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Independent project • 15 hec • First cycle, G2E
Biology with specialisation in Biotechnology – Bachelor's Programme
Examensarbete/Sveriges lantbruksuniversitet,
Institutionen för mikrobiologi: 2014:6 • ISSN 1101-8151
Uppsala 2014

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Credits: 15 hec
Level: First cycle, G2E
Course title: Independent project in Biology - bachelor project
Course code: EX0689
Programme/education: Biology with specialisation in Biotechnology - Bachelor's Programme

Place of publication: Uppsala
Year of publication: 2014
Title of series: Examensarbete/Sveriges lantbruksuniversitet, Institutionen för mikrobiologi
No: 2014:6
ISSN: 1101-8151
Online publication: http://stud.epsilon.slu.se

Keywords: Ecology, biological pesticide, soil fungi, population study, qPCR

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Abstract

Annually insect and arthropod pests cause damage to both crops and cattle. In recent decades there has been an increased attention towards using alternative methods for pest control. The genus *Metarhizium* contains several soil associated entomopathogenic species of fungi which may be used as effective solutions for biological control of invertebrate pests. However, while the pathogenicity has been thoroughly studied, the ecology of this fungal group remains a tentative mystery. The aims of this study were to study the natural abundance of *Metarhizium* in a forest, pasture and agricultural field in Sweden. Out of 36 soil samples two species, *Metarhizium flavoviride var. pemphigi* and *Metarhizium guizhouense* were isolated from forest soil. A cultivation-independent assay revealed that the agricultural field harbored the highest abundance of *Metarhizium*. These findings are most likely due to the effect of local weather conditions at the time of sampling. The results herein indicates that time of sampling is a more important factor than previously anticipated.

Table of Contents

Introduction

Insects annually cause great damage and consequently reduce agricultural production. Pests such as locusts feeding directly on the crop, thus reducing yields, as well as ticks spreading disease to cattle are the cause of severe economic damages (Sonenshine 1991*).* It has become apparent that there is a need for safer pesticides that do not harm the environment and are not prone to side effects (Ren *et al.*, 2014; Zaim & Guillet, 2002). There are several ways to reduce these costs and risks but this study focuses on the use of insect pathogens as biopesticides, an emerging biological control solution.

Biological control is herein defined as the use of one organism (which in this case is an insect pathogen, or entomopathogen) to reduce, contain or inhibit populations of agricultural insect pests. There are several types of biological control, classical which includes the release of novel species to new environments, inoculative which involves an enhancement of local populations of biocontrol organisms, inunduative where the aim is to kill quickly, and conservation which seeks to indirectly enhance natural levels of pathogens and predators by management of the environments (Lomer, 2001; Eilenberg *et al*., 2001). Biological control approaches have several advantages compared to chemical approaches, such as a higher degree of safety for humans and relatively little environmental impact. The use of another organism as a biological control agent can furthermore potentially keep the pest population at a naturally lower, stable density due to natural pathogen/host interaction. However, typically biological solutions act slower and do not eradicate pest populations, making them of less use during an ongoing outbreak. Furthermore, efficiency can depend on weather and host activity and extensive ecological knowledge regarding both pest and pathogen used to control the pest are required to properly deploy biological control.

The genus *Metarhizium* of ascomycete fungi contains entomopathogenic species . Some species of this genus cause Green muscardine disease in insects and arthropods, as first described by microbiologist Elie Metchnikoff in 1880. The host-range of the genus covers insect pests important in commercial agriculture including locusts, grasshoppers, termites, crickets and hemipterans (Zimmermann, 2007). However some species might be more host specific, one example is *Metarhizium acridum,* former *M. anilopsiae var. acridum*, a species which infects only locusts and grasshoppers. This species has been used as a biological control agent commercialized under the trade name Green Muscle or Green Guard (Becker underwood 2014, Matthew & Read, 2007; Esser *et al.*, 2002). Other virulent species such as *M. anisopliae* are also frequently studied with hopes of developing active agents against ticks, mites and malaria carrying mosquitoes (Ren *et al*., 2014; Mugisho *et al*., 2014; Thomas & Read, 2007).

Infection by *M. anisopliae* (and other *Metarhizium* spp.) is accomplished by initial adhesion of conidia to the insect cuticle by proteins encoded by the *Metarhizium* adhesion gene 2 (mad2). This is followed by the development and application of pressure by a specialized type of mycelia, an appressorium, coupled with the release of proteolytic proteins to digest chitin, the main component of the insect's exoskeleton and consequently to gain entrance through the weakened point (Wang & St. Leger, 2014; Hänel, 1982). Once inside the host body, hyphal structures are released which produce toxins that kill the host. Mycelia then invade the tissues and digest the host internally. Finally the fungus penetrates to the surface and develops a dense layer of conidiophores (Hänel, 1982) which passively release conidia back to the soil.

The genus has a worldwide distribution (Schneider *et al.*, 2012; Wang & St. Leger, 2014) and *Metarhizium* spp. can be isolated from soil where it infects its hosts. There is also evidence of species preference to the soils of certain environments, which has been suggested to be the result of species root association preferences for some plants over others (Wyrebek, Bidochka, 2013). Some species have also proven to be rhizosphere competent (Wyrebek *et al.*, 2011). Traits of *Metarhizium*'s lifecycle outside of the host remain more poorly understood and have been suggested as a target for future research to elucidate the ecology of the fungi (Schneider *et al.,* 2011) and their potential biological control effects on insect pests.

In an effort to understand this environmental ecology this study focuses on analyzing *Metarhizium* isolates in soils from three types of environments representing a varying degree of management (from low to high in order): a forest, a pasture and an agricultural field. Samples were taken outside of Uppsala, central Sweden where no studies of local *Metarhizium* populations have been done before. Species composition was analyzed for the forest soil by cultivation on semiselective media and once individual strains were isolated, species were identified by sequencing of the elongation factor 1-alpha (EF-1α) gene. This gene was used since Internal Transcribed Spacer 2 (ITS2) sequences have not proven ideal for identification on species level within the genus *Metarhizium* in past studies (Schneider *et al.,* 2011; Wyrebek *et al.*, 2011).

Furthermore, the abundance of *Metarhizium* was quantitatively analyzed by two methods. The first is the 'classical method' relying on spreading soil on semiselective media and subsequent cultivation and counts of colony forming units (CFU) according to set credentials per g dry weight (dw) soil. The second is a cultivation-independent method developed by Schneider *et al*. (2011). This method depends on quantitative real-time PCR (qPCR) targeting the ITS2 region and comparison to a standard curve. The two quantitative methods were compared to assess the reliability of the end results and the usefulness of both techniques.

Previous abundance studies in similar environments have yielded differing results (Meyling & Eilenberg, 2007). It is therefore difficult to draw general conclusions regarding differences in the abundance levels between e.g. agricultural fields and pastures. However in line with these earlier studies the abundance is expected to be the lowest in the forest environment. It is also expected that the cultivation-independent method will be more sensitive and thus have a lower detection threshold (Schneider *et al.,* 2011.

Regarding strain isolation and characterization several species are expected to be found that have been isolated from forest soils before, e.g. *M. guizhouense, M. brunneum M. flavoviride* (Wyrebek *et al.,* 2011) and *M.robertsii* (Rocha *et al*., 2011) and potentially *M. anisopliae* as well (Bidochka *et al.,* 1998).

Methodology

Sampling conditions and procedure

Soil samples were taken 2 April 2014 from a site near Knivsta, 19 km (12 miles) outside of Uppsala, central Sweden. In the forest, agricultural field and pasture (labelled with "Fr", "Ag" and "Pa" respectively) three 100 m transects (referred to as A, B and C respectively, for a total of 9 transects) were mapped out to cover as much environmental diversity (such as varying degrees of plant habitation and types of plants, open or shadowed soil etc) as possible. The transects were further divided into four equally sized (25m) subtransects which were numerically ordered from the first to last samples taken. Soil cores were taken along every 5 m increment along each transect and were approximately (aprx) 15 cm deep. Samples within the same subtransect were pooled and stored in plastic bags. However, the cores taken from subtransects Forest B2, Forest C3, Pasture B2, Pasture B3, Agricultural field A1 and Agricultural field C4, were stored in individual bags. The soil corer was cleaned with ethanol between samplings. The bags were stored in a freeze box until arriving in the lab where they were stored at +2 ˚ C. In total 60 samples were taken to the laboratory, 10 pooled samples + 10 unpooled samples from 2 subtransects in each environment.

Soil processing and dry weight analysis

Soil was sieved through a 5 mm sieve and stored in the original bags at 2˚ C over the course of 3 days. To determine the soil dry weight, 5 g of soil was dried at 100 ˚ C and re-weighed after 24 h.

Strain isolation and characterization

Plating

5 g sieved soil from each of the individual 60 samples were suspended in 25 ml autoclaved 0.1% Tween80 solution (made from 0.75 g dissolved in 749.25 ml water). The soil

suspensions were incubated for 3 hours and inverted every 30 minutes 7-10 times to thoroughly mix the soil. The tubes were then allowed to sit for 25 seconds for sedimentation before 100 µl of the supernatant was spread on the semiselective agar plates (SM media, see appendix). The plates were incubated at 22˚ C for 16 days.

Re-plating (isolation)

Colonies from the plates were picked based on their morphology, which included dark green to yellow conidia, amorphous colony shape without any defined folding and a colony size over 5cm in diameter (Figure 1).

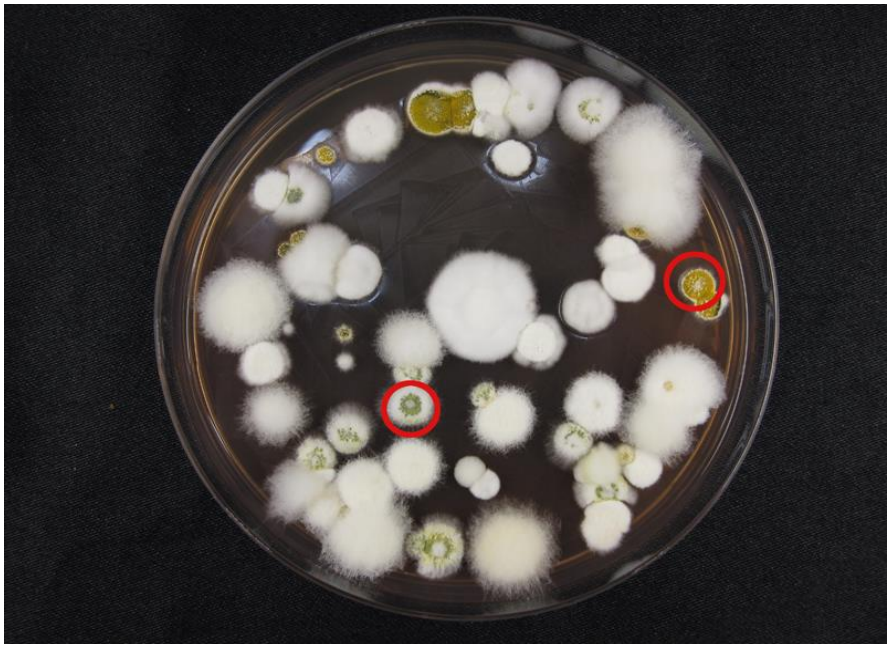

Figure 1 Example of a plate of forest soil spread on SM media that were deemed positive for *Metarhizium*-like colonies (surrounded by a red circle) after 14 days of cultivation. .

Colonies with varying morphology and size were picked with an inoculation loop, transferred to SM plates and incubated at 25˚ C for 11 days. Single colonies were transferred to new SMplates to maintain the cultures.

Mycelia cultivation, DNA Extraction and DNA extract quality check.

For each isolate tentatively identified as *Metarhizium*, 5 ml 0.1% Tween80 was spread over the plate and the colonies were systematically rubbed to release conidia. The solution was pipetted and transferred to liquid SM. The solution was incubated in darkness on a shaker set to 130 rpm at 22˚ C for 2 days.

Mycelium was extracted by transferring the media to a double layer of autoclaved filter papers on a sieve resting in the neck of an E-flask with an outlet. Through under-pressure, the medium was drained into the E-flask, leaving the mycelia granules on the filter papers.

0.3 g mycelium was weighed up into tubes which were immediately submerged in liquid nitrogen and stored at -50˚ C.

Mycelia was lyophilized in open Eppendorf tubes using an Edwards Modulyo Freeze dryer for 2 hours and fungal DNA was extracted using a Qiagen™ DNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Dilutions of the DNA preparations were required to quantitatively estimate the amount of DNA with Pico-Green® (Invitrogen, Carlsbad, CA) using a QubitTM Fluorometer (Invitrogen). A 1:50 dilution was obtained by transferring 2 µl vortexed DNA extract to new tubes containing 98 µl autoclaved water.

A PicoGreen Master Mix (MM) was made by adding (per sample to run+ 2) 1 µl Pico-Green® (Invitrogen) and 199 µl autoclaved TE buffer (pH 8) for each DNA extract. 190 µl MM was then transferred to clear-walled PCR tubes and 10 µl (x in the equation) of the vortexed 1:50 diluted fungal DNA extracts were added. After calibrating the spectrophotometer using two reference solutions the samples were read and the concentration of DNA (in ng/µl solution) was calculated (taking the dilution into account) using the formula.

$$\left(observed\ absorbance * \left(\frac{200}{x}\right)\right) * 1000 * 50$$

The DNA extracts were stored at -25 ˚ C.

DNA quality was verified by gel electrophoresis (Fig 16 in appendix). A 1% agarose gel was made by heating 198 ml 0.5x TBE buffer in which 2 g agarose had been dissolved and incubated at 60 ˚ C until cast. All subsequent gels solutions were made in the same way unless otherwise specified. 4 µl of the DNA solutions were transferred to new tubes and mixed with 1 µl GelRed dye and loaded onto the cast gel and run at 86 V for aprx 45 minutes. The gels (Fig 16 in appendix) were analyzed under UV light. All subsequent gels were run accordingly and referred to as gels unless specified.

PCR amplification and quality check

For strain identification, the EF-1α factor was amplified by PCR and sequenced. The fungal DNA extracts were diluted to 5 ng/µl and used as template. A PCR was set up in 20 µl reaction volumes containing 10 ng sample template, 1x Fermentos Dream Taq Green PCR MM (Thermo Fisher Scientific, Göteborg, Sweden), 0.5 µM EF1T forward primer (ATGGGTAAGGARGACAAGAC; Rehner. S & Buckley. E, 2005) and 0.5 µM EF2T reverse primer (GGAAGTACCAGTGATCATGTT; O'Donnell. K, Cigelnik. E, 1997) per reaction. The PCR program included activation at 95˚ for 900 sec, and then 45 cycles denaturation at 94˚ for 40 sec, annealing at 65˚ for 40 sec and extension at 72˚ for 120 sec, and then a final deactivation step at 72˚ for 600 sec. Aliquots from the PCR products were mixed with dye and loaded onto a gel and run for 45 minutes (see figure 17 in appendix). The remaining solutions were put into the freezer.

Sequence analysis

The amplified DNA fragments were estimated to have a concentration of 30 ng/µl based on the band strengths on the gel. From each PCR product a 1:10 dilution was prepared by transferring 4 µl vortexted PCR product to tubes containing 36 µl sterile water. For each strain, 5 µl from the 1:10 dilution was transferred after vortexing to two PCR tubes to act as sequencing templates. Each template was sequenced from both sides adding 1 µM EF1T forward or EF2T reverse primer in a total volume of 18 µl. The samples were then sent to Uppsala Genome Centre for sequencing.

Both forward and reverse sequences from each strain were aligned and primer sequences were removed. The edited sequences were used in BLAST similarity searches against the NCBI nucleotide database.

Cultivation-dependent method for determining *Metarhizium* community size

Plating

Soil samples were suspended in Tween80 and spread on SM media as described above. The plates were incubated at 22 ˚C for 29 days.

Establishing CFU/g dry weight

After incubation, colonies with *Metarhizium*-like colony-morphology were identified and the number of colony forming units (CFU) was estimated. Colonies were deemed positive if they fulfilled the four criteria described above. The numbers of CFU were divided by the dry weight (dw) of the soil plated on the medium and calculated as (Table 3 in appendix):

$$\frac{soil\ weighed\ up\ (g) * \ dw\ soil\ fraction}{soil\ suspension\ volume\ (ml)} * suspension\ volume\ spread\ on\ plate\ (ml)$$

Cultivation-independent method for determining *Metarhizium* community size

Soil DNA extraction procedure and quality check

DNA was directly extracted from the pooled soil samples by using a PowerLyzer™ Powersoil® DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA). The procedure followed the instructions outlined in the protocols of the kit with the following exceptions: 1, the lysed-cell solution was centrifuged at 13 000 rpm for 3 min instead of 30 sec. 2, the entire DNA-precipitate was loaded onto the spin filter to maximize DNA yields. 3, the elution solution was incubated on the filters for 5 minutes at 37˚ C before the final centrifugation step. Once extracted the DNA preparations were quality-verified on a gel and quantified as described above (Fig 15 in appendix). In between analysis the DNA solutions were stored at – 25˚ C.

Reference cultivation conditions + Mycelia induction

The reference *Metarhizium* strains used for preparation of standards for the qPCR were provided by Dr. Jürg Enkerli from Agroscope Zürich, Switzerland (Table 1).

Table1 Strains used to make the vector reference solutions for use as standards in the qPCR. The table lists the official strain ID's and the corresponding species names.

Strain ID	Species
ARSEF 7487	*M. anisopliae*
ARSEF 2575	*M. robertsii*
ARSEF 2107	*M. brunneum*
CBS 258.90	*M. guizhouense*
ARSEF 7488	*M. lepidiotae*
ARSEF 1914	*M. majus*
CBS 257.90	*M. pingshaense*
ARSEF 2596	*M. globosum*
ARSEF 7487	*M. acridum*

The reference strains were maintained on potato dextrose agar (PDA, see appendix for composition). For re-inoculation and DNA extraction, the spores were harvested and used for mycelium growth induction as described above.

Reference DNA extraction and quality checks

Genomic DNA was extracted from the lyophilized mycelia from reference strains as described above. DNA concentrations of extracts were determined with Pico Green® (Invitrogen) and DNA quality was checked by gel electrophoresis (gel not shown).

Reference DNA PCR and cloning

A PCR was set up in 20 µl reaction volumes containing 2 ng DNA from reference strains, 1x PCR buffer (Qiagen), 0.4 mM dNTPs (Invitrogen), 0.4 mM $MgCl_2$, 2 units of HotStart Taq (Qiagen), 0.2 µM Ma1763 forward primer (CCAACTCCCAACCCCTGTGAAT) and Ma2097 reverse primer (AAAACCAGCCTCGCCGAT, Invitrogen). The PCR program included activation at 95˚ for 900 sec, and then 35 cycles denaturation at 94˚ for 40 sec, annealing at 64˚ for 40 sec and extension at 72˚ for 120 sec, and finally a deactivation step at 72˚ for 600 sec. The PCR products were then incubated overnight at 10˚ C and ran on a gel at 86 V for 45 min (gel not shown) to verify amplification.

The PCR product from reference strain *M. majus (*ARSEF 1914*)* was chosen as standard for the qPCR due to its clear band on the gel (data not shown) and was ligated into the vector using the Topo TA Cloning Kit (Invitrogen). Ligation of the PCR fragment into the vector and subsequent transformation of chemically competent TOP10 *E. coli* cells were accomplished according to the manufacturer's instructions. The transformed bacterial suspension was spread in two different volumes, 25 µl and 50 µl, on Lysogeny-Broth (LB) plates with 50 µg/ml kanamycin and incubated overnight at 37˚ C.

Vector extraction and purification

Colonies were picked and grown in liquid LB medium with 50 µg/ml kanamycin at 37°C overnight. The bacterial cells were separated by centrifugation at 13 000 rpm. The extraction of the vector was then done using the QIAprep Spin Miniprep kit (Qiagen). The plasmid DNA concentrations were estimated using Pico Green® as described above, however the samples were diluted 1:100 by transferring 2 µl solution to tubes containing 198 µl water.

From the estimated concentrations the number of plasmids/µl was calculated from the weight of the plasmid, which in turn was derived from multiplying the weight of a single nucleotide pair with the number of basepairs (bp) of the vector plus the insert.

The plasmid quality was checked by gel electrophoresis: 4 µl of plasmid extract was transferred to new tubes and mixed with 1 µl DNA dye. The vector solutions, together with a 1 kb ladder was loaded on a 1% agarose gel made by adding 0.5 g agarose to a solution of 50 ml 1x TAE buffer gel. After the initial boiling, 1 droplet of ethidium bromide was also added and the gel was cast. The gel ran at 60 V for 2 hours (gel not shown)

qPCR procedure

Once the number of plasmids/µl was determined a stock dilution of 10^6 copies/µl was prepared. This was then used to make a dilution series by taking 5 µl vortexed solution to a new tube containing 45 µl sterile water, serially diluting the samples by a factor of 10 until the series had 7 samples and spanned 10^6 to 10 copies/µl. The soil DNA extracts were diluted to 2ng/µl to function as templates.

A PCR was set-up in 20 µl reaction volumes containing 5 ng soil DNA, 1x PCR buffer (Qiagen) 0.4mM dNTPs (Invitrogen), 2 mM $MgCl_2$ (Invitrogen, 0.6 mg/ml BSA, 0.1x SYBR green, 2 units

of HotStart Taq (Qiagen), 0.6 µM Ma1763 forward primer (CCAACTCCCAACCCCTGTGAAT) and 0.3 µM Ma2097 reverse primer (AAAACCAGCCTCGCCGAT, Invitrogen). The PCR program included activation at 95˚ for 900 sec , and then 45 cycles of denaturation at 94˚ for 40 sec, annealing at 65˚ for 40 sec and extension at 72˚ for 120 sec, and finally deactivation at 72˚ for 600 sec. Samples were loaded onto a gel and run for 45 minutes.

The qPCR assay was duplicated once with new DNA and vector dilutions.

Calculations

Before quantification was possible the dw of soil (g) from which the DNA was extracted was calculated. To this end the concentration of dw soil/DNA extract volume (g/µl) was calculated, and the volume of extract (µl) used in each qPCR reaction. The dw soil/DNA extract volume was calculated as (raw data presented in Table 6 in the appendix):

$$\frac{(Soil\ weight\ (g) * Soil\ dw\ fraction)}{DNA\ extract\ volume\ in\ PCR\ reaction\ (\mu l)}$$

The amount of extract used for each qPCR reaction was then calculated as;

$$\frac{Initial\ DNA\ amount\ (10\ ng)}{DNA\ concentration\ raw\ extract\ (ng/\mu l)}$$

Results

Strain isolation and characterization

After isolation, 11 distinct isolates were tentatively identified as *Metarhizium* based on colony appearance and therefore sequenced. All the EF-1α sequences had expected sizes, ranging from 600 to 700bp and clear nucleotide identity with no apparent impurities on the pyrograms (data not shown). Out of the 11 isolates, two distinct sequences of different species origin were identified with the BLAST similarity search. Eight were positive for *M. flavoviride* var. *pemphigi* while three were assigned to *M. quizhouense*. Species identification was verified by sequence identities ≥99%, E-values at 0.0 and max scores >1300 (complete BLAST results in Table 2). All isolates originated from three subtransects from the forest: nine from B3, one from C1 and one from C3. Both species occurred in both transects and both species are identified from plate B3. By visual comparison of all strains according to identified species, it is clear that colony morphology supports the grouping as clear morphological differences are directly observable (Figure 2). Distinguishing traits such as darker, more prolific- and homogenous distribution of conidia of the *M. guizhuense* isolates

compared to the *M. flavoviride var. pemphigi* isolates gives clear indication that the two groups represent different species.

Table 2: BLAST similarity search results of the sequenced EF1-α gene from the isolated forest strains. Included are, for the 11 strains, data of the subtransect from which the strains were isolated, the suggested species identity and the datasets obtained from the BLAST search. BLAST data include the amount of reference sequence covered by the sample (query covery), the likelihood of random sequence matches (E-value), and nucleotide sequence similarity with reference (sequence identity). Finally the reference sequence is listed under NCBI accession nr.

Sample	**Isolate subtransect of origin**	**Sequences***	**Species**	**query covery**	**E-value**	**Sequence Identity**	**NCBI Accession nr**
Strain1	Forest B3	See appendix*	*M. flavoviride var. pemphigi*	100 %	0.0	99 %	HQ412795.1
Strain2	Forest B3	See appendix*	*M. flavoviride var. pemphigi*	100 %	0.0	99 %	HQ412795.1
Strain3	Forest B3	See appendix*	*M. flavoviride var. pemphigi*	100 %	0.0	99 %	HQ412795.1
Strain4	Forest B3	See appendix*	*M. flavoviride var. pemphigi*	100 %	0.0	99 %	HQ412795.1
Strain15	Forest B3	See appendix*	*M. guizhouense*	100 %	0.0	100 %	HQ412787.1
Strain6	Forest C3	See appendix*	*M. guizhouense*	100 %	0.0	100 %	HQ412787.1
Strain7	Forest B3	See appendix*	*M. flavoviride var. pemphigi*	98 %	0.0	99 %	HQ412795.1
Strain8	Forest B3	See appendix*	*M. guizhouense*	100 %	0.0	100 %	HQ412787.1
Strain9	Forest B3	See appendix*	*M. flavoviride var. pemphigi*	100 %	0.0	99 %	HQ412795.1
Strain10	Forest C1	See appendix*	*M. flavoviride var. pemphigi*	100 %	0.0	99 %	HQ412795.1
Strain11	Forest B3	See appendix*	*M. flavoviride var. pemphigi*	100 %	0.0	99 %	HQ412795.1

**see table 5 in appendix for sample sequences.*

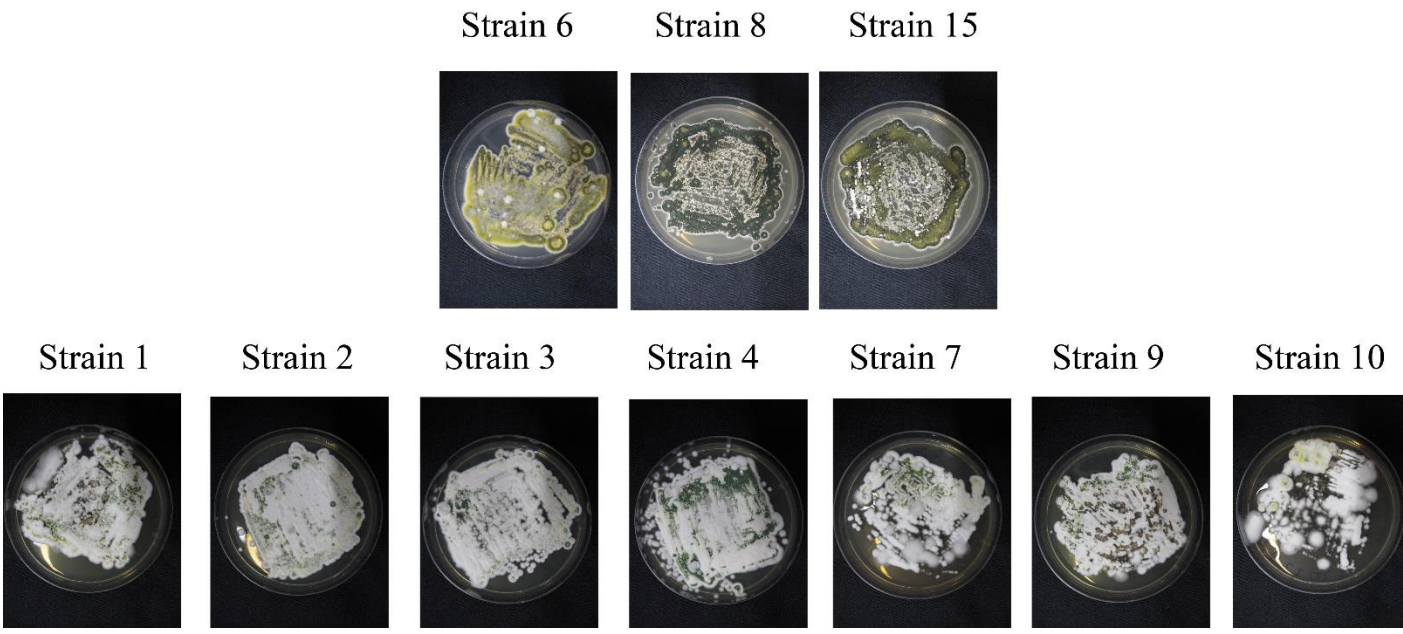

Figure 2 Morphology of all isolated strains. The colonies are organized spatially according to species with strains 6, 8 and 15, identified as *M. guizhouense* on top and strains 1, 2, 3, 4, 7, 9 and 10, identified as *M. flavoviride var. pemphigi* below. Note: one plate, strain 11, positive for *M. flavoviride var. pemphigi* is absent as the strain was discovered after the photographs were taken.

Cultivation-dependent quantification of *Metarhizium* in soil

Soil samples collected in the forest were selected for cultivation-dependent quantification of *Metarhizium*, with three replicates each. Based on the set criteria for *Metarhizium* positive colonies, five plates from two subtransects were positive for *Metarhizium* abundance such as all replicates of subtransect B3 and two replicates of subtransect C3 (Fig 3 for a picture compilation of all the plates). By calculation of the mean it was clear that subtransect B3 had a drastically higher abundance of 675 (±187) CFU/g dw soil compared to subtransect C3 with 103 (±35) CFU/g dw soil. All other subtransects were negative for any colonies with *Metarhizium* like appearance according to the set criteria (fig 4).

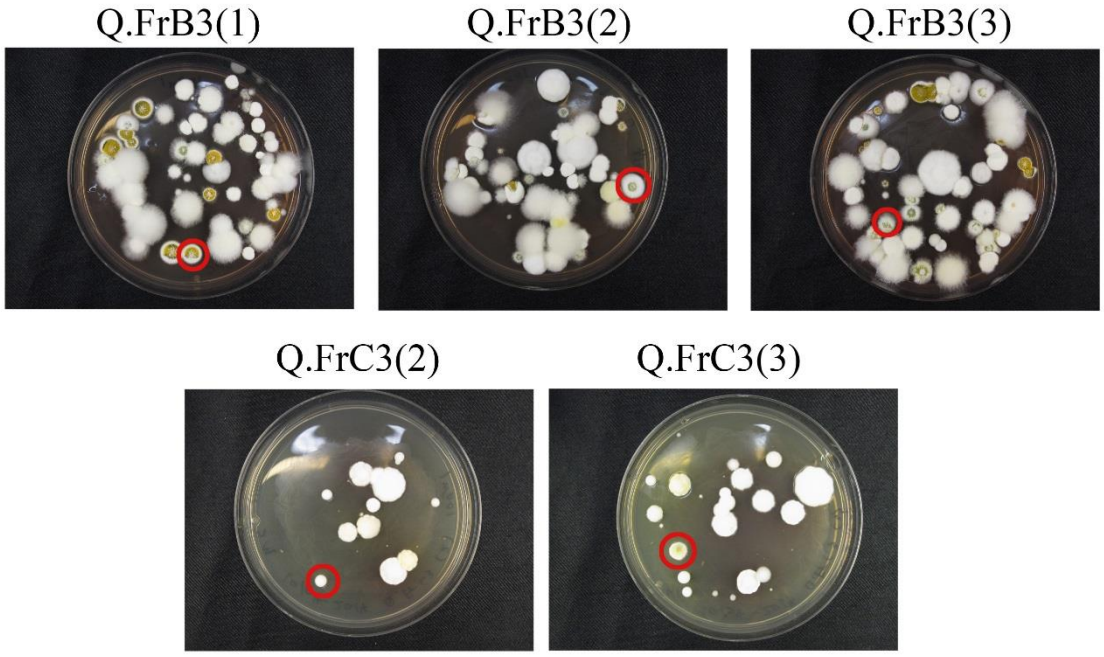

Figure 3: Plates with forest soil spread on SM media that were positive for Metarhizium-like colonies after 14 days of incubation. Examples of *Metarhizium*-like colonies are marked by a red circle. The pictures are organized based on the isolate's origin (subtransect) with plates of subtransect B3 on top and C3 on bottom. Note: as the pictures were taken several weeks in advance not all colonies display the morphology on which the colonies were deemed positive at a later date.

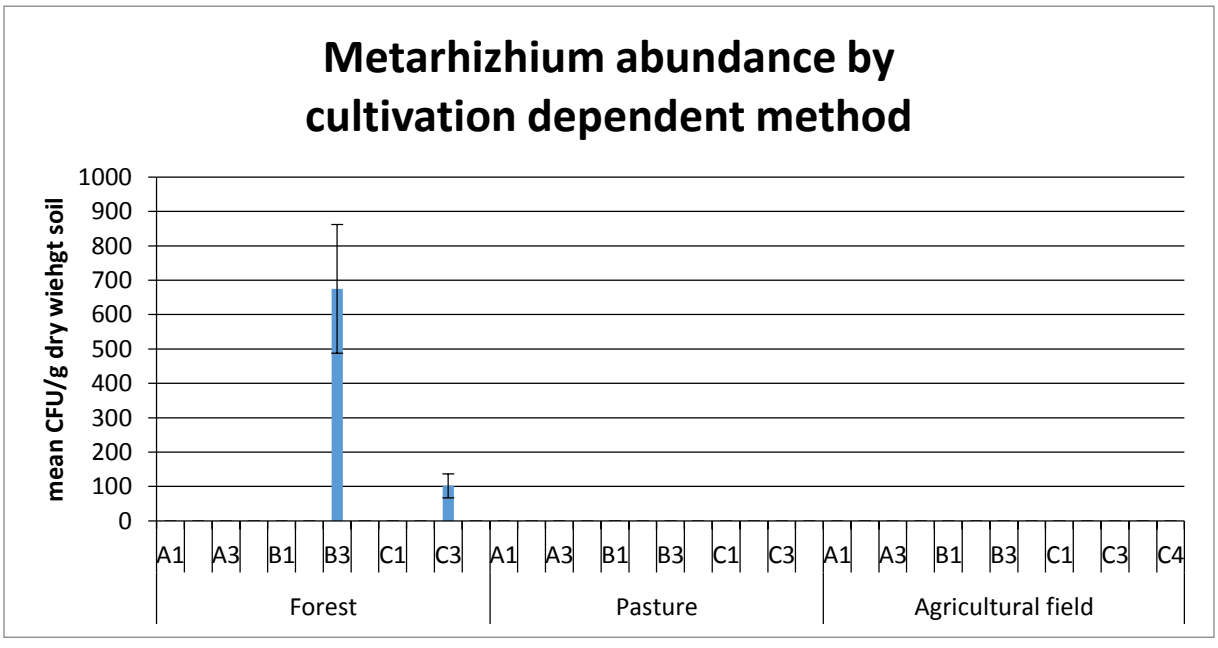

Figure 4 Metarhizium abundance in three different environments in Sweden. Transects are represented by A, B and C and subtransects by number 1-4). A total of 36 soil samples/subtransects for all environments, 12 for each environment.

Cultivation-independent quantification of *Metarhizium* in soil

The copy numbers of ITS fragments as obtained from the qPCR runs were used to calculate the abundance level per g dw soil (Figures 12 and 14 in appendix for standard curves and Figures 11 and 13 for amplification graphs). PCR reactions indicating a level of ITS copies $\geq 10^6$ and $\geq 10^7$ per PCR reaction tube for run 1 and run 2 respectively were classified as errors and discarded. These datasets (see Table 6 in appendix for raw data) were used to make bar plots representing the abundance in each soil sample.

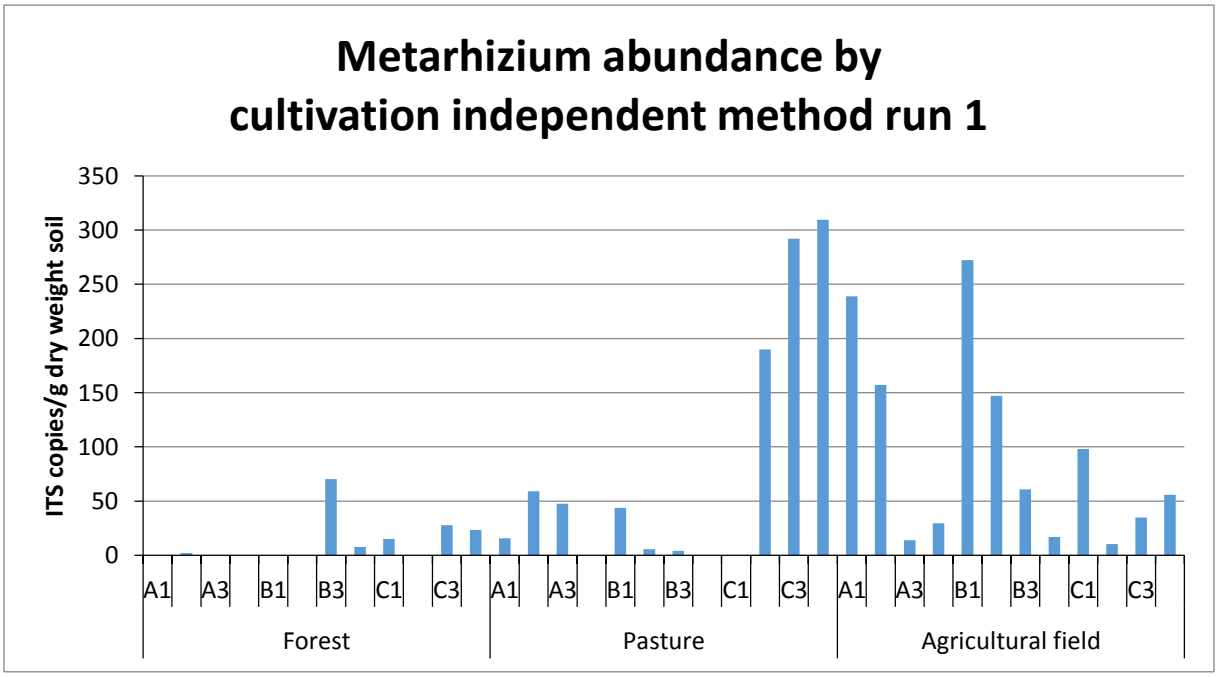

Figure 5: Abundance of Metarhizium ITS2 fragments from the pooled environmental subtransects as detected in the first run of qPCR. Samples are listed on the X axis according to habitat type, transects A-C and subtransects 1-4 (within the same transect) for a total of 36 samples, 12 from each habitat type. The scale has been reduced to 10^{-6} the actual read. See Figure 11 and 12 in appendix for qPCR amplification and standard curve.

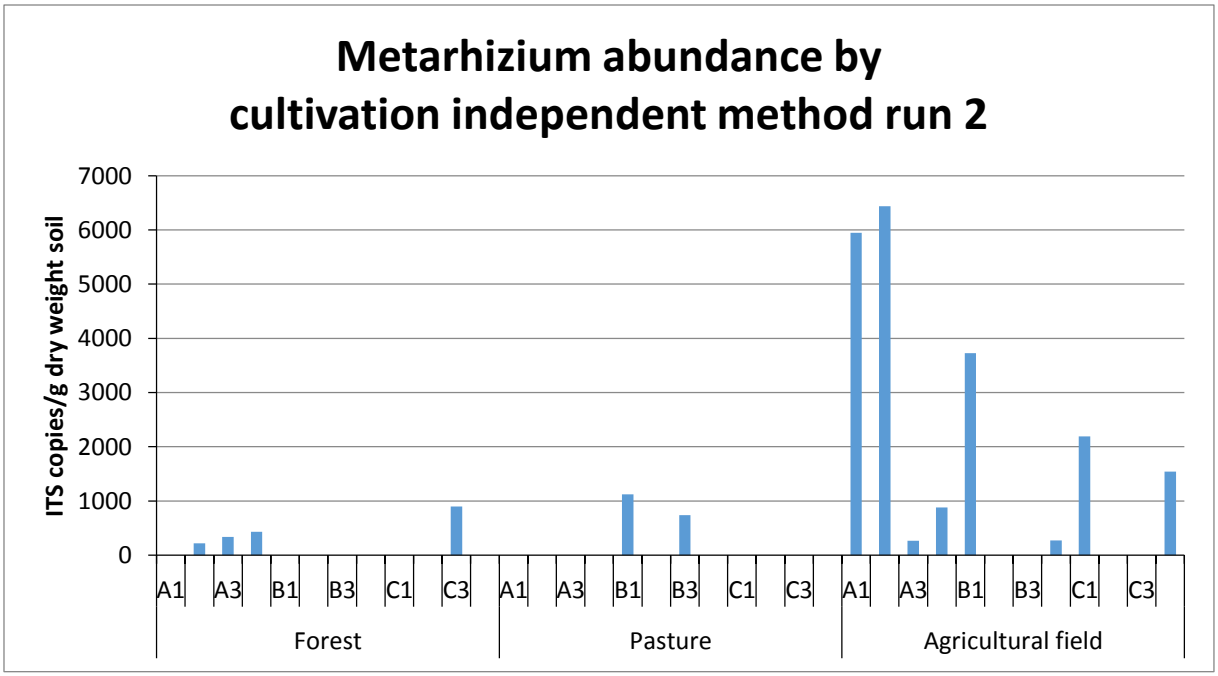

Figure 6: Illustrates the first dataset from table 6, the abundance levels of Metarhizium ITS2 fragments per gram dry weight soil (seen on the y-axis) from the pooled environmental subtransects as detected in the first run of qPCR. Samples are listed on the X axis according to environment type, transects A-B and subtransects 1-4 (within the same transect) for a total of 36 samples, 12 from each environment type. The scale has been reduced to 10^{-7} the actual read. See figure 13 and 14 in appendix for qPCR amplification and standard curve.

For both runs great degrees of heterogeneity can be observed between samples within the same environment, transect and subtransect. *Metarhizium* fungi were detected in several transects where they did not appear from the cultivation-dependent method. Several of these have lower abundance levels than the transect with the lowest (C3) from the cultivation-dependent method. Fewer subtransects indicated *Metarhizium* abundances in the second run of qPCR compared to the first. *Metarhizium* were detected in four samples of forest soil in the second run compared to seven in the first run, but only in two samples of pasture soil compared to nine in the first run and in eight samples from the agricultural field compared to all 12 in the first run.

Abundance levels had a range from $689*10^3$ (Forest A3) to $109*10^6$ (Pasture C3) ITS copies/dw soil for the first run while abundance levels ranged from $219*10^7$ (Forest A2) to $634*10^8$ (Agricultural field A2 ITS copies/dw soil in the second run. For the dataset of the first run the pasture had the highest mean abundance value of $96.7*10^6$ ($\pm 115*10^6$), followed by the Agricultural field of $94.7*10^6$ ($\pm 86.1*10^6$) and last the forest with $13.3*10^7$ ($\pm 20.5*10^7$). For the second run the Agricultural field had the highest mean abundance level $177*10^8$ ($\pm 225*10^8$), followed by the Pasture with $169*10^7$ ($\pm 368*10^7$) and last the forest with $171*10^7$ ($\pm 274*10^7$).

In order to visualize the variation between samples within the same transect, the means and deviation from the means of each transect were calculated and the result was made into new bar plots separately for each qPCR run (Figures 7 and 8).

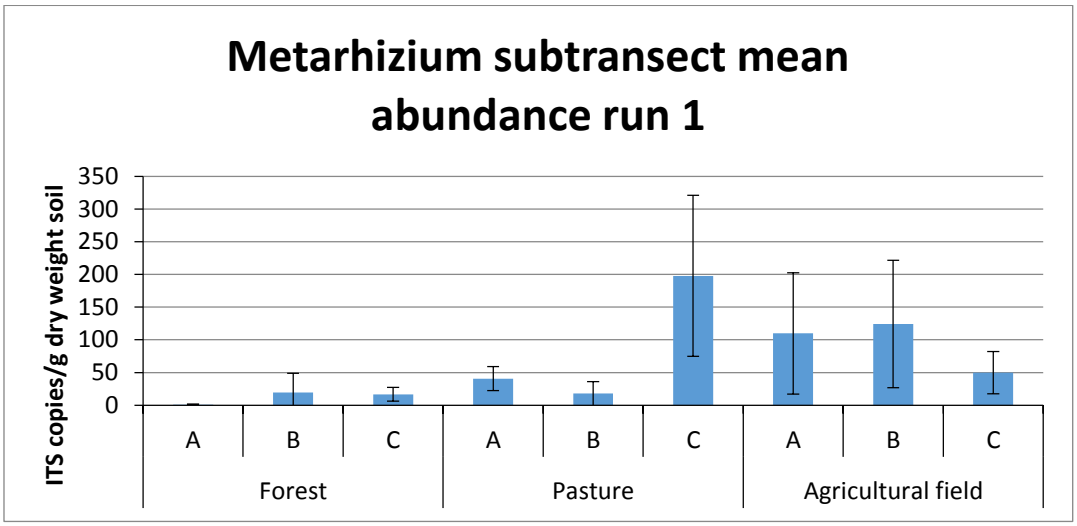

Figure 7: Transect means from table 6, and Figure 4 of the abundance levels of Metarhizium ITS2 fragments per gram dry weight soil for the first run of qPCR. Samples are listed on the X axis according to habitat type, transects 1-C for a total of 9 samples, 3 from each environment type. The scale has been reduced to 10^{-6} the actual read.

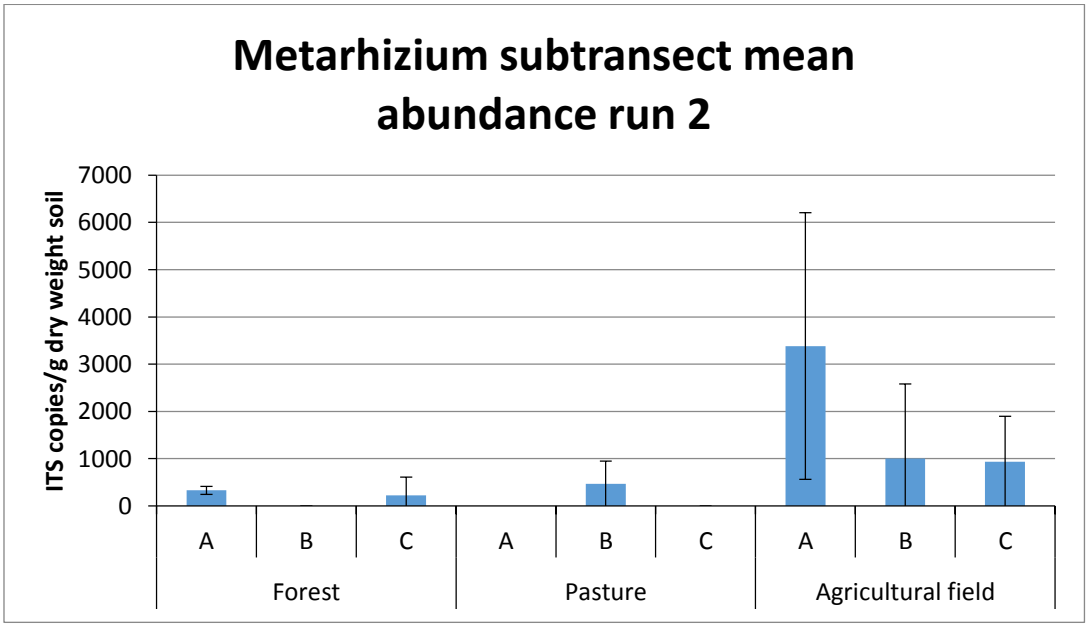

Figure 8: Transect means from table 6, and figure 5 the abundance levels of Metarhizium ITS2 fragments per gram dry weight soil for the second run of qPCR. Samples are listed on the X axis according to habitat type, transects 1-C for a total of 9 samples, 3 from each environment type. The scale has been reduced to 10^{-7} the actual read.

Once again great heterogeneity between transects can be observed for both runs. The abundance for the first run ranged between Forest transect A $690*10^4$ ($\pm 927*10^3$) and Pasture transect C with $197*10^6$ ($\pm 122*10^6$) ITS/g dw soil. For the second run the abundance ranged between Forest transect C with $233*10^6$ ($387*10^6$) and Agricultural field transect A $338*10^7$ ($\pm$ $282*10^7$) ITS/g dw soil.

Discussion

Characterization of *Metarhizium* strains from forest soil

Based on the sequence BLAST results with an identity score of ≥99% and E-value of 0.0 (Table 2), three isolates were conclusively positive for *M. guizhouense* while remaining isolates were positive for *M. flavoviride var. pemphigi*. Colony morphology supported this division. The three isolates identified as *M. guizhouense,* with olive dark/black or yellow conidia were relatively dense and homogenous distributed across the colony with very little apparent white mycelia formation, appeared radically different from *M. flavoviride var. pemphigi* isolates (Fig 2). They had brighter, more heterogeneously distributed, emerald green conidia and more apparent mycelia formation. Rocha *et al*. (2011) described their *M. flavoviride var. pemphigi* isolate similarly; "IP 143, which was identified molecularly as *M. flavoviride var. pemphigi*, has conidia that are brighter green (and without other obvious brown shading) than other *Metarhizium* species." which is a description highly applicable also to the isolates in this study.

Both species have been isolated from the soils of forests by other research groups, e.g. Nishi *et al.* (2011), Rocha *et al.* (2011), and Wyrebek *et al.* (2011), but as far as this author knows not from Swedish soil. This finding is indicating a completely global distribution of both species, which until then had been found in the America's, Oceania and far eastern geographical spans of the globe. However, compared to these other studies, common *Metarhizium* species such as *M. anisopliae, M. robertsii* (Rocha *et al.,* 2011), and *M. brunneum* (Wyrebek *et al.,* 2011) were missing in the forest soil analyzed in the present study. These findings indicate that the abundance of these species is lower in Swedish forest soils as compared to forest soils in Brazil, Japan and Canada. As colonies were chosen for isolation and sequencing based on varying morphology, it is unlikely that any *Metarhizium* species that could propagate on the plates would be left out.

The *Metarhizium* diversity in the present study was still lower compared to other studies. A potential reason for this low number of isolated *Metarhizium* species can be the seasonal timing of sampling, which was performed shortly after subzero conditions had lifted for a week of warmer early spring conditions at around 3-4 °C. These conditions may have suppressed the abundance of *Metarhizium* species that germinate slowly at lower temperatures. This is supported by a study by Nishi *et al.* (2011), that suggested that *M. flavoviride var. pemphigi* has higher germination rates in colder conditions (~10˚ C) compared with other *Metarhizium* species, which could allow it to "push" above the population threshold density for being isolated. The same study found that while *M.*

guizhouense, which was suggested to be better adapted to colder environments by Wyrebek *et al.* (*2011),* does not share this trait. The presence of *M. guizhouense* could however also be attributed to the environment, as the species is found to be strongly associated with tree roots (Wyrebek, 2011), along which the species could possibly hibernate.

The results from this study may support Nishi's conclusion, saying that more *M. flavoviride var. pemphigi* isolates were obtained due to a higher initial abundance in the soil induced by the weather conditions which promoted germination at lower temperatures.

Cultivation-dependent quantification of *Metarhizium* in soil

Two of the subtransects were positive for *Metarhizium* abundance, subtransect B3 with 675 (± 187) colonies/g soil dw and C3 with 102 (±35) colonies/g soil dw (Figure 4). This level is within the range found in other studies, e.g. Schneider *et al.* (2012), who obtained 437 (±1019) CFU/g soil dw. However, they also found significantly higher abundances in the soils of other environments, especially permanent grasslands, similar to the pasture studied herein. Schneider *et al.* (2012) further concluded that regarding the *Metarhizium* abundance, not only habitat type matters but also that there can be big differences among different locations/regions. This variation in abundance might be due to the pathogenic nature of the fungi's life style, resulting in high fungal densities in areas of high density of infected insect cadavers, due to the growth of mycelia in the host and release of mature conidia. It is however unlikely that all soil samples found negative for *Metarhizium*-like colonies can be attributed to "bad luck" when sampling. The forest site sampled could feasibly correspond to a site with lower abundance in the study of Schneider *et al.* However, it seems unlikely that random events can completely explain the absence of CFU on plates from other environments, where several studies have found higher abundances than in forests (e.g. Schneider *et al.* (2012), Perez-Golzález, *et al.* (2014).

The isolates of *Metarhizium* from forest soil were characterized as *M. guizhouense* and especially *M. flavoviride var. pemphigi,* who has been known to germinate at lower temperatures. Thus, the CFU detected from these soils are most certainly of these species. It seems likely that *M. guizhouense*, which is known to associate with tree roots, could maintain a more stable dormant population over the colder winter months and *M. flavoviride var. pemphig* could potentially have been active for a short time due to the increased local temperature. These characteristics would lead to an initially higher population abundance of these two species in the soil at the time of sampling compared to other *Metarhizium* fungi. This in turn would allow them to remain viable during cultivation on plates with other species where nutrition and space is limited. Other species such as *M. anilopsiae* which germinates at higher temperatures would be eliminated by other fungi when cultivation (Nishi *et al.,* 2013). This would be the matter even as *M. anilopsiae* is frequently found in high abundances of agricultural soil (Bidochka *et al.,* 1998) as the initial levels would be too low for the populations sampled to remain viable during cultivation on plates, resulting in the low CFU observed.

Based on these results, future studies should take season and potential weather conditions into account when sampling, plus give more attention to local/regional patchiness.

Cultivation-independent quantification of *Metarhizium* in soil

Comparing the two separate qPCR runs is difficult due to the high degree of variation of scale between to two runs. However, in both runs the degree of heterogeneity among thesubtransects is similar and directly observable and can be attributed to the fungi's ecology.

It is obvious that the highest *Metarhizium* abundance was found in the agricultural field>pasture>forest when comparing both qPCR data sets. In a similar study by Schneider *et al.* (2012) in Switzerland, significantly higher abundances of *Metarhizium* were found in permanent grassland when compared to agricultural fields and forests margins. A Canadian study, on the other hand, found the opposite (Bidochka *et al.,* 1998). The abundance is spatially highly heterogeneous as it can be seen from the error bars of thesubtransect means (Figs 7 and 8). It is possible that a seasonal shift of *Metarhizium* abundance levels may occur. The different environments sampled could have seasonal variation in important variables like temperature which is known to affect *Metarhizium* population densities. For instance the agricultural field might warm up earlier due to the increased solar radiation on the soil, as *M. anisopliae* is frequently found in these soils and is known to germinate at higher temperatures. It is not unlikely that populations would rise as temperatures did, and while initially populations would appear smaller they would rise later in the season due to local climate conditions. Future research is needed to elucidate seasonal shifts in *Metarhizium* abundance. Weather conditions and time of year are still favored factors possibly explaining this phenomenon as it is supported by another study.

When comparing the two quantification methods, it appears that the cultivation-independent technique is more sensitive than the cultivation-dependent method. This finding is clearly demonstrated when comparing the numbers of CFU estimated by cultivation-dependent method (Figure 4) with the copy numbers estimated by cultivation-independent quantification (Figure 5), where in soil samples from transects with less than 100 CFU g^{-1} soil dw *Metarhizium* was still detectable with qPCR.

Final conclusions

The findings of this study indicate that while there is a high degree of variance in *Metarhizium* abundances between sampling sites, agricultural fields have the highest *Metarhizium* abundance followed by pastures and forests. This could be relevant if the presence of *Metarhizium* in the sampled field (and possibly surrounding fields) is high enough for infecting insect pests, and could lead to a decreased need for inoculation with other forms of pesticides.

The cultivation-independent technique for detection and quantification of *Metarhizium* is a more sensitive method to detect populations of *Metarhizium* with low abundances in the

soil of these environments and should be considered for future *Metarhizium* population studies in soil. Potential season-related fluctuations might be the reason of contradictory results regarding *Metarhizium* abundances in agricultural fields of this study when compared to the findings in Schneider et al. (2012), where soil samples were collected in summer. Consequently, a study over the course of a year is required to make any real assessment.

Furthermore, this study successfully identified two species of *Metarhizium* from forest soil from Central Sweden, *M. guizhouense* and *M. flavoviride var. pemphigi.*

References

Becker Underwood website
Date of viewing: 2014/04-19 22:19
Webpage link: http://www.beckerunderwood.com/productsservices/biological-crop-protection/bio-pesticides/green-Muscle

Bidochka. M. J, Kasperski. J.E, Wild, G.A.M., 1998. "Occurrence of the entomopathogenic fungi Metarhizium anisopliae and Beauveria bassiana in soils from temperate and near- northern habitats." Can. J. Bot. 76, 1198–1204

Eilenberg. J, Hajek. A, Lomer. C, (2001) "Suggestions for unifying the terminology in biological control"
Doi: Biocontrol 46, 387–400.

Esser. K, Bennet. J.W, Kempken. F, "The Mycota, Agricultural applications" (2002)
ISBN: 3-540-42628-0

Hänel. H (*1982*) "The life cycle of the insect pathogenic fungus Metarhizium anisopliae in the termite Nasutitermes exitiosus"Mycopathologia80, 137-145(1982)

Lomer. C.J, Bateman. R.P, Johnson. D. L, Langewald. J, Thomas. M (2001) "BIOLOGICAL CONTROL OF LOCUSTS AND GRASSHOPPERS Annual Review of Entomology"
DOI: 10.1146/annurev.ento.46.1.667
.

Metchnikov, E.A. (1880) "Zur Lehre über Insektenkrankenheiten" Zool. Anz. 3, 44–47.

Mugisho. B.D, Knapp. M, Ekesi. S, Chabi-Olaye. D, Iddi. B.H, Kalemba. M.N (2014) "Efficacy of Metarhizium anisopliae in controlling the two-spotted spider mite Tetranychus urticae on common bean in screenhouse and field experiments"
DOI 10.1111/1744-7917.12111

Meyling. N.V, Thorup-Kristensen. K, Eilenberg. J (2011) "Below- and aboveground abundance and distribution of fungal entomopathogens in experimental conventional and organic cropping systems"
doi: 10.1016/j.biocontrol.2011.07.017

Meyling. N.V, Eilenberg. J (2007) "Ecology of the entomopathogenic fungi Beauveria bassiana and Metarhizium anisopliae in temperate agroecosystems: Potential for conservation biological control"
DOI:10.1016/j.biocontrol.2007.07.007

Nishi. O, Hasegawa. K, Liyama, K, Yasunaga-Aoki. C, Shimizu. S (2011) "Phylogenetic analysis of *Metarhizium* spp. isolated from soil in Japan" *Appl. Entomol. Zool.* 46 301-309

Nishi. O, Liyama. K, Yasunaga-Aoki. C, Shimizu.S (2013) "Comparison of the germination rates of Metarhizium spp. conidia from Japan at high and low temperatures" doi:10.1111/lam.12150

O'Donnel K, Cigelnik E (1997), " Two Divergent Intragenomic rDNA ITS2 Types within a Monophyletic Lineage of the Fungus Fusarium Are Nonorthologous" Molecular phylogenetics and evolution (1997) Mol Phylo Evol **7**:103–116, [1055-7903] O'Donnell, K yr:1997 vol:7 iss:1 pg:103 -116

Perez-González. V.H, Guzmán-Francoa A.W, Alatorre-Rosas. R, Hernández-López. J, Hernández-López. A, Carrillo-Benítez. M.G, Jason Baverstock (2014) "Specific diversity of the entomopathogenic fungi Beauveria and Metarhizium in Mexican agricultural soils" Journal of invertebrate pathology (not yet published)

Rehner. S.A (2005) "A Beauveria phylogeny inferred from nuclear ITS and EF1-a sequences: evidence for cryptic diversification and links to Cordyceps teleomorphs"
doi: 10.3852/mycologia.97.1.84

Ren. Q, Sun. M, Guan. G, Liu. Z, Chen. Z, Liu. A, Li. Y, Ma. M, Yang. J, Niu. Q, Liu. J, Han. X, Yin. H, Luo. J (2014) "Susceptibility of the tick Haemaphysalis qinghaiensis to isolates of the fungus Metarhizium anisopliae in China"DOI:10.1007/s10493-014-9790-2

Rocha. L.F.N, Inglis. P.W, Humber. R.A, Kipnis. A and Luz. C (2011) "Occurrence of Metarhizium spp. in Central Brazilian soils" doi: 10.1002/jobm.201100482

Schneider. S, Widmer. F, Jacot. K, Kölliker. R, Enkerli. J, (*2012*) "Spatial distribution of Metarhizium clade 1 in agricultural landscapes with arable land and different semi-natural habitats"

Schneider. S, Rehner. S. A, Widmer. F, Enkerli. J (*2011*) "A PCR-based tool for cultivation-independent detection and quantification of Metarhizium clade 1"

Sonenshine DE (1991) "Biology of ticks, vol. 1." Oxford University Press, New York
Thomas. M.B, Read. A.F (*2007*) "Can fungal biopesticides control malaria?" Nature Reviews Microbiology 5, 377-383 (May issue 2007) doi:10.1038/nrmicro1638

Wang & Raymond J. St. Leger (*2014*) "The Ecological Genomics of Fungi, First Edition" page 243.

Wyrebek. M, J. Bidochka. M.J (*2013*) "Variability in the Insect and Plant Adhesins, Mad1 and Mad2, within the Fungal Genus Metarhizium Suggest Plant Adaptation as an Evolutionary Force"

Wyrebek. M, Huber. C, Sasan. K.R, Bidochka. M.J (2011) "Three sympatrically occurring species of Metarhizium show plant rhizosphere specificity"
Microbiology (*2011*), 157, 2904–2911 DOI 10.1099/mic.0.051102-0

Zaim. M, Guillet. P (2002) "Alternative insecticides: an urgent need"
DOI: 10.1016/S1471-4922(01)02220-6

Zimmermann, G (2007)" Review on safety of the entomopathogenic fungus Metarhizium anisopliae"
Biocontrol Sci. Technol. 17, 879–920

Appendix

Tables

Table 3 The raw data used to calculate the dry matter content of soils.

Drying			
Sample	wet weight	Dry weight	Dry matter fraction*
1	5	3,39	0,678
2	5,01	3,4	0,679
3	5,01	3,43	0,685
4	5,01	3,44	0,687
5	5	3,47	0,694
6	5,02	3,53	0,703
7	4,98	3,54	0,711
8	5	3,63	0,726
9	4,99	3,33	0,667
10	5,06	3,36	0,664
11	5,06	3,73	0,737
12	5,03	3,64	0,724
13	5,03	3,94	0,783
14	5	3,9	0,780
15	5,02	4,05	0,807
16	5,02	3,99	0,795
17	4,99	3,86	0,774
18	4,98	3,87	0,777
19	5	3,95	0,790
20	5,02	3,97	0,791
21	5,01	3,96	0,790
22	5	3,94	0,788
23	5	3,94	0,788
24	4,99	3,83	0,768
25	5,01	3,91	0,780
26	4,99	3,94	0,790
27	5,01	3,92	0,782
28	4,99	3,95	0,792
29	5,01	4,03	0,804
30	5	4,01	0,802
31	4,99	3,94	0,790
32	4,99	3,94	0,790
33	5	3,88	0,776
34	5,02	3,95	0,787
35	5	4,01	0,802
36	5	4,11	0,822

Table 4 Data and calculations of note used to obtain final dataset for quantification using cultivation based methods.

plate	CFU	dw soil spread (g)	CFU (g^-1)	CFU/g dw soil (g^-1)	Plate of origin ~	plate mean (g^-1)	error (g^-1)
Q.FrB3 (1)	11	$142*10^{-6}$	$711*10^{-6}$	$354*10^{-5}$	Fr.B3	$675*10^{-6}$	187
Q.FrB3 (2)	6	$145*10^{-6}$	$711*10^{-6}$	$363*10^{-6}$	Fr.C3	$102*10^{-6}$	35,0
Q.FrB3 (3)	12	$143*10^{-6}$	$711*10^{-6}$	$359*10^{-6}$			
Q.FrC3 (2)	2	$146*10^{-6}$	$737*10^{-6}$	$365*10^{-6}$			
Q.FrC3 (3)	1	$149*10^{-6}$	$737*10^{-6}$	$373*10^{-5}$			

Table 5 Sequences from the Strain characterization samples as obtained from Uppsala Genome Center.

Sample	Sequences*
Strain1	TCACATCAACGTGGTCGTTATCGTAAGTAGCCTTGCTCAATTCGCGTCTTGTAAAAGCCGCTTTACTGACTTGCTCGTCATAGGGGTATGTTTTGGAACCTACGCTTTCGAAG TACTCGAAGTTGATTGATAACTGACCGATCCTCACAGCCACGTCGACTCCGGCAAGTCTACCACCACCTGGTCACTTGATCTACCAGTGCGGTGGTATCGACAAGCGAACCA TTGAGAAGTTCGAGAAGGTAAGCCAAATTCCCTGTTTTAATGATTCCTGCTTATTTGGGCGATGGGAACACTTTTGTTTTCTCACTGCCTGTTGACCATTACCCCTCACTGTC ACACAAAATTTTTCGCGGGGCCTTATCTTGGGCTTTTGGTGGGGCATCGCATACCCCGCCAGCTGTTGAGGTGTCTTTTGCGTGTCTTGCCTGCTGTTAAGAACCACAACGT GACCATCGCCTTCAAAACCCAAAAAAAGATTGGAAACTAATTTGCATCTCTGTATAGGAAGCCGCTGAACTCGGCAAGGGTTCCTTCAAGTACGCATGGGTTCTTGACAAGC TCAAGGCCGAGCGTGAGCGTGGTATCACCATCGACATTGCCCTCT
Strain2	TCACATCAACGTGGTCGTTATCGTAAGTAGCCTTGCTCAATTCGCGTCTTGTAAAAGCCGCTTTACTGACTTGCTCGTCATAGGGGTATGTTTTGGAACCTACGCTTTCGAAG TACTCGAAGTTGATTGATAACTGACCGATCCTCACAGCCACGTCGACTCCGGCAAGTCTACCACCACTGGTCACTTGATCTACCAGTGCGGTGGTATCGACAAGCGAACCA TTGAGAAGTTCGAGAAGGTAAGCCAAATTCCCTGTTTTAATGATTCCTGCTTATTTGGGCGATGGGAACACTTTTGTTTTCTCACTGCCTGTTGACCATTACCCCTCACTGTC ACACAAAATTTTTCGCGGGGCCTTATCTTGGGCTTTTGGTGGGGCATCGCATACCCCGCCAGCTGTTGAGGTGTCTTTTGCGTGTCTTGCCTGCTGTTAAGAACCACAACGT GACCATCGCCTTCAAAACCCAAAAAAAGATTGGAAACTAATTTGCATCTCTGTATAGGAAGCCGCTGAACTCGGCAAGGGTTCCTTCAAGTACGCATGGGTTCTTGACAAGC TCAAGGCCGAGCGTGAGCGTGGTATCACCATCGACATTGCCCTCT
Strain3	TCACATCAACGTGGTCGTTATCGTAAGTAGCCTTGCTCAATTCGCGTCTTGTAAAAGCCGCTTTACTGACTTGCTCGTCATAGGGGTATGTTTTGGAACCTACGCTTTCGAAG TACTCGAAGTTGATTGATAACTGACCGATCCTCACAGCCACGTCGACTCCGGCAAGTCTACCACCACTGGTCACTTGATCTACCAGTGCGGTGGTATCGACAAGCGAACCA TTGAGAAGTTCGAGAAGGTAAGCCAAATTCCCTGTTTTAATGATTCCTGCTTATTTGGGCGATGGGAACACTTTTGTTTTCTCACTGCCTGTTGACCATTACCCCTCACTGTC ACACAAAATTTTTCGCGGGGCCTTATCTTGGGCTTTTGGTGGGGCATCGCATACCCCGCCAGCTGTTGAGGTGTCTTTTGCGTGTCTTGCCTGCTGTTAAGAACCACAACGT GACCATCGCCTTCAAAACCCAAAAAAAGATTGGAAACTAATTTGCATCTCTGTATAGGAAGCCGCTGAACTCGGCAAGGGTTCCTTCAAGTACGCATGGGTTCTTGACAAGC TCAAGGCCGAGCGTGAGCGTGGTATCACCATCGACATTGCCCTCT
Strain4	TCACATCAACGTGGTCGTTATCGTAAGTAGCCTTGCTCAATTCGCGTCTTGTAAAAGCCGCTTTACTGACTTGCTCGTCATAGGGGTATGTTTTGGAACCTACGCTTTCGAAG TACTCGAAGTTGATTGATAACTGACCGATCCTCACAGCCACGTCGACTCCGGCAAGTCTACCACCACTGGTCACTTGATCTACCAGTGCGGTGGTATCGACAAGCGAACCA TTGAGAAGTTCGAGAAGGTAAGCCAAATTCCCTGTTTTAATGATTCCTGCTTATTTGGGCGATGGGAACACTTTTGTTTTCTCACTGCCTGTTGACCATTACCCCTCACTGTC ACACAAAATTTTTCGCGGGGCCTTATCTTGGGCTTTTGGTGGGGCATCGCATACCCCGCCAGCTGTTGAGGTGTCTTTTGCGTGTCTTGCCTGCTGTTAAGAACCACAACGT GACCATCGCCTTCAAAACCCAAAAAAAGATTGGAAACTAATTTGCATCTCTGTATAGGAAGCCGCTGAACTCGGCAAGGGTTCCTTCAAGTACGCATGGGTTCTTGACAAGC TCAAGGCCGAGCGTGAGCGTGGTATCACCATCGACATTGCCCTCT
Strain5	TCACATCAACGTGGTCGTTATCGTAAGTCGCCTGCCTCCATTTCGAACCTTGTAGAAGCTGTTATACTGACTTGCTTGTCGTAGGGGTATGATTCGGAGCCTACACTCTTCG CCGTCTCGAGTTTGTGATAACTGACTGGTCCTCACAGCCACGTCGACTCCGGCAAGTCTACCACCACTGGTCACTTGATCTACCAGTGCGGTGGTATCGACAAGCGTACCA TTGAGAAGTTCGAGAAGGTAAGCCAAACCCCTCCGATGAATGATCTGCTATTGTTTGGCGATGAACATTATTGGGTTTCCCGCTGCCTGTCGGCCATTACCCCTCACTGTGG CACGAAAATTTTCGCGGGGCCTTATCTTGGACTTTGGTGGGGCACCATACCCCGCCAGCTGTCGAGAGTGTCTCTGTGTGTCTCTGGCTGTTGAAACCACAATATTGTCGTT GCTTTCAGAGGGAAAAACATGAAACTAATTTGGATCGCTGTATAGGAAGCCGCTGAACTCGGCAAGGGTTCCTTCAAGTACGCATGGGTTCTTGACAAGCTCAAGGCCGAG CGTGAGCGTGGTATCACCATCGACATTGCCCTCT
Strain6	TCACATCAACGTGGTCGTTATCGTAAGTCGCCTGCCTCCATTTCGAACCTTGTAGAAGCTGTTATACTGACTTGCTTGTCGTAGGGGTATGATTCGGAGCCTACACTCTTCG CCGTCTCGAGTTTGTGATAACTGACTGGTCCTCACAGCCACGTCGACTCCGGCAAGTCTACCACCACTGGTCACTTGATCTACCAGTGCGGTGGTATCGACAAGCGTACCA TTGAGAAGTTCGAGAAGGTAAGCCAAACCCCTCCGATGAATGATCTGCTATTGTTTGGCGATGAACATTATTGGGTTTCCCGCTGCCTGTCGGCCATTACCCCTCACTGTGG CACGAAAATTTTCGCGGGGCCTTATCTTGGACTTTGGTGGGGCACCATACCCCGCCAGCTGTCGAGAGTGTCTCTGTGTGTCTCTGGCTGTTGAAACCACAATATTGTCGTT GCTTTCAGAGGGAAAAACATGAAACTAATTTGGATCGCTGTATAGGAAGCCGCTGAACTCGGCAAGGGTTCCTTCAAGTACGCATGGGTTCTTGACAAGCTCAAGGCCGAG CGTGAGCGTGGTATCACCATCGACATTGCCCTCT
Strain7	CAGTGATCATGTTCTTGATGAAATCACGGTGACCGGGAGCATCTGTGCAAGGGATATTAGCATTCAAATCCGACAAACGAAACCGTGTGGTTTAGTTAGCGCAAGTCGACAT ACCGATGACAGTGACATAGTACTTGGGAGTCTCGAACTTCCAGAGGGCAATGTCGATGGTGATACCACGCTCACGCTCGGCCTTGAGCTTGTCAAGAACCCATGCGTACTT GAAGGAACCCTTGCCGAGTTCAGCGGCTTCCTATACAGAGATGCAAATTAGTTTCCAATCTTTTTTTGGGTTTTGAAGGCGATGGTCACGTTGTGGTTCTTAACAGCAGGCA AGACACGCAAAAGACACCTCAACAGCTGGCGGGGTATGCGATGCCCCACCAAAAGCCCAAGATAAGGCCCCGCGAAAAATTTTGTGTGACAGTGAGGGGTAATGGTCAAC AGGCAGTGAGAAAACAAAAGTGTTCCCATCGCCCAAATAAGCAGGAATCATTAAAACAGGGAATTTGGCTTACCTTCTCGAACTTCTCAATGGTTCGCTTGTCGATACCACC GCACTGGTAGATCAAGTGACCAGTGGTGGTAGACTTGCCGGAGTCGACGTGGCTGTGAGGATCGGTCAGTTATCAATCAACTTCGAGTACTTCGAAAGCGTAGGTTCCAAA ACATACCCCTATGACGAGCAAGTCAGTAAAGCGGCTTTTACAAGACGCGAATTGAGCAAGGCTACTTACGATAACGACCACGTTGATGTGAGTCTTGTCCTC
Strain8	TCACATCAACGTGGTCGTTATCGTAAGTCGCCTGCCTCCATTTCGAACCTTGTAGAAGCTGTTATACTGACTTGCTTGTCGTAGGGGTATGATTCGGAGCCTACACTCTTCG CCGTCTCGAGTTTGTGATAACTGACTGGTCCTCACAGCCACGTCGACTCCGGCAAGTCTACCACCACTGGTCACTTGATCTACCAGTGCGGTGGTATCGACAAGCGTACCA TTGAGAAGTTCGAGAAGGTAAGCCAAACCCCTCCGATGAATGATCTGCTATTGTTTGGCGATGAACATTATTGGGTTTCCCGCTGCCTGTCGGCCATTACCCCTCACTGTGG CACGAAAATTTTCGCGGGGCCTTATCTTGGACTTTGGTGGGGCACCATACCCCGCCAGCTGTCGAGAGTGTCTCTGTGTGTCTCTGGCTGTTGAAACCACAATATTGTCGTT GCTTTCAGAGGGAAAAACATGAAACTAATTTGGATCGCTGTATAGGAAGCCGCTGAACTCGGCAAGGGTTCCTTCAAGTACGCATGGGTTCTTGACAAGCTCAAGGCCGAG CGTGAGCGTGGTATCACCATCGACATTGCCCTCT
Strain9	TCACATCAACGTGGTCGTTATCGTAAGTAGCCTTGCTCAATTCGCGTCTTGTAAAAGCCGCTTTACTGACTTGCTCGTCATAGGGGTATGTTTTGGAACCTACGCTTTCGAAG TACTCGAAGTTGATTGATAACTGACCGATCCTCACAGCCACGTCGACTCCGGCAAGTCTACCACCACTGGTCACTTGATCTACCAGTGCGGTGGTATCGACAAGCGAACCA TTGAGAAGTTCGAGAAGGTAAGCCAAATTCCCTGTTTTAATGATTCCTGCTTATTTGGGCGATGGGAACACTTTTGTTTTCTCACTGCCTGTTGACCATTACCCCTCACTGTC ACACAAAATTTTTCGCGGGGCCTTATCTTGGGCTTTTGGTGGGGCATCGCATACCCCGCCAGCTGTTGAGGTGTCTTTTGCGTGTCTTGCCTGCTGTTAAGAACCACAACGT GACCATCGCCTTCAAAACCCAAAAAAAGATTGGAAACTAATTTGCATCTCTGTATAGGAAGCCGCTGAACTCGGCAAGGGTTCCTTCAAGTACGCATGGGTTCTTGACAAGC TCAAGGCCGAGCGTGAGCGTGGTATCACCATCGACATTGCCCTCT
Strain10	TCACATCAACGTGGTCGTTATCGTAAGTAGCCTTGCTCAATTCGCGTCTTGTAAAAGCCGCTTTACTGACTTGCTCGTCATAGGGGTATGTTTTGGAACCTACGCTTTCGAAG TACTCGAAGTTGATTGATAACTGACCGATCCTCACAGCCACGTCGACTCCGGCAAGTCTACCACCACTGGTCACTTGATCTACCAGTGCGGTGGTATCGACAAGCGAACCA TTGAGAAGTTCGAGAAGGTAAGCCAAATTCCCTGTTTTAATGATTCCTGCTTATTTGGGCGATGGGAACACTTTTGTTTTCTCACTGCCTGTTGACCATTACCCCTCACTGTC ACACAAAATTTTTCGCGGGGCCTTATCTTGGGCTTTTGGTGGGGCATCGCATACCCCGCCAGCTGTTGAGGTGTCTTTTGCGTGTCTTGCCTGCTGTTAAGAACCACAACGT GACCATCGCCTTCAAAACCCAAAAAAAGATTGGAAACTAATTTGCATCTCTGTATAGGAAGCCGCTGAACTCGGCAAGGGTTCCTTCAAGTACGCATGGGTTCTTGACAAGC TCAAGGCCGAGCGTGAGCGTGGTATCACCATCGACATTGCCCTCT
Strain11	TCACATCAACGTGGTCGTTATCGTAAGTAGCCTTGCTCAATTCGCGTCTTGTAAAAGCCGCTTTACTGACTTGCTCGTCATAGGGGTATGTTTTGGAACCTACGCTTTCGAAG TACTCGAAGTTGATTGATAACTGACCGATCCTCACAGCCACGTCGACTCCGGCAAGTCTACCACCACTGGTCACTTGATCTACCAGTGCGGTGGTATCGACAAGCGAACCA TTGAGAAGTTCGAGAAGGTAAGCCAAATTCCCTGTTTTAATGATTCCTGCTTATTTGGGCGATGGGAACACTTTTGTTTTCTCACTGCCTGTTGACCATTACCCCTCACTGTC ACACAAAATTTTTCGCGGGGCCTTATCTTGGGCTTTTGGTGGGGCATCGCATACCCCGCCAGCTGTTGAGGTGTCTTTTGCGTGTCTTGCCTGCTGTTAAGAACCACAACGT GACCATCGCCTTCAAAACCCAAAAAAAGATTGGAAACTAATTTGCATCTCTGTATAGGAAGCCGCTGAACTCGGCAAGGGTTCCTTCAAGTACGCATGGGTTCTTGACAAGC TCAAGGCCGAGCGTGAGCGTGGTATCACCATCGACATTGCCCTCT

*primer sequences which were removed before BLASTing and is not presented in the table

Table 6 Data used to calculate the amount of ITS fragments from raw qPCR data

Environment	Subtransect of origin	Starting quantity (^-1)	Copies per dw soil (mg^1)	Soil for extraction(g)	Soil dry weight fraction*
Run 1					
Forest	A1	$1{,}58*10^6$	$1{,}04*10^9$	0,255	0,678
	A2	$1{,}20*10^3$	$2{,}00*10^6$	0,261	0,679
	A3	$2{,}82*10^1$	$6{,}89*10^4$	0,249	0,685
	A4	0	0	0,253	0,687
	B1	0	0	0,252	0,694
	B2	0	0	0,246	0,703
	B3	$2{,}10*10^4$	$7{,}03*10^7$	0,251	0,711
	B4	$1{,}85*10^3$	$7{,}70*10^6$	0,249	0,726
	C1	$3{,}46*10^3$	$1{,}52*10^7$	0,255	0,667
	C2	0	0	0,247	0,664
	C3	$8{,}83*10^3$	$2{,}78*10^7$	0,250	0,737
	C4	$8{,}14*10^3$	$2{,}34*10^7$	0,250	0,724
Pasture	A1	$1{,}19*10^4$	$1{,}57*10^7$	0,243	0,783
	A2	$3{,}07*10^4$	$5{,}90*10^7$	0,249	0,780
	A3	$1{,}75*10^4$	$4{,}75*10^7$	0,257	0,807
	A4	$4{,}15*10^6$	$1{,}15*10^{10}$	0,249	0,795
	B1	$1{,}96*10^4$	$4{,}37*10^7$	0,254	0,774
	B2	$2{,}68*10^3$	$5{,}68*10^6$	0,250	0,777
	B3	$4{,}37*10^3$	$4{,}09*10^6$	0,252	0,790
	B4	$1{,}25*10^6$	$3{,}77*10^9$	0,255	0,791
	C1	0	0	0,252	0,790
	C2	$6{,}65*10^4$	$1{,}90*10^8$	0,250	0,788
	C3	$9{,}70*10^4$	$2{,}92*10^8$	0,253	0,788
	C4	$1{,}12*10^5$	$3{,}09*10^8$	0,259	0,768
Agricultural field	A1	$1{,}12*10^5$	$2{,}39*10^8$	0,250	0,780
	A2	$6{,}28*10^4$	$1{,}57*10^8$	0,249	0,790
	A3	$1{,}04*10^4$	$1{,}40*10^7$	0,255	0,782
	A4	$2{,}81*10^4$	$2{,}96*10^7$	0,252	0,792
	B1	$1{,}29*10^5$	$2{,}72*10^8$	0,250	0,804
	B2	$5{,}92*10^4$	$1{,}47*10^8$	0,248	0,802
	B3	$2{,}07*10^4$	$6{,}10*10^7$	0,250	0,790
	B4	$7{,}51*10^3$	$1{,}70*10^7$	0,251	0,790
	C1	$4{,}29*10^4$	$9{,}80*10^7$	0,250	0,776
	C2	$5{,}16*10^3$	$1{,}05*10^7$	0,252	0,787
	C3	$1{,}89*10^4$	$3{,}49*10^7$	0,250	0,802
	C4	$2{,}80*10^4$	$5{,}59*10^7$	0,249	0,822
Run 2					
Forest	A1	$7{,}36*10^7$	$4{,}83*10^{10}$	0,255	0,678
	A2	$1{,}31*10^5$	$2{,}20*10^8$	0,261	0,679
	A3	$1{,}37*10^5$	$3{,}36*10^8$	0,249	0,685

	A4	$2{,}45*10^5$	$4{,}33*10^8$	0,253	0,687
	B1	0	0	0,252	0,694
	B2	0	0	0,246	0,703
	B3	0	0	0,251	0,711
	B4	0	0	0,249	0,726
	C1	0	0	0,255	0,667
	C2	0	0	0,247	0,664
	C3	$2{,}85*10^5$	$8{,}96*10^8$	0,250	0,737
	C4	0	0	0,250	0,724
Pasture	A1	0	0	0,243	0,783
	A2	0	0	0,249	0,780
	A3	0	0	0,257	0,807
	A4	$1{,}20*10^8$	$3{,}34*10^{11}$	0,249	0,795
	B1	$5{,}05*10^5$	$1{,}12*10^9$	0,254	0,774
	B2	0	0	0,250	0,777
	B3	$7{,}91*10^5$	$7{,}40*10^8$	0,252	0,790
	B4	0	0	0,255	0,791
	C1	0	0	0,252	0,790
	C2	0	0	0,250	0,788
	C3	0	0	0,253	0,788
	C4	0	0	0,259	0,768
Agricultural field	A1	$2{,}78*10^6$	$5{,}95*10^9$	0,250	0,780
	A2	$2{,}57*10^6$	$6{,}44*10^9$	0,249	0,790
	A3	$1{,}97*10^5$	$2{,}64*10^8$	0,255	0,782
	A4	$8{,}36*10^5$	$8{,}83*10^8$	0,252	0,792
	B1	$1{,}77*10^6$	$3{,}73*10^9$	0,250	0,804
	B2	0	0	0,248	0,802
	B3	0	0	0,250	0,790
	B4	$1{,}19*10^5$	$2{,}71*10^8$	0,251	0,790
	C1	$9{,}59*10^5$	$2{,}19*10^9$	0,250	0,776
	C2	0	0	0,252	0,787
	C3	0	0	0,250	0,802
	C4	$7{,}71*10^5$	$1{,}54*10^9$	0,249	0,822

*See table 2 for dw fraction calculus raw data.

Figures

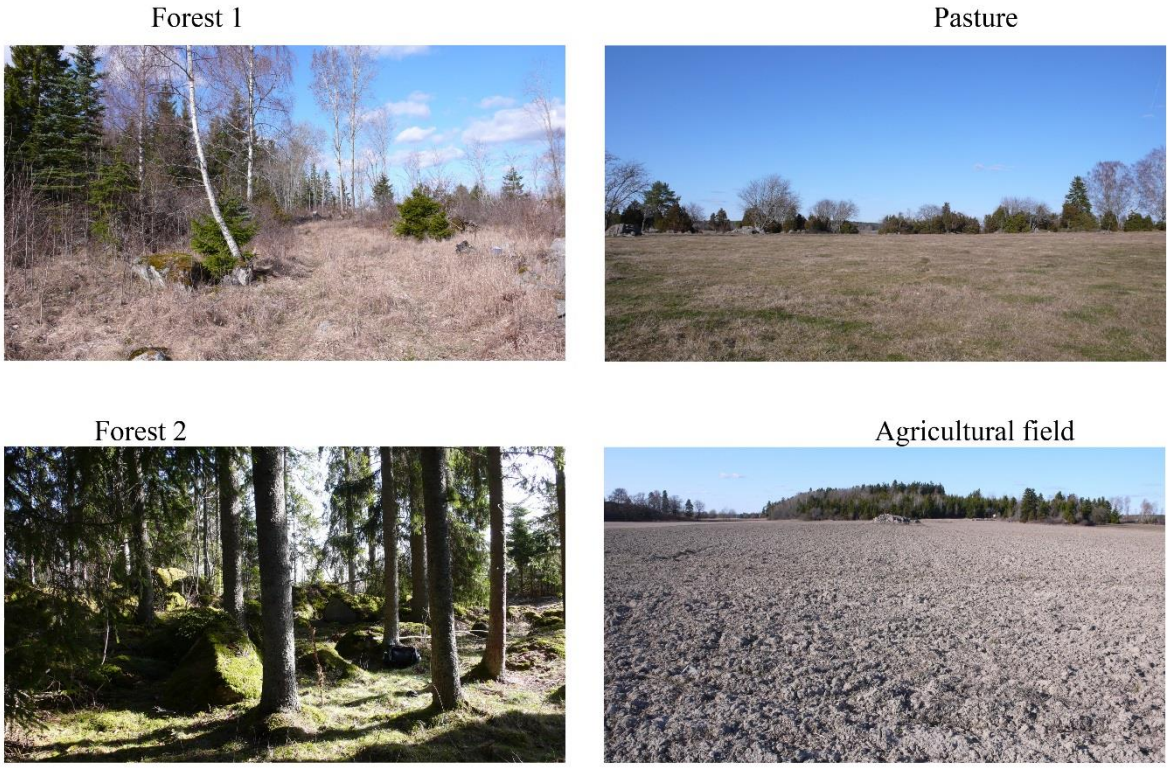

Figure 9 Compilation of pictures taken the day of sampling. Due to the variation of tree abundance in the forest environment two pictures were taken.

Figure 10 A soil core sample from forest soil.

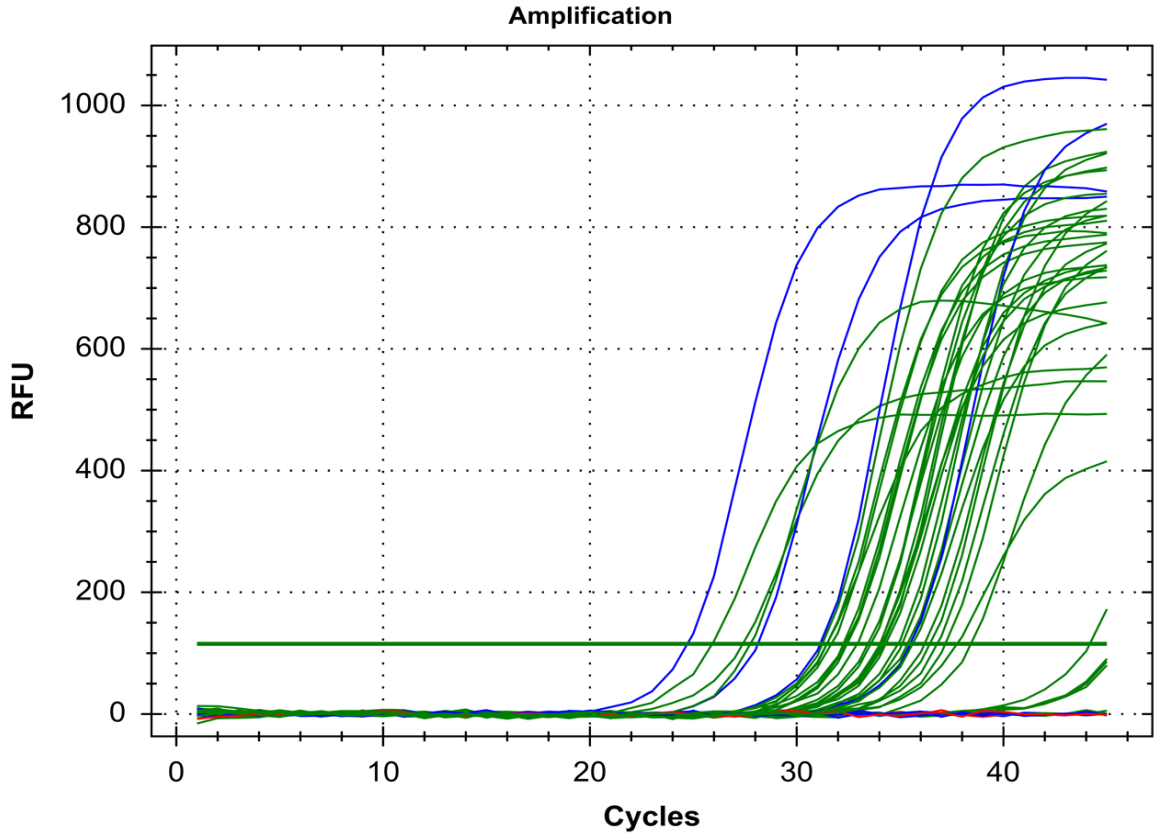

Figure 11 Amplification curves of the first run of qPCR. Blue lines indicate the serial dilution used as a standard while green lines illustrate the amplification of targeted ITS copies present in the soil DNA extract used as a template.

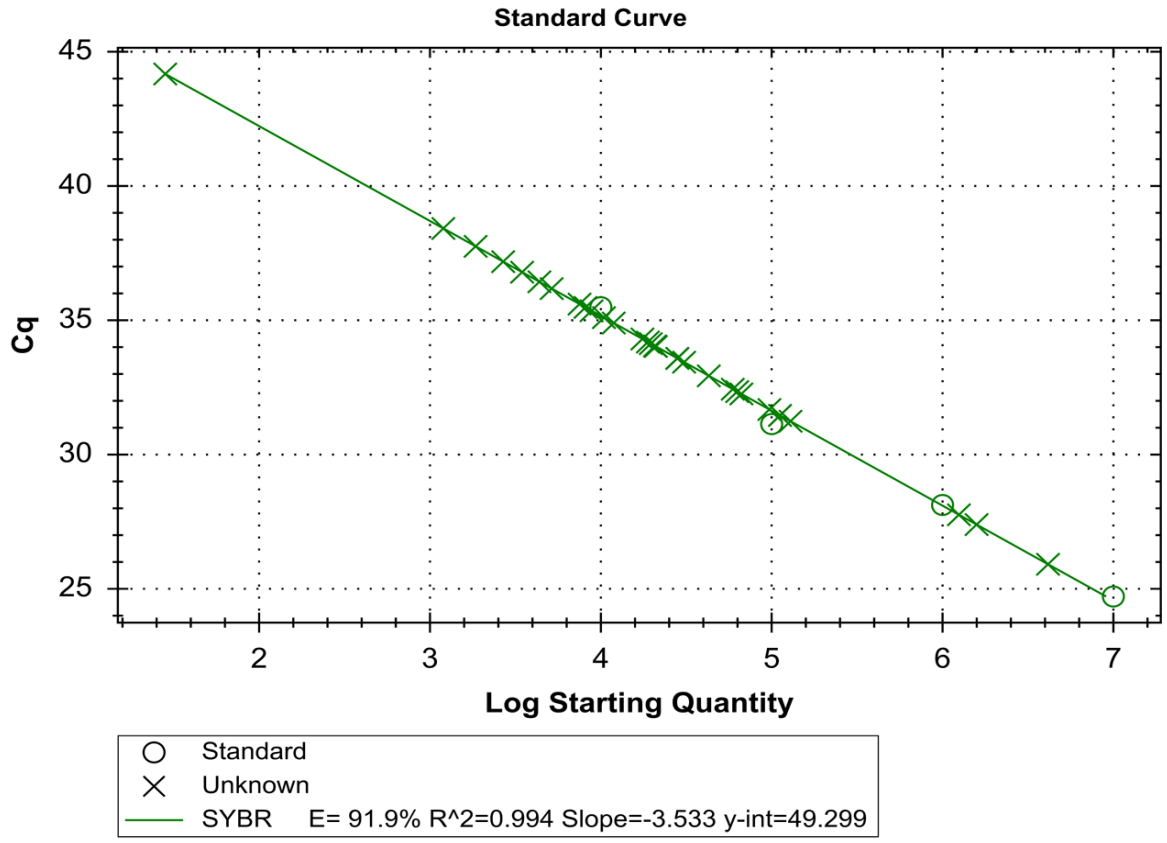

Figure 12. Standard curve for the first run of qPCR based on serial 10 fold dilutions starting with 1.00E+07 (indicated as circles). Data points below 1.00E+04 were excluded from further analysis and *Metarhizium* abundances were considered as negative.

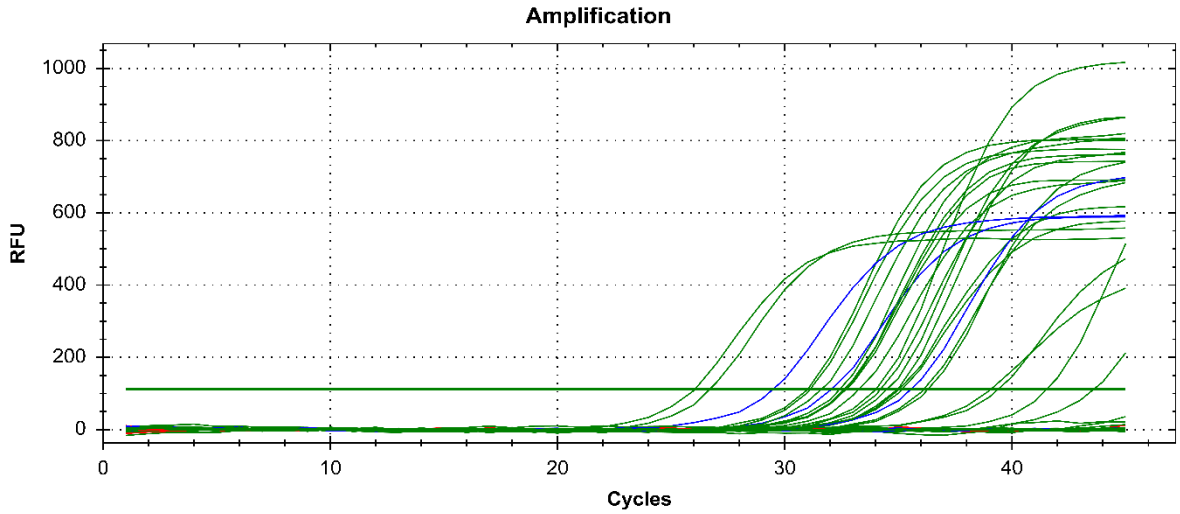

Figure 13 Amplification curves of the second run of qPCR. Blue lines indicate the serial dilution used as a standard while green lines illustrate the amplification of targeted ITS copies present in the soil DNA extract used as a template.

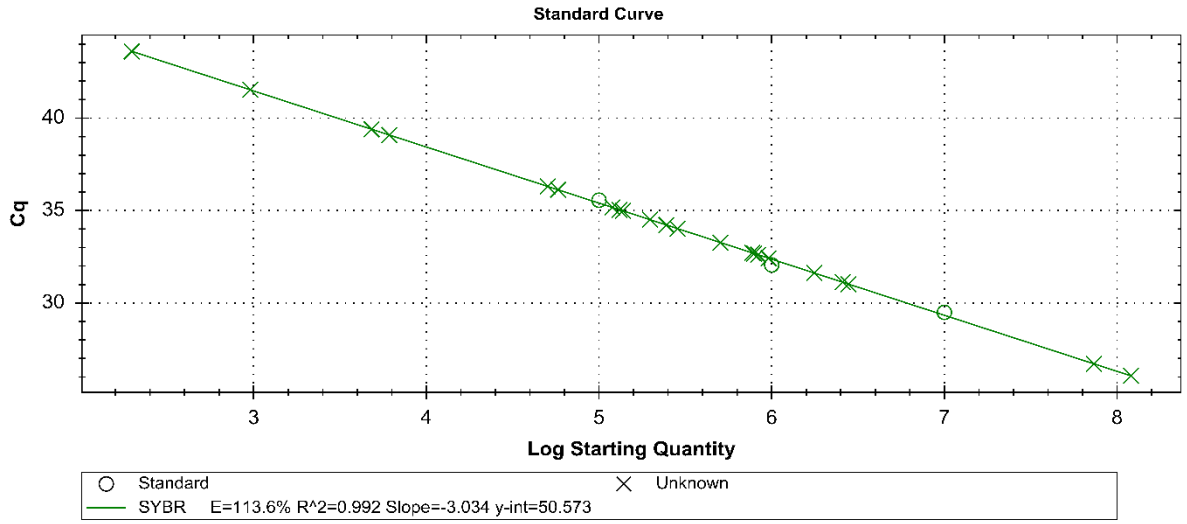

Figure 14. Standard curve for the second run of qPCR based on serial 10 fold dilutions starting with 1.00E+07 (indicated as circles). Data points below 1.00E+05 were excluded from further analysis and *Metarhizium* abundances were considered as negative.

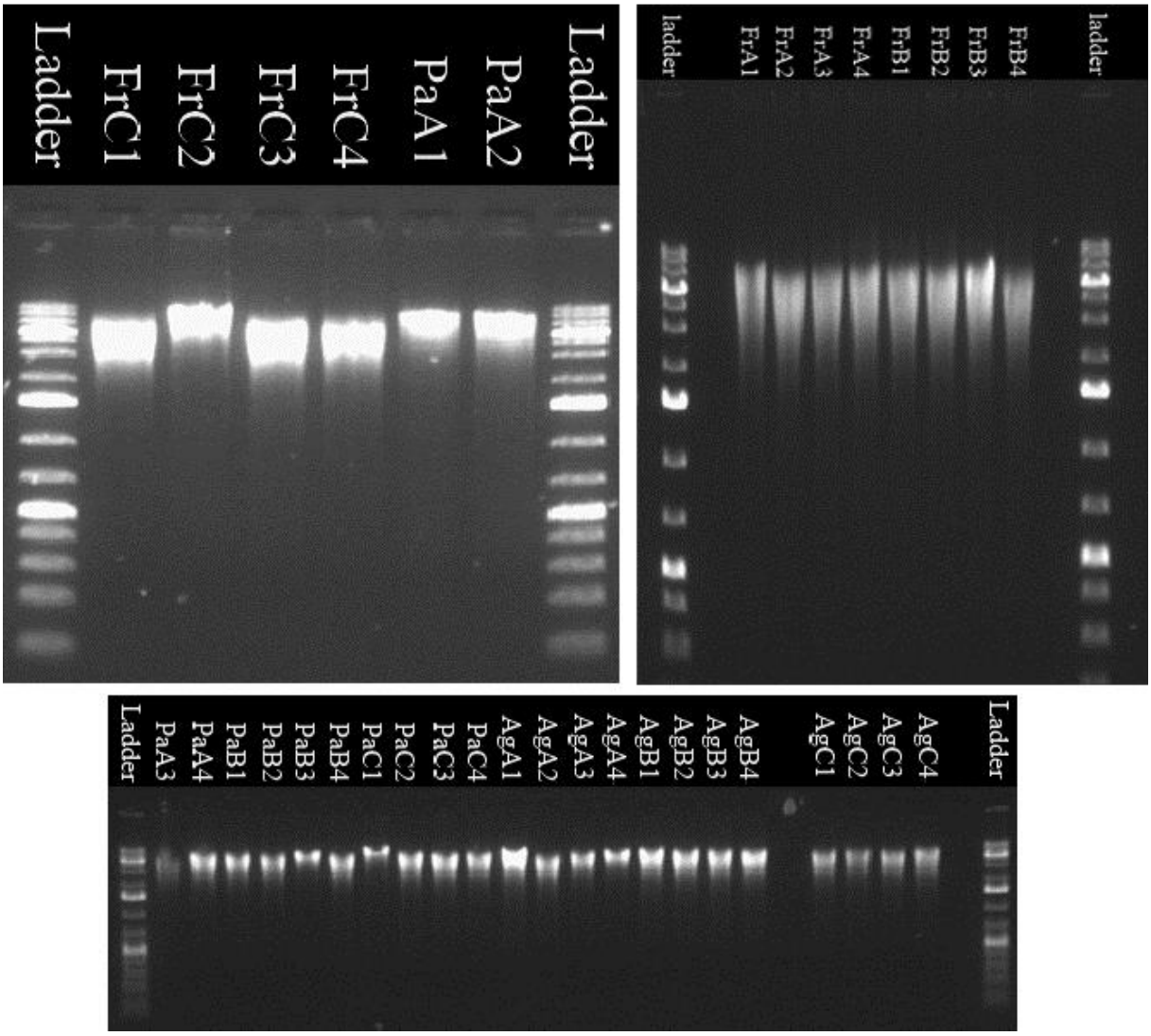

Figure 15 Gel runs for quality verification of soil DNA extracts. Samples were arranged in order of environment, transect, and subtransect. Fr for forest, Pa for Pasture, and Ag Agricultural field. A 1 kb ladder solution was also added to give size estimates and verification of gel functionality. Wells without samples were not loaded. Bright bands on the expected level indicates successful extraction.

Strain DNA extraction gel

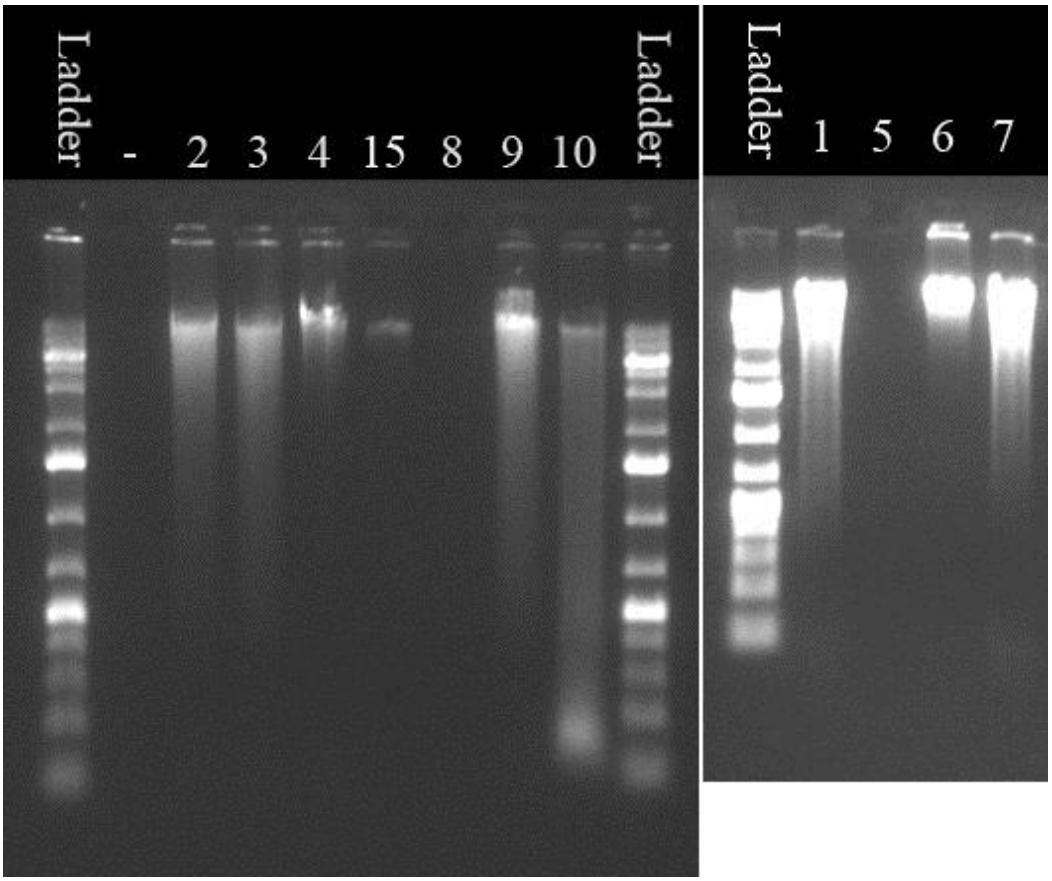

Figure 16 Gels used to verify the quality of isolated strain DNA. Bright bands at similar levels in the gel indicates successful extraction, though amounts varied between samples as seen by relative brightness of the bands. Gel with samples 1-7 was run first to test the extraction protocols and as sample 5 was negative for DNA the isolate of the mycelial replicate, 15, was run afterwards, and was positive. A 1 kb ladder solution was also added to give size estimates and verification of gel functionality. Wells without samples were not loaded.

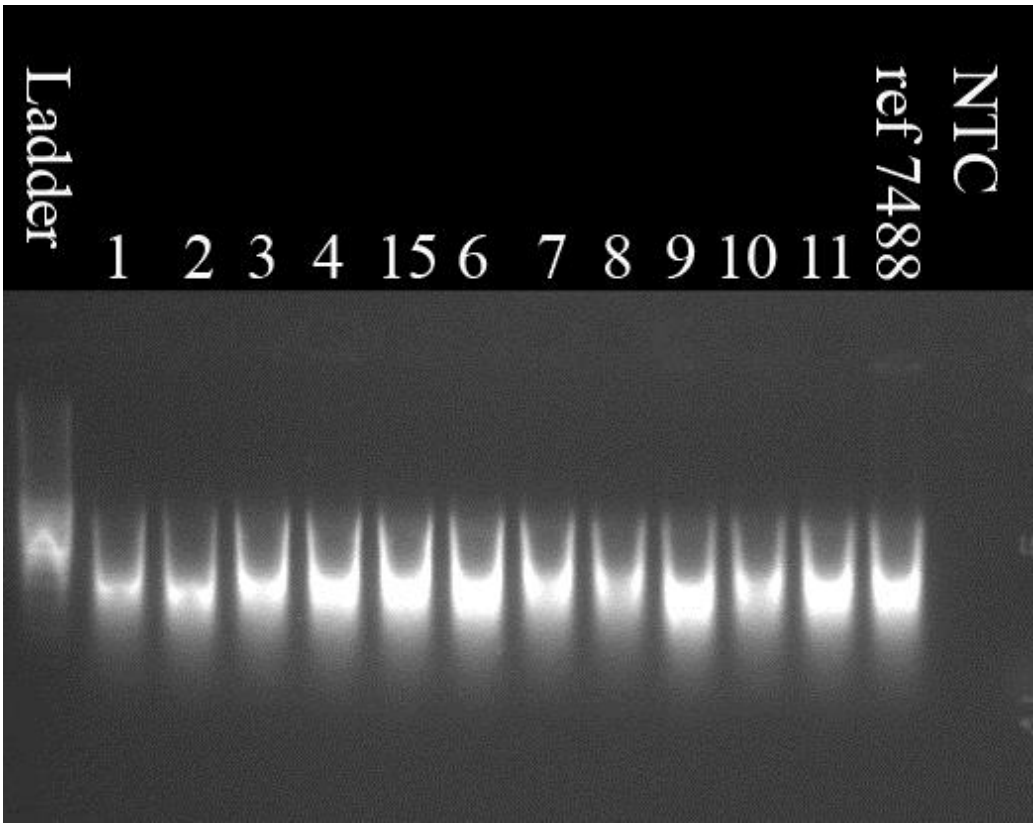

Figure 17 Gel used to verify the quality of PCR products of the EF-1α factor for subsequent sequencing. A 1 kb ladder solution was also added to give size estimates and verification of gel functionality. Bright bands indicates successfully PCR amplified fragments.

Composition of media used

PDA

per L: 4.0g Potato extract OR 200g potato infusion, 20g Dextrose, 15g Agar, dissolve in H_2O until 1L. Bring to boil while stirring to dissolve the powders, autoclave at 121˚ C for 15 minutes or equivalent.

SM

Per L: 5g Peptone, 10g glocuse, 18g Bac-agar, dissolve in H_2O until 1 L and stir. Autoclave until sterile. Make antiobiotic mix with 50mg cycloheximide, 100mg streptomycin and 50mg tetracycline, dissolve and stir in autoclaved H_2O until 100ml. Add antibiotic mix to media when apprx 60˚ C, finish by adding 244 µl Syllit Dodine.

CM

Per L: 0.36g KH_2PO_4, 1.78g Na_2HPO_4: (H_2O), 1g KCl, 0.7g $MgSO_4NO_3$, 5g yeast extract, 10g D-Glucose, 0.6 ml Triton X-100, dissolve in H2O until 1 L. Autoclave until sterile.