Fungal Biology 122 (2018) 231–240

Contents lists available at ScienceDirect

Fungal Biology

journal homepage: www.elsevier.com/locate/funbio

Specificity in *Arabidopsis thaliana* recruitment of root fungal communities from soil and rhizosphere

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ARTICLE INFO

Article history:
Received 30 November 2017
Accepted 23 December 2017
Available online 10 January 2018

Corresponding Editor: Ursula Peintner

Keywords:
Arabidopsis
Archaeorhizomcyetes
Brassicaceae
Ion Torrent
ITS metabarcoding
Rhizosphere

ABSTRACT

Biotic and abiotic conditions in soil pose major constraints on growth and reproductive success of plants. Fungi are important agents in plant soil interactions but the belowground mycobiota associated with plants remains poorly understood. We grew one genotype each from Sweden and Italy of the widely-studied plant model *Arabidopsis thaliana*. Plants were grown under controlled conditions in organic topsoil local to the Swedish genotype, and harvested after ten weeks. Total DNA was extracted from three belowground compartments: endosphere (sonicated roots), rhizosphere and bulk soil, and fungal communities were characterized from each by amplification and sequencing of the fungal barcode region ITS2. Fungal species diversity was found to decrease from bulk soil to rhizosphere to endosphere. A significant effect of plant genotype on fungal community composition was detected only in the endosphere compartment. Despite *A. thaliana* being a non-mycorrhizal plant, it hosts a number of known mycorrhiza fungi in its endosphere compartment, which is also colonized by endophytic, pathogenic and saprotrophic fungi. Species in the Archaeorhizomycetes were most abundant in rhizosphere samples suggesting an adaptation to environments with high nutrient turnover for some of these species. We conclude that *A. thaliana* endosphere fungal communities represent a selected subset of fungi recruited from soil and that plant genotype has small but significant quantitative and qualitative effects on these communities.

1. Introduction

Biotic and abiotic conditions in soil pose major constraints on growth and reproductive success of plants. Meanwhile growth and death of plants create specific belowground habitats for microorganisms, such as rhizosphere soil, root surfaces, dead organic material and internal root compartments, where conditions are distinct from those in soil not colonized by plants (bulk soil). Compared to bulk soil, rhizosphere soils are known for increased levels of nutrient cycling and higher microbial biomass as a consequence of plant-derived carbon fueling this niche (Philippot et al., 2013). Microbial colonization of roots is primarily known from studies of specific pathogenic or mutualistic interactions, resulting in distinct effects on plant health. In addition to these well-studied pathogenic or mutualistic fungi and bacteria, roots also host broader and less well known endophytic microbiomes that affect plant fitness, mainly through their influence on nutrient availability and through indirect pathogen protection (Bulgarelli et al., 2013). Root endophytic communities are primarily recruited from soil. Interestingly, soil also serves as an inoculum for the endophytic microbiome of aboveground plant organs (Bai et al., 2015; Zarraonaindia et al., 2015).

Arabidopsis thaliana (Brassicaceae) is an important model organism for plant biology and genetics. Much of the work on *A. thaliana* has been based on research of domesticated genotypes grown under controlled conditions. However, there has been recent enthusiasm to explore natural genetic variation and to conduct

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https://doi.org/10.1016/j.funbio.2017.12.013

studies under field conditions to address questions of *A. thaliana* ecology and evolution (Trontin et al., 2011; Ågren & Schemske, 2012). Plant traits such as flowering time have been shown to respond in a predictable manner to selected rhizosphere communities (Panke-Buisse et al., 2015). Such belowground feedback between plants and their associated microbiome is potentially an important process in local adaptation, yet the belowground biota associated with *A. thaliana* remains poorly understood. The first characterizations of *A. thaliana* root and soil microbiome identified soil type as the main factor shaping rhizosphere and root endosphere bacterial communities (Bulgarelli et al., 2012; Lundberg et al., 2012). These studies demonstrated that a distinct assemblage of bacteria derived from soil colonizes roots, and that *A. thaliana* genotype had a limited effect on the root endophytic community composition (Bulgarelli et al., 2012; Lundberg et al., 2012). Nonetheless, a significant genotype specific quantitative enrichment was detected for a small number of bacterial taxa inside roots (Lundberg et al., 2012).

A two-step selection process has been proposed to drive shifts in the bacterial community composition from bulk soil to the internal (endosphere) root compartment across many plant species (Bulgarelli et al., 2013). This two-step selection process occurs as follows: Firstly, from bulk soil to rhizosphere, increased carbon and nutrient availability generally favor fast growing bacterial taxa. In addition, differences in plant specific exudates and growth patterns result in plant species-specific assemblages of rhizosphere bacterial communities that are often less diverse than those recorded in bulk soil (Philippot et al., 2013). Secondly, from rhizosphere to endosphere, host specific mechanisms select for certain bacteria adapted to the endosphere environment. In addition, generalist bacteria with saprotrophic capacity are also enriched in the endosphere, likely because roots consist in part of dead woody material which may serve as substrate for these generalists (Bulgarelli et al., 2012).

Soil inhabiting fungi are major constituents of the belowground microbiome and establish a diversity of relationships with plant roots, from pathogenic or parasitic to obligate symbionts. Hitherto, fungal communities associated with roots and rhizospheres of *A. thaliana* are known only from culture-dependent studies. Identification of fungi isolated from roots of *A. thaliana* and *Microthlaspi perfoliatum* (Brassicaceae) demonstrated that root endophytes are common in both host plants, and that the fungal communities are host specific (Keim et al., 2014). Co-cultivation assays demonstrated that many fungal species affected plant growth and health. Similarly, root endophytes isolated from asymptomatic roots were found to affect plant growth in the lab, ranging from positive to negative effects (Junker et al., 2012). Hence, despite being a non-mycorrhizal plant, health and growth of *A. thaliana* depends on the fungi that colonize its roots, particularity in natural settings (Peškan-Berghöfer et al., 2004). This has made *A. thaliana* an increasingly popular model system to study the spectrum of parasitic to mutualistic lifestyles of endophytic fungi (Mandyam et al., 2013; Fesel & Zuccaro, 2016). For such studies, the *A. thaliana* model system has great potential for identifying molecular mechanisms determining fungal root community assembly. However, this field is still in its infancy and the structure and composition of *A. thaliana* rhizosphere and endosphere fungal communities remains largely unknown.

Here we set out to explore the recruitment of root endophytic fungi from bulk soil. We hypothesized that endosphere fungal communities follow the two-step selection pattern identified for bacterial communities (Bulgarelli et al., 2013), with a gradual decreasing richness from soil to rhizosphere to root endosphere. Further we hypothesized that plant genotype would significantly affect the composition of the endosphere mycobiome. For this purpose, we used two wild *A. thaliana* genotypes – one from Sweden and one from Italy. A series of reciprocal transplant experiments has previously demonstrated strong adaptive differentiation between these genotypes (Ågren & Schemske, 2012), and a mapping population derived from their cross has been used to examine the genetic and functional basis of this local adaptation (Ågren et al., 2013). There is evidence that differences in seed dormancy, flowering time and cold tolerance contribute to the adaptive differentiation (Oakley et al., 2014; Postma & Ågren, 2016; Ågren et al., 2017). However, the two populations not only inhabit strikingly different climates that could be expected to influence adaptive population differentiation, but also markedly different soils (volcanic soil in Italy vs. moraine in Sweden). Therefore, differences in selection due to contrasting biotic and abiotic soil conditions may have influenced their divergence. We grew both *A. thaliana* genotypes in the Swedish soil to explore if recruitment of endosphere fungi from soil was affected by plant genotype.

Using high throughput amplicon sequencing of soil fungal communities we specifically tested: (1) whether species richness of fungal communities decreases from bulk soil to rhizosphere to endosphere; (2) whether differential taxon abundance across compartments reflects known trophic status of taxonomically identified OTUs; and (3) whether there is a plant genotype effect on endosphere fungi recruited from a common soil.

2. Materials and methods

2.1. Experimental design

Fifteen replicates of each of two genotypes were grown in glasshouse conditions. The two genotypes were inbred lines collected from two natural *A. thaliana* populations, one from Italy (Castelnuovo di Porto, Italy, 42°07′N; 12°29′E) and one from Sweden (Rödåsen, Sweden, 62°48′N; 18°12′E). The two lines are the parents of a cross that has been used to produce a large RIL population (Ågren et al., 2013), and the seeds planted in the present experiment were the product of 10 generations of selfing in the laboratory. Original seeds were collected in the source populations in April 2003 in Italy and in June 2002 in Sweden. Henceforward, the plant genotypes are referred to as Italian and Swedish, respectively. Plants were grown in 10 × 10 × 14 cm plastic pots filled with topsoil (0–10 cm in depth), collected at the site of the Swedish population on 4th September 2013. The soil was homogenized by sieving at 5 mm and stored at 4 °C for 2 weeks before the experiment.

Bleach sterilized seeds were placed onto soil in each pot. Pots were cold-stratified in the dark at 4 °C for 1 week, and then moved to a growth room (22 °C/16 °C; 16 h/8 h day–night cycle, with 150 μE m^{-2} s^{-1} photosynthetically active radiation). After seed germination, pots were weeded and thinned to nine evenly spaced *A. thaliana* seedlings in each replicate pot.

2.2. Harvest

Plants were harvested after ten weeks, when the Italian genotype had initiated bolting (pre-flowering development), and the Swedish genotype had reached full rosette size (Fig S1A). The harvest protocol largely followed Bulgarelli et al. (2012) and Lundberg et al. (2012) to collect endosphere, rhizosphere and bulk soil compartments from each pot. Bulk soil was separated from rhizosphere soil by lifting the plants onto a sterile surface and manually shaking the soil from the root by tapping each plant individually (Fig S1B). All visible roots and plant material was removed from the soil before collecting a sub-sample in a sterile 50 mL conical tube. Soils were stored frozen at −20 °C until freeze

dried, and then stored at −80 °C. Hereafter these samples are referred to as 'Bulk soil'.

After removing loosely attached soil, the upper 4 cm (from just below the rosette) of the root systems of all plants were collected using a sterile scalpel. The roots from the bottom of the pots and apical roots were discarded. All remaining roots were pooled by pot into a 15 mL conical tube containing 3 mL sterile Silwet L-77 amended PBS buffer (PBS-S; 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.0, 0.02 % Silwet L-77). These roots were then shaken vigorously by hand and vortexed on maximum power for 5 s, then left to settle on the laboratory bench for 2 min. Roots were removed and transferred to a new sterile 15 mL conical tube containing 3 mL PBS buffer with a sterile spatula (see below). Meanwhile the remaining soil retained in the 15 mL conical tube and original PBS buffer was centrifuged (1500×*g* for 20 min). The resulting pellet was freeze dried and stored at −80 °C. Hereafter these samples are referred to as the 'Rhizospheres'.

Root segments were then sequentially washed (vigorous shaking by hand and vortexing on maximum power for 20 s) in sterile 15 mL conical tubes with 3 mL PBS buffer until the buffer was visually clear (ca. 3–4 wash cycles). Once PBS buffer was clear, roots were transferred into sterile 50 mL conical tubes with 5 mL PBS buffer for sonication on ice for 5 × 30 s pulses at ca. 160 W and 30 s breaks (Rapidis 350, Ultrasonics Ltd, Shipley Yorks, England). The sonication probe was sterilized between samples. Efficacy of sonication was visually confirmed with scanning electron micrographs before and after sonication of a sub-sample of roots (Fig S2). The PBS buffer was then discarded and roots were freeze dried and stored at −80 °C. Hereafter these samples are referred to as the 'Endosphere'.

2.3. Pilot study

Prior to this experiment, we ran a pilot study to explore the composition of Actinobacteria communities in belowground compartments of *A. thaliana* to establish that our experimental set up and harvest procedure were comparable to Bulgarelli et al., (2012) and Lundberg et al., (2012). The pilot study ran in accordance with the main experiment described above. Two pots of each plant genotype were grown in Swedish soil, and total DNAs were harvested for the two belowground compartments (Rhizosphere and Endosphere). For details on DNA extraction and Actinobacteria community characterization see Methods S1.

2.4. DNA extraction

Total DNA was extracted from 200 to 250 mg of freeze-dried material of each compartment (Bulk soil, Rhizosphere and Endosphere) using a kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Based on previous standardization and optimization of the protocol (data not shown) we used the lysis buffer SL2 and enhancer SX and a final elution volume of 30 μL. Integrity and concentration of total DNA were checked by electrophoresis in 1 % agarose gel, 0.5 × TAE buffer and Qubit® Fluorometric Quantitation (Life Technologies, California, USA), respectively.

2.5. PCR and sequencing

A fragment of 5.8S, the complete Internal Transcribed Spacer 2 (ITS2) and a fragment of the large subunit (LSU) of the rRNA gene were amplified using primers gITS7 *forward* (Ihrmark et al., 2012) and modified ITS4m *reverse* (5′-TCCTC[C/G][G/C]CTTATTGATATGC-3′) with an adaptor on the reverse primer and sample barcodes and an adaptor on the forward primer (Supplementary Table S1, Rosling et al., 2016). The ITS locus is broadly accepted as the barcode for fungi (Schoch et al., 2012), and there is no significant difference in species discrimination power between the ITS1 and ITS2 regions (Blaalid et al., 2013). Here, modifications on the reverse primer ITS4 were added in order to reduce its bias against the soil fungal class Archaeorhizomycetes (Rosling et al., 2011) (see primer sequence above).

Duplicate PCR amplification was performed for each of three different annealing temperatures, according to Rosling et al. (2016). PCR master mixes were comprised of 10–20 ng of DNA, 1 × SSoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, California, USA) and 0.8 nM of each primer. Amplifications were carried out in a CFR96 Touch™ Real/Time PCR Detection system (Bio-Rad Laboratories, California, USA) with 10 min pre-denaturation at 95 °C, and 1 min denaturation at 95 °C, followed by 45 s at three independent annealing temperatures (50, 54 and 58 °C, to reduce primer bias (Schmidt et al., 2013) and 50 s of extension at 72 °C, with a final 3 min extension at 72 °C). The number of PCR cycles was also adjusted from 23 to 27 in order to avoid multiple cycles of amplification at the plateau phase. We included two positive controls with DNA extracted from a culture of *Neurospora crassa* (Ascomycota). The six PCR reactions of each sample were combined and purified using the ZR-96 DNA Clean & Concentrator™-5 (Zymo Research, The Epigenetics Company, California, USA). DNA concentrations were quantified in duplicates using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies, California, USA) on a TECAN F500 microplate reader and the integrity of the PCR products was checked by electrophoresis in 2 % agarose gel 0.5 × TAE buffer. Aliquots of 35 ng of each sample were pooled for Ion Torrent sequencing in a 318 chip (Life Technologies, California, USA) at Uppsala Genome Center (http://www.igp.uu.se/facilities/genome_center). This sequencing technology has been shown to be suitable for fungal DNA-metabarcoding studies (Brown et al., 2013; Kemler et al., 2013; Tonge et al., 2014).

2.6. Bioinformatics analysis

Three endosphere samples from each of the two plant genotypes had low sequence coverage, and all samples from the corresponding six pots were therefore removed. From the remaining 72 samples (12 replicates × 2 genotype × 3 compartments) we obtained a total of 3 558 577 sequences with a mean read length of 192 bp. These were demultiplexed and quality filtered at $Q \geq 20$ per base using Torrent Suite v4.0.4 (Fig S3A) followed by trimming of the fastq files ($Q \geq 25$ in sequence average, minimum length 150 bp, and maximum length 500 bp) in mothur v1.33.3 (Schloss et al., 2009, Fig S3B). The ITS2 locus was extracted using ITSx v1.0.9 (Bengtsson-Palme et al., 2013) keeping only sequences longer than an 80 bp. Reads assigned to plants and other non-fungal organisms were discarded, resulting in 1 127 434 sequences from the 72 samples and 85 639 sequences from the two positive controls (Fig S3C).

Sequences were clustered into OTUs using a *de novo* approach implemented in TSC (Jiang et al., 2012) under the following parameters: data type (−r = 454); Ion Torrent and 454 Life Sciences sequencing technologies possess similar kind of sequence errors (Yang et al., 2013); clustering algorithm single linkage (−s = sl), clustering algorithm recommended for ITS locus in fungi (Lindahl et al., 2013); at sequence similarity 0.97 (d = 0.03); considered a suitable cutoff for species delimitation in fungi (Blaalid et al., 2013); in conjunction with different cutoffs for high and low abundance sequences (−c = 100, 500 and 1000). We obtained 27 777 OTUs for c = 100, and 15 005 OTUs for c = 500 and 1000, hence the clustering results based on a high and low abundance cutoff of 500 were used for downstream analysis. Singleton and doubleton OTUs were discarded to a final dataset containing 1 199 956 sequences (99 % of all ITS2 sequences), representing 1385 OTUs (9.2 % of the OTUs).

Rarefaction curves were calculated for each compartment and plant genotype separately using *phyloseq* (McMurdie & Holmes, 2013).

In general, a lower number of fungal sequences were obtained from Endosphere samples (total = 104 380; mean = 4349, SD = 1499) than from Rhizosphere samples (total = 525 680; mean = 22 182; SD = 4804) and Bulk soil samples (total = 486 704; mean = 20 279; SD = 6277). Thus, the script *core_microbes.py* in Qiime was used to account for differences in sequence depth among samples. This script applies a rarefaction method to sample the lowest number of sequences found in one sample (n = 1980). Rarefaction diminished the 5 % error rate indicated by the positive controls to 4.3 %. The rarefied dataset contained 142 560 sequences and 780 OTUs and was used for all statistical analysis described below. Because Ion Torrent sequencing technology can cause premature sequence truncation (Salipante et al., 2014), we selected the longest sequence to represent each OTU using the script *pick_rep_set.py* in Qiime v1.9.0 (Caporaso et al., 2010).

OTUs were assigned a taxonomy using BLAST 2.2.29+ (McGinnis & Madden, 2004) against the ITS2 extraction of the 2015-03-02_UNITE + INDS database (Kõljalg et al., 2013) followed by BLASTing to the CBS database (Federhen, 2015). Hits in CBS improved taxonomic assignment of many Ascomycota. After confirming that our approach assigned sequences of the positive controls to the correct taxon (*N. crassa*, 85 267 sequences, 96.2 ± 5.2 %), the sequences from these controls were removed from our dataset. The final taxonomic assignments were corrected by relying on 97, 90, 85, 80, 75 and 70 % sequence identity across the ITS2 region for assigning OTUs to species, genus, family, order, class, or phylum, respectively (Tedersoo et al., 2014). Based on sequence identity we could assign over 60 % of the OTUs (representing 83 % of the sequences) to genus level or above (Fig S4A). However, many of the reference sequences lack taxonomic assignment. When the level of known taxonomy of the references is taken into account we could assign OTUs to the following taxonomic levels: 157 species (54 % of sequences), 62 genus (<1 %), 75 family (1 %), 221 order (20 %), 125 class (11 %), 103 phylum (14 %), and 37 (<1 %) unclassified Fungi (Fig S4B). Nucleotide sequences representing the OTUs that satisfied GenBank requirements are available under the accession numbers KT218967–KT220178. Sequence abundance by genotype, compartments and samples as well as original and trimmed ITS2 sequences are available in the Supplementary Online File 1.

2.7. Statistical analyses

Taxonomic assignment to class level was reliable for the majority of sequences and we therefore ranked the average sequence abundance of classes across all samples. To analyze if fungal community composition differed between genotypes and compartments, principal coordinates analysis (PCoA) based on Bray Curtis distance matrix was performed in the package *ampvis* (http://madsalbertsen.github.io/ampvis/) in R v3.0.2 for the whole dataset as well as for Endosphere compartment separately. To explore statistical significance of genotype and compartment we performed a multivariate analysis of variance using *Adonis* function in Qiime. Further MRPP analysis was performed on all samples and endosphere samples only using the script compare_categories.py, basdd on Bray Curtis distance, also in Qiime. For these chance corrected within-group agreement was expressed by A*.

Observed richness, Chao 1 species richness index and Simpson's diversity index was calculated in Qiime (*alpha_diveresity.py*) for all samples. Following the Adonis test demonstrating a significant effect of compartment, a Tukey's t-test (JMP12; SAS Institute Inc., Cary, NC, USA) was used to test for pairwise differences between compartments. Prior to this analysis, indexes were transformed towards normality (log Chao 1 and exponential Shannon).

Frequent and abundant OTUs were defined as those occurring in at least 60 of the 72 samples (at least 83 % of the samples) and each representing at least 0.5 % of the sequences of the rarefied dataset. These criteria identified 36 OTUs that together represented 85 % of the sequences in the rarefied dataset (Table S2). The STD normalized read counts of these 36 OTUs across compartment and genotype were analyzed by hierarchical clustering and illustrated with a heat map computed from Euclidean distance matrices with non-centroid standard deviation normalization for the two axes, OTU and compartment, using the R packages *ggplots* (http://ggplot2.org) and *ggdendro* (https://cran.r-project.org/web/packages/ggdendro/index.html). OTUs with significantly different abundance across compartments were identified using a Bonferroni test (at $P < 0.05$). In addition, the distribution of read counts across samples for the 36 frequent and abundant OTUs was transformed towards normal distribution using log and square-root transformation when needed, and their distribution was tested in a mixed linear model (MLM) with the two factors, genotype and compartment, followed by pairwise comparisons using Student's t-test or Tukey's t-test, for genotype and compartment respectively using JMP 12.

Abundant Endosphere OTUs were defined as fungal OTUs accounting for at least 0.5 % of sequences and detected in at least 75 % of the Endosphere samples. These criteria identified 28 OTUs that together represented 83 % of the sequences from Endosphere samples in the rarefied dataset (Table S3). Twenty-five of these OTUs are among frequent and abundant OTUs described above. We tested for plant genotype specific distribution of OTUs within the Endosphere compartment using Adonis based on all OTUs found in the Endosphere samples as well as on the 28 abundant Endosphere OTUs alone.

We manually annotated taxonomy assignment of the 39 frequent OTUs (Tables S2 and S3) using the online UNITE database (Kõljalg et al., 2013) thereby verifying that these represent unique Species Hypotheses and had references sequences at above the 97 % homology threshold for species identification. However, sequence homology varies between species and references sequences lacked full taxonomic descriptions, hence we assigned names to the OTUs based on taxonomy of its UNITE Species Hypotheses. Further, we used the online tool FUNGuild (http://www.stbates.org/guilds/app.php) to assign trophic status to the frequent OTUs (Nguyen et al., 2015). FUNGuild takes into account the sequence homology, taxonomy of best available references and published literature on trophic status at that taxonomic level. Based on a combination of these effects trophic status is assigned with different certainty (Possible, Probable and Highly Probable). Trophic status was assigned to 26 of the 39 OTUs (Tables S2 and S3). When manually confirming that the assigned trophic status was reasonable, we found that assignment to Probable saprotrophs was often based on environmental studies finding the taxa in soil samples rather than hard evidence on trophic status. We kept these assignments in all cases except for the Archaeorhizomycetes where evidence of trophic status is still scarce.

3. Results

Results of pilot study on belowground Actinobacteria communities demonstrate that our harvest procedure recovered Endosphere and rhizosphere bacterial communities in accordance with the studies by Bulgarelli et al. (2012), Lundberg et al. (2012). Actinobacteria dominated the communities (Fig S5), since primers specific to this phylum were used in the Pilot study. Rhizosphere separated from Endosphere communities along axis one which describes 35 % of the variation in the Pilot study (Fig S6). Separation between genotypes could not be determined due to the small number of replicates. Rhizosphere samples have higher species

richness and diversity compared to Endosphere samples (Fig S7). Based on these results we conclude that our harvest procedure successfully separated the rhizosphere and pedosphere compartments and recover distinct communities from these.

3.1. Belowground fungal communities associated with A. thaliana

Rarefaction curves for the three compartments indicated that community characterization reached saturation and that sampling was close to exhaustive (Fig S8). For all three compartments combined, we found that Ascomycetes was by far the most abundant (87 % of all sequences) and OTU rich (542 OTUs) phylum, with Basidiomycetes a distant second (8 %; 145 OTUs). Rank abundance demonstrated that members of Ascomycetes, especially Archaeorhizomycetes (46.7 % of all fungal sequences; 34 OTUs), were the most abundant fungal classes, followed by Leotiomycetes, Dothideomycetes, Eurotiomycetes and Sordariomycetes (Fig 1). The next most abundant fungal group was unclassified taxa in the Mucoromycotina and Tritirachiomycetes (Basidiomycota). Fungal communities were significantly different between the three belowground compartments at all assigned taxonomical levels (Adonis: phylum $F = 14.51$, $r^2 = 0.30$; class $F = 72.00$, $r^2 = 0.68$; order $F = 68.69$, $r^2 = 0.66$; family $F = 56.67$, $r^2 = 0.62$, genus $F = 56.52$, $r^2 = 0.62$; $P \leq 0.001$ and OTU $F = 51.65$, $r^2 = 0.60$, $P < 0.001$). The four Ascomycetes classes Eurotiomycetes, Leotiomycetes, Dothideomycetes and Archaeorhizomycetes were equally abundant in Bulk soil samples but were differentially enriched in the two other compartments (Fig. S9). While Leotiomycetes was most abundant in the Endosphere, Archaeorhizomycetes was most abundant in Rhizosphere samples.

Principal coordinates analysis (PCoA) further demonstrated that fungal community composition based on OTUs was distinct in the three compartments ($r^2 = 0.8504$, Adonis $P = 0.001$) (Fig 2). The major separation in community composition was along axis one, which explained 57.8 % of the total variation and separated the Endosphere compartment from the Bulk soil and Rhizosphere compartments. Bulk soil and Rhizosphere samples separated along axis two, which explained 11 % of the total variation. There was no overall significant difference in fungal community between genotypes ($r^2 = 0.0024$, Adonis $P = 0.833$; MRPP, $A^* = -0.0019$, $P = 0.521$) however Endosphere samples separated by genotype (Fig 2).

3.2. Fungal OTU richness is substantially decreased in the Endosphere

Of the 780 fungal OTUs detected in the rarefied dataset, 381 were detected in Endosphere samples while Rhizosphere and Bulk soil samples both had 543 OTUs. Many of these were rare, and only 323 OTUs were detected in five or more samples; among these, nine were unique to Endosphere, while 68 were not detected in Endosphere (Fig S10). Taxa that were restricted to one or two compartments were generally rare and together accounted for 1.4 % of the reads in the rarefied dataset. Nine OTUs were detected exclusively in the Endosphere but these were all found in low abundance and a non-soil origin of these taxa could not be excluded. Fungal species richness, as measured by observed OTUs and Chao 1 OTU richness, was significantly lower in Endosphere samples compared to Bulk soils and Rhizosphere samples (Fig 3). This is in agreement with the major community composition separation between Endosphere and other compartments (Fig 1). Alpha diversity, as measured by Shannon's diversity index, shows a pattern of gradually decreasing diversity from Bulk soil to Endosphere with diversity of all three compartments being significantly

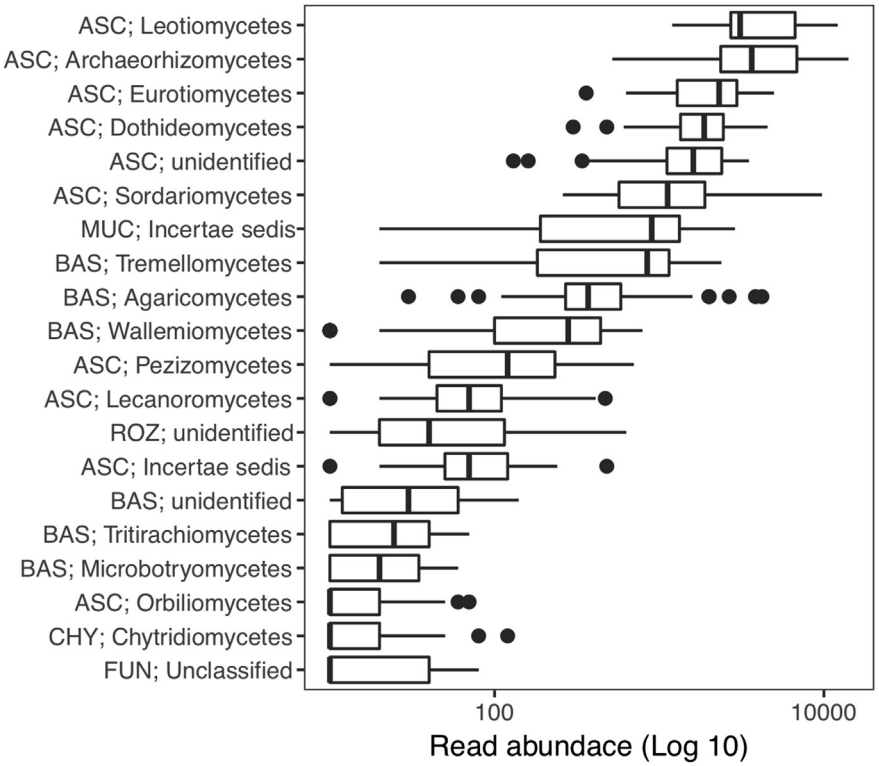

Fig 1. Rank abundance of fungal classes across soil compartment and plant genotype based on average read abundance (sum of all reads in OTUs assigned to the class). Means are represented by a line inside each boxplot. Classes are labelled by Phyla: Ascomycota (ASC), Basidiomycota (BAS), Mucoromycotina (MUC) and Rozellomycota (ROZ) when assigned and by Class Chytridomycetes (CHY) or Kingdom Fungi (FUN) when phyla were unknown.

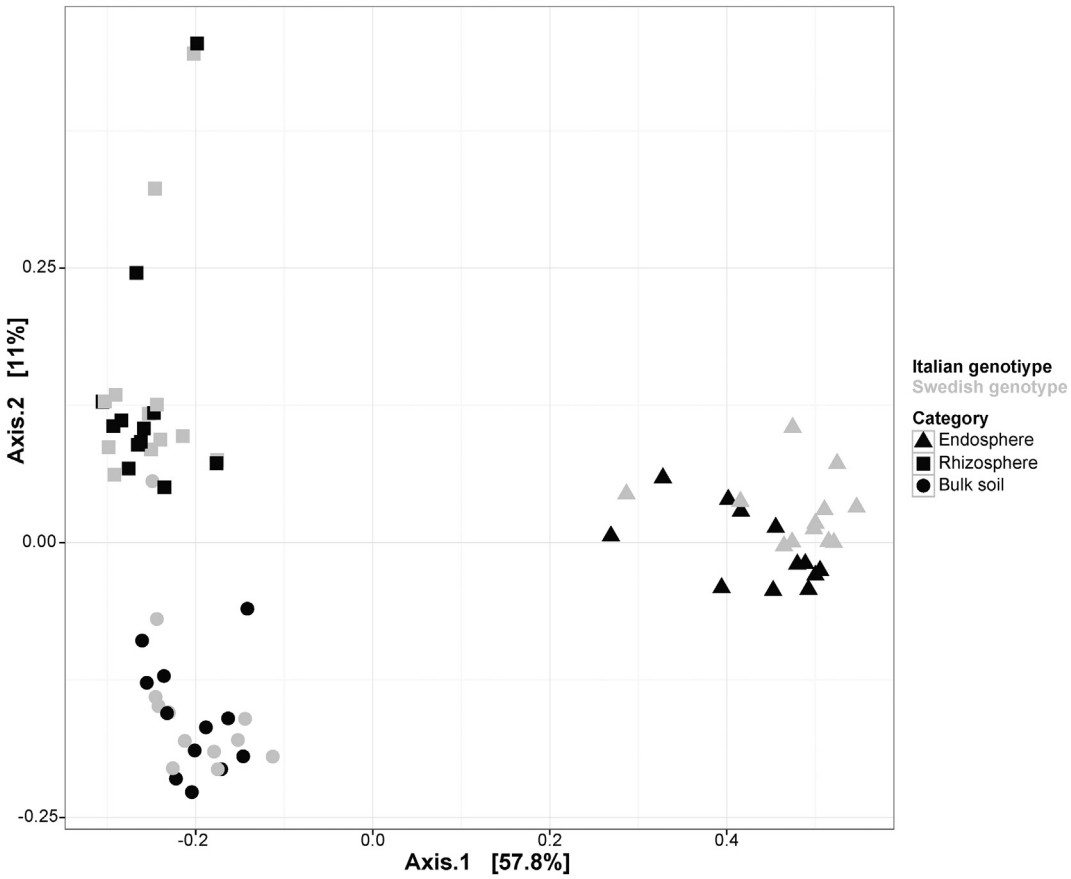

Fig 2. PCoA of fungal community of the three compartments Endosphere (triangle), Rhizosphere (square) and Bulk soil (circle) based on Bray distance matrix of rarefied read counts of 780 OTUs across 72 samples. Different genotypes of the plants are indicated by color, black for Italian and grey for Swedish.

different (Fig 3). Overall, 36 OTUs were identified as the most abundant and frequent OTUs across all compartments, and together these represent over 85 % of the sequences in the rarefied dataset (Fig 4, Table S2). The abundance of 27 of these 36 differed significantly across compartments ($P < 0.0001$) based on both the Bonferroni test and MLM (Fig 4), whereas three more OTUs differed significantly between compartments in the MLM test only (Table S2). There was a small but significant effect of genotype on the abundance of *Oidiodendron griseum* (OTU10) and an unknown Ascomycota species (OTU44). These were both most abundant in association with the Swedish genotype (Table S2).

Twenty-two of the 35 frequent and abundant OTUs were enriched in Bulk soil, of these five were equally abundant in the Rhizosphere. Among soil-enriched taxa, the majority could be assigned to Saprotrophs ($n = 10$), while three are Pathotroph–Symbiotrophs, one was a Symbiotroph, and the rest ($n = 8$) could not be assigned to trophic status (Fig 4, Table S2). The three OTUs that were significantly enriched in Rhizosphere were classified as belonging to the Archaeorhizomycetes, a class for which no trophic status is known. The only other Archaeorhizomycetes among the frequent and abundant OTUs was *Archaeorhizomyces finlayi* (OUT4) which was enriched in Bulk soil (Fig 4). Eleven OTUs were enriched in the Endosphere compartment, with *Wilcoxina rehmii* (OTU14) being equally abundant in the Rhizosphere and *Herpotrichia juniperi* (OTU36) being abundant also in Bulk soil. Among Endosphere enriched taxa four are assigned to Saprotrophs (*Paraconiothyrium brasiliense* (OTU4556), *H. juniperi* (OTU36), *Lachnum pygmaeum* (OTU7359) and *Ilyonectria mors-panacis* (OTU32), three were Symbiotrophs (*W. rehmii* (OTU14), *Amphinema byssoides* (OTU53) and *Phialocephala fortinii* (OTU15)) one was the Pathotroph *Gibberella tricincta* (OTU16) and two (an unknown Ascomycete (OTU3654) and an unidentified *Helotiales* sp. (OTU7)) could not be assigned to a trophic status (Fig 4; Table S2).

3.3. Genotype specific recruitment of endosphere fungi

Unlike fungal communities in Bulk soil and Rhizosphere samples, communities in Endospheres samples separated by plant genotype (Adonis $P = 0.01$; MRPP, $A^* = 0.0279$, $P = 0.012$) (Fig 2). Across the Endosphere samples, 28 OTUs were frequent and abundant, accounting for a total of 83 % of reads recovered from root samples (Fig 5). These included all 11 OTUs that were enriched in the Endosphere (Fig 4). Three of the abundant Endosphere OTUs were significantly enriched in one of the plant genotypes (Fig 5, Table S3). One was significantly more abundant in the Italian genotype – the saprotroph *Ilyonectria mors-panacis* (OTU32). In the Swedish genotype, two OTUs were significantly enriched, an unidentified Helotiales (OTU7) and an *Archaeorhizomycetes* sp. (OTU40), none of which could be assigned to a trophic status.

4. Discussion

By dissecting the belowground compartments of *A. thaliana* plants and characterizing their specific fungal communities, we demonstrate that root endosphere fungal communities are recruited from soil in a way consistent with the two-step selection process proposed for assembly of root endophytic bacterial communities (Bulgarelli et al., 2013). That is, bulk soil communities are

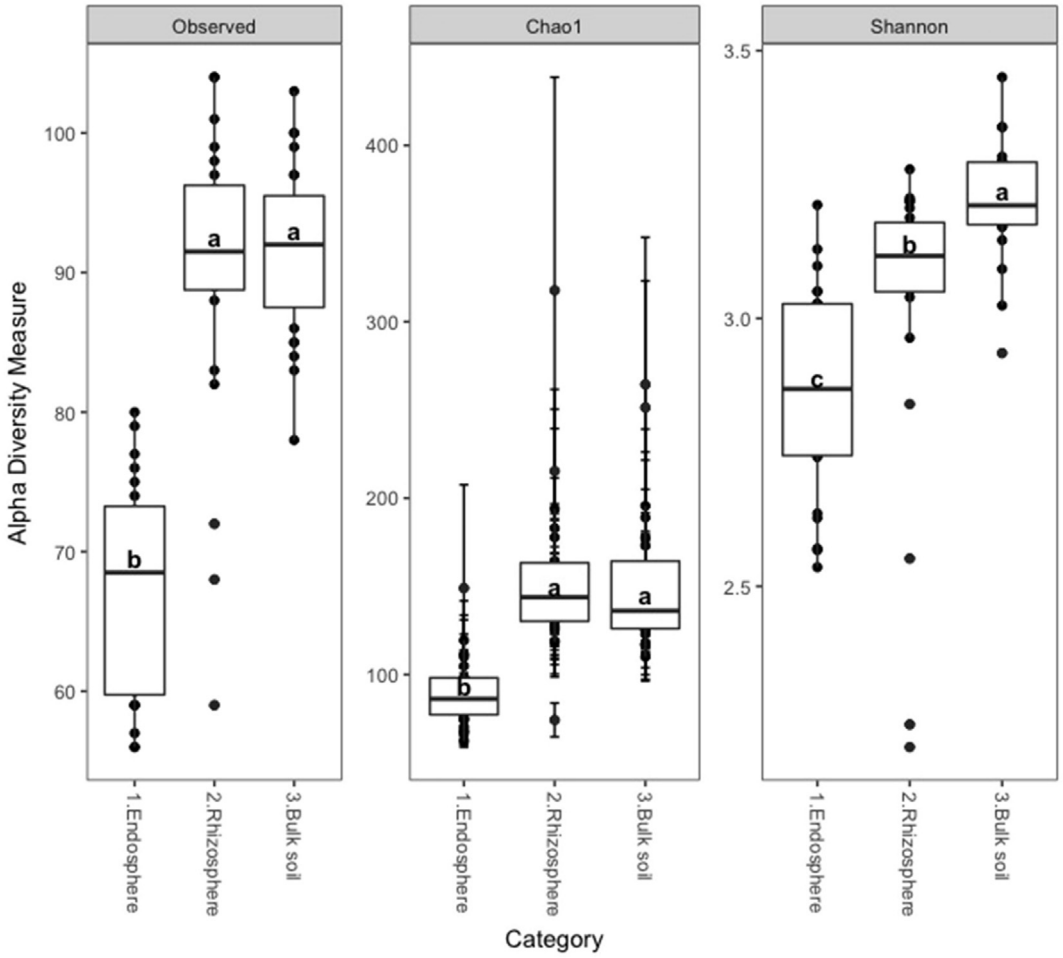

Fig 3. Observed species, Chao 1 and Shannon diversity index calculated using OTUs as a proxy for species across the three compartments Endosphere, Rhizosphere and Bulk soil. Means are represented by a line inside each boxplot. Different letters indicate significant pairwise differences between compartments based on Turkey's t-test.

species rich and diverse and become somewhat less so in rhizosphere soil, finally only a fraction of these colonize the interior root compartment. For root associated fungal communities the selection process is not gradual, but rather reflects a distinct shift in community composition and richness that occurs from exterior to interior root compartments. This pattern is well-known for plants that naturally associate with mycorrhizal fungi (Gottel et al., 2011), where plant hosts offer specific habitats that shape the root endosphere community (Goldmann et al., 2016). While we know that fungi colonize roots and may benefit plant growth also in typically non-mycorrhizal plants, such as *A. thaliana* (Peškan-Berghöfer et al., 2004; Fesel & Zuccaro, 2016), little is known about the endosphere fungal community in this important model species. We found that the *A. thaliana* endosphere was colonized by typical mycorrhizal fungi, such as *W. rehmii*, *Amphinema byssoides*, as well as the common root endophytes *P. fortinii* and the pathogen *G. tricincta*.

Changing composition of bacterial community from soil to endosphere has been associated with a switch in trophic status with more slow-growing saprotrophic groups dominating the bulk soil compartments, faster growing taxa that live on root derived carbon substrates dominating the rhizosphere, and host specific colonization of the endosphere (Bulgarelli et al., 2013; Philippot et al., 2013). While it is likely that the fungal community changes in a similar manner, our data cannot fully resolve such a functional shift from bulk soil to rhizosphere to roots. Our ability to confidently assign trophic status to OTUs in our study was limited by the resolution of the barcoding method used, accuracy of taxonomic assignment to short sequence reads, and a lack of knowledge of trophic status of the identified taxa. We minimized these biases by focusing our functional analysis on the most abundant and frequent OTUs (Tables S2 and S3) for which taxonomic assignment was evaluated using the UNITE database and by manually evaluating the trophic status assigned using FUNGuild (Nguyen et al., 2015). We found that fungal communities in bulk soil samples were dominated by known saprotrophic fungi, and endosphere samples were colonized by several taxa on the pathotroph–symbiotroph spectrum.

Interestingly, taxa that were significantly enriched in the rhizosphere were all identified as belonging to the Archaeorhizomycetes (Rosling et al., 2011). Archaeorhizomycetes is an abundant group of soil and root associated Ascomycetes for which no trophic status is known. The two existing cultures of Archaeorhizomcyetes were isolated from root endospheres and are both slow-growing (Menkis et al., 2014). The observed enrichment in the rhizosphere suggests that some species in this class may be fast growing in natural substrates. One of the Archaeorhizomycetes taxa was the most abundant OTU in this study, representing 15 % of all reads, indicating that bait cultures with non-host plants such as *A. thaliana* may be a feasible method to enrich uncultured fungal species for possible isolation or at least characterization by molecular methods. In the rhizosphere, root exudates and cell debris

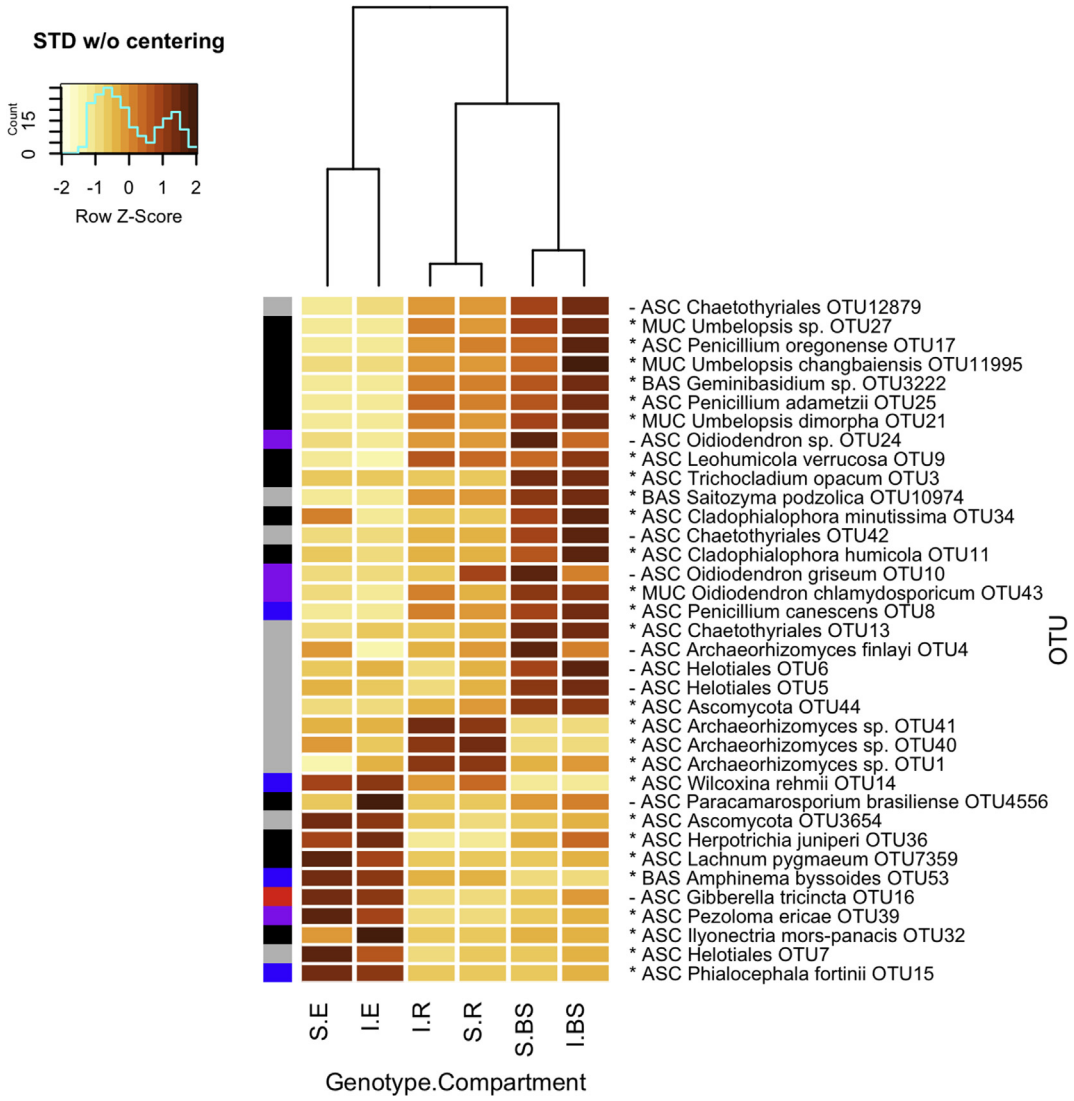

Fig 4. Heat map based on STD normalized read counts of 36 abundant and frequent OTUs observed in at least 60 samples and contributing to at least 0.5 % of the reads in the rarefied dataset. Dendrogram clustering of treatments (plant genotypes Italian (I) and Swedish (S) as well as compartments Endosphere (E), Rhizosphere (R) and Bulk soil (BS)) along the X-axis and OTUs along the Y axis are based on read abundance. OTUs are labeled by Phyla (Ascomycota (ASC), Basidiomycota (BAS), Mucoromycotina (MUC)) and highest assigned taxonomy. Taxa with significantly different distribution across compartments are indicated with * ($P < 0.0001$) in both tests. Color bars to the left indicate assigned trophic status (saprotroph (black), symbiotroph (blue), pathotroph–symbiotroph (purple), pathogen (red) and un-assigned (gray)). Taxa are sorted from top to bottom by decreasing % of its reads found in Bulk soil compartment.

enrich the soil substrate making it a suitable habitat for fast-growing organisms. Species-specific, and even genotype-specific, microbial communities assemble in response to these plant exudates (Broeckling et al., 2008; Sloan & Lebeis, 2015). Rhizosphere microbiomes are more diverse and may be just as important for plant health and development (Chaparro et al., 2014; Panke-Buisse et al., 2015) as the more specific root endosphere community discussed above.

Endophytic fungi isolated from leaves and roots of healthy *A. thaliana* plants (Junker et al., 2012; García et al., 2013; Keim et al., 2014) have been shown to affect plant health when re-inoculated under experimental conditions. Both beneficial and detrimental effects on plant health have been observed, indicating that endophytic fungi function along a parasitic to mutualistic life strategy spectrum, and that the endophyte lifestyle may be opportunistic (Junker et al., 2012; Fesel & Zuccaro, 2016). Most of the Endosphere enriched OTUs detected in our study were assigned to a trophic status along the symbiotic spectrum, but their effects on *A. thaliana* remain unexplored. Despite not being mycorrhizal, consistent plant benefits of the association between *A. thaliana* and the root endophytic fungus *Piriformospora indica* has been demonstrated, and this system has developed into a powerful model for studying the molecular genetics of beneficial plant–fungal interactions (Peškan-Berghöfer et al., 2004). High levels of overlap have been demonstrated for bacterial communities colonizing *Arabidopsis* leaves and roots (Bai et al., 2015), but it remains to be explored if this is also true for fungi.

Similar to observations of endosphere bacterial communities (Bulgarelli et al., 2012; Lundberg et al., 2012), we detected a small but significant effect of plant genotype on the fungal community retrieved from within *A. thaliana* roots. The overall effect of genotype on the endosphere community was mirrored by significant differences in read abundances of three of the most frequent and abundant taxa detected in endosphere samples. It is too early to speculate about possible functional effects of differences in endosphere fungal communities recovered from these different

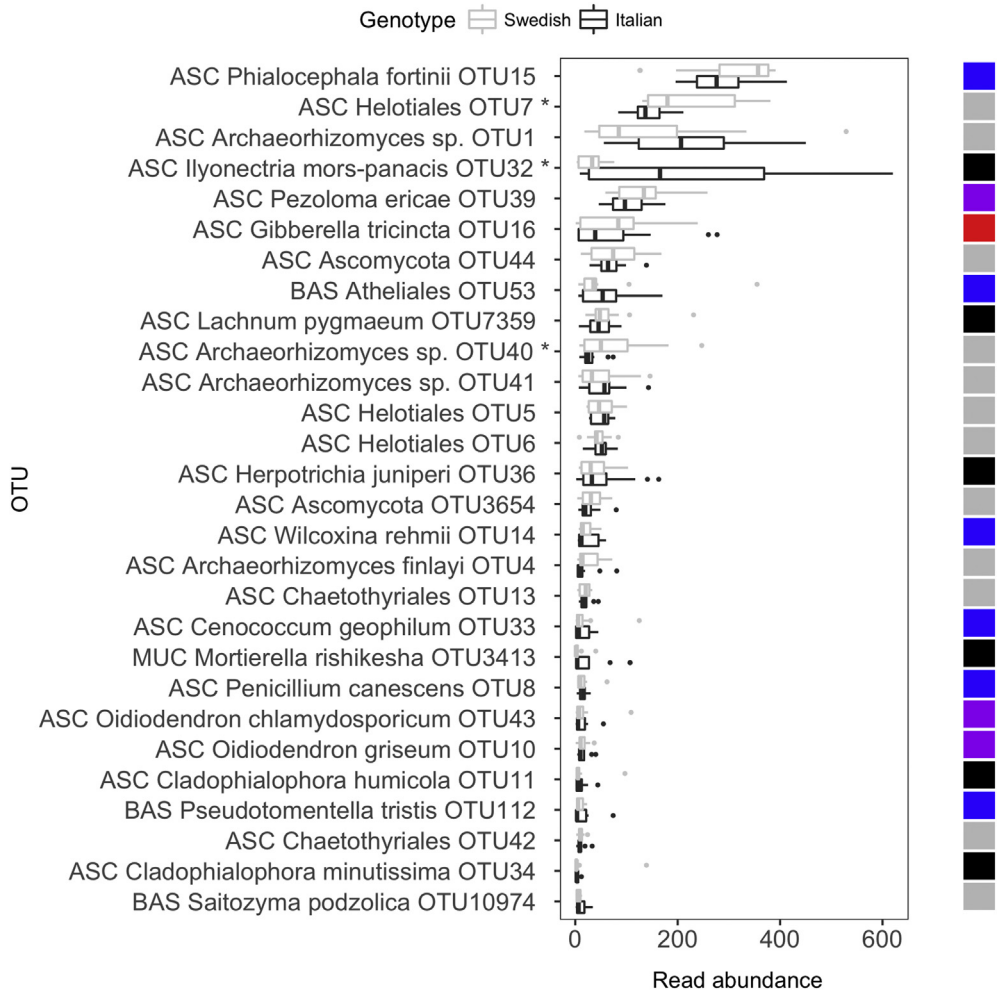

Fig 5. Rank abundance of the 28 abundant Endosphere OTUs that are detected in at least 75 % of the Endosphere compartment and each account for at least 0.5 % of the Endosphere reads. Mean abundance is presented separately for the two plant genotypes Italy (black) and Sweden (grey). OTUs are labeled by Phyla (Ascomycota (ASC), Basidiomycota (BAS), Mucoromycotina (MUC)) and available genus name. Taxa with significantly different distribution between genotypes are indicated with *. Color bars to the right indicate assigned trophic status (saprotroph (black), symbiotroph (blue), pathotroph–symbiotroph (purple), pathogen (red) and un-assigned (gray)).

A. thaliana genotypes. Genotype effects on fungal community composition have been observed in other plant species, for instance cultivar level genotypes have been shown to significantly affect fungal phyllosphere communities in lettuce (Hunter et al., 2015).

5. Conclusion

Plant traits such as flowering time have been shown to be affected by specific soil microbial communities (Panke-Buisse et al., 2015), which indicates the importance of microbiomes in local adaptation. Here we demonstrate that plant genotypes affect recruitment of root endophytic fungal communities in *A. thaliana*, and that roots of this non-mycorrhizal plant are indeed colonized by mycorrhizal fungi. This highlights the potential of this excellent model system for future studies of mechanisms regulating specificity in plant fungal interactions.

Acknowledgements

This work was supported by the Swedish Research Council (349-2007-8731 J.Å., S.L.B. and S.G.E.A. and 2012-3950 A.R.) and Carl Trygger Foundation for Scientific Research (11:387 H.U.) and the Australian Research Council (DE150100542, DP150103414 M.F.B.). We thank Stefan Bertilsson, Department of Limnology, Uppsala University, for facilitating the CFR96 Touch™ Real/Time PCR Detection system as well as Afsaneh Ahmadzadeh and Haleh Hayatgheibi for sample preparation, DNA extraction and purification. We also thank Prof. Jan Stenlid at SLU Uppsala for valuable discussions and Douglas Scofield for helping with bioinformatic analysis.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.funbio.2017.12.013.

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