# Diversity and species composition of fungal endophytes in *Avenella flexuosa* under different sheep grazing regimes.

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**Oslo 2010**

# Forord/preface

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# Abstract

- Fungal endophytes have been shown to be extremely widespread and abundant in terrestrial plants. It is also known that fungal endophytes may affect the grazing intensity of the host plant.

- In this study the occurrence of fungal endophytes in the perennial grass *Avenella flexuosa* was investigated under different grazing regimes in a field site in Norway. The main aim was to reveal to what degree the grazing influenced the fungal diversity and species composition.

- The fungal endophytes were analyzed using both culturing coupled to Sanger sequencing as well as 454 sequencing. The ITS2 rDNA region was used as a fungal DNA barcode.

- Based on the 454 data, a higher number of fungi were detected in stems of grazed compared to non-grazed plants. However, no significant difference was observed in the inflorescences and leaves. A distinct difference in fungal community composition was observed between leaves, stems and inflorescences. Apparently, the fungal community composition varied more in non-grazed compared to grazed plants. Saturation curve analyses indicated that most fungi remained undetected. Noteworthy, a high discrepancy was observed between the two methods, both in OTU number and overlap. From the 37 and 896 operational taxonomic units obtained from cultivation and 454 sequencing respectively. Only three OTUs were recovered by both methods. No known toxin producing endophytes were detected in *A. flexuosa*. Instead, *Phaeosphaeria/Stagonospora* spp. dominated both culture and 454 data, constituting potentially biological important endophytes of this grass. Intriguingly, various basidio-yeasts with high prevalence in the 454 dataset were not recovered by cultivation.

- This study indicates that grazing may have some influence on the diversity and composition of fungal endophytes. However, the different approaches underlines that the results must be interpreted with great care since there are many potential biases inherent both with the culturing and the 454 sequencing approach.

# Introduction

Endophytes (from Greek "endo" - within and "phytos" – plant) constitute a broad range of different organisms including bacteria, algae, insects and fungi (Schulz & Boyle, 2005). The strict definition of the term endophyte has been debated, with some authors stating that the term should be redefined or even abandoned (Wennström, 1994; Hyde & Soytong, 2008). In this study, the following definition of endophytic fungi is used: "*Endophytic fungi are fungi, that for all or part of their lifecycle, invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues, but cause no symptoms of disease*" (Wilson, 1995). Symbiotic interactions between plants and fungi are estimated to be at least 400 million years old (Remy *et al.*, 1994; Krings *et al.*, 2007), and are thought to be one of the most essential evolutionary steps from an aquatic to a terrestrial lifestyle (Selosse & Le Tacon, 1998; Heckman *et al.*, 2001). Endophytic fungi differ from the mycorrhizal fungi in both ecology and infection allocation. While mycorrhizas are restricted to the rhizosphere with particular importance for plant growth in nutrient-stressed situations, endophytes can be found in both above and belowground plant-tissues, and seem to be present even when nutrients are abundant (Smith & Read, 2008). Endophytic fungi are highly diverse, with the reported majority being ascomycetes, of which many lack a known teleomorphic state (Carroll, 1988). Transmission mode of the endophytic fungi is either horizontal, where spores infect a new host through the surface of the plant, or vertical, where the endophytic fungus infects the seeds. Upon seed dispersal and subsequent germination, the continuation of endophytic interaction is ensured.

During the past 25 years, at least two different ways of grouping fungal endophytes have been suggested (White, 1988; Rodriguez *et al.*, 2009). White (1988) highlighted the transmission mode in particular, and distinguished between the endophytes of the Clavicipitaceae (clavicipitaceous endophytes) and the rest (non-clavicipitaceous endophytes). In the four classes of fungal endophytes suggested by Rodriguez *et al.* (2009) the highly diverse nature of the non-clavicipitaceous endophytes has also been taken into account. Class 1 endophytes consist of the clavicipitaceous endophytes that are characterized by vertical and horizontal transmission, narrow host range and colonization limited to the shoot, stem and rhizome. Many members of the class 1 endophytes may also enhance resistance to invertebrate herbivory. This tendency is however not consistent throughout the entire range of class 1 endophytes, and in some cases might be a product of the fungal genotype (Clay, 1990;

Faeth *et al.*, 2006). Class 2 endophytes consist primarily of ascomycetes, but also some basidiomycetes. Both vertical and horizontal modes of transmission are known, and they colonize roots, stems and leaves with extensive colonization in the plant. The biodiversity per plant is in general low, but class 2 endophytes have a broad host range and confer in some cases habitat adapted benefits, for example salt tolerance in plants colonizing coastal beaches (Rodriguez *et al.*, 2008). Studies have also shown that some class 2 endophytes may speed up host defences when exposed to pathogens (Redman *et al.*, 1999). Class 3 endophytes are horizontally transmitted, have a broad host range, low colonization in plants and infection is restricted to shoots. Characteristically, the diversity of these endophytes may be very high even within a single plant. For example plants in tropic habitats may harbour hundreds of fungal endophytic species and studies have shown that leaves may be colonized by up to one fungal isolate per 2 mm$^2$ (Arnold *et al.*, 2000; Gamboa *et al.*, 2003). However, the ecological roles of these fungi are largely unknown. Class 4 endophytes are commonly referred to as dark septate endophytes (DSE). These are characterized by the presence of melanized hyphae, occur only in the root and are horizontally transmitted. Not much is known about their ecology, except that their distribution seems to be almost cosmopolitan and especially common in high-stressed habitats (Rodriguez *et al.*, 2009).

Over the past 30 years, the research field of fungal endophytes has attracted the attention of scientists from a wide variety of disciplines. The main focus has been on important agricultural plants, especially cultivated grasses such as *Festuca arundinacea* and *Lolium perenne* and their symbiotic interactions with endophytic fungi within the family *Clavicipitaceae*, notably the asexual *Neotyphodium* spp and their sexual forms of *Epichloe* spp, as well as the genus *Balansia* (Clay, 1988; Clay, 1990; Schulz & Boyle, 2005). Studies have demonstrated various endophytic interactions ranging from mutualistic to antagonistic, depending on host and endophyte genotype, and environmental conditions (Clay, 1988; Saikkonen *et al.*, 1998; Faeth, 2002; Faeth & Sullivan, 2003; Faeth *et al.*, 2006). Interactions between a very limited number of grass- and fungal species have founded model systems that have dominated the literature since the beginning of the discipline (Cheplick & Faeth, 2009). However, it is problematic to extrapolate from these limited model systems, especially when considering the relatively large proportion of contradictory results in the literature (Saikkonen *et al.*, 2006). To understand how these interactions have evolved, it is important to also investigate native grasses and their natural fungal endophytes (Faeth & Sullivan, 2003; Sullivan & Faeth, 2004).

Already in 1906 the effects of the so called 'sleepy grass' was described, where livestock displayed great reduction of motor function (Bailey, 1903). Later, this effect was attributed to the fungal endophytes of the grass and the toxic compounds they produced (Epstein *et al.*, 1964). Although agronomic grasses infected by *Neotyphodium* are overrepresented in studies dealing with fungal endophytes and their impact on herbivory, some knowledge also exists for wild native grasses and other fungal infective agents (Saikkonen *et al.*, 2000; Rosa *et al.*, 2009). The role of alkaloids mediating herbivory resistance is well documented, and it may seem that native grasses in general harbour lower levels of alkaloids than agricultural grasses (Cheplick & Faeth, 2009). However, also here the results differ. In wild *L. perenne* paramine levels may range from 6.3 to 40 µg per gram dry weight (Bony *et al.*, 2001), and high alkaloid variability have also been found in a variety of different grasses. Leuchtmann *et al.* (2000) demonstrated that different populations may vary in respect to alkaloid levels with as much as four orders of magnitude. Studies with mammalian herbivores have shown that consumption of endophyte infected *F. arundinacea* may cause a wide variety of diseases in domesticated animals including reduced reproduction and milk production, elevated body temperatures, heat stress and fescue toxicoses (Siegel & Bush, 1995). The effects of an endophyte infected diet have also been shown to affect female sexual maturation in the herbivore prairie vole *Microtus ochrogaster* (Fortier *et al.*, 2000).

Research on endophytic fungi has traditionally been based on surface sterilization and culture methods followed by morphological and/or molecular identification of the emerging fungi (Arnold *et al.*, 2000; Schulz & Boyle, 2005). More recently, endophyte studies have also employed direct PCR on plant material with fungal-specific primers, and subsequent cloning and sequencing of the amplified fragments (Flowers *et al.*, 2003; Jumpponen *et al.*, 2003; Bergemann & Garbelotto, 2006). Over the past few years sequencing technology have undergone great advances, so-called next generation (NG) sequencing techniques make sequencing of whole genomes possible in a matter of hours (Aury *et al.*, 2008). Several NG sequencing methods have been developed in the last years, all able to execute millions of parallel sequencing reactions, producing data at ultra high rates (Kircher & Kelso, 2010). The GS FLX Titanium™ upgrade of the 454 system used in this study, offer increased sequence length up to 400 bp, which decrease costs and increases the quality of high-throughput sequence data (Aury *et al.*, 2008; Nilsson *et al.*, 2009). However, these new techniques also create new challenges. Bioinformatics tools for analysing huge amounts of sequence data are presently limited, but this field is progressing fast (De Schrijver *et al.*, 2010; Durling *et al.*, in

prep; Henn *et al.*, 2010; Kumar *et al.*, in prep). Furthermore, erroneous and/or incomplete sequence annotations in GenBank complicate species/operational taxonomic units (hereafter referred to as OTUs) identification based on automated BLAST searches. The rapidly increasing number of submitted sequences through NG sequencing methods will in this respect constitute a constantly increasing challenge in the years to come (Karp, 1998; Karp, 2001; Vilgalys, 2003; Buee *et al.*, 2009).

Each year 2.1 million domestic sheep (*Ovis aries*) are released for summer grazing in Norway. In alpine habitats, the perennial grass *Avenella flexuosa* (Poaceae) accounts for about 30% of their diet and is one of the most important sources of food for sheep, although it is considered as a grass of medium nutritional value (Mysterud & Austrheim, 2005). *Avenella flexuosa* is characterized as a shade tolerant grass adapted to low soil pH, and is abundant in Europe with a main distribution in the cold temperate regions (Högbom & Högberg, 1991; Chiapella, 2007). A few studies of endophytic fungi in *A. flexuosa* have been conducted, and it seems as this grass harbor relative low frequencies of endophytes in general, and that horizontal transmission may be more widespread than vertical transmission (Saikkonen *et al.*, 2000; Göransson *et al.*, 2008).

The principal objective of this study is to investigate the diversity and composition of fungal endophytes in populations of *A. flexuosa* in an alpine habitat under different grazing pressures, by means of traditional culture dependent methods paralleled by 454 sequencing. The aims of this study are to

1. Find potential important endophytes in *A. flexuosa*.

2. Compare culture based methods to a next-generation sequencing technique.

3. Describe the diversity and composition of fungal endophytes in *A. flexuosa*.

4. Investigate whether the diversity of endophytes is affected by herbivory.

## Materials and methods

*Study site*
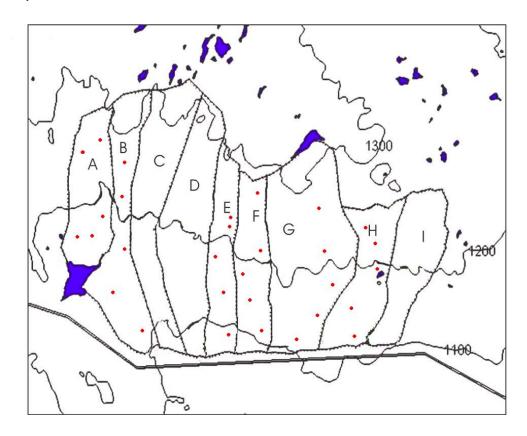


**Figure 1. Schematic overview of the study site. Enclosures B, F and H contain equivalent to 80 sheep/km$^2$. Enclosures A, E and G are control enclosures not containing sheep. Within each enclosure three plants were collected from each of the five plots (red circles), corresponding to a total of 90 collected plants. Plots from enclosures C, D and I were not sampled.**

The study site is situated in Hol municipality, Buskerud County in the southern part of Norway (between $60^o40'$ – $60^o45'$N and $7^o55'$ – $8^o00'$E). The climate of the area is sub-continental alpine, with moderate to low precipitation (Førland, 1993; Mysterud & Austrheim, 2005). The study site was established in 2001 and consists of a 2.7 km$^2$ field site divided in nine enclosures with an average area of 0.3 km$^2$. Three enclosures host 80 sheep per km$^2$ (high grazing pressure, B, F and H: Figure 1), three host 25 sheep per km$^2$ (moderate grazing pressure, C, D and I: Figure 1), and three control enclosures host no sheep (no grazing pressure, A, E and G Figure: 1). The nine enclosures are separated with 110 cm high fencing, for a more detailed description of the study site see (Mysterud & Austrheim, 2005; Mysterud *et al.*, 2007). In 2001, a total of 20 plots of 0.25 m$^2$ were placed at random locations within

each of the nine enclosures in order to investigate effects of sheep grazing on an alpine ecosystem (Mysterud *et al.*, 2007; Mysterud & Austrheim, 2008). In this study, the enclosures with high grazing pressure (enclosure B, F and H) and no grazing pressure (enclosure A, E and G) were used (Figure 1)

*Sampling of plant material*

On August 21$^{st}$ 2008 90 plant individuals of *A. flexuosa* were randomly sampled from the three control enclosures and three high grazing pressure enclosures, hereafter referred to as sample 1. To minimize disturbance of the existing plant community in the preexisting plots that were part of other ongoing studies, plants were collected in close proximity to the preexisting plots, no closer than 1 meter and not further away than 3 meters. Plants were deposited in zip lock plastic bags and kept in a cooling bag for approximately 4 hours before storage at 4$^{o}$C until isolation. At each plot, an additional bulk sample of approximately 15 plants was collected, hereafter referred to as sample 2. Each bulk sample was deposited in a zip-lock plastic bag, kept in a cooling bag for approximately 4 hours before storage at -20$^{o}$C. Plots from different altitudes were sampled to cover a latitudinal gradient for the entire study site (Figure 1).

*Cultivation of fungal endophytes*

The plants from sample 1 were processed within 4 days after sampling. Individual plants were dissected in order to surface sterilize stems, leaves and inflorescences separately. The surface sterilization procedure followed recommendations from Clay and Schulz (*pers comm.)*, and (Schulz *et al.*, 1993) and included the following steps: material was washed for 1 min in 96% EtOH, 2 min in commercial bleach (Klorin-Lilleborg, containing 4.5% NaOCl), 30 s in EtOH and finally rinsed 3 times in autoclaved dH$_2$O. The water baths were changed after each plant, and the EtOH and bleach baths were changed between each plot (i.e. after three plants) in order to minimize potential cross contamination. The cut points were trimmed using a sterile scalpel, so that any EtOH or bleach penetrating through the cut site would not inhibit fungal growth. To check whether the sterilization procedure had efficiently eliminated surface associated fungi, an imprint was made for each sterilized plant tissue part prior to transfer

onto 2% MEA (malt extract agar; 15 g agarose, 20 g malt extract, 1 l autoclaved $dH_2O$, 1000µl of: 25 mg/ml streptomycin and 12.5 mg/ml tetracycline) (Schulz & Boyle, 2005). Agar plates with sterilized plant material were incubated in darkness at 19$^o$C. The plates were inspected regularly for 3 months, and any emerging colonies were isolated in a flow using a sterile scalpel. Axenic culture isolates were maintained on 2% MEA, and a backup of each isolate was stored in 20% glycerol at -80$^o$C.

In many cases, a slimy, pale yellowish helix assumed to be bacterial contamination surrounded the surface sterilized plant tissues on the agar plates despite use of antibiotics in the growth medium. On these plates, growth of filamentous fungi was scarce or completely missing. Hence, culturing of filamentous fungal endophytes seemed therefore to be partly inhibited. Unfortunately, the assumed contamination was not confirmed by microscopy to be bacteria, but appeared macroscopically as bacteria and not yeast. Only four of the 90 imprint controls yielded fungal colonies. These were macroscopically identified, and were not found in any of the other isolation plates from plant material (data not shown).

*Molecular identification of fungal cultures*

Approximately 0.25 cm$^2$ of mycelia from each axenic culture was collected using a sterile scalpel and transferred to an Eppendorf tube containing 600 µl cetyltrimethyl ammonium bromide buffer (CTAB; 20 g/l CTAB, 1.4 M NaCl, 0.1 M tris-HCl, 20 mM Na$_2$EDTA) and frozen at -80$^o$C for minimum 30 min. The tubes were thawed on a heating block, two sterile tungsten carbide beads were added and crushed on a Retch® MM301 machine (Anders Pihl AS, Dale, Norway) for 2 min at 20Hz before another freezing step at -80$^o$C for minimum 15 min. DNA was extracted following a 2% CTAB protocol (Murray & Thompson, 1980) with a few modifications as described by Gardes & Burns (1993).

PCR was performed in 10 µl reactions in the Eppendorf Mastercycler epgradient (Eppendorf, Hamburg, Germany) using 3.2 µl miliQ $H_2O$, 0.2 µl DyNAzyme II (Finnzymes Oy, Espoo, Finland), 1 µl 10X buffer, 2.0 µl of 2µM dNTP, 0.8 µl of 5µM ITS1F and ITS4 primers (White *et al.*, 1990; Bruns & Gardes, 1993) and 2 µl template DNA. In cases where the DyNAzyme II procedure failed to amplify a fungal PCR product, PuReTaq™Ready-To-Go™ PCR beads (GE Healthcare, Little Chalfont, UK) were used instead. The beads were added 20 µl stock DNA and 2 µl of the primers ITS5 and ITS4 (5µM) (White *et al.*, 1990). The PCR program was as follows: denaturation 94$^o$C for 4 min, followed by 35 cycles of

denaturation at 94$^o$C for 25 s, annealing at 50$^o$C for 30 s, extension at 72$^o$C for 2 min followed by a final extension step at 72$^o$C for 10 min. The PCR amplicons were visualized by gel electrophoresis on a 1% agarose gel with SYBR$^®$Safe, using FastRuler$^{TM}$ LowRange DNA Ladder (Fermentas, USA) in order to determine the approximate size of DNA fragments.

The PCR amplicons were purified with Exosap-IT (GE healthcare) in 7 μl reactions using 1 μl PCR product, 1 μl 10X diluted Exosap-IT and 5 μl miliQ H$_2$O. All PCR products were sequenced in both directions using the ABI BigDye Terminator sequencing buffer and v3.1 Cycle sequencing kit on an ABI PRISM 3730 Genetic analyzer (Applied Biosystems, Foster City, CA). Chromatograms were manually investigated using BioEdit Sequence alignment editor v.7.0 (Hall, 1999), and the taxonomic affiliation of the resulting ITS-sequence were searched for using BLAST searches (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi; (Altschul *et al.*, 1997). The sequences were also clustered at 98% cut-off in order to compare the frequency of fungi using cultivation and 454 sequencing using the CLOTU pipeline (Kumar *et al.*, in prep).

*454 sequence analysis*

To compare the culture based approach with a 454 sequencing approach, plants from sample 2 were prepared for 454 sequencing. From each plot, three plants were randomly selected and separated by stem, leaf and inflorescence. To remove potential surface associated fungi, stems and leaves were rinsed with sterile paper soaked in EtOH (96%) three times repeatedly. Finally, the stems and leaves surfaces were wiped again three times using a sterile paper soaked in sdH$_2$O. Surface sterilization of the inflorescence was performed in a 50 ml falcon tube containing approximately 25 ml EtOH on a rotating table for 2 min. Excess EtOH was removed with a pipette. The flowers were rinsed twice in 25 ml of sdH$_2$O following the same procedure. Sterilization efficiency was investigated using a scanning electron microscope of a small sub-set of the treated plants and no fungal spores or hyphae were observed on the investigated plants (data not shown). The stems, inflorescences and leaves from three plants were pooled according to tissue type in 2 ml Eppendorf tubes and stored at -80$^o$C, yielding a total of 90 samples.

*1. DNA isolation.*

The material was freeze-dried, weighed and transferred to 2 ml Precellys MK28 tubes (Bertin technologies Montigny, France) containing 7 stainless steel beads. Inflorescences and stems were crushed for 180 s at 6 500 Hz using the Precellys24® homogenizer (Bertin technologies Montigny, France), but due to the fragile nature of *A. flexuosa* leaves, these were crushed for 60 s at 6500 Hz. DNA was isolated following the CTAB DNA extraction protocol described in GMO-CRL (2006) with some modifications: 1400 µl preheated CTAB buffer was added with the crushed plant material, together with 10 µl RNase (10mg/ml), followed by 30 min incubation at 65$^{\circ}$C. Ten µl Protinase K (20mg/ml) was added and incubated at 65$^{\circ}$C for 30 min. Samples were then frozen at -80$^{\circ}$C for a minimum of 1 hour, thawed at 65$^{\circ}$C and centrifuged at 12 000 *g* at room temperature. The supernatant (~1 000 µl) was transferred to a new tube and 500 µl chloroform was added and the solution was thoroughly mixed. Samples were centrifuged for 15 min at 12 000 *g*, and 800 µl of the aqueous upper phase were transferred to a new tube containing 500 µl chloroform and thoroughly mixed and centrifuged for 5 min at 12 000 *g*. The aqueous upper phase (~600 µl) was transferred to a new tube, and 1 200 µl of CTAB precipitation buffer was added and the solution was incubated at room temperature for 1 h. The solution was centrifuged at 13 000 *g* for 5 min, the supernatant was discarded, and the precipitate was dissolved in 350 µl 1.2 M NaCl. Then, 350 µl chloroform was added, and the solution was thoroughly mixed. The aqueous upper phase (~300 µl) was transferred to a new tube, and 180 µl isopropanol was added. The solution was mixed gently, and incubated at room temperature for 20 min. Further, the solution was centrifuged for 10 min at 12 000 *g* to pellet the DNA. The supernatant was then removed, and 500 µl 70% EtOH was added and mixed gently to rinse the pellet. The tubes were then centrifuged for 10 min at 12 000 *g*, the supernatant was discarded and the pellet was vacuum dried. The DNA pellets were dissolved in 100 µl TE buffer (pH 7.5) and stored at -20$^{\circ}$C until further use. To control for potential carry-over contamination an extraction blank control were included in the DNA-isolation and subsequent PCR analysis. Approximate concentrations were obtained using the NanoDrop® ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

The total genomic DNA yield for the 90 plant tissue samples subjected to 454 sequencing analyses were in general high for inflorescences (457.2 ng/µl, SD ± 299.8), low for leaves (31.2 ng/µl, SD ± 25.7), and intermediate for stems (173.8 ng/µl, SD ± 118.9); see supplementary material Table S1 for details).

## 2. Real-time PCR

Initial PCR tests were performed on the 90 DNA samples described above using the fungal specific primer ITS1F in combination with ITS4 (see below). Based on the individual band-intensity, the DNA samples were divided into three dilution categories: strong, medium and weak. Samples from each of the three categories were diluted 500X, 50X and 10X, respectively. Real-time PCR (RT-PCR) was performed in order to assess the overall amount of fungi and to approximately determine, for each of the DNA-samples, the cycle threshold (Ct-value) and the exponential phase of the PCR reactions. RT-PCR amplifications were performed with QuantiTect SYBR Green PCR Kit (Quiagen) on Stratagene Mx3000P QPCR System (Stratagene) in a total volume of 25 µl using 2.5 µl forward and reverse primers (5 µM of ITS1F and ITS4) with 12.5 µl SYBRGreen master mix™, 5 µl template DNA that was diluted accordingly to the dilution scheme (see supplementary material Table S1), and 2.5 µl miliQ $H_2O$. Data were analyzed using the Stratagene MxPro™ software. For samples where PCR inhibition was suspected based on abnormal shape of the curve and/or unexpected high Ct-values, a new RT-PCR run was performed based on further diluted samples. If the shape of the curve and Ct-value improved this was interpreted as evidence of inhibition in the first RT-PCR run. By investigating when samples reached the exponential phase in the RT-PCR reaction and adjusting the number of cycles in the initial PCR (see below), we assumed that major differences in ITS copy numbers between samples were evened out. Thus, samples containing only a few ITS copies would have an increased probability of being detected in the 454 sequence run. The Ct-values for the individual samples ranged from 29 to 45 (mean: 36.7, SD ± 2.99, supplementary Table S1) indicating a relative low concentration of fungal DNA. Dilution series of a selection of DNA extracts indicated some, but no major, PCR inhibition (data not shown).

## 3. Initial PCR

Based on the Ct-values from the RT-PCR we made 14 categories (Ct-value± 0.5 cycle) (supplementary material Table S1). Initial PCR products were obtained using the ITS1F-ITS4 primer pair as describes above. The amplifications were performed using a MJResearch PCT-200 Thermal Cycler (MJ Research, Waltham, MA) in a total reaction volume of 30 µl, using 1.5 µl 5 µM of each primers, 3 µl 2 µM dNTPs, 0.3 µl Phusion™ High-Fidelity DNA

Polymerase (Finnzyme, Oy), 6 µl 5X Phusion™ HF buffer, 14.7 µl miliQ $H_2O$ and 3 µl template DNA (diluted according to the RT-PCR results). The PCR program included denaturation at $89^oC$ for 30 s, followed by the adjusted number of cycles according to Ct-values from the RT-PCR (see supplement material Table S1), including the following steps: denaturation at $89^oC$ for 10 s, annealing $55^oC$ for 20 s, extension $72^oC$ for 20 s followed by a final extension step at $72^oC$ for 7 min. Due to the exonuclease activity of Phusion™ High-Fidelity Polymerase samples were immediately frozen before continuing with nested-PCR.

### 4. *Nested PCR*

To accurately produce amplicons containing the 454 adaptor sequence, a nested-PCR approach was used. A Titanium™ primer is 55 bp long, and consists of four parts: 1) a 21 bp adaptor sequence (5'-CGTATCGCCTCCCTCGCGCCA-3'), 2) a 4 bp key-sequence (5'-TCAG-3') used to calibrate the nucleotide fluorescence signal, 3) a 10 bp unique mid-sequence identification tag, and 4) a barcoding forward primer (ITS 3; 5'-GCATCGATGAAGAACGCAGC-3', in this study). The reverse primer consists of a different adaptor sequence (5'-CTATGCGCCTTGCCAGCCCGC-3'), and the reverse barcoding primer is ITS 4 (5'-TCCTCCGCTTATTGATATGC-3'), as well as the key and tag sequences. The reaction was performed using the MJResearch PTC-200 Thermal Cycler, and a total reaction volume of 20 µl, using 2 µl of 5µM ITS3/4 tagged primers, 2 µl 2 µM dNTP, 0,2 µl Phusion™ High-Fidelity DNA Polymerase (Finnzyme Oy), 4 µl 5X Phusion™ HF buffer, 7.8 µl miliQ $H_2O$ and 4 µl of template DNA from the initial PCR. The PCR program included denaturation at $89^oC$ for 30 s followed by 20 cycles of denaturation at $89^oC$ for 10 s, annealing $55^oC$ for 20 s, extension $72^oC$ for 20 s before a final extension step at $72^oC$ for 7 min. A total of 100 samples were processed including the 90 original samples and 10 additional samples of random replicates of the former 90 in order to test the consistency of the 454 sequencing. The 100 samples were tagged with 20 different Titanium™ tags, thus 5 samples were tagged with the same Titanium primer. These were later separated in 5 different lanes on Titanium™ plate. To remove the polymerase, short sequences and primers from the samples the Wizard® SV Gel and PCR Clean-Up System (Promega) were used following the manufacturers protocol. In order to assure an optimal sequencing run, DNA quantification using fluorometry was performed on a FLUOstar Optima plate reader (BMG Labtek GmbH) with the Quant-iT Picogreen dsDNA assay kit as recommended by the manufacturer

(Invitrogen). The results from the quantification were used to standardize concentrations of the samples (see supplementary material Table S1 for details).

After the nested PCR step (20 cycles), PCR amplification yielded with few exceptions strong PCR bands. In the final Picogreen quantification of PCR product prior to 454 sequencing, the average concentration was 8 ng/µl, 45 of the 100 samples had concentrations above 10 ng/µl. All DNA extraction controls and PCR controls remained negative.

Sequencing was done on a Genome Sequencer FLX$^{TM}$ (454 Life Sciences Corp., Bradford, CT, USA) at The Norwegian High-Throughput Sequencing Centre (Oslo, Norway).

*454 sequence quality control and clustering*

The five lanes yielded a total of 64.1 Mbp, with an average sequence length of 414.2 bp. Using the CLOTU pipeline (Kumar *et al.*, in prep), sequences with the correct forward and reverse primers, tags and adaptor B were identified. Only sequences with correct primers and tags were kept for further analysis. For quality control sequences shorter than 250 bp and sequences with unresolved bases (N's) were removed (see supplementary material Table S2). Duplicate sequences were removed before sequence clustering in CLOTU was tested with 4 different sequence similarity cut-off levels (96%, 97%, 98% and 99%; supplementary material Table S3). After identification of OTUs at the 98% cut-off level, the sequences from the 10 replicated samples were removed from in further analyses. BLAST searches were conducted using one representative sequence from each OTU of the 203 (i.e. all OTUs with more than one unique read) most common OTUs. One of these represented an obvious contaminant and was excluded in all, but the 454 sequencing consistency analysis.

*Statistical analyses*

*1. Ordination*

Generalized Non-metric Multidimensional Scaling (GNMDS) ordinations were performed using R (R Core Developmental Team, 2010) on (a) presence-absence OTU plot matrix, where the occurrences of fungal OTUs were summarized across the plant tissues, in order to investigate fungal diversity in the plots and (b) presence-absence OTU tissue type matrix,

where fungal OTUs from the same plant tissue were summarized within enclosures, to investigate if fungal OTUs differed in the plant tissue-types. In the GNMDS ordination 1000 permutations were computed and the two axes with the lowest stress were used. Procrustes comparison were performed between the two best solutions=1.382e-08, (symmetric correlation=1, 0, significance=0.001) and the best and worst solution=1.652, (symmetric correlation = 0.2668, significance=0.549). A verimax rotation was performed so that the axes were sorted by decreasing explained variation. This in addition to half-change scaling of the axes, single axis interpretation of the ordination is possible. Half-change scaling will scale the axis so that 1.0 unit on the GNMDS axis on average will correspond to a halving of fungistic similarity between ordination plots.

### 2. *OTU richness estimates and shared species*

The OTU plot matrix was used in the computation of accumulation curves. The method described by Ugland *et al.* (2003) takes into account small scale spatial relationships that shape microbial ecosystems by dividing a sampled area into smaller sub-areas. By first calculating a species-accumulation curve for randomized samples of all the single sub-areas followed by a species-accumulation curve for all combinations of two sub-areas, this is repeated for all the defined sub-areas. Finally, a total species (T-S) curve is calculated based on the terminal points of the sub-area accumulation curves. This can then be extrapolated to assess the total number of species in the study area (supplementary material Table S4). This method have proven more reliable than traditional richness estimators such as Chao (1984), that tend to overestimate species richness in heterogeneous samples and/or in situations where the presence of rare species are high (Ugland *et al.*, 2003). Shared OTU statistics was computed in EstimateS version 8.2.0 (Colwell, 2006).

### 3. *Vectorized General Linear Models*

To reveal which factors affecting the number of putative endophytes in the plant parts, as measured by 454 sequence data, a Vectorized General Linear Models (VGLM) analysis was conducted, where the number of OTUs per plant part was used as response. The main challenge in the analysis is the large number samples with zero OTUs. Plotting revealed an intermediate group containing 15-30 OTUs, however the remaining plots had a very high

frequency of fungal OTUs (>80: see supplementary material Figure S5). For count data, a Poisson distribution would be the first choice. However, the large number of zeroes is unlikely to fit a standard Poisson distribution. Datasets containing a larger proportion of zero observations than standard distributions (such as Poisson) are referred to as 'zero-inflated' (Martin *et al.*, 2005). We therefore used VGLM with a zero inflated Poisson distribution. The VGLM analysis estimates the likelihood of having 0 or ≥1 OTUs using logistic regression. Further, for values ≥1 the variation is analyzed with a Poisson regression, thus providing two parameter estimates for each term. Due to a high degree of correlation between some of the environmental variables (see supplementary material Figure S6) calcium, magnesium, nitrogen and potassium were summarized to comprise a complex variable referred as base-cation. All variables were log transformed to stabilize the variance. To find the most parsimonious model, we used an AIC forward selection procedure (Burnham & Anderson, 2002). VGLM is implemented in the VGAM package in R, version 2.11.1 (R Development Core Team 2010).

### 4. *Neighbour-joining analyses*

A separate sequence alignment of the ITS2 region, consisting of 24 reference sequences of known endophytes from the genus *Phaeosphaeria/Stagonospora* retrieved from GenBank, and five sequences generated from cultivation and 454 sequencing were analyzed using Neighbour-joining (NJ: consult supplementary material Table S7 for reference sequences). This was done to more certainly assess taxonomic placement of OTUs within the genus *Phaeosphaeria*. Neighbour-joining analyses were conducted in MEGA (Tamura *et al.*, 2007), bootstrap test were set at 1000 replications using default settings and gaps were treated as missing data.

# Results

*Sequence data*

## 1. Culture data

Isolation of putative endophytes from surface sterilized tissues of *A. flexuosa* yielded 83 axenic cultures where corresponding ITS2 sequences clustered into 37 OTUs based on 98% sequence similarity cut-off. Of these were 64.8% singletons. The frequency distribution of the OTUs is shown in Figure 2a and their taxonomic affinity at the order level in Figure 4a.

## 2. 454 sequence data

After filtering out putative low quality 454 reads using CLOTU; 169 887 reads were reduced to 152 984 (see read statistics in supplementary Table S2). Removal of duplicate reads resulted in a total of 33 003 unique ITS2 sequences that were subjected to clustering analyses. At a 98% cut-off level, the 454 sequences yielded 4 763 clusters, of which 3 790 (~80%) were singletons. In the further statistical analyses, singletons were removed, which resulted in a total of 897 OTUs (see supplementary material Table S3 for details). Noteworthy, the second most common OTU was identified as an obvious contaminant, namely *Agaricus bisporus*, a non-native fungus to Norway. The contamination source was tracked back to the laboratory where DNA extraction of *A. bisporus* had been performed at high volumes. This OTU was removed from downstream analyses. Hence, the total number of OTUs used in all further analyses was 896. Figure 2b displays the frequency distribution of the 202 most common OTUs (i.e. all OTU with more than one unique read).

Of the 896 OTUs, as many as 806 (89.9%) were only recovered in a single sample plot, while 90 (10%) OTUs were found in two or more plots. Only 22 OTUs (2.4%) were found in five or more plots.

To assess the consistency of the 454 sequencing, ten samples were sequenced in replicates (run1 and run2). The second run yielded 76 novel OTUs not recovered in any other sample, and were thus removed in downstream analyses. Regarding the 20 most common OTUs found in the replicate runs, the correlation was high ($r^2$ =0.64, Figure 3). However, run1 and run2 shared only 34 of the 195 total OTUs (17%) while they had 59 and 102 unique OTUs respectively. The Bray Curtis similarity index was 0.297. Number of reads for the two runs can be found in supplementary material Table S8.
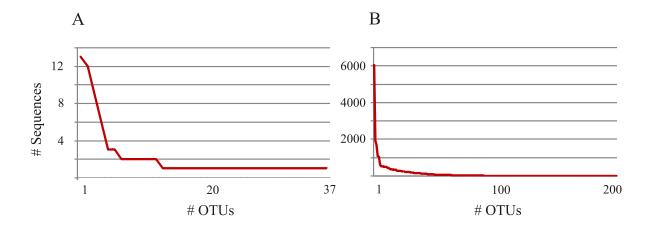
**Figure 2. Number of occurrences of OTUs obtained from (a) culture derived ITS2 sequences and (b) 454 sequences using 98% sequence identity as cutoff during clustering. (a) Twenty-four of the 37 ITS2 sequences were singletons. (b) Only the 202 most frequent OTUs are shown.**
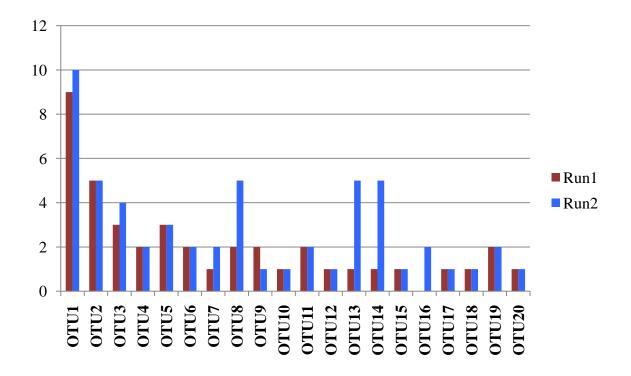


**Figure 3. Number of occurrences of the 20 most common OTUs obtained in replicate 454 sequencing runs of ten samples. The numbers of OTUs across the two runs are highly correlated ($r^2$=0.64).**

*Taxonomic affiliation and key species*

The most frequently recorded genera using culturing were *Davidiella* (anamorph: *Cladosporium*), *Phaeosphaeria* (anamorph: *Stagonospora*), *Aureobasidium, Sirococcus, Kabatiella* and OTUs from the family *Sarcosomataceae* (see Table 1), all ascomycetes. The relative abundance of fungal orders inferred from the culture data is shown in Figure 4a. Pleosporales was the most frequent order containing OTUs from the genera *Phaeosphaeria, Pyrenophora* and *Saccharicola*. The second most frequent order was Diaporthales with putative species from the genera *Sirococcus*, *Leucostoma* and *Valsella*. Dothidiales includes the genera *Aureobasidium* and *Kabatiella*.

The most common OTUs (i.e. highest occurrence in the plots) using 454 sequencing were recognized within the ascomycete genera *Phaeosphaeria*, *Botryotinia* (anamorph: *Botrytis*), *Godrinia*, and the basidiomycete genera *Filobasidiella* (anamorph: *Cryptococcus*), *Tremella*, *Sakaguchia,* and *Exobasidium*. Information about taxonomic affiliation for the 20 most frequently detected OTUs using 454 sequencing are shown in Table 1. The relative abundance of the most common fungal orders inferred from the 454 sequence data is shown in Figure 4b. Tremellales was the most frequent order containing OTUs from the genera *Filobasidiella*, *Dioszegia* and *Tremella*. Pleosporales was the second most abundant order, containing only OTUs from the genus *Phaeosphaeria*. Three OTUs from the fungal genera *Phaeosphaeria, Sirococcus* obtained by culturing were recovered by 454 sequencing (Table 1). The remaining OTUs did not overlap between the two approaches.

The Neighbour-joining tree in Figure 5 depicts the clustering of the most frequently detected OTU (*Phaeosphaeria/Stagonospora* sp) with closely related *Phaeosphaeria* taxa and corresponding *Stagonospora* anamorphs retrieved from GenBank. Two OTUs, (454 OTU-1 and culture OTU-6: Table 1) clustered in a 99% bootstrap supported clade along with a range of closely related species, including *P. alpina, P. padellana* and *P. oreochloae*. One OTU, namely OTU-4, clustered together with a confirmed beneficial endophytic isolate of *Stagonospora* sp. (Ernst *et al.* 2003). The culture derived OTU-5 showed closest affiliation to several closely related *Phaeosphaeria* taxa, including *P. lindii, P. vagans* and *P. luctusoa,* and a sequence from a putative endophytic fungus, however the support was low. One OTU only recovered in one plot, and thus not included in Table 1, namely OTU-S, grouped together with an insufficiently identified sequence, *Phaeosphaeria* sp. in a 97% bootstrap supported clade.

**Table 1. Information about the best BLAST hits for the 8 and 20 most frequent OTUs obtained from cultures and 454 sequence data, respectively.**

| *OTU#* | Taxonomic affiliation[1] | Order | AN[2] | QC[3] | SI[4] | OiP[5] |
|---|---|---|---|---|---|---|
| | ***Culture data*** | | | | | |
| 1 | *Davidiella macrospora* | Capnodiales | EU167591 | 100 | 100 | 20 |
| 2 | *Aureobasidium pullulans* | Dothideales | EU547495 | 99 | 91 | 16.7 |
| 3 | ***Sirococcus conigenus*[6]** | Diaporthales | AY168971 | 55 | 98 | 16.7 |
| 4 | *Stagonospora sp.* | Pleosporales | AJ496626 | 100 | 97 | 16.7 |
| 5 | ***Phaeosphaeria pontiformis*[6]** | Pleosporales | AJ496632 | 96 | 97 | 6.7 |
| 6 | ***Phaeosphaeria alpina*[6]** | Pleosporales | AF439471 | 82 | 98 | 6.7 |
| 7 | *Sarcosomataceae sp.* | Pezizales | AY465503 | 87 | 97 | 6.7 |
| 8 | *Kabatiella caulivora* | Dothideales | EU167576 | 98 | 92 | 6.7 |
| | ***454 data*** | | | | | |
| 1 | *Phaeosphaeria padellana* | Pleosporales | AF439496 | 92 | 95 | 93 |
| 2 | *Cryptococcus* aff. *Victoriae* | Tremellales | FN298668 | 100 | 95 | 57 |
| 3 | *Tremella foliacea* | Tremellales | AF042450 | 99 | 84 | 53 |
| 4 | *Exobasidium pachysporum* | Exobasidiales | AB180352 | 95 | 95 | 53 |
| 5 | *Botryotinia fuckeliana* | Helotiales | FN812726 | 100 | 98 | 50 |
| 6 | *Sakaguchia dacryoidea* | Erythrobasidiales | AF444597 | 100 | 83 | 33 |
| 7 | *Godronia* sp. | Helotiales | EF672237 | 100 | 96 | 30 |
| 8 | Uncultured basidiomycete | N/A | EF504571 | 100 | 95 | 30 |
| | (*Cryptococcus victoriae*)[7] | | AF444645 | 100 | 80 | 27 |
| 9 | *Lophium mytilinum* | Mytilinidiales | EF596819 | | | |
| 10 | *Rhodotorula nothofagi* | Sporidiobolales | AY383749 | 86 | 96 | 23 |
| 11 | Uncultured fungus clone | N/A | EU516879 | 100 | 97 | 23 |
| | (*Mortierella elongata*)[7] | | AB476416 | 100 | 86 | 23 |
| 12 | *Sporobolomyces phyllomatis* | Erythrobasidiales | AF444515 | | | |
| 13 | *Bensingtonia changbaiensis* | Agaricostilbales | AY233339 | 99 | 88 | 23 |
| 14 | *Exobasidium rhododendri* | Exobasidiales | EU784219 | 91 | 91 | 23 |
| 15 | *Cadophora melinii* | Helotiales | DQ404352 | 100 | 90 | 23 |
| 16 | *Bensingtonia yamatoana* | Agaricostilbales | AF444634 | 100 | 96 | 23 |
| 17 | *Mrakiella cryoconiti* | Cystofilobasidiales | GQ911549 | 100 | 97 | 20 |
| 18 | *Cladophialophora minutissima* | Chaetothyriales | EF016382 | 98 | 90 | 20 |
| 19 | *Cryptococcus hungaricus* | Tremellales | AF272668 | 98 | 96 | 20 |
| 20 | *Mortierella macrocystis* | Mortierellales | AJ878782 | 99 | 94 | 17 |

[1] Species name of the sequence retrieved as the most similar sequence in EMBL/GenBank to the given OTU. When possible, the best annotated and presumed reliable sequence was chosen.

[2] GeneBank accession number (AN).
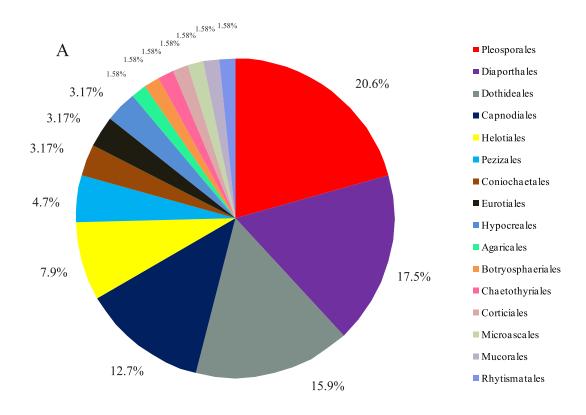
[3] Query coverage (QC): The percentage overlap between query sequence and GenBank sequence.

[4] Sequence identity (SI): The percentage similarity between query sequence and GenBank sequence

[5] Occurrence in plots (OiP): The percentage of plots the OTU was recovered. Culture data includes all OTUs found in more than one plot.

[6] Culture OTUs recovered in the 454 sequencing run, in bold.

[7] Due to insufficiently identified ITS sequence in Genbank, the web-application *emerencia* was applied in an attempt to better assess taxonomic affiliation (Nilsson *et al.*, 2005).

**A**

Pleosporales — 20.6%
Diaporthales — 17.5%
Dothideales — 15.9%
Capnodiales — 12.7%
Helotiales — 7.9%
Pezizales — 4.7%
Coniochaetales — 3.17%
Eurotiales — 3.17%
Hypocreales — 3.17%
Agaricales — 1.58%
Botryosphaeriales — 1.58%
Chaetothyriales — 1.58%
Corticiales — 1.58%
Microascales — 1.58%
Mucorales — 1.58%
Rhytismatales — 1.58%

Legend:
- Pleosporales
- Diaporthales
- Dothideales
- Capnodiales
- Helotiales
- Pezizales
- Coniochaetales
- Eurotiales
- Hypocreales
- Agaricales
- Botryosphaeriales
- Chaetothyriales
- Corticiales
- Microascales
- Mucorales
- Rhytismatales

**B**

27.4%
19.5%
18.5 %
9.5%
5.0%
3.2%
3.0%
2.5%
2.4%
1.8%
1.4%
1.3%
1.0%
1.0%
0.7%
0.6%
0.5%

Legend:
- Tremellales
- Pleosporales
- Erythrobasidiales
- Exobasidiales
- Cystofilobasidiales
- Agaricostilbales
- Envir samples uncult fungus
- Helotiales
- Pucciniales
- Sporidiobolales
- Taphrinales
- Diaporthales
- Chaetothyriales
- Rhytismatales
- Agaricales
- Mortierellales
- Less frequent OTUs

**Figure 4.Taxonomic distribution at the order level of ITS2 sequences derived from (a) cultures and (b) 454 sequences. Only OTUs including more than 100 unique reads are included in (b).**
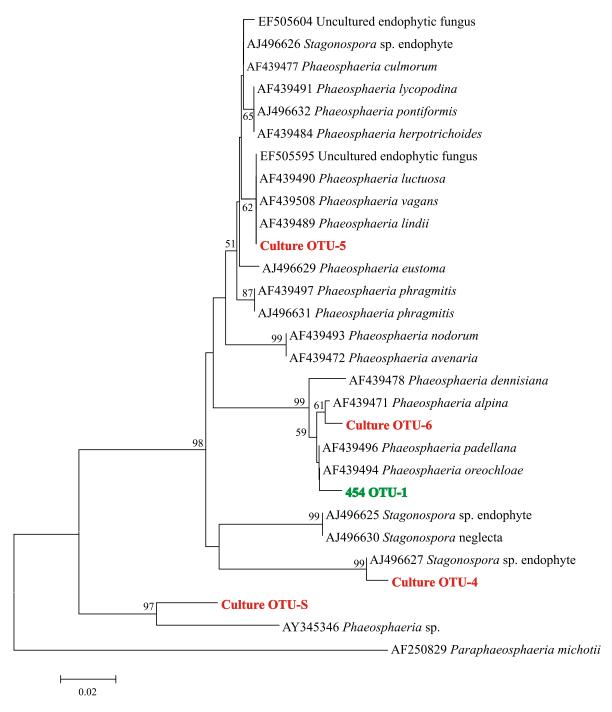
**Figure 5. Neighbour-joining tree of the ITS2 region from known *Phaeosphaeria* reference species, endophytic isolates and sequence data from 454 sequencing (in green) and culturing (in red). Numbers adjacent to branches show the bootstrap values (≥50%) of 1000 replicates. The culture derived OTU-6 and 454 derived OTU-1 both grouped in a 99% bootstrap supported clade along with fungi isolated from plants. The culture derived OTU-5 grouped together with *P. vagans* and *P. lindii*, however due to low bootstrap support the topography is unresolved. The culture derived OTU-4 clustred with 99% support with a confirmed beneficial endophytic isolate of *Stagonospora* sp. OTU-S, an isolate only found in one plot, grouped together with an insufficiently indentified sequence in a 97% supported clade. *Paraphaeosphaeria michotii* was used as outgroup.**

The number of OTUs varied greatly across the investigated plots using both cultivation and 454 sequencing (Figure 6). For example, in enclosure F the number of OTUs detected using 454 sequencing ranged from 5 to 143 across plots. The total number of OTUs detected in the different enclosures ranged from 182 to 220 (Figure. 6b). Cultivation recovered only a fraction of the number of OTUs retrieved through 454 sequencing.
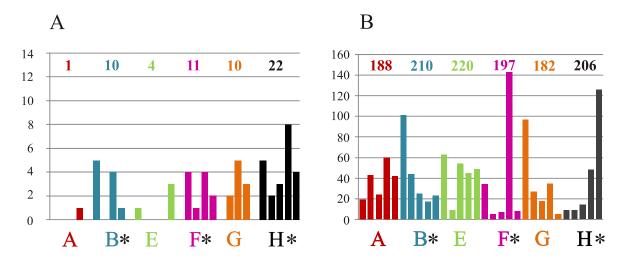


**Figure 6. Number of OTUs recorded in the different plots and enclosures (A-H) by (a) the culture based approach and (b) 454 sequencing. The total number of OTUs per enclosure is indicated above the chart. Grazed enclosures indicated with an asterisk (*). Note the different scaling on the y-axis.**

Noteworthy, the OTU-accumulation curves for the main localities showed no sign of reaching an asymptote (see Figure 7). Using a log linear function of the total species curve, 6391 OTUs was estimated to appear if $10^6$ plots had been sampled (see supplementary material Table S4).

Figure 8 and 9 show the frequency distribution of the 20 most common OTUs, observed using 454 sequencing, across non-grazed and grazed plots and across plant tissue types (inflorescences, leaves and stems), respectively. Regarding grazing, zero of the 20 most common OTUs were found to have a distribution significantly different from even (chi-square test, $p < 0.05$). Correspondingly, three OTUs showed a non-even distribution across plant tissue types (Figure 8 and 9).

Using a VGLM analysis, the number of OTUs per plant unit was related to the various explanatory factors, including grazing. Supplementary material Table S9 lists the observed mean of OTUs in the tissue typed. The number of OTUs in the inflorescences was at the same in grazed (observed mean=15.8) and control plots (observed mean=15.0). In stems, the number of OTUs were significantly higher in grazed plots (observed mean=20.86) compared to control plots (observed mean=7.8). The model seems to predict the number of OTUs well in the stems and inflorescences since a similar number of OTUs is seen for observed (supplementary material Table S9) and predicted mean (Figure 10). However, no reliable estimate of number of OTUs from leaves was obtained, likely due to rather extreme scatter of data points. In Table 2, the model predicted that leaves had a significantly higher number of fungal OTUs than inflorescences in control plots. However, the mean number of observed OTUs (=19.06) deviated from the predicted mean number of OTUs (~16.5, Figure 10), it thus seems as though the model did not fit with the leaf data well. Based on these observations, the results were not analyzed further. A significantly lower number of OTUs were detected in plots with higher concentrations of phosphorus and in plots rich in organic matter (i.e. loss of ignition). The complex variable base-cation, on the other hand, was positively related to number of OTUs (see Table 2).
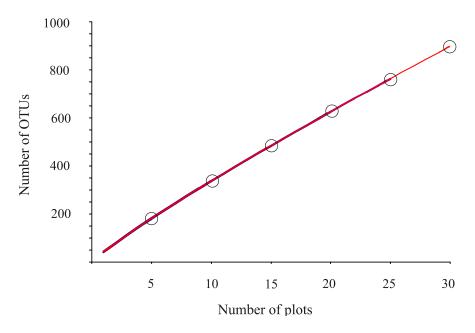


**Figure 7. Accumulation curves of OTU-plot data, based on the six sub-areas (/enclosures). The terminal points of the six accumulation curves serve as the basis for the T-S curve, indicated with circles. The end point of the curve corresponds to the 896 OTUs found in the study.**
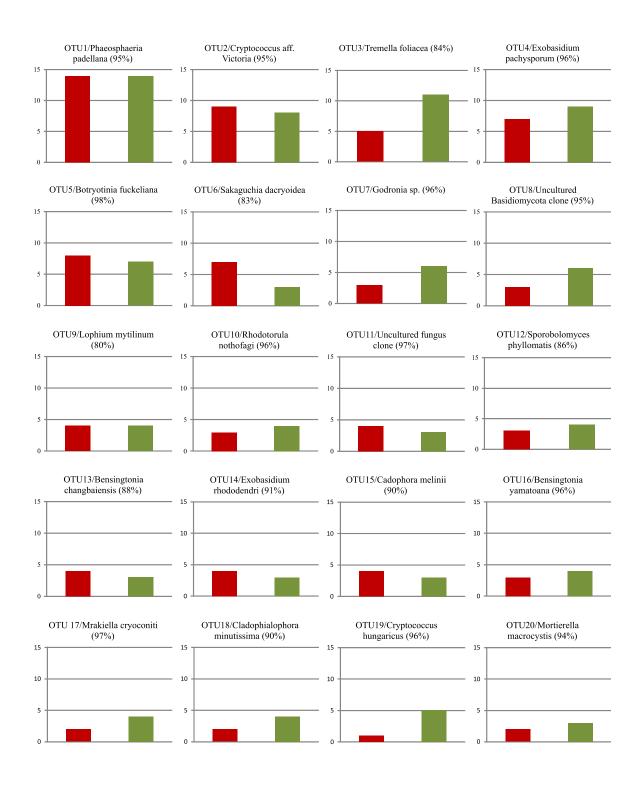
**Figure 8. Number of occurrences of the 20 most common OTUs in control (red) versus grazed (green) plots, as observed by 454 sequencing. No OTU had a significant deviation from even distribution (chi-square test, p<0.05). Information about the best BLAST hits of the OTUs are shown in parenthesis.**

**Figure 9. Number of occurrences of the 20 most common OTUs in the different tissue types; inflorescences (red bars), leaves (blue bars) and stems (yellow bars), as observed by 454 sequencing. The asterisk indicate a significant deviation from even distribution (chi-square test, p<0.05). Information about the best BLAST hits of the OTUs are shown in parenthesis.**

**Table 2. Results of the Vectorized General Linear Model (VGLM) analysis.**

Most parsimonious model:

Species ~ tissue + treatment + tissue:treatment + phosphorous + base-cation + loss on ignition

| Coefficients[1] | Value | SE | Lower 95% | Upper 95% |
|---|---|---|---|---|
| Intercept: 1 | -2.6579 | 2.3632 | -7.3842 | 2.0684 |
| Intercept: 2 | 2.3883 | 0.2140 | **1.9602** | **2.8163** |
| Tissue type (Leaves vs. inflorescences): 1 | 1.6305 | 1.1894 | -0.7482 | 4.0093 |
| Tissue type (Leaves vs. inflorescences): 2 | 0.4220 | 0.0879 | **0.2462** | **0.5978** |
| Tissue type (Stems vs. inflorescences): 1 | 0.7653 | 1.2857 | -1.8061 | 3.3367 |
| Tissue type (Stems vs. inflorescences): 2 | -0.6408 | 0.1132 | **-0.8673** | **-0.4143** |
| Treatment (Grazed vs. control): 1 | -10.9798 | 141.2906 | -293.5610 | 271.6014 |
| Treatment (Grazed vs. control): 2 | -0.1038 | 0.0965 | -0.2968 | 0.0891 |
| Phosphorous: 1 | -0.5208 | 2.0173 | -4.5553 | 3.5138 |
| Phosphorous: 2 | -0.6581 | 0.1851 | **-1.0282** | **-0.2880** |
| Base-cation: 1 | -0.0569 | 0.4719 | -1.0007 | 0.8869 |
| Base-cation: 2 | 0.2972 | 0.0489 | **0.1993** | **0.3951** |
| Loss on ignition: 1 | 0.6201 | 1.5198 | -2.4195 | 3.6596 |
| Loss on ignition: 2 | -0.7371 | 0.1734 | **-1.0838** | **-0.3903** |
| Leaves:treatment: 1 | 12.1732 | 141.2924 | -270.4116 | 294.7580 |
| Leaves:treatment: 2 | -0.1982 | 0.1405 | -0.4791 | 0.0828 |
| Stems:treatment: 1 | 11.0224 | 141.2944 | -271.5665 | 293.6112 |
| Stems:treatment: 2 | 1.1630 | 0.1425 | **0.8780** | **1.4480** |

[1] Logistic regression estimates indicated by: 1. Poisson regression estimated indicated by: 2. Intercept: Baseline was inflorescences from control enclosures. Bold values are significant at the 0.05 level.
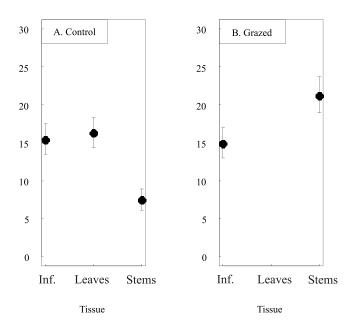
**Figure 10. Predicted mean number of fungal OTUs in the three tissue types (Inflorescences, Leaves and Stems), in (a) control and (b) grazed enclosures. In grazed plots the number of OTUs in stems is significantly higher than in stems from control plots. Due to extreme standard error in the leaves in (b) it is impossible to display the plot.**

*Community composition*

Shared number of OTUs, obtained in EstimateS, across enclosures is shown in Table 3. OTUs recovered through cultivation show a very low number of shared OTUs across enclosures, ranging from zero to four. Similarly, for the 454 data, a relative low proportion of shared OTUs are observed, ranging from 14 to 41 (for shared OTUs and Bray-Curtis similarity indices for all plots, see supplementary material Table S10).

Using the presence/absence matrix of the 454 data, a GNMDS ordination was performed to visualize the variation in composition of OTUs in the 15 grazed and 15 control plots. The grazed and control plots showed no separation along GNMDS axis one (Figure 11). However, along GNMDS axis two the grazed plots appeared more aggregated compared to the control plots, indicating less variation in the fungal community composition along this complex gradient. A GNMDS ordination was also conducted on a presence/absence matrix where data from the six enclosures was summarized according to tissue-types (Figure 12). Noteworthy, the inflorescences separated markedly from leaves and stems along GNMDS axis one in fungal community composition. The stems also grouped somewhat together while the variation in composition of OTUs was greater in among the leaves.

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**Table 3) Number of shared OTUs, obtained in EstimateS between the enclosures of culture data (lower left) and 454 data (upper right).**

| Cultures[2] | 454[1] | | | | | |
|---|---|---|---|---|---|---|
| | A | B* | E | F* | G | H* |
| A | - | 25 | 17 | 14 | 20 | 31 |
| B* | 0 | - | 21 | 21 | 33 | 41 |
| E | 0 | 1 | - | 14 | 15 | 20 |
| F* | 0 | 3 | 2 | - | 14 | 21 |
| G | 0 | 1 | 1 | 3 | - | 32 |
| H* | 0 | 4 | 2 | 4 | 4 | - |

Number of shared species in enclosures A-H, derived from [1]454 sequence and [2]Culture data. Results from grazed enclosures are marked with an asterisk.
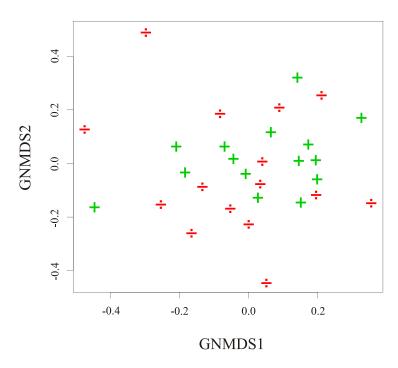


**Figure 11. GNMDS ordination plots of grazed (+) and control (÷) plots and based on 454 sequencing. OTU occurrence were summarized within each plot creating a species-plot matrix. Axes are half-change scaled, and sorted by decreasing explained variation.**
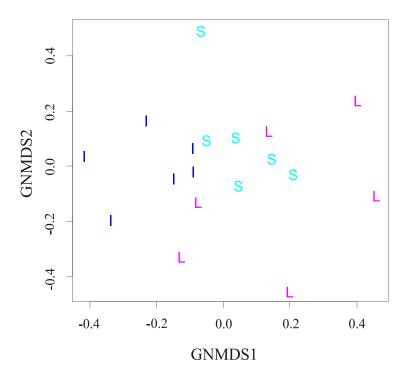
**Figure 12. GNMDS ordination of an OTU-tissue-type matrix, OTUs were summarized within tissue-types so that each plot symbolizes the total OTU diversisty of a given tissue-type in one enclosure. Inflorescenses (I), leaves (L) and stems (S). Axes are half-change scaled, and sorted by decreasing explained variation.**

## Discussion

In this study both culturing and 454 sequencing were used to assess putative fungal endophytes in *A. flexuosa* under different grazing regimes. A surprisingly high discrepancy between the methods was observed: cultivation detected only 37 OTUs while 454 sequencing recovered almost 900 fungal OTUs with only three OTUs overlapping between the methods. Total species richness based on the 454 sequence data was estimated to be ~6000 fungal OTUs. The vast majority of OTUs recovered by both methods were rare, but a few dominating OTUs may represent important endophytes or phylloplane fungi associated with *A. flexuosa* in alpine areas of Norway. None of the clavicipitaceous toxin producing grass endophytes were detected. The putative grass endophytes detected in this study may therefore fit within the endophyte class 2 and/or class 3 according to the categories suggested by Rodriguez *et al.* (2009). Grazing seemed to increase the OTU diversity in the grass stems.

*What can be observed from culturing versus 454 sequencing?*

Cultivation and 454 sequencing showed little overlap in their ability to detect various fungal OTUs, with three OTUs shared between the two methods. A far higher fungal diversity was detected using 454 sequencing, and some fungal groups, e.g. putatively yeast forming basidiomycetes were not detected using culturing. Since only OTUs with more than one unique read were compared with OTUs recovered through cultivation, it is possible that the overlap would have increased if all 454 sequences had been investigated. Several other factors may also have lead to these discrepancies, including initial differences in the fungal endophyte community in individual plants selected for culture and 454 sequencing, exclusion by competition during growth in culture or lack of ability to grow *in vitro*. Some fungi also require selective media and will not grow on conventional media such as malt extract agar (Guo *et al.*, 2001). Furthermore, Arnold *et al.* (2010) stressed the importance of monitoring the isolation plates for as long as 12 months insuring a complete coverage of late emerging endophytes. In this study, only fungi emerging during a three month period were analyzed. In addition, some slow growing endophytes may be outcompeted by co-occurring fungi that grow faster (Guo *et al.*, 2001). Decreasing the size of the plated plant parts (to ~4 mm$^2$) may reduce overgrowth of the slower growing fungi by fast growing and early emerging fungi (Arnold & Lutzoni, 2007). There are several mechanisms affecting the amplification of

template DNA, such as length differences between the amplicons. Pure stochastic effects will also influence downstream sequencing analyses (Walsh *et al.*, 1992). Bellemain *et al.* (2010) investigated taxonomic biases in barcoding primers *in silico* and showed that ITS2 may favor the amplification of ascomycetes due to the generally shorter ITS2 sequences of ascomycetes compared to basidiomycetes. Furthermore, the researchers stressed that the use of specific primers (such as ITS1F) must be done with care since mismatches between the commonly used ITS barcoding primers and target sequences are widespread. The combination of cultivation and 454 sequencing biases mentioned above probably account for the low degree of overlap between the two methods. Noteworthy, some yeasts may have been mistaken as bacteria, and thus not analyzed further.

The culturing approach used in this study probably underestimated the endophyte diversity greatly. On the other hand, numerous methodological factors may have lead to an overestimation of fungal diversity using 454 sequencing. If phylloplane fungi, coincidental spores or other fungal contaminations have entered the DNA extraction, the two PCR steps prior to 454 sequencing will multiply such fungal sequences, in particular if the portion of true endophyte fungal DNA in the initial sample was low. However, inspection of selected plant surfaces using scanning electron microscopy revealed no fungal spores or hyphae. Real time-PCR and Picogreen quantifications both indicated a relatively low abundance of fungi associated with *A. flexuosa*. More than 13% of the samples had no amplification before cycle 40, which is commonly regarded as a negative result (Vrålstad *et al.*, 2009). Thus, these initial tests pointed to a relatively low abundance of fungi in the plant tissues. The introduction of a second, nested PCR, in this study consisting of 20 additional cycles, will have increased the copy number of any fungal ITS copy present in the initial DNA sample drastically, even for samples harboring only a few fungal genomes. The detection of the obvious laboratory contaminant *Agaricus bisporus* as one on the most frequent OTUs underlines the vulnerability of the method.

*454 sequencing consistency*

The replicated 454 runs of ten samples revealed that there was an overall low correspondence of which OTUs were detected across the two runs (only 17% overlap). Regarding the number of reads there were also some differences, this is probably due to stochastic effects involved

in the 454 sequencing run. However, detection of additional OTUs through increased sequence depth is expected as the accumulation curves showed no signs of flattening. Even though the overlap in the total number of OTUs were low, the consistency between the more common OTUs was good. All but one of the twenty most common OTUs was recovered in both runs, although some of the OTUs at different frequencies. This indicates that 454 method works well for common and presumably biological important species. However, it is important to use 454 sequencing in parallel with cultivation considering the low overlap between the methods.

*Taxonomic coverage*

A large proportion of the 454 sequences had taxonomic affinity to basidiomycetes in the genera *Tremella, Cryptococcus, Exobasidium, Rhodotorula* and *Bensingtonia*. These genera include many well known plant associated fungi, including potential endophytes (Fonseca *et al.*, 2000; Xin *et al.*, 2009; Olstorpe *et al.*, 2010). Many of the OTUs recovered could potentially represent fungi with a yeast phase. Whether these fungi represent endophytes or partly phylloplaneous fungi is difficult to resolve. Nutrient leakage, either from fungal pathogens or the plant itself, may facilitate the growth of epiphytic yeasts that in turn form a protective barrier against new infective agents (Allen *et al.*, 2004). Some epiphytic yeasts can penetrate the plant via the stomata or damages in the cuticle, and this potential infection pathway may be the start of an endophytic interaction (Isaeva *et al.*, 2010). Many of the basidiomycete genera detected in this study were also detected by Jumpponen & Jones (2009) in a study of fungi associated with the phyllosphere of *Quercus macrocarpa*.

Both the results obtained by cultivation and 454 sequencing indicated that fungi within the genus *Phaeosphaeria* play an important role as endophytes of *A. flexuosa*. This was the only fungal genus recognized as highly prevalent using both approaches. *Phaeosphaeria* species are primarily known as inconspicuous ascomycetes some of which cause blotch diseases in cereals (Camara *et al.*, 2002; Ueng *et al.*, 2003; Wang *et al.*, 2007). During the past years, several studies have also reported on the anamorphic state of *Phaeosphaeria*, namely *Stagonospora*, as endophytes of grasses (Ernst *et al.*, 2003; Tao *et al.*, 2008). Ernst *et al.* (2003) revealed that several *Stagonospora* anamorphs were abundant in *Phragmites australis* (Poaceae). They showed experimentally that these endophytes were vertically transmitted and also contributed to increased aboveground biomass of their host.

Since *Phaeosphaeria* spp. and their *Stagonospora* anamorphs include several known endophytes, the OTUs detected in this study could then also represent genuine endophytes and not e.g. phylloplane epiphytes or surface contaminants. The consistency observed between culturing and 454 sequences in detecting *Phaeosphaeria* support this. *Phaeosphaeria* and *Stagonospora* OTUs were more frequently detected in inflorescences compared to leaves and stems. Ernst *et al.* (2003) suggested that hyphae from endophytic *Phaeosphaeria* penetrate the shoot during the summer months to establish a vertical transmission mode. This is in accordance with the findings in this study, where *Phaeosphaeria* was recovered in 83% of the inflorescences using 454 sequencing.

*Effects of grazing on OTU diversity*

Large differences in the number of fungal OTUs between plots was observed using both cultivation and 454 sequencing, which suggests that some undetected small-scale spatial mechanisms probably affect the fungal diversity. For example, studies have shown that local microclimate can be an important factor affecting plant-fungal associations (Pan *et al.*, 2008).

Stems from grazed plots harbored a significantly higher diversity of fungal OTUs compared with stems originating from control plots. Regarding inflorescences, grazing did not affect the number of OTUs. The higher fungal richness of stems in grazed plots could be due to competitive exclusion of endophytic fungi in the less disturbed plots. Alternatively, the plants harboring a high diversity of endophytes may be better adapted to grazing, and hence species rich plants will be favored in grazed plots. Herrera *et al.* (2009) observed that communities of root associated fungi within *Bouteloua gracilis*, a native North American grass, included fungi that had previously been ascribed as coprophilic. Spores from an endophytic fungus may survive digestion and in turn infect neighboring plants through damages obtained through grazing. It is important to have in mind that, in this study, sampling of the plants was not done prior to the release of sheep, thus the observed increase in OTUs could potentially be due to horizontal infection through grazing derived damages, and not through selection via higher adaptation to grazing.

Further, plots with high levels of phosphorus harbored plants with a lower number of fungal OTUs. It may be speculated that any competitive advantages endophyte infected plants may have in nutrient low plots might be evened out in nutrient high plots, where nutrient availability is not a limiting factor. In plots rich in organic matter (i.e. loss on

ignition) a decrease in fungal diversity is observed. Likewise, Regvar *et al.* (2010) investigated fungal colonization of root fungi and observed that root associated dark septate endophytes decreased at high levels of organic matter. The complex variable base-cation however seems to be an important factor for plants harboring a high diversity of fungi. One possible reason might be that harboring fungal endophytes comes at a cost, and a higher demand for nitrogen and cofactors may be a limiting factor. Especially the production of alkaloids have been investigated, even under artificially increased resource availability, plants harboring alkaloid-producing fungi experienced decreased growth and seed production. This was probably due to the metabolic costs of hosting toxin producing fungi (Faeth *et al.*, 2010). However, due to the large differences in diversity within enclosures and the poor fit of data to the model, conclusions based on this analysis must be made with caution.

The accumulation curves showed no sign of reaching an asymptote, indicating that only a small fraction of the diversity was recorded and that more intensive sampling is needed to recover the overall diversity in the area. The high heterogeneity between both plots and plants, in addition to the large number of rare OTUs, all contribute to the near linear shape of the accumulation curves. This trend is very commonly observed in surveys of fungal diversity, especially when environmental sequencing methods are applied (Arnold *et al.*, 2000; U'Ren *et al.*, 2009). It is important to bear in mind the inherent methodological biases with 454 sequencing and downstream analyses that may lead to an overestimation of OTUs (Kunin *et al.*, 2010; Tedersoo *et al.*, 2010).

*Effects of grazing on OTU composition*

A trend of what could be a shift in fungal species composition was observed in grazed plots. The shift regarding allocation of fungal OTUs in tissue types might indicate a complex interaction between fungal OTUs, host plant and grazing.

For OTU composition in the tissue types, the inflorescences groups fairly consistently along GNMDS axis 1. The presence of vertically transmitted fungi might account for this grouping. Several OTUs with taxonomic affiliation to the genus *Phaeosphaeria* was found in the inflorescences. Other fungi with similar ecology could be present in *A. flexuosa*, however, this was not investigated. Or additionally, the surface sterilization of the inflorescences might not have been as successful as the sterilization of the leaves and stems, thus any remaining contaminants might increase the similarity of the samples and grouping them together.

The grazed and control plots did not group consistently along GNMDS axis 1. However, along GNMDS axis 2, grazed plots showed a narrower range than control plots. This could indicate that along this axis grazed plots are more similar to other grazed plots, while control plots on the other hand show a wider spread in the ordination, indicating a larger dissimilarity with regards to OTU composition. One possible explanation for this trend might be that some fungi associated with *A. flexuosa* are more adapted to grazing and thus are selected for in grazed plots. However, this signal is weak, and we can only speculate that the observed trend could be an indication of a compositional shift of fungal OTUs in grazed plots. Noteworthy, observing a significant difference in species composition might prove to be difficult if a horizontal transmission mode is common. Any trends of a compositional shift might be masked by continuous infections throughout the growing season by horizontally transmitted endophytes.

*Methodological aspects and concluding remarks*

This study demonstrates that the use of environmental high-throughput sequencing to study putative endophytic fungal communities must be done with great care. For example, 454 sequencing do not separate between endophytic and phylloplane fungi on the plant surface and may therefore give the rise to a high number of non-endophytic OTUs. To ensure that a fungus indeed is not a surface contaminant, methods such as fluorescent probing of fungal specific (1→3)-ß-d-glucans may be applied (Johnston *et al.*, 2006). Actions were made to remove surface contaminants, and when considering the low abundance of common contaminants and cosmopolitan fungi (i.e. *Aspergillus* and *Penecillium*) in the 454 dataset, the surface sterilization seems to be, at least, partly successful. In retrospect, increased focus on yeasts and slow growing fungi in the cultivation experiment, as well as using a broader range of growth media, would probably have recovered more fungal OTUs. This was unfortunately not realistic within the time frame of this study.

In addition to the extensive spatial variation and patchiness in the distribution of fungi, as observed in this study, there might be important temporal changes in fungal species composition during the season. Sampling a biological system in one day, as done in this study, is analogous to a photographic snap-shot. Several studies have shown that there are temporal variations in plant fungal associations (Kemp *et al.*, 2003; Li *et al.*, 2005).

The ongoing methodological revolution in microbial ecology gives great promises for future studies of fungal endophytes in their natural habitat. Using 454 sequencing technology to study endophytic fungi have clear limitations and pitfalls, but probably also a great potential if the method is used appropriately. The method will recover the most common phyllosphere fungal OTUs with high accuracy, including endophytes. In combination with more specific methods, 454 sequencing may prove a very important tool for investigating endophyte communities, in particular in plants where the knowledge of associated fungal endophytes are poor or completely missing. This study is among the first targeted studies on putative endophytes in wild *A. flexuosa* populations.

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# Supplementary material

**Table S1. Sample information for all samples including; concentrations, RT-PCR data and read statistics. Grazed plots are in bold.**

| Plot[1] | Tt[2] | ID[3] | C[4] | Coordinates[5] | | Alt[6] | Weight[7] | xDilution[8] | CT[9] | Cat.[10] | Cycle Cat[11] | UR[12] | DR[13] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **H20** | I | 1 | 1014.2 | 6729160 | 32 W 0442726 | 1120 | 113.1 | 50 | 40.3 | 12 | 40 | 1 352 | 3 045 |
| **H20** | L | 2 | 0.4 | | | | 14.7 | 10 | 37.71 | 10 | 38 | 570 | 2 907 |
| **H20** | S | 3 | ~0 | | | | 136.4 | 500 | 37.8 | 10 | 38 | 324 | 796 |
| **H15** | I | 4 | 298 | 6729381 | 32 W 0442762 | 1172 | 42.1 | 500 | 39.97 | 12 | 40 | 761 | 2 933 |
| **H15** | L | 5 | 56.3 | | | | 14.3 | 500 | 34.77 | 7 | 35 | 0 | 0 |
| **H15** | S | 6 | ~0 | | | | 145.9 | 500 | 36.16 | 8 | 36 | 863 | 2 887 |
| **H01** | I | 7 | 44.1 | 6729589 | 32 W 0442924 | 1193 | 31.1 | 10 | 45 | 17 | 45 | 826 | 2 697 |
| **H01** | L | 8 | 6.4 | | | | 73.8 | 50 | 40.91 | 13 | 41 | 0 | 0 |
| **H01** | S | 9 | 136.8 | | | | 13 | 500 | 41.96 | 14 | 42 | 0 | 0 |
| **H10** | I | 10 | 231.6 | 6729741 | 32 W 0442914 | 1227 | 66.2 | 500 | 41.97 | 14 | 42 | 389 | 2 656 |
| **H10** | L | 11 | 88.5 | | | | 28.7 | 50 | 34.83 | 7 | 35 | 0 | 0 |
| **H10** | S | 12 | 89.4 | | | | 186.2 | 50 | 42.18 | 14 | 42 | 348 | 1 361 |
| **H07** | I | 13 | 119.9 | 6729824 | 32 W 0442903 | 1239 | 57.8 | 100 | 40.7 | 13 | 41 | 572 | 2 608 |
| **H07** | L | 14 | 11.7 | | | | 7.9 | 500 | 37 | 9 | 37 | 0 | 0 |
| **H07** | S | 15 | 270.4 | | | | 14.1 | 500 | 38.31 | 10 | 38 | 93 | 623 |
| G01 | I | 16 | 397.7 | 6729975 | 32 W 0442550 | 1269 | 77.5 | 100 | 40.71 | 13 | 41 | 437 | 1 442 |
| G01 | L | 17 | 11.2 | | | | 16.4 | 10 | 37.1 | 9 | 37 | 669 | 2 149 |
| G01 | S | 18 | 65.3 | | | | 199.6 | 500 | 34.03 | 6 | 34 | 0 | 0 |
| G10 | I | 19 | 364.6 | 6729647 | 32 W 0442525 | 1228 | 45.1 | 50 | 39.98 | 12 | 40 | 554 | 1 971 |
| G10 | L | 20 | 34 | | | | 15.9 | 500 | 29 | 1 | 29 | 1 | 1 |
| G10 | S | 21 | 179.6 | | | | 125 | 50 | 41.15 | 13 | 41 | 354 | 1 556 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| G13 | I | 22 | 187.6 | 6729476 | 32 W 0442579 | 1175 | 53.9 | 50 | 40.92 | 13 | 41 | 621 | 2 251 |
| G13 | L | 23 | 9.8 | | | | 10.2 | 500 | 36.61 | 9 | 37 | 1 | 1 |
| G13 | S | 24 | 11 | | | | 134.4 | 500 | 35.95 | 8 | 36 | 0 | 0 |
| G16 | I | 25 | 198.8 | 6729315 | 32 W 0442529 | 1144 | 72 | 50 | 38.17 | 10 | 38 | 136 | 426 |
| G16 | L | 26 | ~0 | | | | 12 | 10 | 32.64 | 5 | 33 | 514 | 2 147 |
| G16 | S | 27 | 185 | | | | 199.6 | 50 | 35.62 | 8 | 36 | 423 | 2 214 |
| G20 | I | 28 | 336.2 | 6729159 | 32 W 0442405 | 1122 | 35.7 | 500 | 41.87 | 14 | 42 | 260 | 1 870 |
| G20 | L | 29 | ~0 | | | | 11.9 | 10 | 39.09 | 11 | 39 | 0 | 0 |
| G20 | S | 30 | 375.2 | | | | 139.2 | 50 | 38.46 | 10 | 38 | 1 | 1 |
| **F11** | I | 31 | 1160.6 | 6729193 | 32 W 0442188 | 1110 | 103.7 | 500 | 39.06 | 11 | 39 | 4 | 11 |
| **F11** | L | 32 | 11.1 | | | | 25.9 | 10 | 39.14 | 11 | 39 | 0 | 0 |
| **F11** | S | 33 | 10.5 | | | | 195.4 | 10 | 36.99 | 9 | 37 | 222 | 1 301 |
| **F09** | I | 34 | 27.3 | 6729364 | 32 W 0442093 | 1133 | 68 | 500 | 32.16 | 4 | 32 | 2 | 2 |
| **F09** | L | 35 | 0.7 | | | | 14.1 | 10 | 37.07 | 9 | 37 | 280 | 2 123 |
| **F09** | S | 36 | 9.1 | | | | 188.9 | 10 | 38.68 | 11 | 39 | 0 | 0 |
| **F16** | I | 37 | 516.2 | 6729487 | 32 W 0442033 | 1151 | 81.3 | 100 | 36.95 | 9 | 37 | 921 | 2 958 |
| **F16** | L | 38 | ~0 | | | | 11 | 10 | 37.17 | 9 | 37 | 0 | 0 |
| **F16** | S | 39 | 16.9 | | | | 195.9 | 10 | 37.59 | 10 | 38 | 369 | 1 939 |
| **F18** | I | 40 | 281 | 6729700 | 32 W 0442164 | 1218 | 43.7 | 50 | 38.11 | 10 | 38 | 199 | 937 |
| **F18** | L | 41 | 2.3 | | | | 15.9 | 10 | 38.38 | 10 | 38 | 0 | 0 |
| **F18** | S | 42 | 456.6 | | | | 164 | 50 | 36.26 | 8 | 36 | 319 | 1 152 |
| **F02** | I | 43 | 542.4 | 6730019 | 32 W 0442154 | 1274 | 51.7 | 50 | 32.24 | 4 | 32 | 420 | 2 569 |
| **F02** | L | 44 | 2 | | | | 13.5 | 10 | 35.41 | 7 | 35 | 48 | 341 |
| **F02** | S | 45 | 21.4 | | | | 123.3 | 10 | 37.56 | 10 | 38 | 429 | 991 |
| E07 | I | 46 | 1250.8 | 6729851 | 32 W 0441943 | 1227 | 73.1 | 100 | 37.22 | 9 | 37 | 327 | 1 604 |
| E07 | L | 47 | 31.5 | | | | 11.1 | 50 | 39.36 | 11 | 39 | 0 | 0 |
| E07 | S | 48 | 192.2 | | | | 192.8 | 50 | 39.56 | 12 | 40 | 333 | 1 608 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| E09 | I | 49 | 440.5 | 6729802 | 32 W 0444937 | 1215 | 25.1 | 100 | 37.64 | 10 | 38 | 171 | 2 337 |
| E09 | L | 50 | 37.2 | | | | 12.9 | 10 | 36.52 | 9 | 37 | 519 | 2 623 |
| E09 | S | 51 | 147.2 | | | | 78.2 | 500 | 33.28 | 5 | 33 | 2 | 4 |
| E13 | I | 52 | 381.8 | 6729607 | 32 W 0441851 | 1183 | 42.2 | 10 | 42.16 | 14 | 42 | 364 | 2 077 |
| E13 | L | 53 | 31.7 | | | | 16 | 10 | 33.42 | 5 | 33 | 389 | 1 713 |
| E13 | S | 54 | 293.7 | | | | 148.7 | 500 | 36.5 | 8 | 36 | 478 | 1 903 |
| E15 | I | 55 | 978.6 | 6729469 | 32 W 0441911 | 1158 | 71.7 | 50 | 37.94 | 10 | 38 | 377 | 1 509 |
| E15 | L | 56 | 69.1 | | | | 25.9 | 100 | 33.32 | 5 | 33 | 0 | 0 |
| E15 | S | 57 | 247.1 | | | | 196.5 | 10 | 38.32 | 10 | 38 | 241 | 3 662 |
| E18 | I | 58 | 396.6 | 6729212 | 32 W 0441965 | 1097 | 58.3 | 50 | 36.31 | 8 | 36 | 355 | 2 791 |
| E18 | L | 59 | 42.1 | | | | 23.1 | 10 | 32.98 | 5 | 33 | 328 | 2 037 |
| E18 | S | 60 | 165.4 | | | | 142.4 | 500 | 33.65 | 6 | 34 | 311 | 2 597 |
| **B16** | I | 61 | 475.9 | 6729533 | 32 W 0441228 | 1177 | 46.1 | 500 | 35.55 | 8 | 36 | 315 | 1 559 |
| **B16** | L | 62 | 37.2 | | | | 13.7 | 50 | 31.69 | 4 | 32 | 0 | 0 |
| **B16** | S | 63 | 186.2 | | | | 233.1 | 500 | 33.09 | 5 | 33 | 373 | 1 372 |
| **B15** | I | 64 | 399.2 | 3729719 | 32 W 0441235 | 1167 | 58.2 | 500 | 37.34 | 9 | 37 | 519 | 2 838 |
| **B15** | L | 65 | 21.9 | | | | 10 | 50 | 35.15 | 7 | 35 | 338 | 1 701 |
| **B15** | S | 66 | 176.4 | | | | 161.7 | 50 | 34.4 | 6 | 34 | 415 | 1 591 |
| **B09** | I | 67 | 342 | 6730052 | 32 W 0441229 | 1224 | 30.8 | 10 | 38.11 | 10 | 38 | 265 | 1 195 |
| **B09** | L | 68 | 26.7 | | | | 11.9 | 10 | 35.59 | 8 | 36 | 421 | 2 476 |
| **B09** | S | 69 | 201.4 | | | | 103.1 | 10 | 38.86 | 11 | 39 | 683 | 3 085 |
| **B05** | I | 70 | 354 | 6730339 | 32 W 0441265 | 1274 | 68.8 | 50 | 31.89 | 4 | 32 | 172 | 1 521 |
| **B05** | L | 71 | 33.1 | | | | 12.3 | 10 | 35.82 | 8 | 36 | 711 | 3 764 |
| **B05** | S | 72 | 180.5 | | | | 164.1 | 10 | 37.86 | 10 | 38 | 760 | 2 356 |
| **B18** | I | 73 | 521.3 | 6729292 | 32 W 0441400 | 1117 | 42.7 | 50 | 33.97 | 6 | 34 | 165 | 1 610 |
| **B18** | L | 74 | 73.9 | | | | 20.8 | 50 | 34.14 | 6 | 34 | 1 | 1 |
| **B18** | S | 75 | 355 | | | | 137.3 | 10 | 36.66 | 9 | 37 | 459 | 1 283 |

| Plot[1] | Tt[2] | ID[3] | C[4] | Coordinates[5] | | Alt[6] | Weight[7] | Dilution[8] | CT[9] | Category[10] | Cycle category[11] | UR[12] | DR[13] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A04 | I | 76 | 284.5 | 6730355 | 32 W 0441095 | 1263 | 48.5 | 500 | 35.74 | 8 | 36 | 519 | 3 435 |
| A04 | L | 77 | 58.6 | | | | 19 | 50 | 31.93 | 4 | 32 | 0 | 0 |
| A04 | S | 78 | 238.1 | | | | 193.4 | 50 | 31.49 | 3 | 31 | 190 | 1 716 |
| A06 | I | 79 | 395.5 | 6730297 | 32 W 0440965 | 1254 | 48.3 | 500 | 37.64 | 10 | 38 | 296 | 900 |
| A06 | L | 80 | 71.9 | | | | 28.5 | 10 | 33.9 | 6 | 34 | 706 | 2 772 |
| A06 | S | 81 | 161.2 | | | | 148.5 | 500 | 37.11 | 9 | 37 | 183 | 1 608 |
| A14 | I | 82 | 508.1 | 6729996 | 32 W 0441119 | 1193 | 82.9 | 50 | 35.78 | 8 | 36 | 507 | 2 055 |
| A14 | L | 83 | 47.3 | | | | 33.4 | 10 | 35.2 | 7 | 35 | 457 | 2 195 |
| A14 | S | 84 | 341.7 | | | | 208.1 | 10 | 37.4 | 9 | 37 | 570 | 1949 |
| A12 | I | 85 | 691.8 | 6729831 | 32 W 0441043 | 1189 | 54 | 500 | 37.8 | 10 | 38 | 324 | 2 382 |
| A12 | L | 86 | 55.4 | | | | 24.7 | 10 | 33.64 | 6 | 34 | 531 | 1 643 |
| A12 | S | 87 | 168.1 | | | | 151.7 | 10 | 36.53 | 9 | 37 | 498 | 1 724 |
| A19 | I | 88 | 576.2 | 6729829 | 32 W 0440958 | 1185 | 127.5 | 500 | 39.35 | 11 | 39 | 0 | 0 |
| A19 | L | 89 | 45 | | | | 17.8 | 10 | 31.61 | 4 | 32 | 429 | 1 439 |
| A19 | S | 90 | 161.1 | | | | 330 | 500 | 32.89 | 5 | 33 | 447 | 1 679 |

[1] Plot identification, grazed plots in bold.

[2] Tt (Tissue type, I=Inflorescences, L=leaves, S=Stems)

[3] ID (Sample identity number)

[4] C (Nanodrop derived genomic DNA concentrations).

[5] Coordinates of the plots.

[6] Alt (Altitude of the sampled plots, in meters above sea level).

[7] Weight of the freeze dried plant material.

[8] Dilution regime based on the weak/medium and strong categories made prior to RT-PCR.

[9] CT (Cycle threshold, the number of cycles the samples in question were detected in the RT PCR).

[10] The category the samples in question were placed in.

[11] Cycle category, samples differing with ±0.5 Ct, were run with the same number of initial PCR cycles.

[12] UR (Unique reads, the number of reads after the removal of duplicate reads)

[13] DR (Duplicate number of reads)

**Table S2. CLOTU pipeline parameters, assessing the quality of 454 sequences.**

| CLOTU parameters | | Number of sequences removed prior to clustering | Single parameter comparison[1] |
|---|---|---|---|
| Total number sequences | **169 887** | | |
| Sequences with Tag mismatch | | 3 663 | 3 663 |
| FWD prim mismatch: Errors > 2 | | 72 | 831 |
| REV prim mismatch: Errors > 2 | | 6 405 | 5 960 |
| Chimeric sequences | | 1 068 | 1 077 |
| Sequences with N's | | 4 199 | 8 189 |
| Sequences with length < 250 | | 350 | 1 005 |
| Adaptor error | | 1 146 | |
| Total number of sequences removed | | 16 903 | |
| Total number of sequences to cluster | | 152 984 | |

[1] Single parameter comparison show how many sequences are under each of the CLOTU parameters, as some of the sequences might be included in several of the CLOTU parameters. Thus, summarizing sequences in the single parameter comparison is meaningless as is it only provides an overall view of the quality of the 454 run.

**Table S3. Clustering statistics and the number of singletons for the 454 data at four different cutoff levels.**

| Cutoff level | 96% | 97% | 98% | 99% |
|---|---|---|---|---|
| Clusters to BLAST[1] | 686 | 851 | 897[1] | 1 255 |
| Singletons | 2 583 | 3 234 | 3 790 | 5 926 |

[1] The 76 novel OTUs generated in the replicated runs were removed in this table, including these OTUs the number of clusters to blast at 98% cutoff was=973

**Table S4. Extrapolated number of species derived from accumulation curve Figure 11.**

| Plots/plants[1] | 30/ 90 | 100/ 300 | $10^3$/ $3*10^3$ | $10^4$/ $3*10^4$ | $10^5$/ $3*10^5$ | $10^6$/ $3*10^6$ |
|---|---|---|---|---|---|---|
| Estimated number of species | 2 291 | 2 765 | 3 672 | 4 578 | 5 485 | 6 391 |
| Observed number of species | 896[2] | | | | | |

[1] The number before the slash indicates the number of plots samples, and the number after the slash indicate the number of plants samples.
[2] *A. bisporus* removed from the dataset.
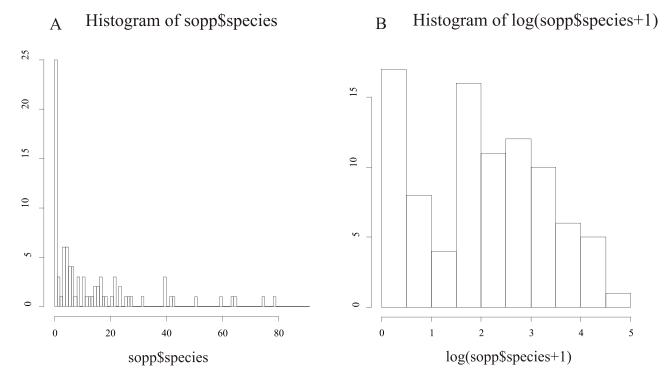


**Figure S5. (a) Histogram of OTU frequency in *A. flexuosa* in the 90 samples derived from 454sequencing. And (b) log transformed histogram of the same data. Data does not fit well with a Poisson distribution.**
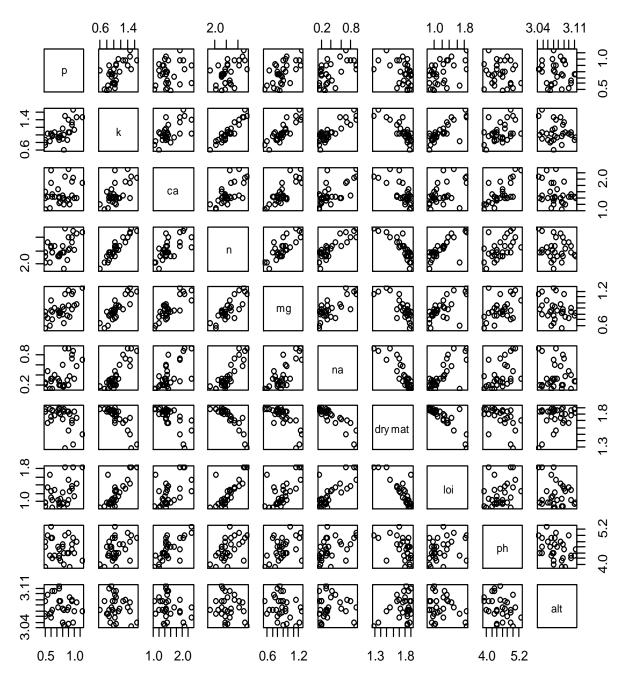
**Figure S6) Scatter plot of the log transformed environmental variables. Due to strong correlation between the variables calcium, magnesium, nitrogen and potassium these were summarized and combined into a complex variable named base-cation**

**Table S7) Reference sequences for Neighbor-joining analyses.**

| Accession nr | Species | Isolate / sample | Host | Origin / Isolation source | Reference |
|---|---|---|---|---|---|
| AF250829 | *Paraphaeosphaeria michotii* | CBS 652.86 | *Typha latifolia* | | Camara *et al.* (2002) |
| AF439471 | *Phaeosphaeria alpina* | CBS456.84 | *Phleum alpinum* | Switzerland | Camara *et al.* (2002) |
| | Culture OTU-6 | | *Avenella flexuosa* | Norway | |
| AF439472 | *Phaeosphaeria avenaria* | ATCC 18596 | *Triticum sp.* | USA | Camara *et al.* (2002) |
| AF439477 | *Phaeosphaeria culmorum* | CBS569.86 | *Arrhenatherum elatius* | Switzerland | Camara *et al.* (2002) |
| AF439478 | *Phaeosphaeria dennisiana* | CBS598.86 | *Minuartia sedoides* | Switzerland | Camara *et al.* (2002) |
| AF439484 | *Phaeosphaeria herpotrichoides* | CBS588.86 | *Melica ciliata* | Switzerland | Camara *et al.* (2002) |
| AF439489 | *Phaeosphaeria lindii* | CBS 607.86 | *Equisetum hyemale* | Switzerland | Camara *et al.* (2002) |
| AF439490 | *Phaeosphaeria luctuosa* | CBS577.86 | *Zea mays* | Switzerland | Camara *et al.* (2002) |
| AF439491 | *Phaeosphaeria lycopodina* | CBS599.86 | *Lycopodium annotinum* | Switzerland | Camara *et al.* (2002) |
| AF439493 | *Phaeosphaeria nodorum* | MPSC 39 | *Lolium perenne* | Denmark | Camara *et al.* (2002) |
| AF439494 | *Phaeosphaeria oreochloae* | CBS457.84 | *Sesleria disticha* | Switzerland | Camara *et al.* (2002) |
| AF439496 | *Phaeosphaeria padellana* | CBS606.86 | *Trisetum distichophyllum* | Switzerland | Camara *et al.* (2002) |
| | 454 OTU-1 | | *Avenella flexuosa* | Norway | |
| AF439497 | *Phaeosphaeria phragmitis* | CBS578.86 | *Phragmites australis* | Switzerland | Camara *et al.* (2002) |
| AF439508 | *Phaeosphaeria vagans* | CBS604 | *Calamagrostis arundinacea* | Sweden | Camara *et al.* (2002) |
| AJ496625 | *Stagonospora sp. endophyte* | 4/99-1 | *Phragmites australis* | Germany / seed | Ernst *et al.* (2003) |
| AJ496626 | *Stagonospora sp. endophyte* | 4/99-5 | *Phragmites australis* | Germany / seed | Ernst *et al.* (2003) |
| | Culture OTU-4 | | *Avenella flexuosa* | Norway | |
| AJ496627 | *Stagonospora sp. endophyte* | 4/99-18 | *Phragmites australis* | Germany / seed | Ernst *et al.* (2003) |
| AJ496629 | *Phaeosphaeria eustoma* | CBS337.86 | *Phragmites australis* | | Ernst *et al.* (2003) |
| AJ496630 | *Stagonospora neglecta* | CBS 343.86 | *Phragmites australis* | | Ernst *et al.* (2003) |
| AJ496631 | *Phaeosphaeria phragmitis* | CBS 578.86 | *Phragmites australis* | | Ernst *et al.* (2003) |
| AJ496632 | *Phaeosphaeria pontiformis* | CBS589.86 | *Phragmites australis* | | Ernst *et al.* (2003) |
| | Culture OTU-5 | | *Avenella flexuosa* | Norway | |
| | Culture OTU-S | | *Avenella flexuosa* | Norway | |
| AY345346 | *Phaeosphaeria sp.* | Genomic DNA | *Hennediella heimii* | Antarctica | Bradner (2003) |
| EF505595 | *Uncultured endophytic fungus* | Genomic DNA | *Zea mays* | leaves, stems, kernels | Pan *et al.* (2008) |
| EF505604 | *Uncultured endophytic fungus* | Genomic DNA | *Zea mays* | leaves, stems, kernels | Pan *et al.*(2008) |

**Table S8. Read statistics from run1 and run2, based on duplicate sequences.**

| Replicated samples | # reads Run1[1] | # reads Run2[1] |
|---|---|---|
| Sample 9/91 | 0 | 1 |
| Sample 10/92 | 2 656 | 2 984 |
| Sample 12/93 | 1 361 | 2 286 |
| Sample 28/94 | 1 870 | 1 684 |
| Sample 52/95 | 2 077 | 2 754 |
| Sample 45/96 | 991 | 1 115 |
| Sample 49/97 | 2 337 | 4 043 |
| Sample 55/98 | 1 509 | 1 666 |
| Sample 57/99 | 3 662 | 1 049 |
| Sample 67/100 | 1 195 | 4 222 |
| Total reads[2] | 17 685 | 21 804 |

[1]Based on the number of duplicate reads
[2]The summarized number of reads for run1 and run2.

**Table S9. Observed number of mean OTUs in tissue types, in control and grazed plots.**

| Tissue type | Control plots | Grazed plots |
|---|---|---|
| Inflorescences | 15.4 | 15 |
| Leaves | 19.1 | 9.4 |
| Stems | 7.8 | 20.8 |

**Table S10. Bray-Curtis similarity and OTU frequency of all sample plots**

| | A04 | A06 | A12 | A14 | A19 | B05 | B09 | B15 | B16 | B18 | E07 | E09 | E13 | E15 | E18 | F02 | F09 | F11 | F16 | F18 | G01 | G10 | G13 | G16 | G20 | H01 | H07 | H10 | H15 | H20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A04 | 1 | 2 | 5 | 3 | 4 | 5 | 4 | 4 | 2 | 3 | 3 | 1 | 3 | 4 | 2 | 3 | 2 | 1 | 2 | 3 | 2 | 3 | 2 | 3 | 2 | 2 | 3 | 2 | 4 | 5 |
| A06 | 0.063 | 1 | 5 | 7 | 7 | 7 | 5 | 3 | 3 | 6 | 3 | 1 | 4 | 4 | 3 | 1 | 0 | 2 | 6 | 2 | 2 | 4 | 5 | 5 | 3 | 3 | 6 | 6 | 7 | 13 |
| A12 | 0.222 | 0.147 | 1 | 3 | 8 | 5 | 3 | 4 | 2 | 2 | 2 | 2 | 3 | 3 | 2 | 3 | 2 | 1 | 1 | 3 | 1 | 2 | 2 | 3 | 1 | 1 | 7 | 1 | 3 | 2 |
| A14 | 0.074 | 0.135 | 0.07 | 1 | 6 | 11 | 5 | 3 | 4 | 5 | 4 | 2 | 6 | 7 | 4 | 2 | 0 | 2 | 7 | 3 | 3 | 8 | 4 | 5 | 2 | 2 | 1 | 6 | 8 | 9 |
| A19 | 0.129 | 0.165 | 0.239 | 0.117 | 1 | 7 | 5 | 5 | 2 | 3 | 3 | 1 | 4 | 4 | 2 | 2 | 3 | 1 | 4 | 2 | 5 | 3 | 3 | 3 | 2 | 3 | 7 | 4 | 7 | 7 |
| B05 | 0.082 | 0.097 | 0.079 | 0.135 | 0.097 | 1 | 9 | 6 | 4 | 7 | 4 | 1 | 8 | 6 | 5 | 3 | 1 | 4 | 7 | 5 | 3 | 7 | 7 | 7 | 3 | 4 | 3 | 7 | 12 | 19 |
| B09 | 0.123 | 0.114 | 0.086 | 0.094 | 0.115 | 0.122 | 1 | 6 | 2 | 6 | 4 | 1 | 7 | 5 | 5 | 5 | 1 | 3 | 5 | 4 | 3 | 5 | 5 | 5 | 2 | 3 | 2 | 4 | 8 | 10 |
| B15 | 0.174 | 0.087 | 0.157 | 0.069 | 0.147 | 0.094 | 0.169 | 1 | 4 | 5 | 3 | 1 | 5 | 4 | 2 | 3 | 3 | 2 | 2 | 6 | 4 | 3 | 4 | 6 | 2 | 2 | 4 | 3 | 5 | 9 |
| B16 | 0.105 | 0.098 | 0.093 | 0.101 | 0.067 | 0.067 | 0.063 | 0.182 | 1 | 4 | 2 | 1 | 4 | 4 | 3 | 2 | 0 | 3 | 3 | 4 | 1 | 5 | 3 | 3 | 3 | 2 | 2 | 3 | 3 | 6 |
| B18 | 0.136 | 0.179 | 0.082 | 0.118 | 0.091 | 0.111 | 0.174 | 0.2 | 0.19 | 1 | 3 | 1 | 8 | 5 | 5 | 2 | 0 | 3 | 3 | 3 | 1 | 3 | 9 | 7 | 2 | 3 | 2 | 4 | 7 | 14 |
| E07 | 0.071 | 0.056 | 0.045 | 0.064 | 0.057 | 0.048 | 0.073 | 0.067 | 0.049 | 0.068 | 1 | 1 | 3 | 3 | 2 | 2 | 0 | 1 | 3 | 2 | 2 | 2 | 2 | 3 | 2 | 2 | 1 | 3 | 4 | 3 |
| E09 | 0.069 | 0.038 | 0.118 | 0.057 | 0.039 | 0.018 | 0.037 | 0.057 | 0.074 | 0.061 | 0.027 | 1 | 2 | 1 | 1 | 1 | 0 | 1 | 2 | 1 | 0 | 3 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 |
| E13 | 0.08 | 0.082 | 0.075 | 0.103 | 0.082 | 0.102 | 0.14 | 0.123 | 0.11 | 0.203 | 0.05 | 0.063 | 1 | 5 | 4 | 4 | 1 | 3 | 3 | 4 | 1 | 5 | 6 | 8 | 2 | 3 | 3 | 5 | 8 | 11 |
| E15 | 0.121 | 0.09 | 0.085 | 0.131 | 0.091 | 0.081 | 0.11 | 0.111 | 0.125 | 0.143 | 0.055 | 0.036 | 0.099 | 1 | 5 | 3 | 1 | 1 | 6 | 3 | 2 | 2 | 4 | 5 | 1 | 1 | 2 | 4 | 4 | 7 |
| E18 | 0.057 | 0.065 | 0.053 | 0.072 | 0.043 | 0.066 | 0.105 | 0.053 | 0.088 | 0.135 | 0.035 | 0.034 | 0.076 | 0.104 | 1 | 2 | 0 | 2 | 2 | 2 | 0 | 3 | 2 | 3 | 2 | 2 | 1 | 2 | 3 | 4 |
| F02 | 0.109 | 0.026 | 0.1 | 0.042 | 0.052 | 0.044 | 0.125 | 0.098 | 0.075 | 0.068 | 0.04 | 0.045 | 0.089 | 0.074 | 0.047 | 1 | 1 | 1 | 2 | 3 | 1 | 3 | 2 | 3 | 1 | 1 | 2 | 1 | 2 | 2 |
| F09 | 0.16 | 0 | 0.133 | 0 | 0.128 | 0.019 | 0.04 | 0.194 | 0 | 0 | 0 | 0 | 0.033 | 0.039 | 0 | 0.05 | 1 | 0 | 0 | 1 | 2 | 1 | 1 | 1 | 0 | 1 | 2 | 0 | 2 | 3 |
| F11 | 0.074 | 0.08 | 0.063 | 0.059 | 0.041 | 0.073 | 0.115 | 0.121 | 0.24 | 0.194 | 0.028 | 0.125 | 0.097 | 0.038 | 0.07 | 0.048 | 0 | 1 | 3 | 3 | 0 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 6 |
| F16 | 0.025 | 0.065 | 0.012 | 0.069 | 0.043 | 0.057 | 0.053 | 0.024 | 0.037 | 0.036 | 0.029 | 0.026 | 0.03 | 0.063 | 0.021 | 0.022 | 0 | 0.04 | 1 | 1 | 3 | 4 | 3 | 3 | 2 | 2 | 1 | 6 | 6 | 11 |
| F18 | 0.207 | 0.077 | 0.176 | 0.086 | 0.078 | 0.09 | 0.148 | 0.343 | 0.296 | 0.182 | 0.055 | 0.111 | 0.125 | 0.109 | 0.068 | 0.136 | 0.143 | 0.375 | 0.013 | 1 | 0 | 2 | 3 | 4 | 2 | 2 | 3 | 2 | 3 | 5 |
| G01 | 0.034 | 0.029 | 0.016 | 0.038 | 0.072 | 0.03 | 0.042 | 0.065 | 0.017 | 0.017 | 0.025 | 0 | 0.013 | 0.028 | 0 | 0.015 | 0.039 | 0 | 0.025 | 0 | 1 | 3 | 1 | 1 | 1 | 1 | 1 | 3 | 3 | 4 |
| G10 | 0.128 | 0.114 | 0.077 | 0.182 | 0.087 | 0.109 | 0.139 | 0.113 | 0.222 | 0.118 | 0.044 | 0.167 | 0.122 | 0.055 | 0.078 | 0.097 | 0.063 | 0.118 | 0.047 | 0.111 | 1 | 1 | 4 | 2 | 3 | 2 | 2 | 6 | 12 | 10 |
| G13 | 0.105 | 0.164 | 0.093 | 0.101 | 0.1 | 0.117 | 0.159 | 0.182 | 0.167 | 0.429 | 0.049 | 0.074 | 0.164 | 0.125 | 0.059 | 0.075 | 0.087 | 0.16 | 0.037 | 0.222 | 0.017 | 0.178 | 1 | 6 | 2 | 2 | 3 | 5 | 9 | 12 |
| G16 | 0.107 | 0.127 | 0.098 | 0.103 | 0.077 | 0.101 | 0.123 | 0.194 | 0.111 | 0.233 | 0.06 | 0.044 | 0.176 | 0.122 | 0.07 | 0.085 | 0.049 | 0.093 | 0.034 | 0.178 | 0.015 | 0.063 | 0.222 | 1 | 2 | 2 | 3 | 5 | 6 | 10 |
| G20 | 0.16 | 0.125 | 0.067 | 0.061 | 0.085 | 0.056 | 0.08 | 0.129 | 0.261 | 0.138 | 0.058 | 0.143 | 0.067 | 0.039 | 0.073 | 0.05 | 0 | 0.333 | 0.027 | 0.286 | 0.02 | 0.188 | 0.174 | 0.098 | 1 | 3 | 2 | 4 | 3 | 3 |
| H01 | 0.138 | 0.115 | 0.059 | 0.057 | 0.118 | 0.072 | 0.111 | 0.114 | 0.148 | 0.182 | 0.055 | 0.111 | 0.094 | 0.036 | 0.068 | 0.045 | 0.143 | 0.25 | 0.026 | 0.222 | 0.019 | 0.111 | 0.148 | 0.089 | 0.429 | 1 | 2 | 3 | 4 | 5 |
| H07 | 0.207 | 0.231 | 0.412 | 0.029 | 0.275 | 0.054 | 0.074 | 0.229 | 0.148 | 0.121 | 0.027 | 0.111 | 0.094 | 0.073 | 0.034 | 0.091 | 0.286 | 0.25 | 0.013 | 0.333 | 0.019 | 0.111 | 0.222 | 0.133 | 0.286 | 0.222 | 1 | 2 | 3 | 4 |
| H10 | 0.118 | 0.211 | 0.051 | 0.16 | 0.143 | 0.121 | 0.136 | 0.15 | 0.188 | 0.211 | 0.077 | 0.174 | 0.145 | 0.133 | 0.063 | 0.041 | 0 | 0.19 | 0.076 | 0.174 | 0.054 | 0.293 | 0.313 | 0.2 | 0.421 | 0.261 | 0.174 | 1 | 10 | 8 |
| H15 | 0.118 | 0.154 | 0.082 | 0.147 | 0.156 | 0.16 | 0.172 | 0.135 | 0.091 | 0.194 | 0.071 | 0.07 | 0.155 | 0.085 | 0.061 | 0.048 | 0.075 | 0.073 | 0.063 | 0.105 | 0.041 | 0.32 | 0.273 | 0.143 | 0.113 | 0.14 | 0.105 | 0.323 | 1 | 17 |
| H20 | 0.068 | 0.154 | 0.026 | 0.096 | 0.083 | 0.167 | 0.117 | 0.118 | 0.083 | 0.187 | 0.032 | 0.03 | 0.122 | 0.081 | 0.045 | 0.025 | 0.046 | 0.09 | 0.082 | 0.074 | 0.036 | 0.131 | 0.167 | 0.123 | 0.046 | 0.074 | 0.059 | 0.114 | 0.195 | 1 |

Matrix consisting of Bray-Curtis similarity indices (lower left) and the number of shared OTUs between plots (upper right). This table is based on the 454 sequence data.