet unidentfied congeners of paspaline and emindole SB (fig. 8b and 9b)

Representative chromatograms of the genotypes from the HPLC-MS analyses are shown in figure 9.

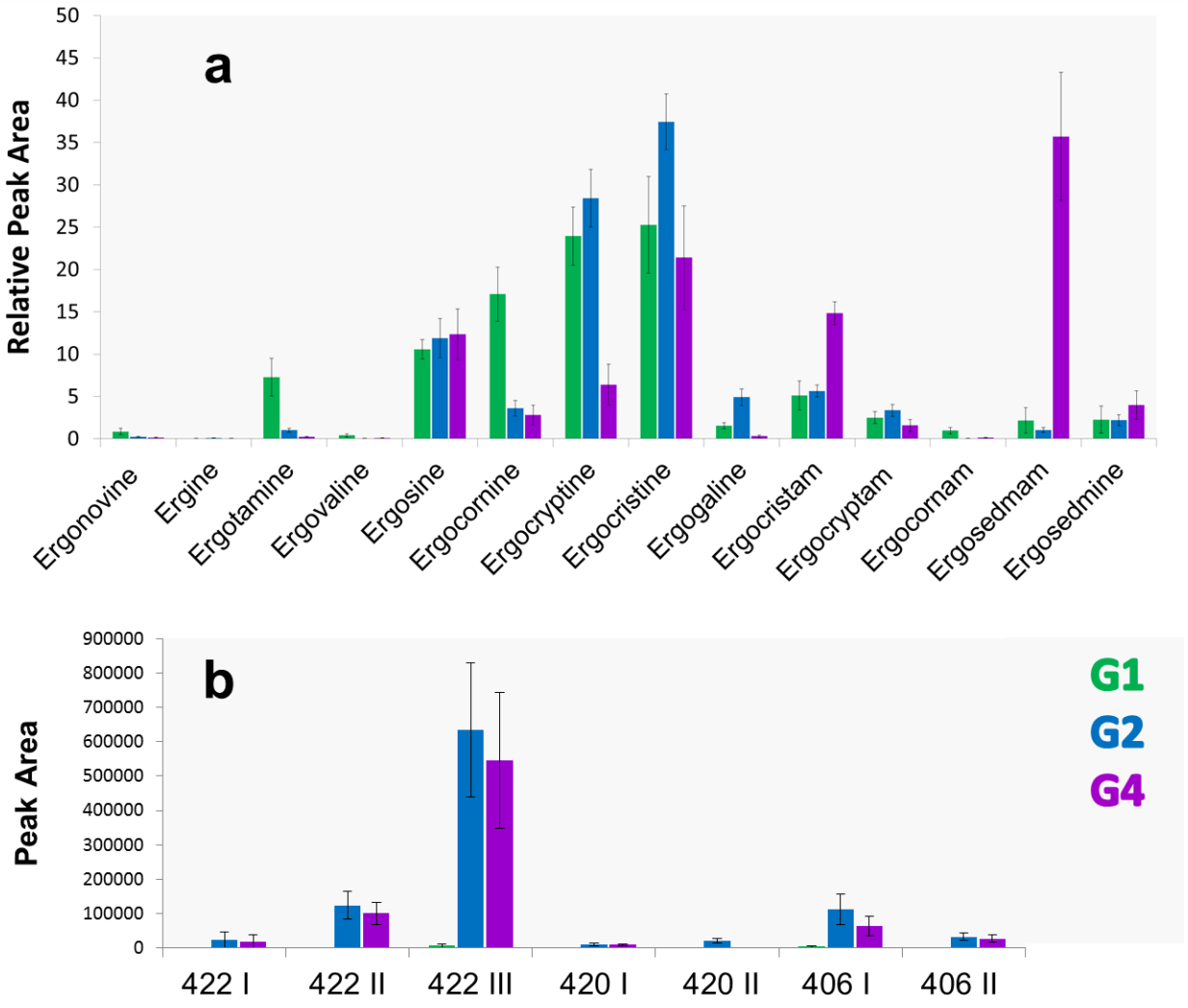


Figure 8. Indole-alkaloids

Histograms showing relative concentrations of ergot alkaloids (a) and indole-diterpenoids normalised to sclerotia weight (b).

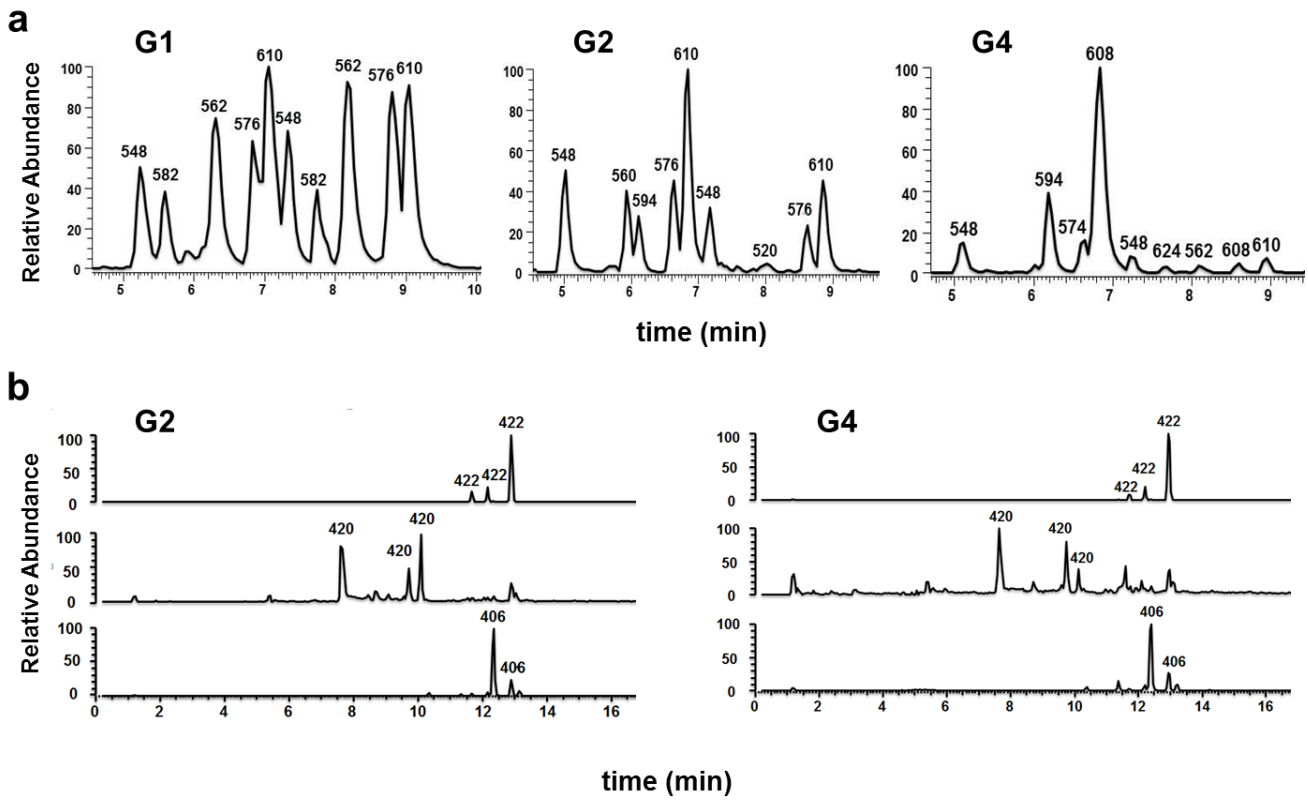


Figure 9. Representative chromatograms

Representative HPLC-MS chromatograms from extracts of sclerotia representing the *C. purpurea* genetic groups G1, G2 and G4, showing ergot alkaloids (a) and indole-diterpenoids (b). Peak labels represent the m/z of mass spectral base peaks and are: ergosine/-inine, 548; ergotamine/-aminine, 582; ergocornine/-inine, 562; ergocryptine/-inine, 576; ergocryptam, 560; ergocristam, 594; ergocristine/-inine, 610; ergosedmine/-inine, 624; ergosedmam, 608. The major m/z 422 indole-diterpenoid corresponds likely to emindole SB, while most of the other indole-diterpenoid compounds are yet unidentified.

A PCA plot, constructed based on the alkaloid profiles (fig 10a) clearly shows that the genetic groups cluster together. The PCA1 axis divides G1 from G2/G4 and PCA2 separates G2 from G4.

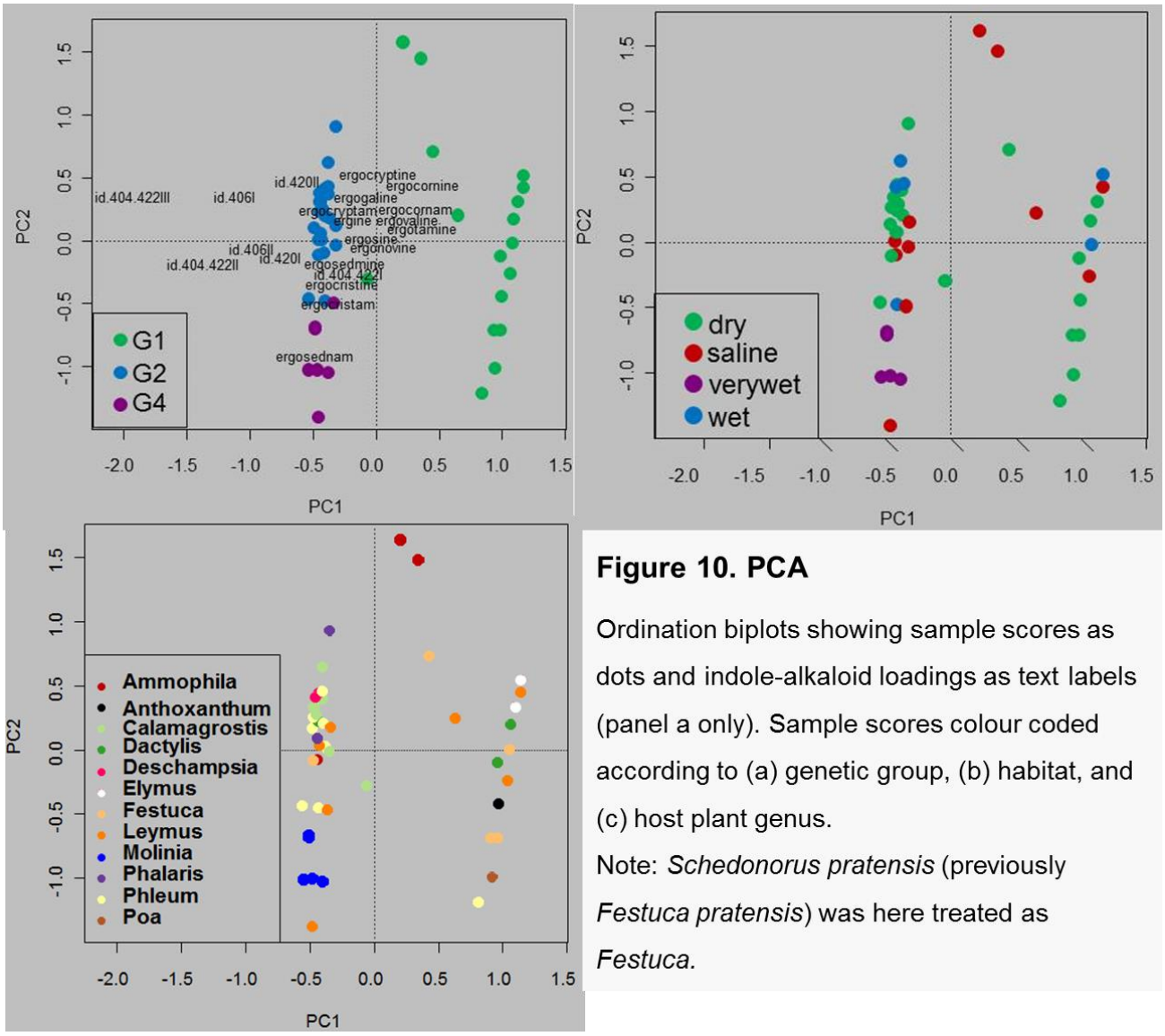


Figure 10. PCA

Ordination biplots showing sample scores as dots and indole-alkaloid loadings as text labels (panel a only). Sample scores colour coded according to (a) genetic group, (b) habitat, and (c) host plant genus.

Note: *Schedonorus pratensis* (previously *Festuca pratensis*) was here treated as *Festuca.*

3.3 Ecological characteristics

3.3.1 Host

Host preference to genotype

The haplotype networks (fig 6 b) illustrates that there was some difference in host affinity between the three genetic groups (fig.6 b). In my study, G1 was associated with *Poa pratensis*, *Schedonorus pratensis*, *Anthoxanthum odoratum*, *Dactylis glomerata*, *Elymus repens*, *Ammophila arenaria, Festuca rubra, Leymus arenarius and Phleum pratense,* while G2 was associated with *Calamagrostis epigejos, D. glomerata*, *A. arenaria, F. rubra, Calamagrostis arundinacea*, *L. arenarius, Phalaris arundinacea, Deschampsia cespitosa and P. pratense.* Hence, the plant species; *P. pratensis*, *S. pratensis*, *A. odoratum* hosted exclusively the genetic group G1,while the plant species *C. arundinacea, D. cespitosa* and *P. arundinacea* hosted exclusively the genetic group G2. On the other hand, G4 was only found on two host plant species. Most importantly, *Molinia caerulea* hosted G4 from all locations where this grass species occurred, and G4 appeared in a few cases also on *L. arenarius* that additionally hosted both G1 and G2.

Co-occurrence

At several locations and hosts the sampled sclerotia belonged not only to a single genetic group, but to two or three (table 2). Even on the same host plant different genetic groups were detected (table 2). Noteworthy, during both seasons of this study (2011 and 2012), the three genetic groups G1, G2 and G4 were present on the same host plant *L. arenarius* in the saline location at Farsund in South Norway, demonstrating sympatry in both space and time.

Table 2. Co-occurrence of genetic groups

Summarised co-occurrences of G1, G2 and G4 on the same host plants within the same habitat.

Season	Genetic group	Host	Location	Habitat
2011	G1/G2	*D. glomerata*	Maridalen	dry
2011	G1/G2	*A. arenaria*	Jæren	saline
2012	G1/G2	*P. Pratense*	Solvik	dry
2011	G1/G2/G4	*L. arenarius*	Farsund	saline
2012	G1/G2/G4	*L. arenarius*	Farsund	saline

Alkaloids vs. host plant

The PCA plot could not clearly identify any effect of the host plant on the alkaloid profiles (fig. 10c). However, the plot showed in a few cases a tendency of clustering alkaloids by some of the host plants. The alkaloid profiles from *Calamagrostis* (*C. arundinacea* and *C. epigejos)* clustered together, as did the five alkaloid profiles from *Molinia* (*M. caerulea*).

Bwplots generated for a selection of seven indole-alkaloids dominant for the G1 group (fig. 11) show some correlation between host and alkaloid composition. However, the bwplots also show substantial differences in the composition of the alkaloid mixture in sclerotia from the same genetic group when it parasitizes different grass species. In a few cases, certain ergot alkaloids and/or indole-diterpenoids were even missing in G1 sclerotia from some hosts while they were present in G1 sclerotia from other. The most prominent examples are shown in Figure 11. Here, ergonovine was produced in G1 sclerotia from *D. glomerata, F. rubra* and *A. odoratum*, and present only in trace amounts in G1 sclerotia from all other hosts. Ergocornine was largely present in all G1 sclerotia with lesser amounts in those that originated from *F. rubra, P. pratensis, P. pratense and A. odoratum*. Ergotamine was also detected in all G1 sclerotia, though to a lesser extent than ergocornine, and mostly in sclerotia from on *A. arenaria* and *L.*

arenarius. Ergovaline was found in highest quantities in sclerotia found on *A. arenaria* in a saline habitat, while only present in trace amounts in G1 sclerotia from other hosts. The same picture applied for ergocornam. Thus, *A. arenaria* seemed to have a large quantitative impact on the production of certain ergot alkaloids, and perhaps also on the composition. This was also the case for some of the indole-diterpenoids (m/z 420 II and m/z 406 I).

No significant effect of host plant genus on content of ergot alkaloids and indole-diterpenoids was observed (ANOVA, $p<0.05$) However, when the host genus *Ammophila* was selected out for separate analyses, a significant differences in the ergot alkaloids ergovaline ($p = 0.006$) and ergocornam ($p = 0.0008$), and for the indole- diterpenoids with m/z 420 II ($p = 0.004$) and 406 I ($p = 0.058$), were observed.

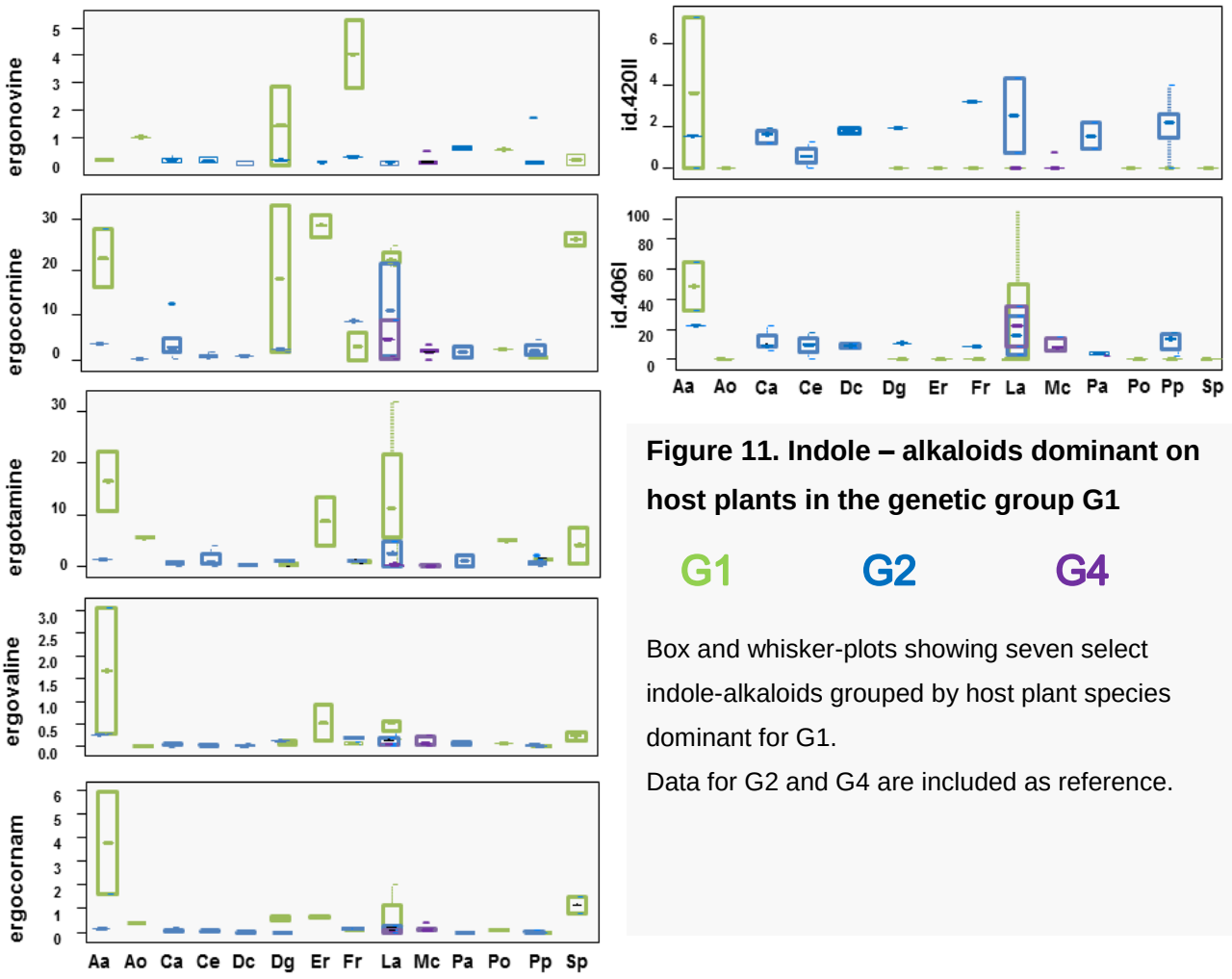


Figure 11. Indole – alkaloids dominant on host plants in the genetic group G1

G1 G2 G4

Box and whisker-plots showing seven select indole-alkaloids grouped by host plant species dominant for G1.
Data for G2 and G4 are included as reference.

3.3.2 Habitat

The relationship between habitat and genetic groups were visualised by superimposing this information onto the haplotype networks (fig. 6 c). In the networks the different habitat types were distributed in a rather chaotic manner, reflecting little of the previously described preference of G1 for dry habitats, and of G2 for wet habitats. The G1 samples in this study were mostly collected in dry habitats, but also a large portion originated from saline and wet habitats. The G2 samples were also represented by a larger amount originating from dry habitats (fig. 6 c). The genetic group G4 was more homogenous in that respect, occurring mostly in very wet habitats and occasionally in saline environments. The habitat affiliations of the genetic groups are also visualized in a mosaicplot, which more clearly shows the similarity in the habitat distribution of G1 and G2 (fig. 12).

Likewise, in the PCA plot, there is no clear clustering of habitat types according to alkaloid profiles. The exception is again the very wet habitat (fig. 10 b), which is associated with the genetic group G4.

3.3.3 Floating ability

As shown in table 3 and the haplotype network (fig. 6 d), there was a clear correspondence between the ability to float and genetic affiliation; sclerotia from G1 generally sank, while the sclerotia from G4 were consistently floating (table 3). The sclerotia from the G2 group were more variable in their ability to float. Most G2 sclerotia showed the ability to float, while a large portion also sank. However, the ability of the G2 sclerotia to either float or sink was not significantly related to host ($p = 0.64$) or habitat ($p = 0.062$).

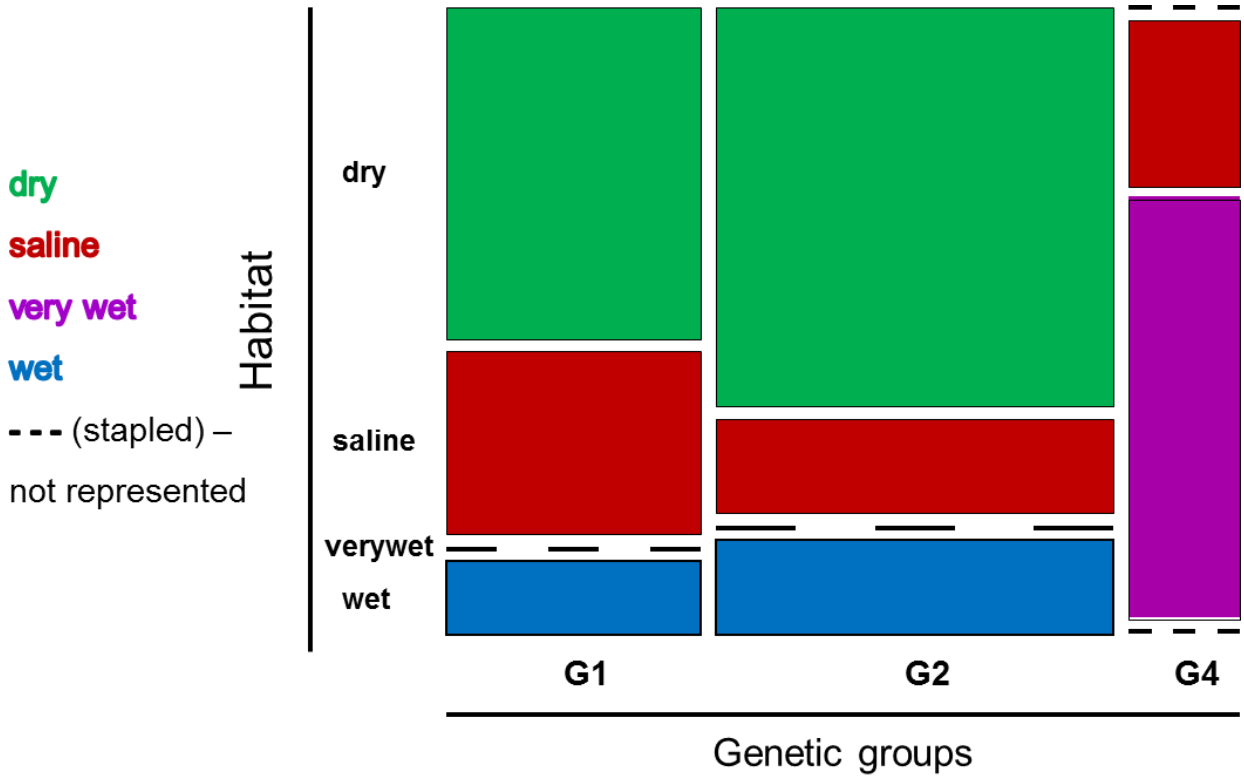


Figure 12. Mosaic plot showing the distribution of the habitats across genetic groups

The genetic group G4 prevalence in very wet habitats, and its absence in dry habitats, is clearly evident.

Table 3. Floating ability

Overview of the floating ability to the sclerotia.

Season	Genetic group	Location	Plot	Habitat	Host	Sinking	Floating
2011	G2	Maridalen	1	dry	*Phleum pratense*	0	12
2011	G2	Maridalen	2	dry	*Phleum pratense*	4	12
2011	G1	Maridalen	3	dry	*Schedonorus pratensis*	13	0
2011	G1	Maridalen	3	dry	*Dactylis glomerata*	5	0
2011	G2	Maridalen	3	dry	*Dactylis glomerata*	5	18
2011	G1	Maridalen	3	dry	*Elymus repens*	8	0
2011	G2	Tryvann	1	dry	*Phleum pratense*	20	20
2011	G2	Tryvann	2	dry	*Phleum pratense*	16	20
2011	G4	Korsvoll	1	very wet	*Molinia caerulea*	0	8
2011	G4	Korsvoll	2	very wet	*Molinia caerulea*	0	20
2011	G4	Korsvoll	3	very wet	*Molinia caerulea*	0	15
2011	G2	Korsvoll	4	wet	*Phleum pratense*	0	5
2011	G1	Jæren	1	saline	*Ammophila arenaria*	8	0
2011	G2	Jæren	1	saline	*Ammophila arenaria*	2	3
2011	G1	Jæren	2	saline	*Ammophila arenaria*	10	0
2011	G1	Farsund	1	saline	*Leymus arenarius*	3	1
2011	G1	Farsund	2	saline	*Leymus arenarius*	2	0
2011	G2	Farsund	2	saline	*Leymus arenarius*	1	0
2011	G4	Farsund	2	saline	*Leymus arenarius*	0	2
2011	G2	Farsund	3	saline	*Calamagrostis epigejos*	20	0
2012	G2	Solvik	1	dry	*Phalaris arundinacea*	0	15
2012	G1	Solvik	1	dry	*Dactylis glomerata*	20	0
2012	G1	Solvik	1	dry	*festuca rubra*	20	0
2012	G1	Solvik	1	dry	*Poa pratensis*	15	0
2012	G1	Solvik	1	dry	*Anthoxanthum odoratum*	15	0
2012	G4	Solvik	2	very wet	*Molinia caerulea*	0	20
2012	G2	Solvik	3	dry	*Calamagrostis arundinacea*	14	15
2012	G1	Solvik	3	dry	*Phleum pratense*	5	0
2012	G2	Solvik	3	dry	*Phleum pratense*	14	12
2012	G2	Solvik	3	dry	*Calamagrostis epigejos*	15	19
2012	G2	Maridalen	4	dry	*Calamagrostis arundinacea*	0	20
2012	G4	Maridalen	5	very wet	*Molinia caerulea*	0	20
2012	G2	Korsvoll	5	wet	*Calamagrostis arundinacea*	0	15
2012	G2	Korsvoll	5	wet	*deschampsia cespitosa*	6	5
2012	G1	Korsvoll	6	wet	*Elymus repens*	6	0
2012	G2	Korsvoll	7	wet	*Phleum pratense*	0	10
2012	G1	Korsvoll	8	wet	*Schedonorus pratensis*	14	0
2012	G2	Tryvann	3	dry	*deschampsia cespitosa*	0	8
2012	G2	Tryvann	3	dry	*festuca rubra*	0	10
2012	G2	Tryvann	4	dry	*Calamagrostis arundinacea*	0	15
2012	G1	Farsund	4	saline	*Leymus arenarius*	4	3
2012	G2	Farsund	4	saline	*Leymus arenarius*	0	3
2012	G4	Farsund	4	saline	*Leymus arenarius*	0	5

4. Discussion

In this study, three genetic groups G1, G2 and G4 were detected in Norway, and a clear link between alkaloid profiles obtained from the sclerotia and phylogenetic affiliation was observed. The genetic group G4 tentatively represents a new 'cryptic species' within the *C. purpurea* complex. The G4 group was predominantly associated with *Molinia caerulea* that is often found as the sole grass species in very wet habitats in Norway. The indole-diterpenoid profile obtained from sclerotia of the G4 group largely resembled G2. However, the ergot alkaloid profile of G4 sclerotia was unique due to a high presence of lactam ergot alkaloids. In both the G2 and G4 groups, several unidentified indole-diterpenoids were discovered. Moreover, a clear difference in the ability to float was observed in the genetic groups G1 and G4. The following text will discuss the main findings of my study.

4.1 Genetic diversity of *C. purpurea* in Norway

According to the literature, three different genotypes (G1-G3) exist in the *C. purpurea* complex (Douhan et al., 2008; Pazoutova et al., 2002a; Pazoutova et al., 2000). In my work they are referred to as "genetic groups", as genetic variation was observed within each group (see below). Briefly, while the G1 group is linked to dry habitat and contain the ergot alkaloids subgroups ergotamines and ergotoxines, the G2 group has been linked to wet and shady habitats and produce ergosine, ergocristine, and ergocryptine (Pazoutova et al., 2000). The G3 group, containing ergocryptine and ergocristine, is more specialised, occurring only on the host genera *Spartina* and *Distichlis* that is found in salt-marsh habitats (Fisher et al., 2005a; Pazoutova et al., 2000; Pazoutova et al., 2002b).

The groups G1 and G2 were detected in this study. In addition, a new genetic group was detected which is referred to as G4. The absence of the G3 group is not surprising since the known hosts of G3 is missing from Northern Europe (Nehring et al., 2012) and the saline habitats visited during this study are far less extreme than the salt marches where G3 has been

found in (Fisher et al., 2005a; Fisher et al., 2005b; Pazoutova et al., 2000; Pazoutova et al., 2002b).

Although additional genetic data would have been preferable, both the multi-locus phylogeny, as well as the haplotype networks support that G4 makes up a separate well-defined group. The only conflicting result is that G4 and G2 share one haplotype, but some shared genetic variation is expected in recently diverged lineages due to e.g. incomplete lineage sorting. The chemical (alkaloid profile) and partly the ecological data support that G4 is a recently evolved, but unique group of *C. purpurea*. G4 is characterised by its preference of *Molinia caerulea* which grows in very wet habitats, and its characteristic alkaloid profile that differ from G2 in the production of ergosedmam (see below).

If G1-G4 belongs to the same biological species, more introgression and recombination, resulting in higher levels of incongruences in the phylogenetic analyses, would have been expected. Hence, I hypothesise that all four genetic groups represent different biological species, as has also been suggested for G1-G3 earlier (Nehring et al., 2012). So-called 'cryptic species', as G1 – G4 seem to represent, is commonly observed in fungal morphotaxa (Dettman et al., 2006).

4.2 Indole – alkaloid profiles of the *C. purpurea* genetic groups

The results presented here give strong support for the alkaloid chemistry being a function of the genetic groups.

Several of the findings in this study regarding the composition of the ergot alkaloid profile in *C. purpurea* sclerotia were in agreement with earlier results. In agreement with Pazoutova et al (2000), ergocristine, ergocryptine and ergosine were the major ergopeptines in G2 sclerotia. However, in the present study the three analogues were present in the order ergocristine>ergocryptine>ergosine, and not ergocristine>ergosine>ergocryptine as reported by Pazoutova et al (2000). According to earlier reports, sclerotia of the G1 genetic group contain mixtures of ergotamines (e.g. ergotamine, ergosine, ergovaline) and ergotoxines (e.g.

ergocristine, α/β-ergocryptine, ergocornine, ergogaline). This is to some degree in accordance with the results of my study, i.e. the relative occurrence of ergotamines and ergotoxines other than ergocristine, ergosine and ergocryptine was significantly higher than in sclerotia of the G2 genetic group. Still, ergocristine and ergocryptine were the dominant ergopeptines in G1 sclerotia. Also, even though the ergot alkaloid profile of G4 sclerotia differed strongly from that of G1 and G2 sclerotia in that it contained mostly lactam ergot alkaloids, the major ergopeptine in these sclerotia was still ergocristine. This is unexpected since G4 sclerotia apparently contained the lactam congener of ergosedmine ("ergosedmam") as the principal ergot alkaloid. It could therefore be expected that the major ergopeptine in these sclerotia is ergosedmine. However, G4 sclerotia were found to contain only minor amounts of this analogue. Previous studies also showed that sclerotia from the G3 genetic group contained ergocristine as one of the major ergot alkaloids (Pazoutova et al., 2002a; Pazoutova et al., 2000).

The G2 and G3 genetic groups have been defined as chemoraces by producing solely ergosine and ergocristine (G2), and ergocristine and ergocryptine (G3). This is not supported in my study as G2 sclerotia contained several other ergopeptines, although in lower relative concentrations than sclerotia from the G1 genetic group.

The indole-diterpenoids is a group of alkaloids that has not received much attention in *C. purpurea*, though more research has been carried out on indole-diterpenoids from other *Claviceps* species and the sister genera *Neotyphodium* and *Epichloe* ((Cawdell-Smith et al., 2010; Imlach et al., 2008; Saikia et al., 2008; Uhlig et al., 2009). Previously, it has been shown that several of these are potentially tremorgenic as a result of inhibition of BK channels in mammals and insects (Imlach et al., 2008; Knaus et al., 1994). The present study showed that the G1 group produce limited indole-diterpenoids compared to G2 and G4. While previous work (Uhlig et al., unpublished results) has tentatively identified the major m/z 422 indole-diterpenoid as emindole SB, and another m/z 422 indole-diterpenoid as paspaline, all other indole-diterpenoid structures remain unknown. Emindole SB and paspaline are early biosynthetic precursors of many known indole-diterpenoids, and their presence in *C. purpurea* indicates the expression of

genes necessary for indole-diterpenoid production. However, biosynthetically more complex indole-diterpenoids, e.g. paspalitrems, have not yet been detected in *C. purpurea.*

Pazoutova et al (2002a; 2000) hypothesised that the alkaloid profiles are not influenced by the host plant, though they can influence the ratio of the various kinds of alkaloids. My results merely support this view, as there were no clear association between the overall alkaloid profiles and host plant. Likewise, no clear correlation between the alkaloid profiles and host plants were observed in this study. However, exceptions were observed. Most noteworthy was that G1 sclerotia of the saline adapted grass *A. arenaria* differed significantly from G1 sclerotia from other grass species for the presence in several indole-alkaloids. The indole-alkaloid composition and quantity could for this specific case seem to depend on the host plant, but the saline habitat or other factors could be involved, preventing clear conclusions.

The consistent presence of indole-diterpenoids in sclerotia from the G2 and G4 genetic groups, but only traces of these compounds in sclerotia from the G1 genetic group show that these are suitable as chemical markers to distinguish G1 from the other two genetic groups. At the same time, dominance of ergosedmam is a strong marker for the G4 genetic group. Thus, the profiles of indole-alkaloids in *C. purpurea* sclerotia can be used to assign them to a specific genetic group, i.e. G1, G2 or G4 (fig. 13).

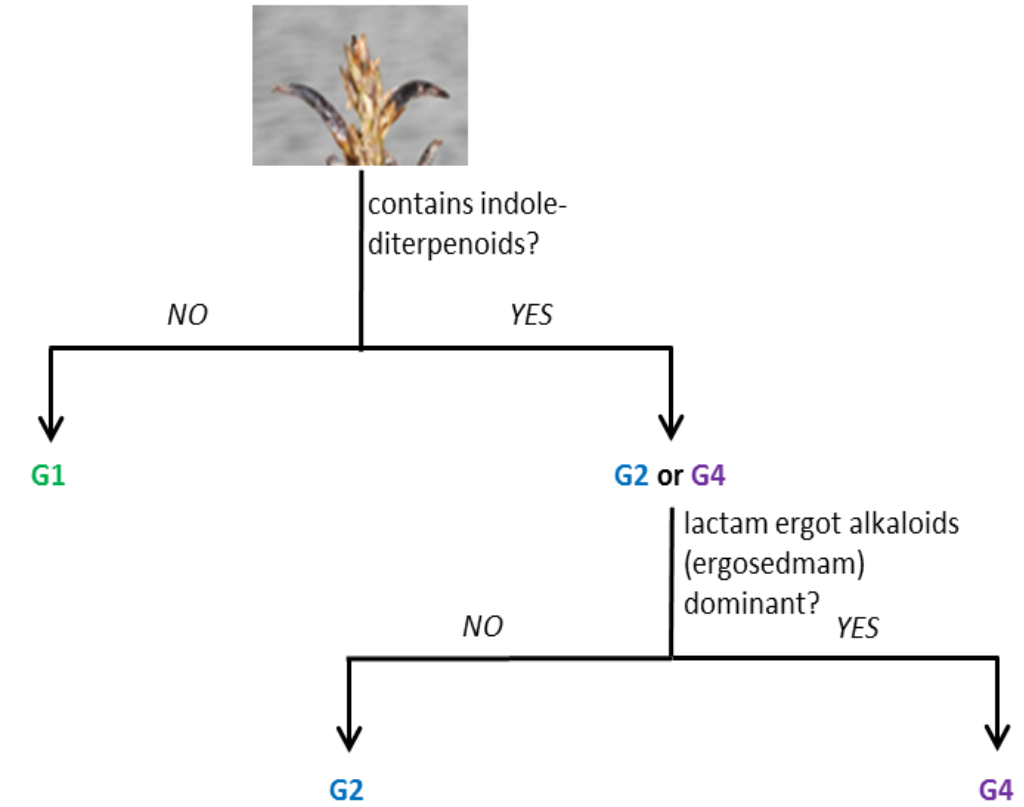


Figure 13. Scheme for affiliation of *C. purpurea* sclerotia to genetic groups based on chemical traits

4.3 Host specificity of *Claviceps purpurea*

Pazoutova et al (2002a; 2000) suggested that the genetic groups G1-G3 possessed variable degrees of host specialisation. In these studies they found that the plant species *A. arenaria, Dactylis* sp*, F. rubra* and *Phleum* sp. were common hosts both for G1 and G2, while *Calamagrostis* and *Phalarodies* hosted only G2. Moreover, G3 has been recorded only on the genera *Spartina* and *Distichlis* (Fisher et al., 2005b; Pazoutova et al., 2000; Pazoutova et al., 2002b). My results are to some extent corresponding with these earlier observations, since G1 and G2 were detected on numerous hosts, indicating low degree of host specialisation. G4, on the other hand, was mainly associated with *Molinia caerulea*. However, a few G4 samples were also found on *L. arenarius*, which also hosted G1 and G2. The data accumulated so far suggests that G3 and G4 are more host specific compared to G1 and G2.

Host specialisation is often seen in biotrophic parasitic fungi such as rusts and smuts due to the evolutionary arm-race between host and parasite. I may therefore speculate that G3 and G4 have more parasitic nature than G1 and G2, which could to a larger extent have some beneficial role for their host plants, which has been suggested for *C. purpurea* (Lane et al., 2000; Wäli et al., 2013). However, experimental studies are needed to obtain a deeper understanding on this topic.

4.4 Habitat preferences and floating ability

This study demonstrates that the different genetic groups of *C. purpurea* can co-occur within one habitat and even on one single host plant. For example, *Leymus arenarius* hosted all three genetic groups in one location. There were also several examples of co-occurrences of G1/G2 on various host plants and habitats. This supports that a completed speciation has happened, since genetic recombination otherwise would be expected. The co-occurrences of the different genetic groups in the same habitats and on the same host plants also indicate that interspecific competition might occur, especially between G1 and G2. Experimental studies are also needed here to confirm this hypothesis.

Previously a correlation between genetic affiliation and habitat preference has been observed (Pazoutova et al., 2002a; Pazoutova et al., 2000). G1 was in these studies linked to dry open fields, G2 to wet and shady forests and G3 to saline coastal marshes. In opposition to these studies, my results do not provide a clear link between genetic affiliation and habitat preference. Although speculative, this may partly be due to the spatial structure and high heterogeneity of the habitats in Norway. In some of the areas investigated, there is a mix of dry and wet areas. In contrast, *M. caerulea* that hosted G4 was consistently found as the sole grass species in very wet habitats, such as the waterfront of lakes and rivers, or in boreal forest swamps and marches where the *M. caerulea* roots are usually totally submerged in water. In such respect, this habitat is more extreme than the general “wet and shady” habitat preferred by G2 (Pazoutova et al., 2002a; Pazoutova et al., 2000).

A clear correlation between the sclerotia ability to float and phylogenetic affiliation were observed, where all G4 sclerotia floated and 97% of the G1 sclerotia sank. The different levels of floating are caused by differences in the intercellular spaces (Duncan et al., 2002). The floating ability of G4 is clearly an adaptation to water-dispersal while this adaptation is not necessary in G1, which mainly is found in drier habitats. A high variation in floating ability was observed in G2, even of the sclerotia collected from different specimens of the same host plant. But, overall there was a far more even distribution of floating (62%) and sinking (38%) sclerotia in G2, compared to G1 and G4. This could be because G2 is adapted to mixed habitats in Norway. In both seasons of the study (2011 and 2012), neither plant species nor habitat was decisive for the floating ability of G2 sclerotia. More experimental work is needed to better understand the complex floating pattern within G2.

4.5 Concluding remarks

Taken together, the different types of data (genetic, chemical and ecological) suggest that the different genetic groups detected in *C. purpurea* (G1-G4) correspond to four different biological species. However, data from additional genetic loci (such as genome wide population genetic markers), more host plants and a wider geographic area, are needed to finally conclude on this topic.

The results obtained in this study calls for various experimental studies to e.g. reveal the effects and functions of the different detected indole-alkaloids and further, to analyse the degree of host specialisation and ecology of the different genetic groups. The *C. purpurea* complex stands out as a very rewarding study system for evolutionary studies on speciation and adaptation. It is also a source of unexplored metabolites for further potential toxicological and/or medical interest.

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Supplementary

Graphs, figures and tables

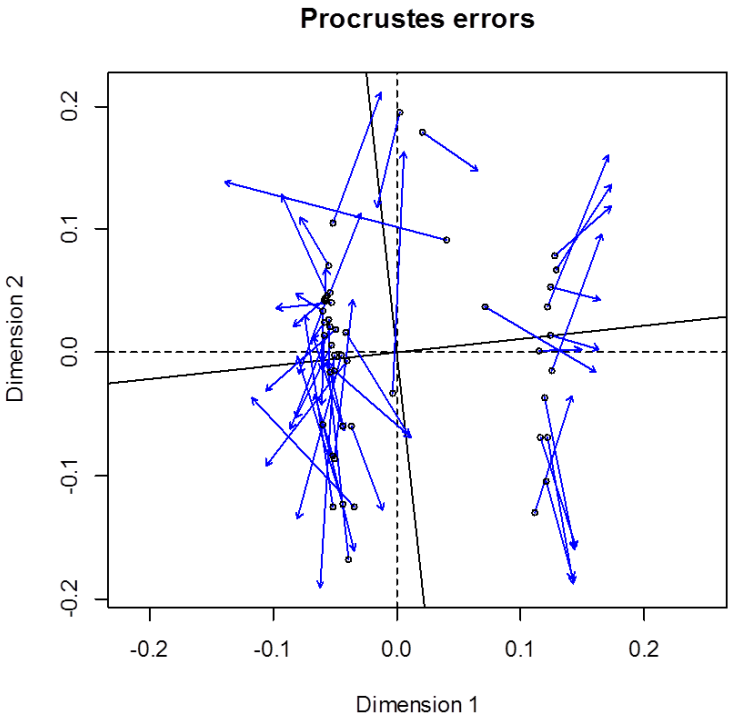
Supplementary table 1. Overview of mass peaks and retention times for the ergot alkaloids

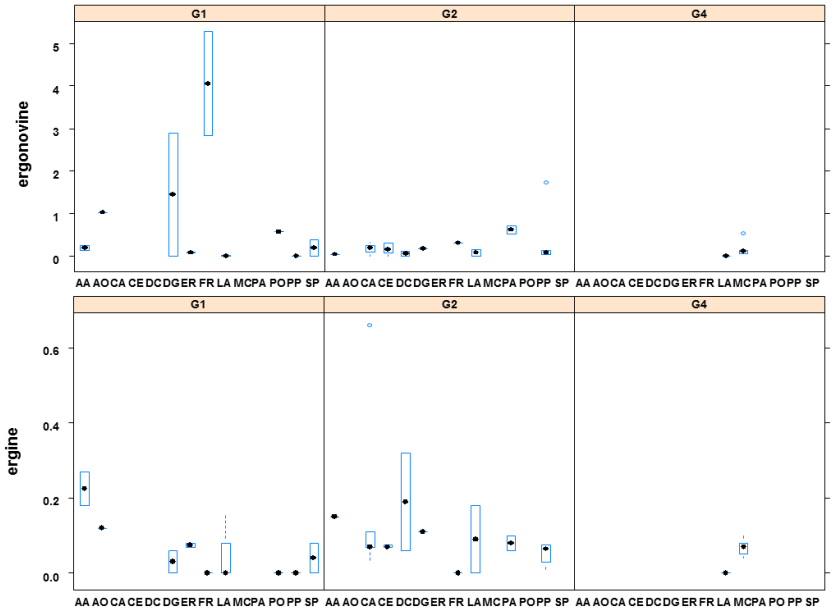
Also shown are the amino acids the ergot alkaloids fragments to.

	m/z	RT	Fragments to amino acid	
Simple lysergic derivatives				
Ergonovine /-inine	326.4	1.0/2.3		
Ergine /-inine	268.5	1.9/0.9		
Ergotamines				
Ergotamine/ -inine	582.6	5.5/7.7	m/z 320	alanine
Ergovaline/ -inine	534.5	4.4/6.6		
Ergosine/ -inine	548.6/548.5	5.1/7.3		
Ergotoxines				
ergocornine/ -inine	562.5	6.1/8.1	m/z 348	valine
Ergocryptine/ -inine	576.6	6.7/8.7		
Ergocrystine/ -inine	610,6/610,5	6.9/9.0		
Ergogaline/ -inine	590.5	7.3/9.3		
Ergotoxams				
Ergocristam	594.5	6.2		
Ergocryptam	560.5	5.7/6.0		
Ergocornam	546.5	5.1		
Ergosednam	608.5	6.8		
Ergoannines				
Ergosedmine	624.5	7.8/10.6	m/z 362	isoleucine

Supplementary figure 1. Procrustes error

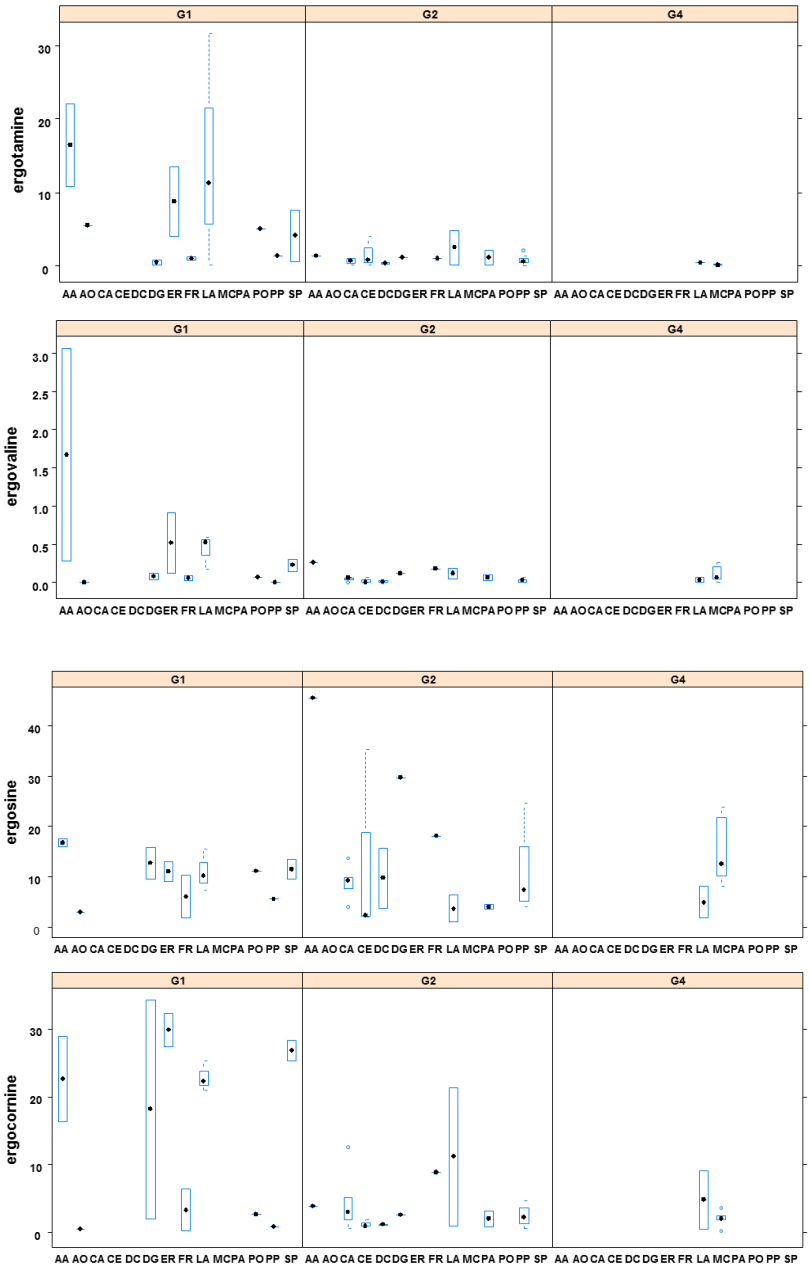
The effects of the transformations on the positions of the indole-alkaloid loadings in the PCA analysis.

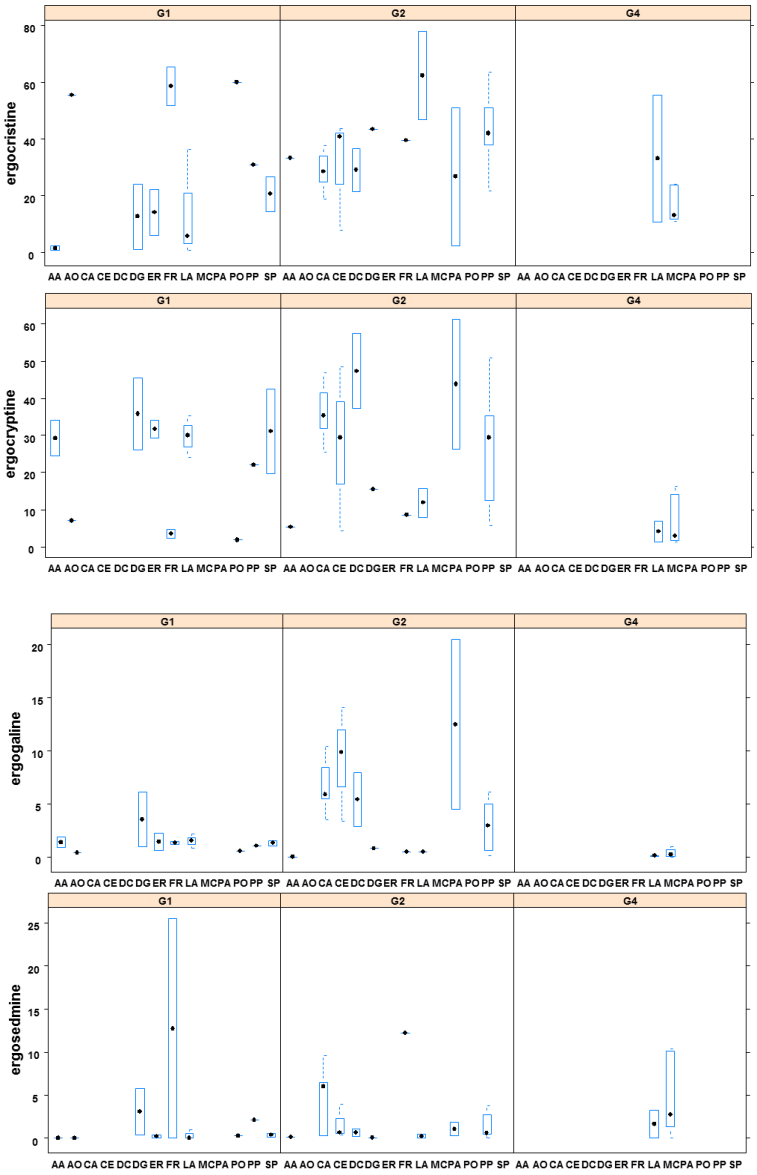


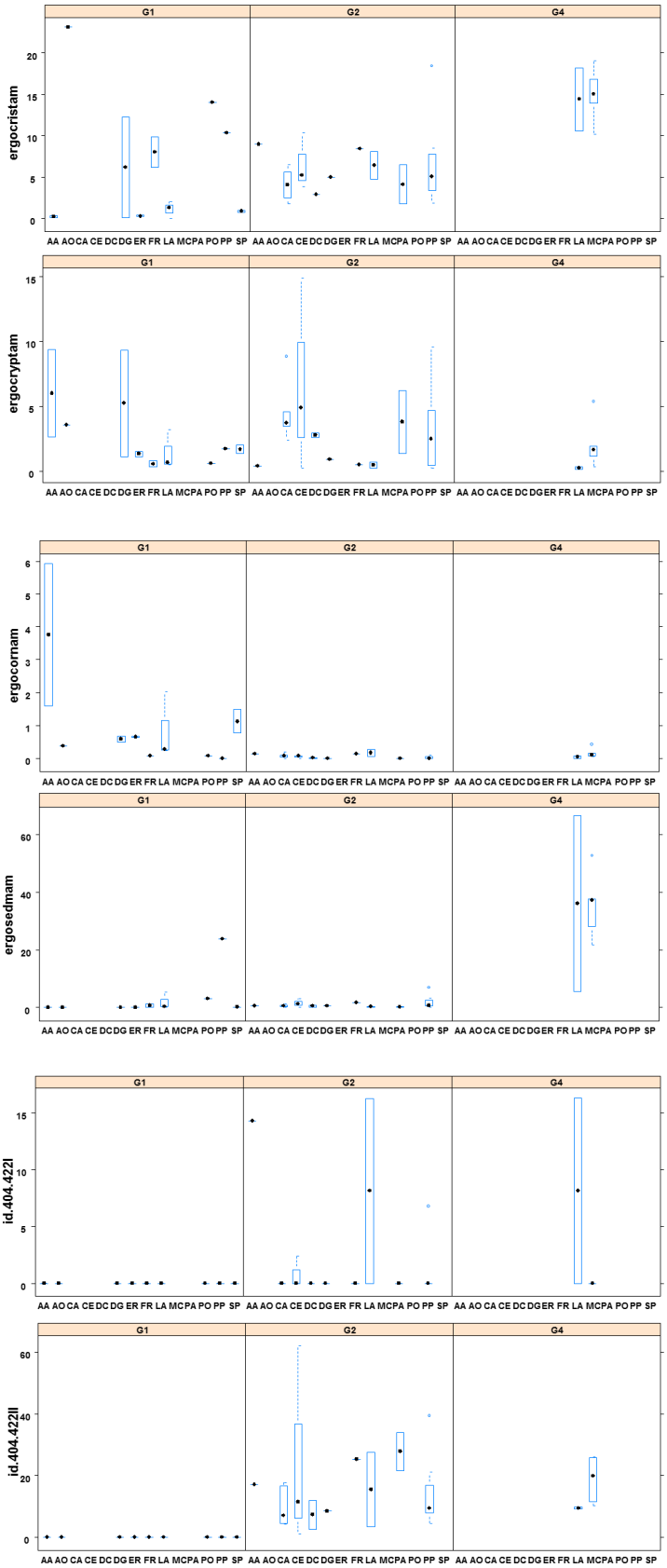


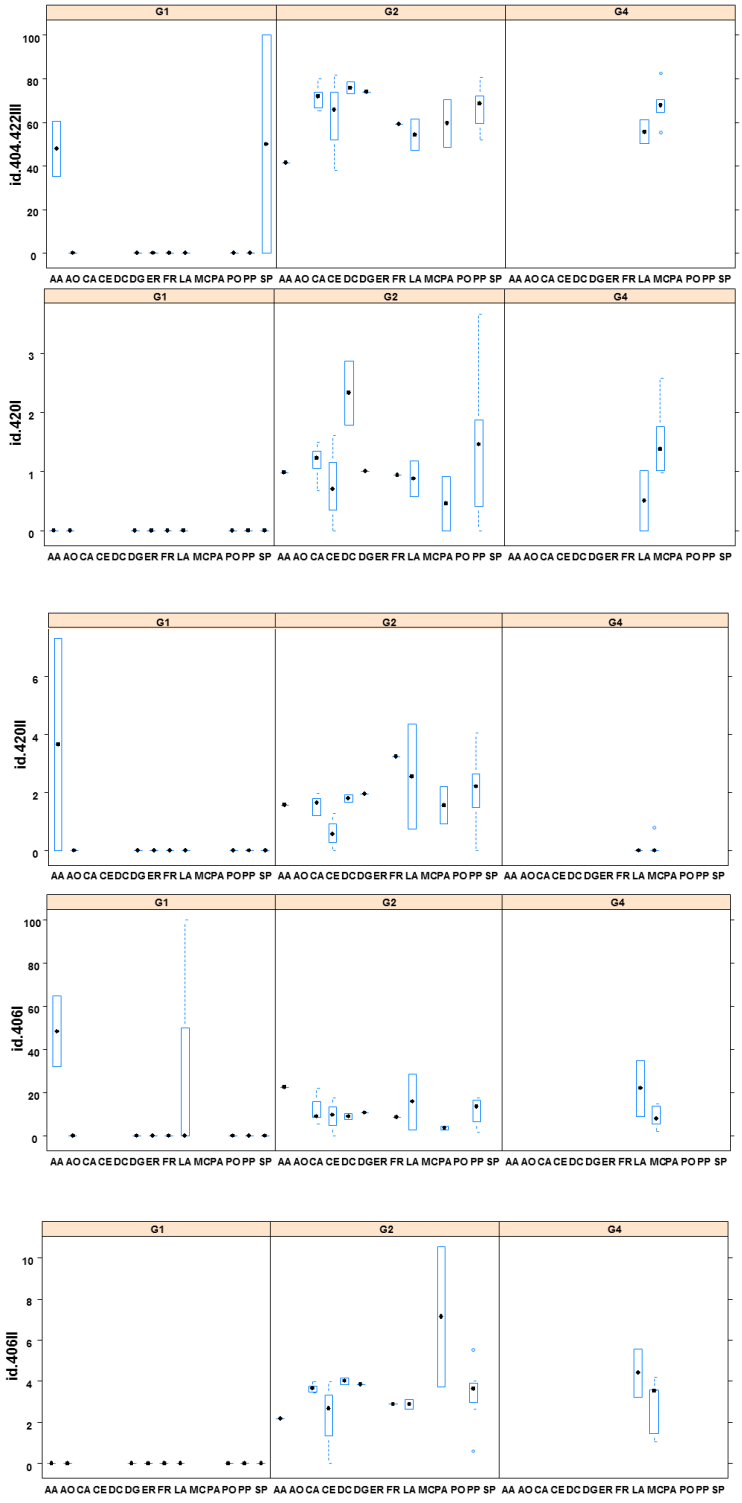
Supplementary figure 2. Complete bwplots

The original bwplots for all ergot-alkaloids and indole-diterpenoids related to genetic group and host species. The plots were generated in R, of which a selection was presented in figure 11.


G1
G2
G4
ergotamine
ergovaline
ergosine
ergocornine
AA AO CA CE DC DG ER FR LA MCPA PO PP SP


G1
G2
G4
ergocristine
ergocryptine
ergogaline
ergosedmine
AA AO CA CE DC DG ER FR LA MCPA PO PP SP


G1
G2
G4
ergocristam
ergocryptam
ergocornam
ergosedmam
id.404.422I
id.404.422II
AA AO CA CE DC DG ER FR LA MCPA PO PP SP


G1
G2
G4
id.404.422III
id.420I
id.420II
id.406I
id.406II
AA AO CA CE DC DG ER FR LA MCPA PO PP SP

The R script

```
# writing in the dataset and starting a script
d <- read.table("….txt", header = TRUE)

#loading in the package lattice
library(lattice)

# Indole-alkaloids plotted by host and
#genetic group generating the bwplots
#The ergot alkaloids
bwplot ( ergonovine     ~ host | genotype, data = d)
bwplot ( ergine         ~ host | genotype, data = d)
bwplot ( ergotamine     ~ host | genotype, data = d)
bwplot ( ergovaline     ~ host | genotype, data = d)
bwplot ( ergosine       ~ host | genotype, data = d)
bwplot ( ergocornine    ~ host | genotype, data = d)
bwplot ( ergocristine   ~ host | genotype, data = d)
bwplot ( ergocryptine   ~ host | genotype, data = d)
bwplot ( ergogaline     ~ host | genotype, data = d)
bwplot ( ergosedmine    ~ host | genotype, data = d)
bwplot ( ergocristam    ~ host | genotype, data = d)
bwplot ( ergocryptam    ~ host | genotype, data = d)
bwplot ( ergocornam     ~ host | genotype, data = d)
bwplot ( ergosedmam     ~ host | genotype, data = d)

#the indole-diterpenoids
bwplot ( id.404.422I    ~ host | genotype, data = d)
bwplot ( id.404.422II   ~ host | genotype, data = d)
bwplot ( id.404.422III  ~ host | genotype, data = d)
bwplot ( id.420I        ~ host | genotype, data = d)
bwplot ( id.420II       ~ host | genotype, data = d)
bwplot ( id.406I        ~ host | genotype, data = d)
bwplot ( id.406II       ~ host | genotype, data = d)

-------------------------------------------------------------------------------

#creating a mosaic plot
#manuallly fixed afterwards in powerpoint,
#to change colours and headings
mosaicplot(genotype ~ habitat, data= d, col = 1:4)

#PCA
# extracting the alkaloids from the dataset
alk <- d[, 11:31]

#loading in the package vegan
library(vegan)
```

```
# Presence of zeros excludes log-transformation to reduce skewness
# Prescaling individual variables by their standard deviations is
# not recommended when all variables are in the same units (as here)

pca.1 <- rda(alk) # Raw data
plot(pca.1)
# Two distinct clusters along the axis 1
# Very high loading on the most abundant indole-alkaloid (id.404.422III)

pca.2 <- rda(sqrt(alk))
plot(pca.2)
# Square root transformation reduces skewness
# and makes the influence of id.404.422III less extreme
# Otherwise same pattern in pca.1 and pca.2
# (symmetric Procrustes correlation = 0.88)
plot(protest(pca.1, pca.2))

pca.3 <- rda(sqrt(sqrt(alk)))
plot(pca.3)
# Fourth root transformation reduces skewness even more
# and makes the influence of id.404.422III even less extreme
# Otherwise same pattern in pca.1 and pca.3
# (symmetric Procrustes correlation = 0.77)
plot(protest(pca.1, pca.3))

plot(pca.3, type = "n")
#makes an empty pca plot, with pca.3 which was fourth root
#transformed to reduce the effect of the largest indole-diterpenoid,
#the pca plot is in itself generated from the distribution of the indole
#alkaloids (see function "alk" above)
points(pca.3, pch = 20, col = (2:4)[d$genotype], cex = 2)
#sets the variable, genetic group, as points on the plot according to their
distribution
text(pca.3, display = "species", col = 1, cex = 0.8)
#sets the variable, indole-alkaloids, as text, superimposed on the points
legend("bottomleft", levels(d$genotype), pch = 19, col = 2:4, cex = 1)
#makes a box in the bottomleft corner with the genetic groups and their
respective colours

# habitat
plot(pca.3, type = "n")
points(pca.3, pch = 20, col = (10:13)[d$habitat], cex = 2)
text(pca.3, display = "species", col = 1, cex = 0.8)
legend("bottomleft", levels(d$habitat), pch = 20,
col = 10:13, cex = 1)

#for the host PCA plot downloaded and installed the package colour brewer,
which enables different colours on points of choice
library(RColorBrewer)
```

```
display.brewer.all() # shows available colours combinations in that package
plot(pca.3, type = "n")
points(pca.3, pch = 20, cex = 2, col=brewer.pal(12,"Paired")[d$genus])
text(pca.3, display = "species", col = 1, cex = 0.8)
legend("bottomleft", levels(d$genus), pch = 20, cex=1,
col=brewer.pal(12,"Paired"))

-------------------------------------------------------------------------

#ANOVA (variance analysis)

#the G2 genetic group's ability to float
#a new dataset with the selected G2 values for
#floating, habitat, and host
f <- read.table ("…txt", header = T)
#floating ability of G2 tested against habitat and host
anova(m1 <- lm(pct.floats ~ habitat, data = f))
anova(m2 <- lm(pct.floats ~ host, data = f))

#a new dataset with the selected G1 values for
#indole-alkaloids and genus
d1 <- read.table ("….txt", header = T)
#The various genera tested against
#selected indole-alkaloids
anova(lm(ergonovine ~ genus, data = d1))
anova(lm(ergocornine ~ genus, data = d1))
anova(lm(ergotamine ~ genus, data = d1))
anova(lm(ergovaline ~ genus, data = d1))
anova(lm(ergocornam ~ genus, data = d1))
anova(lm(id.420II ~ genus, data = d1))
anova(lm(id.406I ~ genus, data = d1))

#Ammophila selected out from the various
#genera of dataset d1
d1$is.ammophila <- factor(d1$genus == "ammophila")
# Ammophila tested against the same indole-alkaloids
anova(lm(ergonovine ~ is.ammophila, data = d1))
anova(lm(ergocornine ~ is.ammophila, data = d1))
anova(lm(ergotamine ~ is.ammophila, data = d1))
anova(lm(ergovaline ~ is.ammophila, data = d1))
anova(lm(ergocornam ~ is.ammophila, data = d1))
anova(lm(id.420II ~ is.ammophila, data = d1))
anova(lm(id.406I ~ is.ammophila, data = d1))
```