Towards understanding the intricate interaction between *Epichloë* spp. and forage grasses in Sweden

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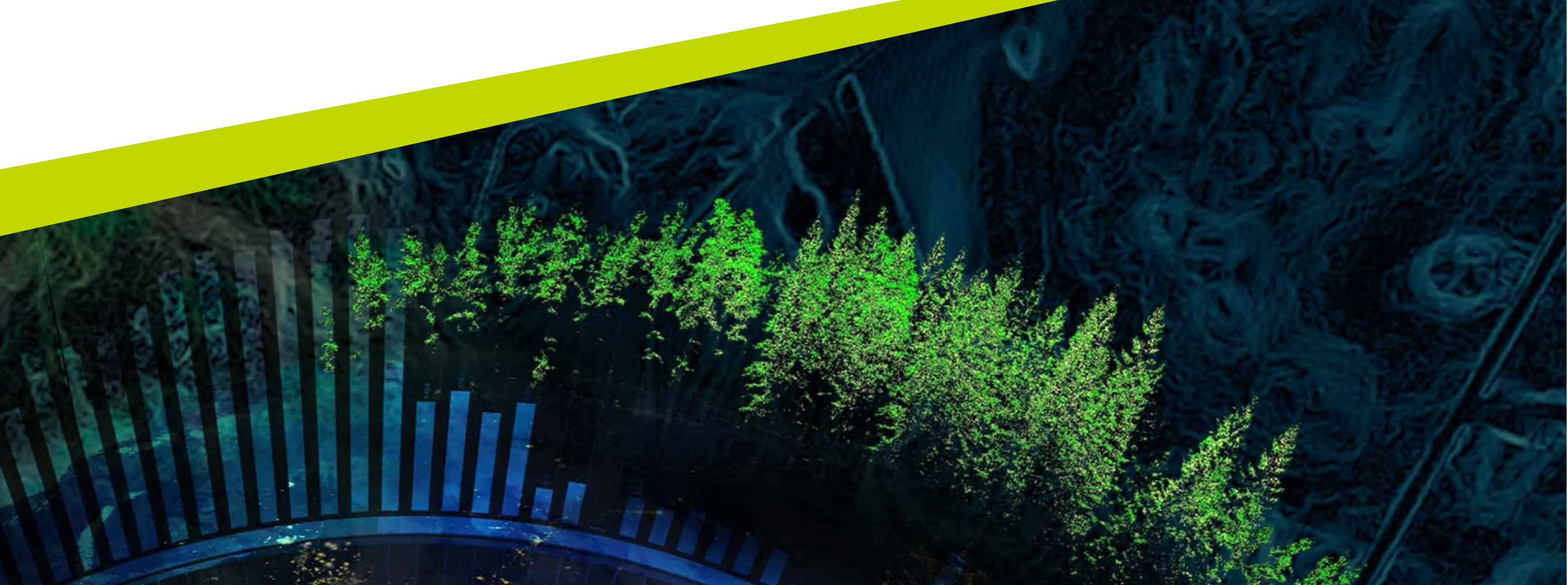

Towards understanding the intricate interaction between *Epichloë* spp. and forage grasses in Sweden.

Mot en ökad förståelse för den komplexa interaktionen mellan Epichloë spp. *och vallgräs i Sverige.*

Nic Johansson

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Abstract

Ataxia, tremors and even death are the possible consequences for both horses and cattle when their feed contains endophyte-derived toxins from *Epichloë* spp. In Sweden and elsewhere, the dangers of the toxins lolitrem B and ergovalin derived from *Epichloë* spp. are not fully understood. In pregnant mares, toxin-contaminated feed can cause hormonal imbalances affecting progesterone, prolactin, and relaxin, which can be a serious health risk for the animal since these hormones are critical for healthy foaling. Besides the animal perspective, *Epichloë* spp. can also be of major concern in seed crop production, as the visible symptoms of fungal stromata inhibit host inflorescence, ultimately resulting in seed yield losses of 9-100%.

In order to increase the knowledge about *Epichloë* spp. in Sweden, molecular diagnostics and consultations with the agricultural sector were carried out. By isolation and molecular diagnosis using ITS primers, one *Epichloë* sp. was identified from a total of 82 isolates. The cultivated isolates originated from known hosts of *Epichloë* spp. such as timothy, meadow fescue and cocksfoot. The sampling sites were located in the counties of Uppland and Västmanland. Apart from one *Epichloë* sp., 25 fungal isolates remain as no hits, unclassified or uncultivated fungi when performing BLASTn, leaving completely new species to be investigated for future work. In addition, several genera of fungi commonly found in cereals were observed. These included *Alternaria, Epiccocum, Cladosporium, Parastagonospora, Fusarium, Colletothricum*, *Phaeosphaeria* and *Microdochium.*

During consultations with experts within the sector, it became clear that in Sweden there is currently no possibility to test for possible harmful endophyte-derived toxins from *Epichloë*-infected forages, leaving animal owners uncertain whether the problems would increase in the future. In order to fill the current knowledge gaps and to gain a better understanding of the problems associated with *Epichloë* spp. in Sweden strong collaborations within animal husbandry, plant pathology and toxicology are needed. The overall understanding of the epidemiology, presence and life cycle of *Epichloë* spp. in forage grasses remain low in Sweden despite the significant amount of ley grown.

Keywords: Epichloë, *Choke disease,* Phleum pratense, Dactylis glomerata, Festuca pratensis*, Timothy, Meadow fescue, Cocksfoot, Choke*

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Abbreviations

HPLC	High-Performance Liquid Chromatography
ITS	Internal Transcribed Spacer
NMR	Nuclear Magnetic Resonance
PDA	Potato Dextrose Agar
PCR	Polymerase Chain Reaction
SFO	Sveriges Frö- och Oljeväxtodlare
SLU	Sveriges Lantbruksuniversitet (Swedish University of Agricultural Sciences)
SVA	Statens Veterinärmedicinska Anstalt (Swedish Veterinary Agency)
YMS	Yeast Malt Sucrose
VSC	Växtskyddscentralen (Plant protection extension – Swedish Board of Agriculture)

1. Introduction

1.1 *Epichloë* spp. - interdisciplinary bridge between plant pathology, animal husbandry and seed production

Through evolution, the fungal family of *Clavicipitacea* and genus *Epichloë* have formed a symbiotic relationship with many of the important forage grasses of today, such as cocksfoot, tall fescue, perennial ryegrass, timothy and meadow fescue (Bushman et al. 2018; Bylin 2014; Sundström 2020; Vikuk et al. 2020; Wang et al. 2019). This plant-fungal interaction is often invisible to the eye since *Epichloë* spp. are endophytes (Leuchtmann et al. 2014). Fungal endophytes colonize the plant host for at least a part of their life cycle, and many of the temperate grasses are reliant on such mutualistic symbiosis with *Epichloë* spp. for increasing their persistence when grazed or to endure abiotic stress (Johnson & Caradus 2019). *Epichloë* endophytes may become visible in the field during the sexual part of the life cycle as they shape yellow or white stromata. The formation of stromata may hinder the inflorescene of the host and create symptoms known as 'choke disease' (Figure 1). Among the described *Epichloë* spp. of today, approximately one third of them are known to shape visible stromata during the sexual part of the life cycle (Leuchtmann et al. 2014).

Figure 1. The formation of visible stromata referred to 'choke disease' or 'choke' on the flag-sheath in Timothy. Source: Swedish Board of Agriculture – plant protection centers)

In the last decades, the impact and consequences of choke as a disease has been highlighted in studies related to yield loss in seed crop production. In France, growing cocksfoot (*Dactylis glomerata*) for seed production became unprofitable for farmers due to yield loss when the infection rate reached 30% (Raynal 1991).

Furthermore, the economic losses in seed crop production in Czech Republic were estimated to be 100 000$ in 2008 (Cagaš & Macháč 2012). In Oregon (USA) estimated seed yield losses due to choke disease varied between 9 and 30% in Orchardgrass (Pfender & Alderman 2003). The economic losses in that specific region were estimated to $0.8 million (Pfender & Alderman 2006). Yet, many questions remain open regarding the biological and economic consequences of these plant-fungal interactions (Johnson & Caradus 2019).

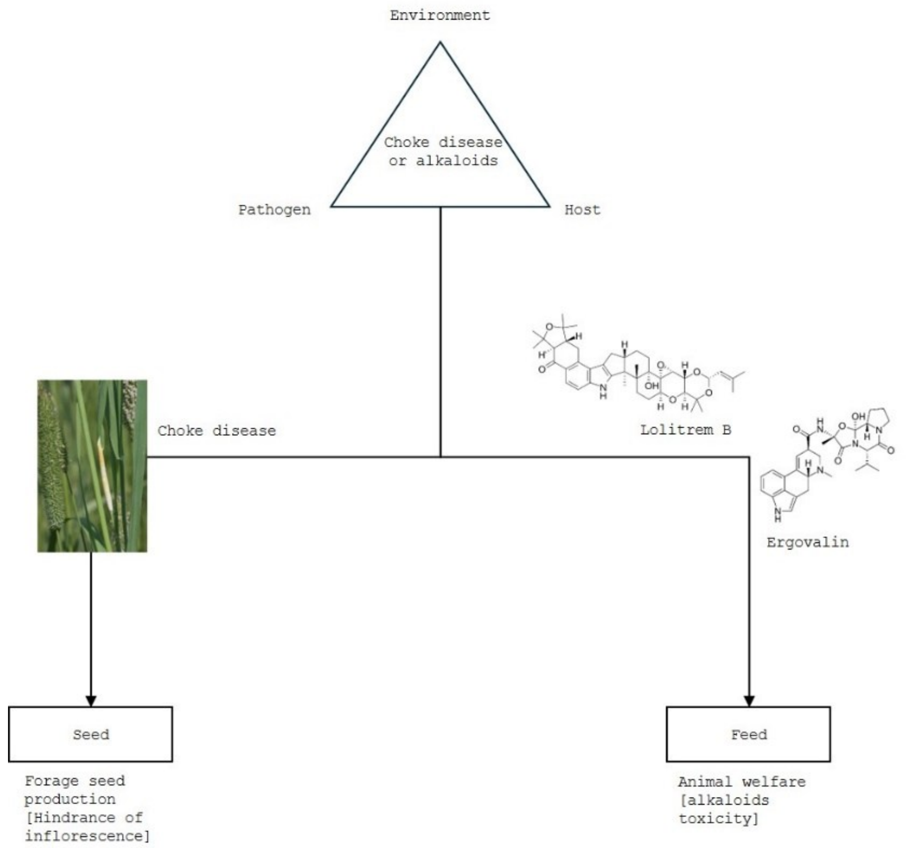


Figure 2. Overview of the interdisciplinary aspects related to the management of *Epichloë* endophytes.
In the upper part, a disease triangle represents the factors favoring the choke disease or the production of alkaloids in forages. In the lower part, the two branches represent two different problems resulting from forage contamination with *Epichloë* endophytes, and their impact.

This is particularly important since specific *Epichloë* spp. do not always cause visible symptoms that correlate with the production of toxic alkaloid substances such as lolitrem B and ergovalin (Craig et al. 2014; Goehring et al. 2005). In nature, the production of alkaloids is a beneficial defence mechanism which protects the plant from herbivores (Shardl et al. 2013). However, forage crops containing *Epichloë*-associated fungi can lead to feed contamination with those same protective alkaloids, which actually are toxic for horses and ruminants. It has been reported that *Epichloë festucae* var. *lolii* in perennial ryegrass (*Lolium perenne*), and toxins from *Epichloë coenophialum* can cause severe health issues for horses such as diarrhea, ataxia, and tremor (Goehring et al. 2005). For mares in gestation, feed from forages containing toxin-producing endophytes can cause hormonal imbalance affecting progesterone, prolactin, and relaxin. This is a serious health hazard for the animal since these hormones are critical for healthy foaling (Monroe et al. 1988).

Due to this, the recommendation in USA is that mares in gestation are fed with forage free from symptoms from 300 days to foaling to avoid complications (Boosinger et al. 1995). There are still uncertainties since other studies show that the uptake of ergotalkaloids are quickly absorbed in the blood, while no reproductivity issues were noted (Youngblood et al. 2004). Ultimately, this highlights the complexity of assessing and appreciating the full effects of endophyte-derived alkaloids in forage quality and ultimately, animal health (Klotz & Mcdowell 2017). In Ireland, cases of possible endophyte infected forage have been highlighted in a report where 13 stud farm had problems with infertility, red-bag delivery, abnormal bleeding after foaling, and the development of oedema in horses. The relationship between the feed and the symptoms could not be satisfactorily established, although the effects of endophytes could not be ruled out (Canty et al. 2014).

1.2 Life cycle and systematics of *Epichloë* spp.

The general life cycle of *Epichloë* spp. has two main cycles and is species dependent. Some undergo both sexual and asexual life cycles, whilst some are obligate asexual or sexual. The asexual life cycle do not result in visible symptoms. The existing endophyte will continue colonizing the host throughout the inflorescence and the formation of new seed embryo. Asexual endophytes are only transmitted by clonal propagation (Chung & Shardl 1997). In the sexual life cycle of *Epichloë* spp., the symptoms are expressed as stromata. Which is the formation of a cushion-like plate of solid mycelium which hinders the development of inflorescence. From the stromata, spores harboring the opposite mating type are transferred by flies. The vector will transfer spermatia to stroma of an opposite mating type. Through karyogamy and meiosis on a mature stroma, an ascus will develop and eject ascospores. Germination of ascospores occur on the grass florets, thus infecting the host ovule, resulting in endophyte presence in the seed. (Figure 3). There are still several knowledge gaps regarding infection, growth, and asexual and sexual transmission of specific *Epichloë sp*. (Bushman 2018). However, *Epichloë typhina* belong to the fungal species which forms the presexual structure known as stroma. A transfer of spermatia of opposite mating type leads to the thickening of the stroma, ascospores will be produced if the parental fungi are of the same mating population. In nature, flies are the vectors which transfer spermatia between the stromata (Chung & Schardl 1997).

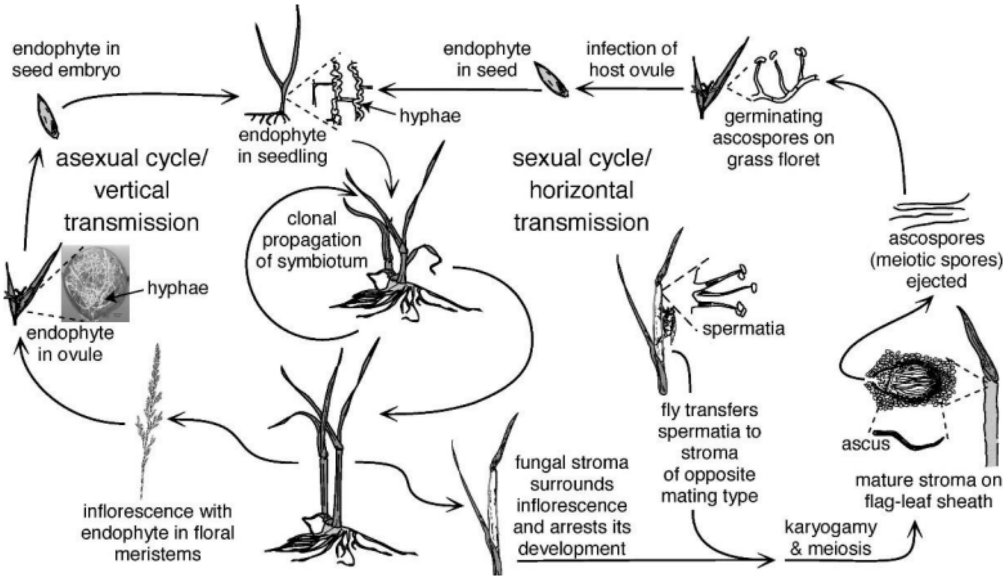


Figure 3. A general illustration of the sexual and asexual life cycles of *Epichloë* spp. Some *Epichloë* sp. undergo both sexual and asexual life cycles, whilst some are obligate asexual or sexual. *Source*: Schardl (2010).

1.3 Understanding the negative impact of *Epichloë* spp. in Sweden: previous and current issues.

In the past years, ergot alkaloids from endophyte-infected ryegrass and fescue have been suggested as the plausible cause for the death of several mares and foals on stud farms in Sweden as Ireland (Darenius et al. 2011). Mares had a high frequency of early foal death, abortions, prolonged gestation, reproductive issues, low or no milk production, red bag delivery, and a withheld after birth. When the forage feed was replaced with new fresh feed, the problems disappeared (Darenius et al. 2011). The overall guidelines from the Swedish Veterinary Agency (SVA) states that endophytic fungi such as *Epichloë* spp. can have significant effects on the wellbeing of horses. The produced alkaloids, and possibly other toxins may result in heath conditions such as loss of appetite, poor growth, increased body temperature, and complications with reproduction (Swedish Veterinary Agency 2024).

Awareness of these toxins arises from time to time in agricultural trade journals when symptoms of choke caused by *Epichloë typhina* are more abundant in forage fields, ley, or pastures (HästSverige 2020). Among the Swedish seed producers, the awareness of choke has been highlighted over the years with severe outbreaks since the formation of stromata hinders the inflorescence, ultimately resulting in no seeds to harvest, according to the Swedish seed & oilseed association (SFO) (SFO 2025).

The agricultural sector's focus on the topic reached a peak in 2019 since it was considered a year with a significant impact on the sector. At the time for the outbreak, many questions were raised such as: (i) What factors led to outbreaks of choke disease in timothy (ii) Is the fungus seed borne? (iii) Is it safe for livestock to be fed timothy from fields with choke disease? (Sundström 2020). In an effort to address these questions, a student thesis work was carried on at SLU (Sundström 2020). Besides this report, there are still little or few efforts addressing the issue of Swedish conditions in a systematic manner, to bring more knowledge to the sector and prevent a similar or worse situation than that in 2019. Since forage crops and pastures account for roughly 50% of the arable land in Sweden (Swedish Board of Agriculture 2024), a major outbreak could potentially limit the supply of both seed and feed in Sweden. Therefore, the aim of this thesis is to further investigate the interaction between *Epichloë* spp. and forage grasses species in Sweden in an attempt to produce new knowledge for the sector and identify new research directions.

1.4 Objectives

This project was directly motivated by the increasing need of knowledge on the topic that has been expressed by the Swedish Board of Agriculture and the Swedish Veterinary Agency (SVA). We began our investigation by differentiating a hypothesis

- We expect to find different species of *Epichloë* and not only *Epichloë typhina* in samples of timothy, meadow fescue and cocksfoot.

In this 30-credits independent project we aimed at:

i. Advancing our understanding of *Epichloë* spp. in timothy and other known hosts by performing molecular diagnostics.
ii. Better understanding the impact of *Epichloë* spp. by consulting experts and stakeholders within the forage production and animal feed sectors.

2. Methods

2.1 Sampling and experimental procedure

The experimental setup was based on material from two different origins/types. One set of samples were timothy forage collected from fields showing the formation of stromata. Another set of samples were timothy, meadow fescue, and cocksfoot samples collected from an unmanaged timothy breeding plot (Pär Ingvarsson, SLU), where meadow fescue and cocksfoot were naturally occurring (Figure 3).

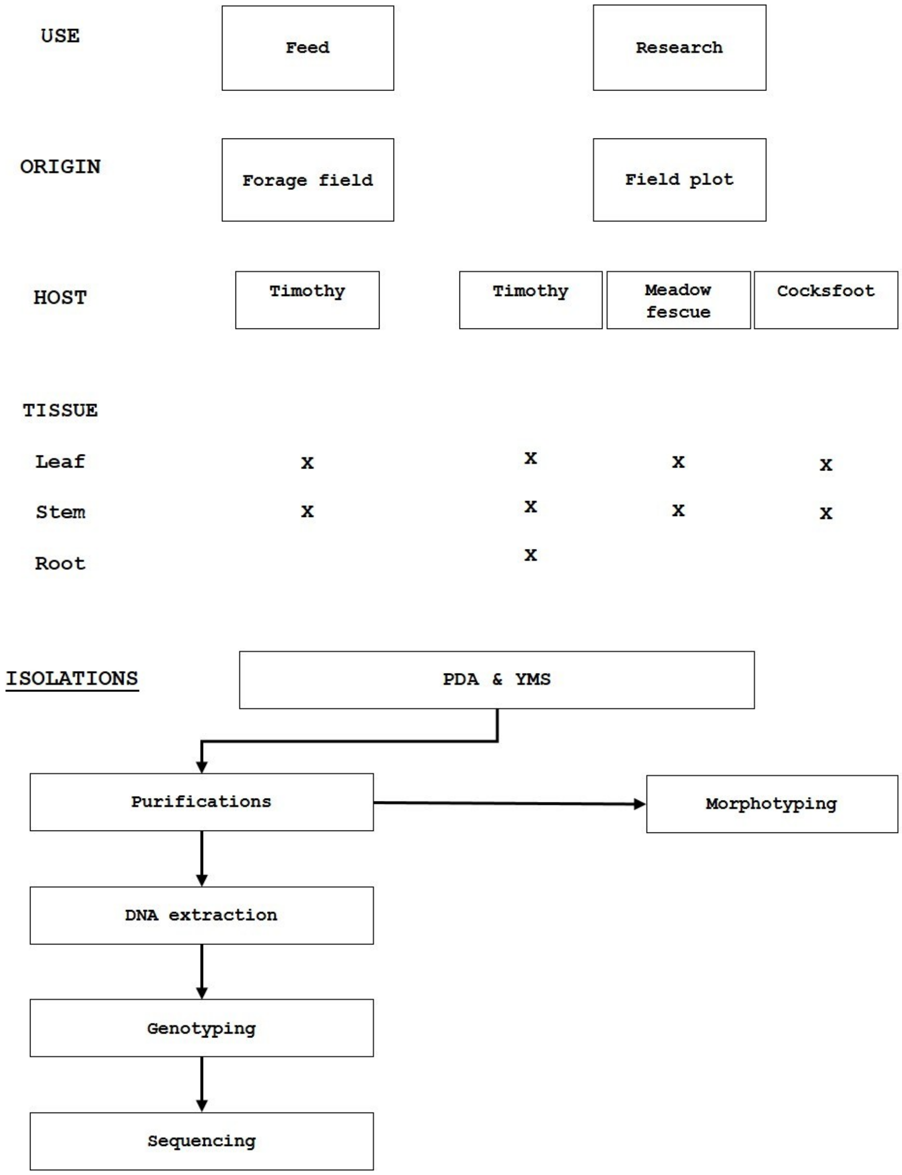


Figure 3. The workflow from field to sequencing of collected and provided samples. The intended use of the sampling sites were for designed for feed and research purposes. The origin were a forage field and an experimental field plot. One set of timothy were collected in a forage field, while another set of timothy, meadow fescue and cocksfoot were sampled from a breeding plot. Tissues were taken from the leaf, stems and roots (field

plot timothy only). An isolation step were performed on PDA (Potato Dextrose Agar) & YMS (Yeast Malt Sucrose) followed by purification steps, morphotying, DNA extraction, genotyping and sequencing.

Frozen timothy plants with visible stromata were provided by Lina Norrlund from the Swedish Board of Agriculture. The provided samples originated from Uppsala, near Ekolsund (collected on 23rd of June 2023) and Västerås, near Hallstahammar (collected on the 22nd of July 2020). Additional plant material constituting of timothy, cocksfoot and meadow fescue were collected from an research breeding plot on the 28th October 2024 (Figure 4). The breeding plot had not been maintained or managed two years prior to the sampling.

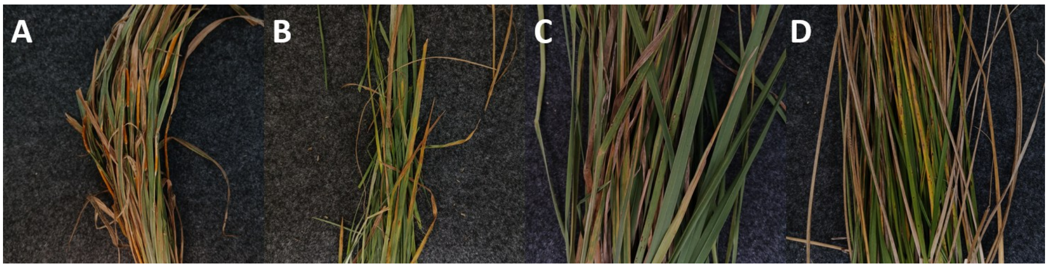


Figure 4. Examples of forage samples analyzed in this study
(A) Frozen timothy samples from Lina Norrlund with stromata **(B)** Timothy plants from field plot **(C)** Cocksfoot from field plot. **(D)** Meadow fescue from field plot.

2.2 Fungal colony isolation and purification

Stems with symptoms and asymptomatic leaf were cut into pieces of 0.5 – 1.0 cm^2 and labelled with plant and tissue type. Two stems and two leaf samples were cut from each of the five hosts. In addition, timothy roots from the breeding plot were cleaned from dirt and dried, and small segments of ca 1 cm were cut out for further mycological work. Two root samples were taken from each timothy plant. Isolations were performed in a sterile microbiological laminar flow hood. All tissues were washed in 70% ethanol for 1 min, then washed twice in Milli-Q for one minute. The samples were dried on a disk of Whatman paper. Once dried, each sample was placed on a 90 mm petri dish with Potato Dextrose Agar (PDA, BD Difco PDA 19.5g, Agar 4g, Water 1000ml) and Yeast Malt Sucrose (YMS, Yeast 4g, Malt extract 4g, Sucrose 4g, Agar 16g, water 1000ml). Media were supplemented with kanamycin at a final concentration of 50 µg/ml to suppress bacterial growth. After each isolation, samples were given a specific ID based on species of origin, tissue of origin, and media of isolation to ensure traceability. Incubation of the isolated samples were carried out at ca. 20 °C in the dark. After approximately 7 days, or when fungal colonies were visible, the purification steps were repeated until a single colony with a single morphotype was obtained.

2.3 Morphotyping

Once the purified fungal isolates were fully grown, an additional plating to a large petri dish was performed. Pictures of the morphotypes were taken at a stage where the petri dish was largely covered by the fungus. Then, colonies were divided into three groups based on the color of the mycelium (Figure 5). Here, our assumption was that possible *Epichloë* spp. could be identified based on the morphotypes reported in the literature. However, all morphotypes were proceeded for DNA extraction irrespective of the morphotype.

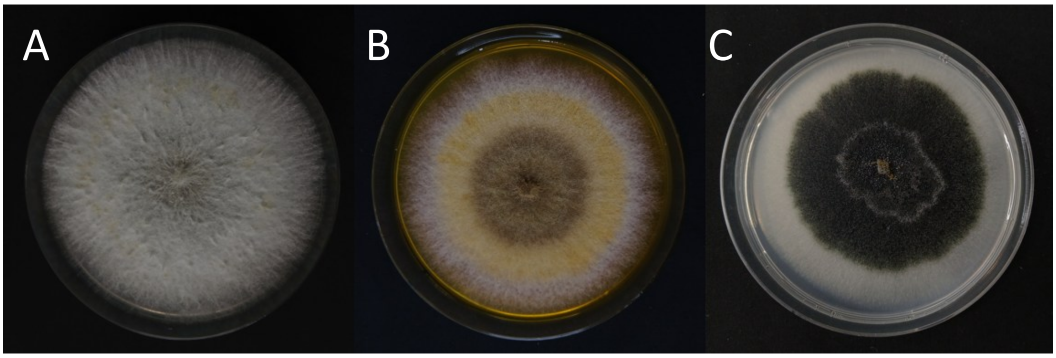


Figure 5. Fungal colonies representing the three morphotype groups
From left to right: white morphotype **(A),** yellow/brown morphotype **(B),** dark morphotype **(C).**

2.4 DNA extraction from fungal isolates

Fungal DNA was extracted by carefully scraping mycelium grown on agar plates and put in a labelled 2 ml capped tube. The acquired mycelia were homogenized by physical grinding using sterilized sand and a sterile drill bit. The DNA extraction was performed according to the manual NucleoSpin Plant II Genomic DNA Purification kit with a lysis buffer system based on CTAB method (Macherey-Nagel, Germany). DNA concentration was measured using a NanoDrop spectrophotometer.

2.5 Genotyping and DNA sequencing

Molecular identification of the fungal genus was performed using Polymerase Chain Reaction (PCR) on the extracted genomic DNA, followed by amplicon sequencing of the conserved nuclear locus internal transcribed spacer (ITS) rDNA gene. For ITS PCR, primer pair ITS1 and ITS4 were used (see Appendix), and amplification was carried out in 25 µL with the DreamTaq Green PCR Master Mix (2X) chemistry (ThermoScientific, USA). The PCR amplification specifications were made in accordance with (Thünen et al, 2022) (see Appendix). Amplicons were visualized on a 1% agarose gel produced by mixing 1.3g of agarose to 130 ml of Tris-acetate-EDTA (TAE) buffer. Melted gels were supplemented with 5,5 µl of the Midori Green Advance DNA dye (NIPPON Genetics, Germany). A mix of 4 µl

PCR product and 4 µl loading dye was loaded in each well. Additionally, 5 µl of Thermofisher Generuler 1kb DNA ladder was added into the first and last well of each gel. The gel was run at 150 volts for ca. 20 minutes. A photo of each gel was taken on a gel documentation system upon exposure to UV. PCR products were sent for SANGER sequencing to Macrogen Europe. The online database BLAST (blastn) was used to identify the fungal genus by DNA sequence homology.

2.6 Consultation with experts within the sector to unveil issues, needs, and gaps related to *Epichloë* spp. in Sweden

In order to collect new information apart from already conducted studies in Sweden (Bylin 2024; Sundström 2020), consultations with experts within government, university, and seed associations were performed. The outline of these consultations focused on the issues, needs, and gaps linked to *Epichloë* spp. in Sweden. Since it is an interdisciplinary topic, both the animal perspective and crop production perspective were highlighted. The main questions for the consultations were:

-What are the current main issues related to *Epichloë* spp. and choke disease in Sweden and elsewhere?
-What are the current needs to get a better understanding of the pathogen and what advice can the sector give today to producers or animal keepers?
-What are the current gaps in Sweden, what is needed today and, in the future, to solve the issues related to *Epichloë* spp*?*

The method of this consultation is thereby, formulated as interview/survey in social sciences. The consultations do not follow the methods of qualitative interview study. The aim was to unveil possible unpublished information within the sector as a basis for further investigation of the issue.

3. Results

3.1 Summary of isolated fungal colonies from different forage hosts

A total of 82 fungal isolates were obtained from the three different sample origins. The most abundant host of origin was timothy (field), followed by meadow fescue (field), timothy (forage) and cocksfoot (field) (Figure 6, upper panel). Two isolates have been classified as unknown origin since the labelled plate were not complete. The division of origin by tissue shows that most successfully cultured fungal isolates originated from the leaves, then stem and root (Figure 6, upper panel). Additionally, 1 sample lacked the label of origin by tissue resulting in a total of three unknowns of all 82. Among the 50 white-colored morphotypes of group 1 which we suspected being *Epichloë* spp., the host origin was similar to each other, only cocksfoot showing a smaller fraction. Within group 1 most of the most common tissue where the fungal colonies were isolated from was leaf. Among the orange and yellow morphotypes of group 2, the host division of the 19 acquired isolates show a smaller fraction from the frozen timothy samples intended for forage. The proportion of isolates from roots is also higher in comparison to Group 1. Finally, for group 3 which had a dark/brown morphotype most of them originated from timothy collected in the field, with a larger proportion of them originating from the leaves.

3.2 Molecular analysis of fungal colonies

The 82 isolates were divided into three batches and proceeded for DNA extraction and PCR. The setup of each PCR was grouped into a first batch of 30 (excluding negative control) consisting of the first differentiated white morphotypes. The second batch was a mix between both dark/orange morphotypes since they were an additional set of 30. The third batch consisted of the remaining 22 white morphotypes. The concentration of the amplified DNA showed in most of the samples to be in between 0-20 ng/µL. Few of the samples showed higher DNA concentrations than 50 ng/µL. A total of 11 samples did not show any bands when performing the gel electrophoresis. Majority of the failed PCRs belonged to either the lower or the higher spectra in terms of DNA concentration. The success rate of 71/82 at the gel electrophoresis indicated that the amplification of DNA through PCR worked well. The negative control consisted of MilliQ water and no signs of contamination were shown in the gel electrophoresis. The ladder shows that the bands were seen between 500 and 750 bp marks, which is the correct expected size for a fungal ITS locus (Figure 7).

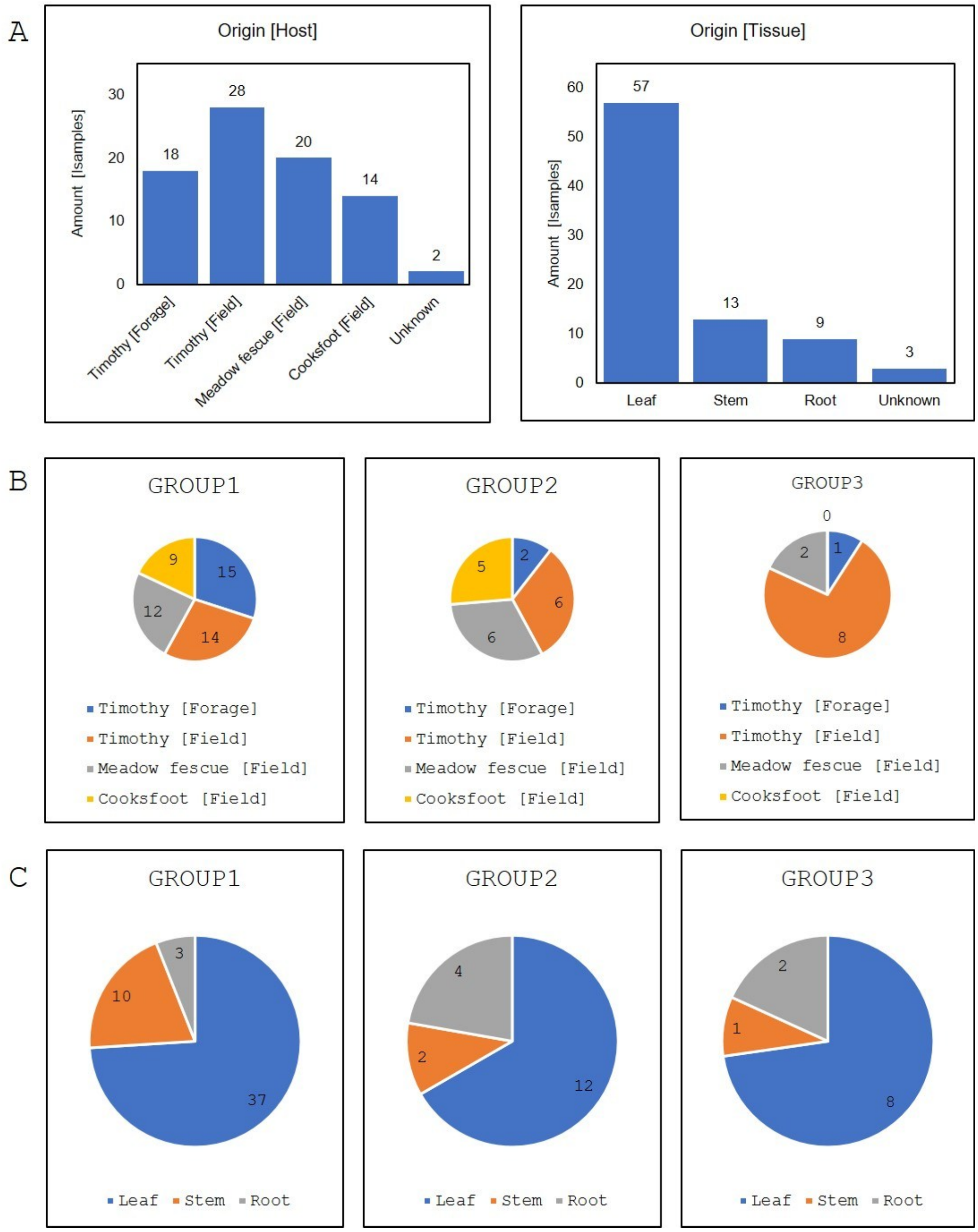


Figure 6. Summary of the origin and sample distribution of the isolated fungal colonies and morphotypes. Group 1 consists of all white morphotypes. Group 2 are all yellow/brown morphotypes and group 3 are all the dark morphotypes. **Upper (A)** panel shows the overall distribution of all 82 colonies by host and tissues. **Middle (B)** panel show distribution of the different morphotypes by host of origin. **Lower (C)** panel shows distribution of the different morphotypes by tissue of origin based on leaf stem or root.

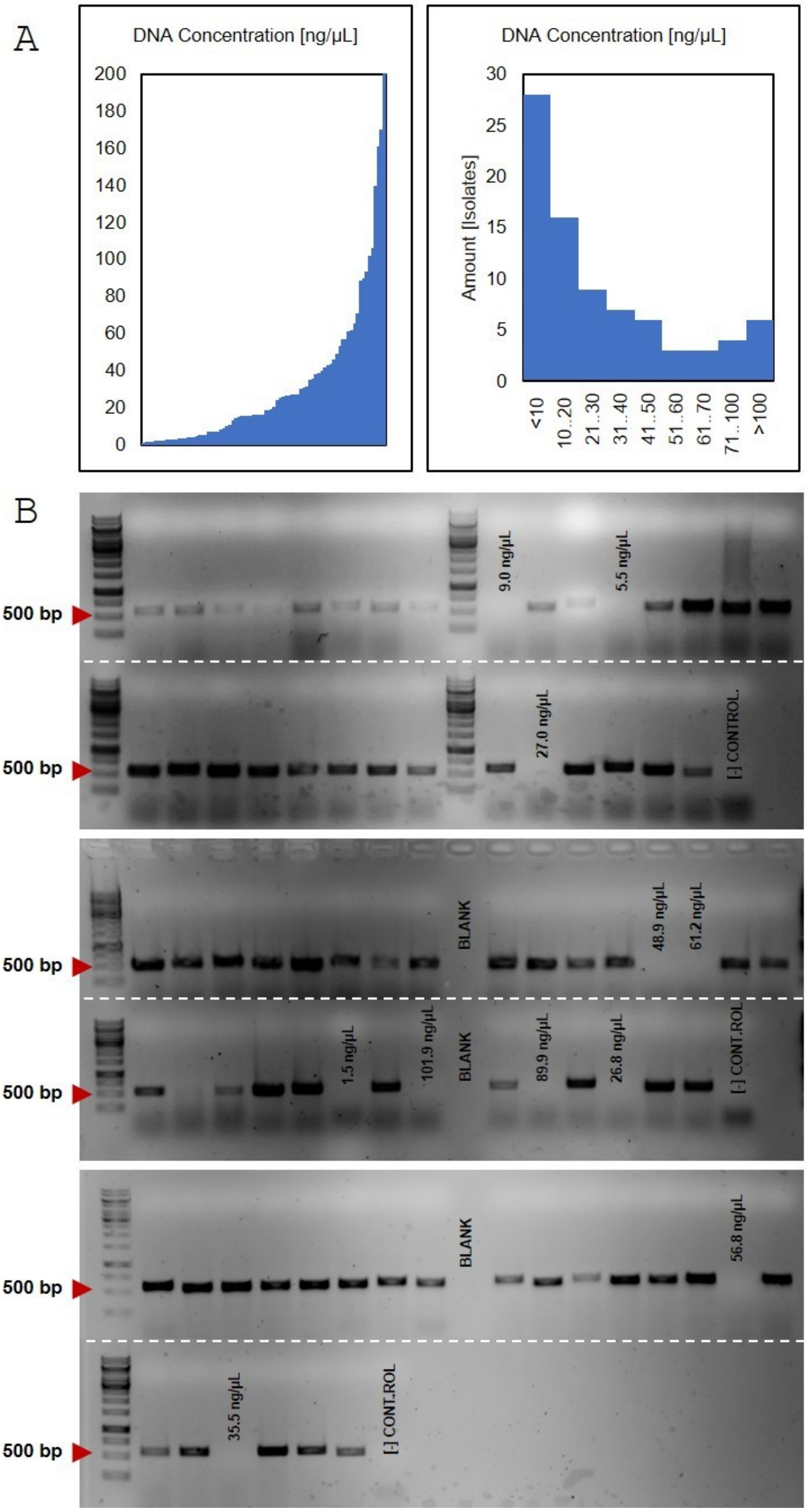


Figure 7. Summary of the results for DNA extractions and PCR amplifications
The upper panel (A) shows the distribution of DNA concentrations from all 82 samples. **The lower panel (B)** shows the gel electrophoresis results from all 82 samples. Blanks, negative control and the 11 failed bands are presented.

3.3 Analysis of fungal genus diversity

A total of 28 different fungal genera associated with forage grasses could be identified in this study (Figure 8). The majority of the isolated colonies correspond to fungi from the genus of *Alternaria*, which includes important plant and postharvest pathogens (Woudenberg et al. 2015). *Cladosporium* spp. involves fungal species which are the causal agent of leaf spots or other lesions (Bensch et al. 2012). The *Epicoccum* genus also involves several species which are known plant diseases, some also of interest to develop biological control products (Taguiam et al. 2021).

We also found fungi from the genus *Parastagonospora* which include fungal species that are directly or indirectly accountable for yield losses on cereal crops such as oats, barley and wheat (Cunfer 2000). Additionally, *Fusarium* spp. were also present. This genus includes species that affect both yield and quality of cereal crops (Nguyen et al. 2025). The genus *Collectotrichum* includes several plant pathogens of importance which can affect the production of strawberries, maize and sorghum (Dean et al. 2012). The *Phaeosphaeria* genus contains species which are globally occurring in wheat (Mcdonald et al. 2012) and *Microdochium* involve species which are important plant pathogens in grasses and cereals (Liang et al. 2019). Overall, these results indicate that there are fungal genera present in the investigated forage hosts which are also present in other agricultural crops.

The results also depict that the fungal genus composition differs a lot between the host species. The genus *Cladosporium* is most frequent in timothy, whilst the genus *Epiccocum* is the most frequent in meadow fescue, and *Alternaria* spp. in cocksfoot. Additionally, there were 11 no hits, 7 unclassified, and 7 uncultured fungi (Figure 8). In total, 25 out of 82 samples could not be attributed to a known fungal genus. This result suggests that there are fungal genera which are present in these grasses which have not previously been studied to a point where the genera can be distinguished in the reference BLASTn database (Figure 8).

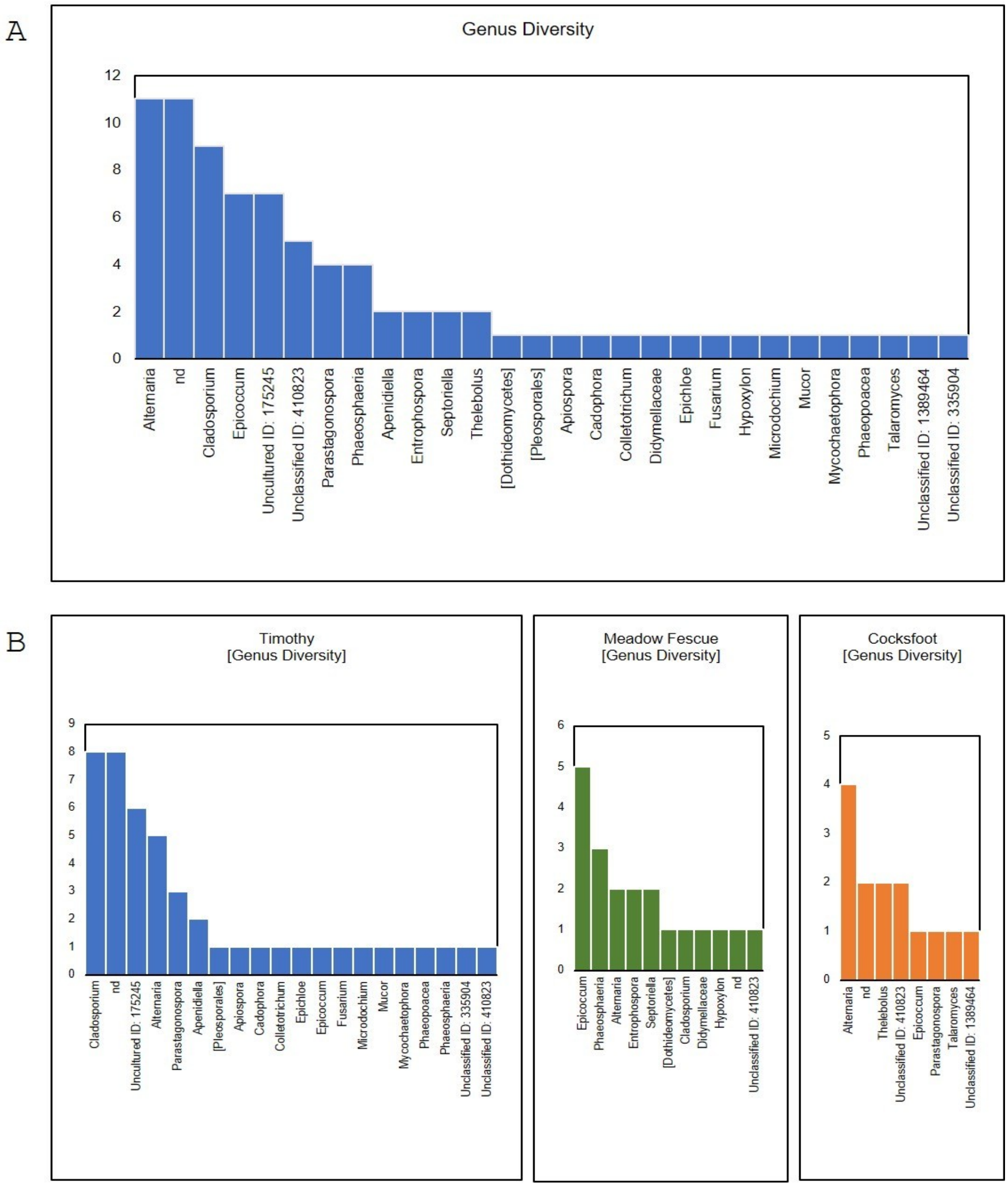


Figure 8. Analysis of genus diversity of fungi isolated from forage. **Upper panel (A)** shows the different genera isolated from three forage grass hosts in this study and the corresponding amount of isolates. **Lower panel (B)** shows genus distribution over the three hosts and the corresponding amount of isolates.

3.4 Consultations with experts within the Swedish agricultural sector

Consultations with experts within the Swedish agricultural sector on the topic of *Epichloë* spp. were performed in parallel with the laboratory work. Discussions were carried out with the following institutions/experts:

- **Plant Protection Extension, Swedish Board of Agriculture (VSC)**
- Lina Norrlund, agricultural advisor.
- **Swedish Seed and Oilseed Association (SFO)**
- Jörgen Persson, representative for SFO and expert in forageseed production
- **Swedish University of Agriculture, Department of Applied Animal Science and Animal Welfare (THV)**
- Cecilia Müller, associate professor in equine nutrition.
- **Swedish Veterinary Agency (SVA)**
- Gunnar Andersson, researcher in food safety, epidemiology and veterinary forensics.
- Erik Nordkvist, researcher at the department of chemistry, environment and feed safety

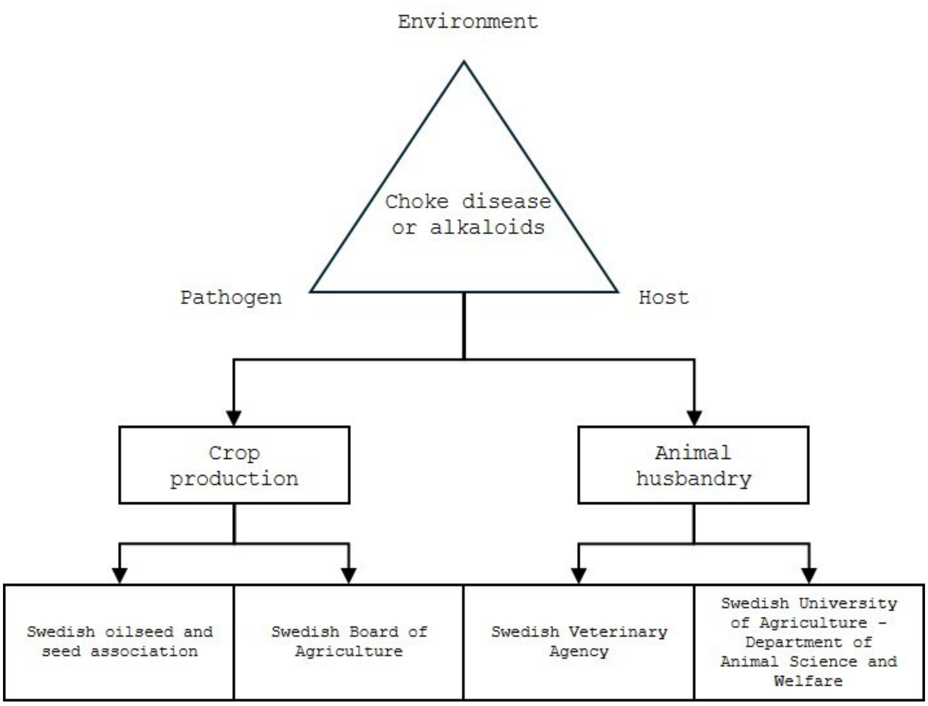


Figure 9. Key sectors and institutions to build an understanding of the situation of *Epichloë* spp. in Sweden. The crop production and animal husbandry are the two main perspectives related to the issue. Consultations were performed with representatives or experts belonging to Swedish oilseed and seed association, Swedish Board of Agriculture, Swedish Veterinary Agency and the Swedish University of Agriculture.

Consultation with the Swedish Board of Agriculture
Lina Norrlund (LN) – Agricultural advisor at the Plant Protection Extension (Uppsala)

According to LN the topic of choke disease is complicated, since the fungus lives inside the plant (i.e. it is mostly invisible), however it is fairly commonly present in pastures and leys kept for several years - not only with timothy, but also with other grass species. Regarding symptoms, LN states that symptoms occur late in the season, often at the stage where the fiber content is high in the plant, making it less tasty for grazing animals. However, how the choke disease appears in the field in the first place is for LN unknown.

The need for knowledge is framed within the possible production of mycotoxins and the reduction of seed yield for seed producers. LN states that toxicity is about amounts, and thus the question is how we can assess what is considered safe or not for feed? Today, the agricultural sector is aware that caution should be raised when it comes to pregnant mares, but issues related to choke in Sweden have not been addressed outside the seed and horse sector. If there are symptoms in the field, the countermeasure and recommendation is to plough the field or ley.

According to LN, there are knowledge gaps that need to be filled regarding to the localization of the toxins in the plant: are they only found in the spikes or systemically in the host? By increasing the knowledge through data collection, and scouting of fields to increase the opportunity to make observations, perhaps predictions of occurrence can be made. Questions remain on how the collection of data should be made (e.g. by counting shoots per plant? per area?) and by whom, is there even a need? LN says. Further investigations by scientists are needed since observation of field plots of interest organizations is not enough to provide all answers. LN concludes by stating that the key to success within this topic is cooperation between both plant and animal sciences.

Consultation with the Swedish Oilseed and Seed Association
Jörgen Persson (JP) – Representative and expert in seed crop production Sveriges Frö och Oljeväxtodlare (SFO).

According to JP there are currently no major issues in Swedish seed production linked to choke. It pops up from time to time. However, JP states that there is no longer any available insecticides for treatments against timothy flies any. Due to this, the longevity of the timothy seed crop has shortened from three/four years in general to two years, since a shorter rotation decreases the risk of choke disease. The awareness of the issue among seed growers has not increased according to JP, in Sweden seed production is often most often located in less productive areas. Same agricultural practices result in approximately the same yield no matter the location, which makes seed production a more suitable choice in marginal lands since the yield potential for other crops is lower.

The general recommendation is still, if there is choke in the field, simply plough it. There are no pesticides available, and for seed production it seems that only timothy is the problem in Sweden. JP also mentions the fact that there is little or no knowledge about how the pathogen appears in the field initially, the conducive conditions for disease outbreak, how to minimize the problem except for ploughing, and what are the effects on crop rotation? JP also asks if it is possible to sell straw from seed crops, if the straw also contains toxic compounds from endophytes, and what are then the threshold values and toxicity limits?

Consultation with animal nutritionist at the Swedish University of Agriculture

Cecilia Müller (CM) – Associate professor in equine nutrition at the Department of Animal Science and Welfare

The main issue according to CM is that there is little or no knowledge at all about timothy and choke disease, it is an empty field in Sweden. In the United States, the stakeholders are fully aware of the problems and do not use endophyte infected feed for gestating mares since it can lead to premature placental separation (aka Red bag delivery). The toxin involved is ergovalin which has been studied in tall fescue. Ergovalin causes the blood vessels to contract, ultimately creating problems for the gestating mare. It is probable that important hormones are affected by either endophytes or ergovalin. The affected hormones are key for healthy gestation. Still, specific knowledge about what is causing these issues and why is non-existent.

CM states that another issue is linked to lolitrem B in perennial ryegrass. A major difference between ergovalin and lolitrem B is its faster decay rate. Problems with lolitrem B become more frequent during fall than in the spring, but it can be hard to notice when observing the horses. The more obvious condition is ryegrass staggers, which can affect all animals. High amounts will end the life of the animal due to ataxia (wiggeling, disorientation) and severe alteration of the nervous system. The problems increase with long-lasting leys and forage harvests late in the season.

CM states that there is no capacity to test for these toxins in Europe, it is possible to send them to Oregon (USA), however it is challenging since the toxins quickly decay. The risk for toxin related feed is the same for every animal, the reality depends on the production methods on the farm. There are currently no standardized methods to do feed analysis in Sweden, the possibilities are there but a method is not registered. Analysis needs to be performed through high-performance liquid chromatography (HPLC) and nuclear magnetic resonance spectroscopy (NMR). CM highlights the fact that there are probably more mycotoxins which can be worth investigating, with different effects on different animal species. NMR is considered a good detection method since more substances become available, however an expert within analytical chemistry is required. The current situation is that there is no knowledge regarding the specific toxin(s) and the cause of the problem.

CM addressed the current gaps as follows: today there is no answer to the question of whether the quality issue with the feed is dangerous or not. Cecilia quickly nuances and says that there are of course other things which could affect the state of the horse, however for those specific problems there are existing tests and methods. For example, it is possible to test viruses, but not for toxins. In terms of toxin amounts, the concentrations which can be considered safe unknown. American studies suggest 500 pbm for ergovalin, 2 pbm for lolitrem B based on a few studies that exists. It is also probable that issues can result from interactions between toxins, not one toxin alone. Species and environmental conditions can perhaps also have an effect. The overall knowledge gap in Sweden is high, it is considered a ‘minor problem’ when applying for financial support.

Consultations with the Swedish Veterinary Agency (SVA)

Gunnar Andersson (GA) **-** Researcher in food safety, epidemiology and veterinary forensics.

Erik Nordkvist (EN) - Researcher at the department of chemistry, environment and feed safety

As previously stated by CM (SLU), GA and EN (SVA), Sweden does not have the analytical capacities in place to test for endophyte-derived toxins. Projects and suitable methods are therefore needed. Since methods are absent, a principle of caution is recommended facing choke infected feed. However, it is not always easy to say since the production of toxins and symptoms are not correlated. GA and EN believe there must be some sort of systematic gathering or experimental data regarding choke disease to evaluate the positive qualities to the disease as well as the negative ones. From the perspective of SVA, most of the concern has been focused on horses, and so little to no contacts have been initiated by cattle owners or seed producers regarding testing feed for endophyte-derived toxins. GA and EN state that there are no epidemiological patterns observed so far, but the topic is highlighted from year to year. They also describe that when the problem comes to be known by the veterinarians in the field (i.e. when diagnosing animals) the veterinarians conclude that there must be something wrong with the feed (i.e. the nature of the problem with the feed is not investigated).

In terms of needs, GA and EN states that experiments in toxicology need to be made to understand the interaction between the fungi, plant and the presence of mycotoxins. Overall, different interests in the topic must be identified from relevant disciplines, to enable a cooperation between plant pathologists and animal scientists. Understanding the interactions between the host and the pathogen/endophyte is complicated and requires more research. GA and EN end by saying that speculation is not good, there has to be tools, and a push from several sectors for things to happen.

4. Discussion

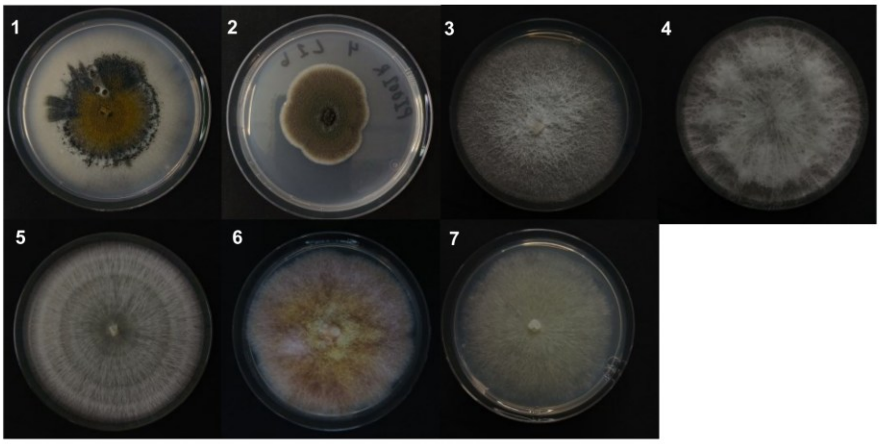
4.1 Advancing our understanding of *Epichloë* spp*.* in timothy and other known hosts by performing molecular diagnostics.

In total, only one of the 82 isolates were confirmed to be *Epichloë* sp. (Figure 9). Apart from one single isolate, there were 11 isolates with no hits, 7 unclassified fungi (5 of them indicate fungal endophyte) and 7 uncultured fungi. This means that 25 of the 82 samples remain unknown and that the general ITS marker did not provide enough phylogenetic resolution (Figure 8). An additional step could be done by testing an *Epichloë* specific marker such as 003tef1-exon1d-1 (Forward) and 004-exon6u-1 (Reverse) to perform a more targeted approach. Unfortunately, this was not possible within the existing timeframe of this thesis.

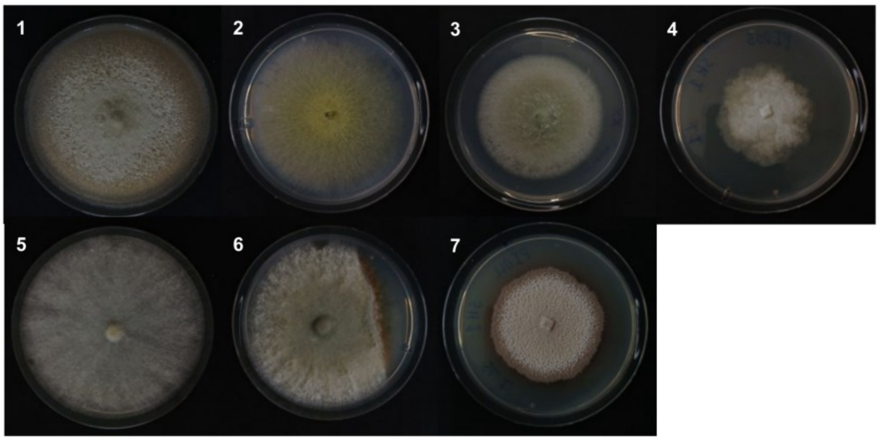
Questions can also be raised whether the method used to break the dormancy of *Epichloë typhina* in the frozen samples with visible stromata was sufficient. A different thawing procedure or changes of the incubation temperature could be necessary for future work with frozen material. Furthermore, the differentiation of *Epichloë* sp. by color are also to be considered a sub-optimal method since the acquired white morphotypes looked very similar without belonging to the same genus, and misidentifications could easily be made in comparisons with morphotypes published in the literature.

In this study both fresh and frozen material were used for isolation. The sampling date of the field plot gives an indication of which fungal genera are present at this particular site and this particular moment. The sampling site had not been in use for the last 2-3 years. The field collected samples of timothy, meadow fescue and cocksfoot should therefore not be considered as cultivated ones. The discarded area therefore is similar to a meadow pasture where plants naturally set their seeds and propagate. To better increase the understanding of the fungal genera present, a systematic sampling of several different locations is required.

A. Uncultured fungi



B. Unclassified fungi



C. No homology found in the databases

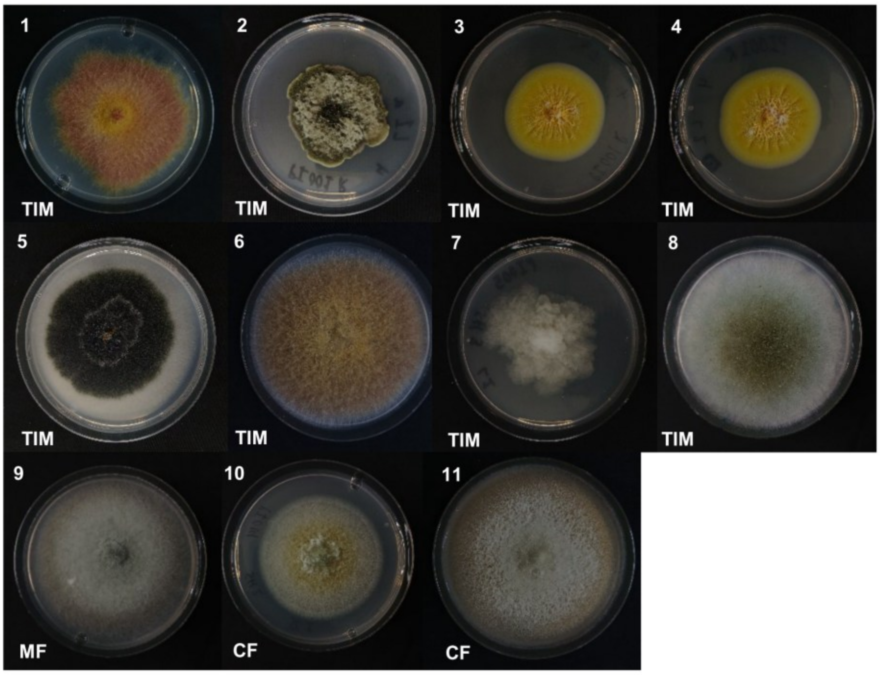


Figure 10. Colony morphotypes of fungi of unknown genus (uncultured, unclassified, or not hit)

Overall fungal genus diversity within the three grass species unveils 28 genera (Figure 9). Most of the isolated known colonies correspond to fungi from the genera *Alternaria*, *Cladosporium* and *Epicoccum* which all belong to genera of fungal species which can also colonize of be problematic in cereal production (Woudenberg et al. 2015; Bench et al. 2012; Taguiam et al. 2021). Apart from these, the less frequent genera such as *Parastagonospora, Fusarium, Collectotrichum, Phaesphaeria* and *Microdochium* include fungal species that either directly or indirectly affect yields or quality in cereals and grasses (Cunfer 2000); Nguyen et al. 2025; Dean et al. 2012; Mcdonald et al. 2012; Liang et al. 2019). Thereby the results suggest that the forage grasses are possible hosts for potential crop pathogens, which raises questions about how this can affect other crops when forage is included in a rotation or present in the farm landscape.

To summarize, a more targeted approach with specific primers is needed to better understand which species are present since 25 samples are remain unknown. Furthermore, additional tests must be made to fully understand if it is feasible to work with frozen samples since none of the samples with visible stromata indicated any presence of *E. typhina.*

4.2 Understanding the issues, needs and gaps related to *Epichloë* sp. in Sweden.

Based on the consultations, it is clear that the issues linked to *Epichloë* spp. in Sweden are complex and have many layers. The possible production of mycotoxins by *Epichloë* spp. can have severe effects on the wellbeing of horses, which considered as an important issue in the animal sciences (CM). However, from a seed production perspective there are at the moment no major issues in Sweden related to choke. This statement puts light on endophyte-derived toxins rather than the production of the seed (JP).

The current needs are highlighted within the frame of mycotoxin contamination and the capacity to test whether the toxicity levels are safe or unsafe as feed (LN;GA;EN;CM). There is a clear need for registered methods since testing can today not be performed in Sweden (CM), while project funding therefore proves important to develop suitable methods to be able develop such capacities (GA & EN). This will also mean that it will be possible to test the straw as JP mentioned. Furthermore, there is an expressed need for more data collection and monitoring of fields (LN). How this should be performed in practice is yet to be defined. In total, this highlights the fact that our current knowledge is not enough to understand the epidemiology and the conducive conditions favoring the production and accumulation of endophyte-derived toxins in the field. Further, knowledge gaps include the location of the toxins, whether they are in the spikes or systematically within the plant (LN). Additionally, the safe concentrations of toxicity for timothy and *Epichloë typhina* is also unknown. The impact of the environment variation in the host species are also unknown according to CM. Therefore, we need to fill in the gaps, and the key to that is cooperation between animal science and plant pathologists as stated by LN, GA & EN and CM.

To summarize, the seed focus is not the most important focus important today. However, it might be in the future. Especially since the sector lacks an understanding of why choke disease occurs. Being able test whether the feed is dangerous or not for animals is the larger concern since it could become a larger problem in the future during conditions of drought. Conditions of drought would decrease the yield of cultivated and managed forages. Ultimately it could force the sector to accept forages from areas which are not as well controlled such as extensive pastures or meadows. The ability to test would then be critical to ensure that the new "imported" forage is safe to use on the farm (this is not the case currently). This leads to the conclusion that *Epichloë* spp. are not only of interest in relation to animal husbandry and seed crop production but is also linked to feed security.

5. Conclusion

Based on results from the experimental part of this work, we can indicate that one *Epichloë* sp. exist in one fresh collected sample without any symptoms of choke. We also know that 25 of the 82 cultivated isolates in this practical could not be assigned to a genus, which suggest there is still a diversity of fungal species associated with grasses that remains unknown. Of the confirmed fungal genera, many can also be found in cereals, which suggest that forages and cereals may also share endophytes and pathogens. We also highlight that despite the typical *Epichloe* morphotypes reported in the literature, identification of these fungi with morphotyping did not work as well as assumed.

Results from consulations highlighted the main issues with *Epichloë* spp. lean towards the animal perspective, however knowledge of plant-fungal interactions, conducive conditions, epidemiology and underlying mechanisms of disease expression are also of interest. The overall situation in seed crop production is not a major problem today, even if it exists in long laying seed crops of timothy. A problem could however still arise in the future. No other seed crop seems to be affected in Sweden at the moment. The possibility to test the feed for mycotoxins is of great importance to prevent and measure toxin concentrations. Overall, the current knowledge in Sweden is scarce and gaps remain.

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Popular science summary

Forage crops are important in Sweden as they are the main source of feed for horses, cows and sheep. Like other crops, they can be infected with various fungi. Some are completely harmless, others harmful. Fungi of the genus *Epichloë* have proved to be problematic as some *Epichloë* spp. have the ability to produce toxic compounds. *Epichloë* spp. are endophytic fungi living in the tissues of their host and the toxins they produce can, in extreme cases, be fatal to grazing animals. The tricky part about these endophytes is that their presence is mostly invisible. When visible, they are recognized by the solidified yellow/orange cylindrical mycelium known as stromata. The development of stromata hinders inflorescence development, which can be problematic in seed crop production as it reduces seed yield.

In Sweden, timothy, cocksfoot and meadow fescue are known hosts of the fungal endophyte *Epichloë typhina*. Current knowledge of the effects of *Epichloë s*pp. on Swedish forage grasses is scarce but remains problematic for two reasons, (i) the possible production of endophyte-derived toxins that may be toxic to animals, (ii) seed yield loss due to obstruction of the inflorescence by the formation of stromata. At present, little is known about the conditions that favour *Epichloë* spp. in Sweden, their occurrence and possible effects on forage. At present, the problems associated with *Epichloë* spp. in Sweden are of minor concern. However, in order to better understand, evaluate and develop this matter for future problems, strong collaborations within animal nutrition, plant pathology and toxicology is recommended. In addition, the lack of ability to test the feed for toxins in Sweden will be of immediate concern in the event of drought or in situations where the agricultural sector may be forced to use feed from extensive leys and less controlled areas. Areas where symptoms associated with certain *Epichloë* spp. are generally more abundant. However, there are many issues that need to be addressed and knowledge gaps that need to be filled. Both from a practical management point of view and at a scientific level, to ensure better guidelines and recommendations within the sector.

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Appendix

Table 1. Primers used for molecular identification of fungal isolates

Locus	Primer	Orientation	Sequence (5'-3')	Length (bp)
ITS	001ITS1	Forward	TCCGTAGGTGAACCTGCGG	19
ITS	002ITS4	Reverse	TCCTCCGCTTATTGATATGC	20

Table 2. Reaction mixture for PCR amiplification

PCR Reaction	Volume (x 1 reaction)
Milli-Q H_2O	16,5 µl
10X DreamTaq Buffer	2,5 µl
dNTP mix, 2mM	1 µl
Forward primer 10 µM	0,5 µl
Reverse primer 10 µM	0,5 µl
Template DNA	4 µl
DreamTaq DNA polymerase	0,2 µl

Table 3. PCR amplification specifications

PCR	Time
94℃ (warmup)	1 min
Times 40 cycles	
94℃	30s
56℃	30s
72℃	1min
72℃	5min

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