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Red clover root-associated microbiota is shaped by geographic location and choice of farming system

Shridhar Jambagi [1], Kristian Persson Hodén [1], Linda Öhlund [2], Christina Dixelius [1,*]

[1]Department of Plant Biology, The Swedish University of Agricultural Sciences, Uppsala BioCenter, Linnean Center for Plant Biology, PO Box 7080, S-75007 Uppsala, Sweden
[2]Lantmännen Ekonomisk Förening, Udda Lundqvists väg 11, Lantmännen Lantbruk, S-26881 Svalöv, Sweden

*Corresponding author. Department of Plant Biology, Swedish University of Agricultural Sciences, Uppsala BioCenter, Linnean Center for Plant Biology, PO Box 7080, Uppsala, Sweden. E-mail: Christina.Dixelius@slu.se

Abstract

Aims: This study evaluated the red clover (*Trifolium pratense*) root-associated microbiota to clarify the presence of pathogenic and beneficial microorganisms in 89 Swedish field sites.

Methods and results: 16S rRNA and ITS amplicon sequencing analysis were performed on DNA extracted from the red clover root samples collected to determine the composition of the prokaryotic and eukaryotic root-associated microbe communities. Alpha and beta diversities were calculated and relative abundance of various microbial taxa and their co-occurrence were analyzed. *Rhizobium* was the most prevalent bacterial genus, followed by *Sphingomonas*, *Mucilaginibacter*, *Flavobacterium*, and the unclassified *Chloroflexi* group KD4-96. The *Leptodontidium*, *Cladosporium*, *Clonostachys*, and *Tetracladium* fungal genera known for endophytic, saprotrophic, and mycoparasitic lifestyles were also frequently observed in all samples. Sixty-two potential pathogenic fungi were identified with a bias toward grass pathogens and a higher abundance in samples from conventional farms.

Conclusions: We showed that the microbial community was mainly shaped by geographic location and management procedures. Co-occurrence networks revealed that the *Rhizobium leguminosarum* bv. *trifolii* was negatively associated with all fungal pathogenic taxa recognized in this study.

Significance and impact of study:

The earlier known root rot and clover rot fungal pathogens remain the main challenge for red clover cultivation.

Keywords: co-occurrence networks, farming systems, geographic locations, *Rhizobium*, root-associated microbiota, *Trifolium pratense*

Introduction

The sustainable use of resources for food and feed production is of increasing importance to meet demands from growing populations, shrinking agricultural land, and requests for high-quality and nutritious products (FAO 2018, Fróna et al. 2021). A number of factors determine the productivity of each crop, and different management regimes are applied worldwide. Conventional agriculture systems are commonly associated with high resource and economical inputs but also with high losses in terms of nutrient leakage and the release of greenhouse gases (Vermeulen et al. 2012, Struik and Kuyper 2017, Tian et al. 2020). In return, yields are higher from conventional compared to organic farming (Christache et al. 2018). The balance between the choice of agricultural management (technical-based), biological preconditions (soil type, water, temperature, and light), and farming traditions, among other factors, varies greatly among countries and regions.

The implementation of sustainable cropping systems highlight the importance of integrating legumes into crop rotation schemes (Ferreira et al. 2021). *Fabaceae* is a species-rich plant family, but only a very small fraction of the ∼19 000 species has been domesticated and used as a source for grains and forages. Concerns regarding climate change and the use of natural resources, such as in the production of mineral fertilizers, have revived interest in increasing the use of forage legume crops. Soybean is not grown in northern Europe, and the import of soybean products is not regarded as a sustainable alternative to improve animal feed states. Red clover (*Trifolium pratense*) is a perennial legume species that is grown as pure stands or intercropped with forage grasses to optimize the nutrient values of animal feed (Marshall et al. 2017). Out of the land used for agricultural activities in Sweden, 15% is used for pasture, and out of the remaining share 42% is devoted to leys and the rest is arable land for crop production (Official Statistics of Sweden Annual Report 2020; www.scb.se); thus, red clover is by far the dominant legume crop in Sweden. Red clover is associated with many advantages and ecosystem services, including nitrogen fixation, improved soil structure, and promotion of genetic diversity through insect pollination (McKenna et al. 2018).

Current breeding goals of red clover comprise factors that influence yields, nutritional values, and persistence to withstand a range of stresses. Leys with red clover are aimed for two or three years of growth, hence the persistence of the red clover plants throughout the entire time period is important. The role of microbial soil biodiversity is here believed to be crucial but the knowledge of its status and impact is limited. Fungal diseases are caused by *Sclerotinia trifoliorum* (clover rot) and *Fusarium* spp. (root rot) are observed in several

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locations (Yli-Mattila et al. 2010) but not in all fields, highlighting the likelihood of new or hitherto unknown pathogenic microbes in the soil.

In this work, we hypothesized that a higher microbial diversity is present in the southern part of the country, including an enrichment of potential pathogens and clear intra- and interkingdom competing patterns. The southern region of Sweden has a longer vegetation period than other parts of the country and hence an increased disease pressure. We further hypothesized that the choice of farm management would generate minor effects on the *Rhizobium* community. To address these questions regarding microbial community compositions and co-occurrence patterns in relation to geographic location and farming systems, red clover root samples were collected from south to north in selected places throughout Sweden.

Materials and methods

Field sample collection

Eighty-nine field sites with red clover encompassing conventional and organic farms located between 55°50′ N and 66°48′ N were visited during the growing season of 2019. In total, 260 samples were collected: 214 samples from 72 conventional and 46 from 17 organic farming sites. From each field site, 2–4 samples were taken, separated by a maximal distance. Plants from pure red clover stands where leaf stage 6 had passed and stem elongation started were selected for the analysis. Each sample consisted of 2–5 root systems of red clover with their surrounding soils. All samples were stored at −20°C until DNA extraction.

Sample preparation

The samples were prepared as previously outlined (Hu et al. 2018) with a few modifications. The soil attached to the root system was carefully removed without damaging the roots. Root systems with closely adhering soil particles (rhizospheric soil) were used for microbiota profiling. From 2 to 4 randomly chosen root systems per sample, 5–6 cm root pieces (crown part) were cut into two halves and transferred into 50-ml Falcon tubes (Fig. S1). The root pieces were thoroughly rinsed three times each with 30 ml sterile ice-cold H_2O. The liquid fractions were collected and centrifuged for 15 min at 4500 × *g* and 4°C, and the resulting pellets (∼150 mg) were used for DNA extraction. The samples represent the root-associated microbiota (RAM).

Microbial community profiling

DNA was extracted using a NucleoSpin Soil Kit (Macherey-Nagel, Germany) following the manufacturer's protocols. The quality and quantity of the sample DNA were checked on a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). The 16S rRNA sequences were generated in two steps (Berry et al. 2011). First, the PCR conditions and primers were applied as previously outlined (Takahashi et al. 2014, Saghaï et al. 2022). V3-V4 hypervariable region of the 16S rRNA gene was amplified using primers Pro341F (5′-CCT ACG GGN BGC ASC AG-3′) and Pro805R (5′-GAC TAC NVG GGT ATC TAA TCC-3′) with overhang adapters (forward: TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG, reverse: GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G) to allow the subsequent addition of multiplexing index-sequences. Duplicated reactions were performed to reduce PCR biases. Amplified products were cleaned using Sera-Mag beads, pooled and used as a template for the second PCR step using the same conditions (Saghaï et al. 2022). Duplicated amplicons were purified using an EZNA CyclePure Kit (Omega Bio-Tek, USA) and pooled into two libraries. The quality and quantity of the libraries were measured using a Nanodrop, Qubit 4 fluorometer (Invitrogen), and Agilent 2100 Bioanalyzer. Sequencing was performed on an Illumina MiSeq with paired-end 250-bp reads using the MiSeq reagent kit v2 at SciLife Laboratory, National Genomics Infrastructure (NGI), SNP&SEQ Technology Platform, Uppsala, Sweden. Demultiplexing of the reads was performed using Illumina software (version 2.6.2.1).

Eukaryotic ITS2 rDNA fragments were prepared by amplification using previously described conditions (Halvarsson and Höglund 2021), including primers and barcode information (Ihrmark et al. 2012). Primers fITS7 (5′-GTGARTCATCGAATCTTTG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used for amplification of ITS2 region. PCR was conducted in duplicate for each sample. Three purified amplicon pools were assembled by combining the barcoded DNA samples. The amplicon size distribution was prechecked using an Agilent 2100 Bioanalyzer. The libraries were prepared using the PacBio amplicon library protocol (Pacific Biosciences, Inc.) and sequenced on a Sequel instrument using three SMRT cells (SMRT Cell 1 M v3) by SciLife Laboratory NGI (Uppsala, Sweden). PacBio circular consensus sequence reads were generated using SMRT Link v 8.0.0.79519 (Pacific Biosciences).

16S rRNA gene and ITS2 rDNA read processing

Paired 16S rRNA amplicon sequencing reads were processed by applying the DADA2 method (Callahan et al. 2016) in QIIME2 version 2020.8 (https://qiime2.org/) by filtering and trimming to a fixed length of 250 bp, including quality checking and chimera removal. Next, filtered sequences were clustered into amplicon sequence variants (ASVs) at 100% identity. Taxonomic assignment of ASVs was performed using SILVA database release 138 (Quast et al. 2012) in QIIME2 with the q2-feature-classifier plugin. The QIIME2-compatible preformatted SILVA reference sequence and taxonomy files were processed using RESCRIPt (http://doi.org/10.5281/zenodo.3891931). The output was used to train the taxonomic classifier by extracting the hypervariable V3-V4 region of the 16S rRNA gene. Multiple sequence alignment and phylogenetic reconstruction were carried out using MAFFT and FastTree (Price et al. 2009, Katoh and Standley 2013). Taxonomy-based filtering of ASV sequences was performed using the q2-taxa plugin to remove ASVs representing mitochondrial and chloroplast sequences. Samples were rarefied to an even depth of 11 184 reads to compensate for different sequencing depths.

ITS2 amplicon data were processed using various tools implemented in the PipeCraft v1.0 platform (Anslan et al. 2017). PacBio circular consensus sequence reads were quality filtered with Mothur 1.36.1 (Schloss et al. 2009) (qwindowaverage = 30, qwindowsize = 50, minlength = 100, maxambig = 0, maxhomop = 12). The resulting reads were demultiplexed based on the unique sequence identifiers using Mothur by allowing one mismatch to the barcode (bdiff = 1) and two mismatches to the primer sequences (pdiff = 2). Putative chimeric sequences were removed using vsearch (v1.11.1; github.com/torognes/vsearch) *de novo* filtering and reference-

based filtering against the UNITE reference dataset v7.1 (Nilsson et al. 2019). Additionally, primer artifacts were filtered out using the PipeCraft built-in module. Full-length ITS2 sequences specific to fungi and oomycete kingdoms were extracted using ITS Extractor v1.0.11 (Bengtsson-Palme et al. 2013) and clustered into operational taxonomic units (OTUs) at 97% sequence similarity using CD-HIT v4.6 (Fu et al. 2012). Single OTUs were removed from further analyses. Prior to clustering, homopolymers > 8 bp were collapsed to 8 bp in all reads to reduce the weight to indels. Taxonomic assignment of OTUs was performed using BLASTn (Camacho et al. 2009) (*e*-value = 10; word size = 7; reward = 1; penalty = −1; gap opening cost = 1; gap extension cost = 2) against the UNITE + INSD reference dataset (Cochrane et al. 2016, Nilsson et al. 2019). For unidentified OTUs, manual BLASTn searches were performed in the GenBank database (www.ncbi.nlm.nih.gov). OTUs with an *e*-value < e-50 of the BLASTn search results, BLAST id% >70% and query coverage >55% were considered reliable for taxonomy assignments. OTU tables were further rarefied to an even sampling depth of 112 reads.

Statistical analysis

All statistical analyses were performed in R (https://www.r-project.org). QIIME2 and PipeCraft output and sample metadata files were imported into R v4.1.0. Microbial diversity and community composition were analyzed at the ASV or OTU level with the phyloseq (McMurdie and Holmes 2013) and vegan (Oksanen et al. 2013) packages in R. The Shannon index was calculated for bacteria/archaea and fungal/protist samples as a measure of the alpha diversity (within sample). Significant differences were tested using the Wilcoxon rank-sum test ($P < 0.05$). To estimate beta diversity (between-sample), principal coordinate analysis (PCoA) ordination based on Bray–Curtis dissimilarities was assessed in all samples with permutational multivariate analysis of variance (PERMANOVA) using the *adonis* function in the R package vegan with 999 permutations. Significant differences in the microbial community between the groups of samples were calculated using PERMANOVA pairwise comparisons with random subsampling of 50 samples from each group and repeated 10 times to ensure that PERMANOVA results were robust despite unequal group size. Similarly, for nonmetric multidimensional scaling (NMDS) ordination based on binary Jaccard distances, analysis of similarity (ANOSIM), a rank-based test (from R package vegan), was carried out on the pairwise differences between the groups of samples to complement PERMANOVA (with 999 permutations). Significance was set at $P = 0.001$. *P*- values were corrected for multiple comparisons using the false discovery rate (FDR) with the Benjamini and Hochberg correction method (Benjamini and Hochberg 1995).

The relative abundance of taxa was depicted using a stacked bar representation with the bacterial ASV and fungal OTU mean abundance values expressed as percentages by employing the phyloseq package (McMurdie and Holmes 2013). Shared taxa of bacteria, archaea, fungi, and protists among country regions and farming systems were defined using the Venn Diagram R package (Chen and Boutros 2011). To identify taxa significantly different between geographical regions and farming systems, differential abundance analysis was carried out using the DESeq2 v1.32.0 (Love et al. 2014) (included within phyloseq package). Taxa were considered to differ significantly between geographical regions at a significance level of adjusted *P*-value < 0.01 and between farming systems at adjusted *P*-value < 0.05. For significance testing, DESeq2 uses a Wald test. The Wald test *P*-values were adjusted for multiple testing using Benjamini and Hochberg correction method (Benjamini and Hochberg 1995). The FungalTraits database (Põlme et al. 2020) was used to assign traits (trophic modes) to OTUs assigned to the fungal kingdom.

Microbial co-occurrence network analysis

Co-occurrence networks were constructed and analyzed using the R package NetCoMi v1.0.2 (Peschel et al. 2021) with the functions *netConstruct()* for network construction, *netAnalyze()* for network analysis, and *netCompare()* for network comparison. Associations were estimated based on Pearson's correlation coefficient (Pearson 1909) as an association measure with centered log-ratio (clr) transformation from the SpiecEasi package (Kurtz et al. 2015) as a normalization method. Data were filtered to include at least 50 sequences for both the 16S rRNA and ITS2 rDNA gene sequence datasets. Clusters were identified using the default "cluster_fast_greedy" algorithm (Clauset et al. 2004). Networks were generated using the 50 most abundant taxa to facilitate the visual interpretation of the interactions. Rhizobium–fungal pathogen co-occurrence networks were created using species-level data from the *Rhizobium* genus and potential pathogenic groups of fungal genera. Henceforth, potential pathogenic or potentially beneficial microbes are denominated pathogenic or beneficial in the text.

Rhizobium isolation

To clarify the identity of the Rhizobium species in our dataset, bacteria from red clover nodules were isolated. In total, four independent bacterial samples were prepared. Each bacterial sample comprised of ten pink nodules, 1–3 mm in diameter, excised from two to three red clover roots from the same root sample. The nodules were rinsed in tap water, surface sterilized by submersion in 95% ethanol for 30 s, followed by 10% sodium hypochlorite for 90 s and rinsing with autoclaved water. The following procedures were performed under sterile conditions. The surface-sterilized nodules were suspended in 100 µl H_2O and crushed with a plastic rod until a milky suspension formed. This suspension was cultivated on tryptone yeast and selective yeast mannitol agar (YEMA) plates incubated at 28°C in the dark (Fred et al. 1932). Colonies were picked after 48 and streaked onto YEMA and Jensen's nitrogen-deficient media plates (Jensen 1951) with and without 0.025 g l^{-1} Congo red dye (Sigma–Aldrich) and incubated at 28°C in the dark. Isolates matching the criteria for growth on nitrogen-deficient media and lack of dye absorption were restreaked to obtain pure cultures.

Rhizobium DNA, PCR, and sequencing

Single colonies were grown on YEMA plates, and DNA was isolated (Coton and Coton 2005). Two fragments of the 16S ribosomal RNA gene were amplified using Phusion High-Fidelity PCR (Thermo Fisher Scientific) with primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Coton and Coton 2005), and 799F (5′-AACMGGATTAGATACCCKG-3′) and 1193R (5′-ACGTCATCCCCACCTTCC-3′) (Chelius *et al.* 2001, Huws et al. 2007). Amplified products were sequenced

using Sanger technology (Eurofins Genomics, Ebersberg, Germany). The obtained sequences were analyzed for quality using SnapGene software (from Insightful Science; available at snapgene.com) and assembled with Lasergene MegAlign Pro software (DNASTAR, Inc.). The taxonomy of the assembled sequences was determined using a BLASTN search at NCBI with a minimum of 99% identity.

Red clover screening with fungal pathogens

Red clover seedlings genotype SW1 were grown in a hydroponic setup with medium described by Bindschedler et al. (2008) combined with pathogen screens developed for chickpea (Amalraj et al. 2019). Boxes with 3-week old plants were inoculated with ∼30 000 spores ml $^{-1}$ of *Fusarium avenaceum*, *F. culmorum*, *F. tricinctum*, *Gibellulopsis nigrescens*, and 2 × 10 ^{6} cfu ml $^{-1}$ of *S. trifoliorum*, *Cylindrocarpon destructans*, *Dactylonectria hordeicola*, *Neosetophoma samarorum*, and *Neonectria major*. Sporulating fungal strains were grown in Czapek dox broth (Difco) and non-sporulating in potato dextrose broth (Sigma-Aldrich) medium for spore/mycelia production and challenged with sterile red clover roots to promote pathogenicity prior use in the screening work. Control plants were treated with sterile distilled water. The root responses were monitored daily. Disease symptoms were scored on a scale from 0 to 3 (Table S1) up to 7 days post inoculation and a disease index (DI) was calculated according to (Happstadius et al. 2003) using the formula $DI = (0 \times N_0) + (1 \times N_1) + (2 \times N_2) + (3 \times N_3)/(N_0 + N_1 + N_2 + N_3)$ where N_n = number of plants in the respective class. Twenty red clover plants were used for each fungal species, and the experiments were replicated three times.

Results

Samples from organic farms contain broad biodiversity

Out of 260 samples collected from 89 Swedish field sites, 46 were derived from 17 organic farms, representing a similar proportion of organic farmland as the official statistics for the entire country (www.scb.se). However, the organic farms are not equally distributed in the country, generating a bias of those samples toward central and northern regions in our datasets. DNA isolation was performed mainly as previously described (Hu et al. 2018) to generate information on RAM. In total, 9 944 450 high-quality amplicon sequences from the V3-V4 region of the 16S rRNA gene and 321 703 quality-filtered ITS reads were generated by Illumina MiSeq and PacBio SMRT sequencing (Table S2). The 16S rRNA gene sequences were sorted into 53 990 bacterial and 74 archaea ASVs after rarefying to 11 184 sequences. The curated ITS dataset was assigned to 2756 fungal and 26 protist OTUs. The dataset was further divided according to geographic origin. The southern region has intense agriculture similar to northern Germany. The northern region reaches the Arctic polar circle with few crops available for agricultural production and where leys are more common. Between those two regions are the central plains.

No significant differences were detected in the alpha diversity (within-sample diversity) of bacteria/archaea communities at the ASV level when comparing samples among the northern, central, and southern regions of Sweden (Wilcoxon rank-sum test, FDR, $P < 0.05$; Fig. 1a; Table S3). The only exception was that the samples from the central region were more diverse than those from the northern and southern regions, which were equally diverse. Furthermore, diversity was significantly greater in organic than in conventional farms (Fig. 1b; Table S3). Fungal/protist diversity at the OTU level did not significantly differ between regions or between farming systems ($P < 0.05$). Nevertheless, samples from the southern region were less diverse than those from the northern and central regions, and samples from organic farms were more diverse than those from conventional farms in our dataset (Fig. 1a and b). The bacteria/archaea alpha diversity was ∼2-fold higher than that of fungi/protists. To assess beta diversity (between-sample diversity) among different groups of geographic locations and farming systems, a PCoA plot based on Bray–Curtis dissimilarity (Fig. 2) and a NMDS plot based on binary Jaccard distances (Fig. S2) were generated. Bray–Curtis provides a measure of community composition differences between samples based on occurrence data (abundance), while the Jaccard distance is based on presence/absence data. We used both methods to measure beta diversity to know if they produced different results. Furthermore, to clarify if the biased sample numbers affected PERMANOVA results, pairwise comparisons were repeated 10 times with random subsampling of 50 samples from each group. All these steps generated consistent results in all comparisons performed. To visualize the results, PCoA and NMDS plots were generated by including all samples from each group. The PCoA plot revealed significant differences in microbial community composition between samples from northern, central, and southern regions (PERMANOVA, $P = 0.001$, Fig. 2a). No significant differences were observed between conventional and organic farms (PERMANOVA, $P = 0.008$, Fig. 2b; Table S4). Similar results were generated using an NMDS plot (Fig. S2; Table S5). Overall, microbial alpha biodiversity was lowest in samples from conventional farms (Fig. 1b; Table S3). This finding may result in less available nutrients for cereal crops, particularly if microbes involved in nitrogen cycling are affected (Saghaï et al. 2022).

RAM composition is conditioned by geographic location and farming system

The RAM communities were further analyzed at the lowest level of taxonomic classification with an abundance value set to > 0.001 to assess the distribution of unique and shared taxa between different regions and farming systems. Bacterial taxa were more abundant, followed by fungal, archaea, and protist taxa (Fig. S3). Likewise, most unique taxa representing these four main microbial groups were found in the northern region and in samples from conventional farmland.

Next, we examined the top 15 most abundant taxa for each microbial group at the phylum, genus, and lowest taxonomic levels (Figs. 3 and 4; Figs. S4–S6). As expected, *Aumllorhizobium–Neorhizobium–Pararhizobium–Rhizobi* (hereafter *Rhizobium*) was the most prevalent bacterial genus in all samples, followed by *Sphingomonas*, *Mucilaginibacter*, *Flavobacterium*, and the unclassified *Chloroflexi* group KD4-96. Although abundance of *Rhizobium* genus did not significantly differ between regions, it was more abundant in samples from the north (Fig. 3a). No significant difference was observed in samples from the two farm management systems (Fig. 3c). Taxonomic assignment of ASVs

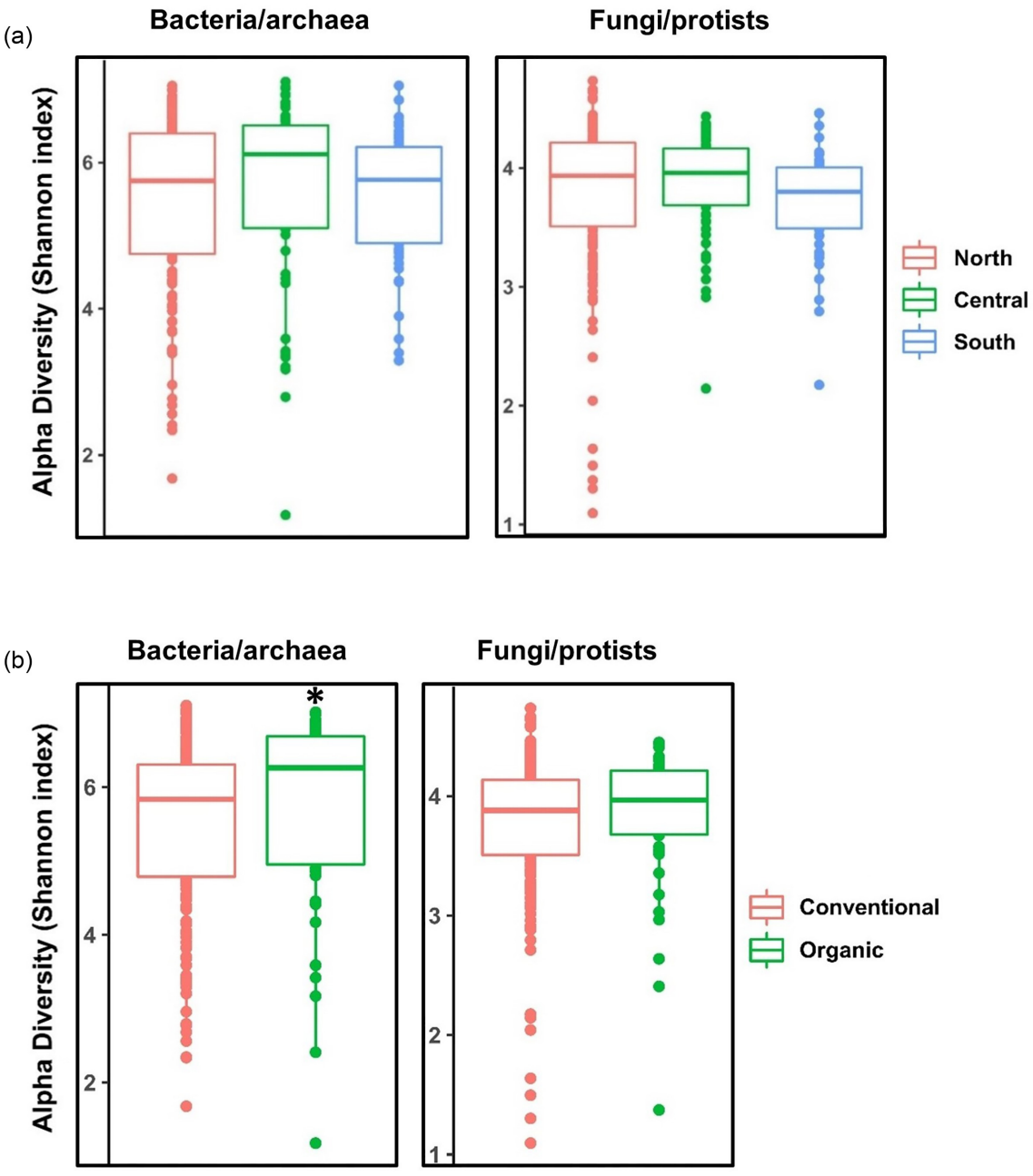

Figure 1. Microbial alpha (within-sample) diversity across different geographic locations and management systems in Sweden. (a) Shannon's alpha diversity of both bacteria/archaea and fungal/protist communities in different regions. (b) Bacteria/archaea and fungal/protist alpha diversity in relation to management systems. The star indicates a significant difference between management systems (Wilcoxon rank-sum test, FDR $P < 0.05$).

using SILVA database in QIIME2 did not provide information on species of unclassified *Rhizobium* enriched in the RAM samples (Fig. 4a). BLAST searches and multiple sequence alignment showed 100% sequence identity to the 16S ribosomal RNA sequence of a *Rhizobium leguminosarum* bv . *trifolii* strains, which were independently isolated from nodules on the collected materials. The abundance of *R. leguminosarum* bv . *trifolii* showed a decreasing trend from north to south but no major influence of farming systems was observed (Fig. 4a and c). Genera known to harbor both pathogenic and plant growth-promoting bacterial species, such as the unclassified groups of *Sphingomonas*, *Pseudomonas*, *Flavobacterium*, *Mucilaginibacter*, and *Aquabacterium*, with the latter being present in water and soil, were abundant in all samples. We also searched for the prevalence of other potential nitrogen-fixing bacteria in our dataset. Several bacterial genera, such as *Devosia*, *Mesorhizobium*, *Labrys*, *Bradyrhizobium*, *Microvirga*, *Phyllobacterium*, and *Bradyrhizobium* were found less frequent in all samples from different geographic locations and farm management systems (Table S6).

Among fungi, *Leptodontidium*, *Cladosporium*, *Clonostachys*, and *Tetracladium* genera known for endophytic, saprotrophic, and mycoparasitic lifestyles according to information from the FungalTraits database were frequently observed in all samples but with different distributions between regions and farming systems (Fig. 3b and d). At the lowest

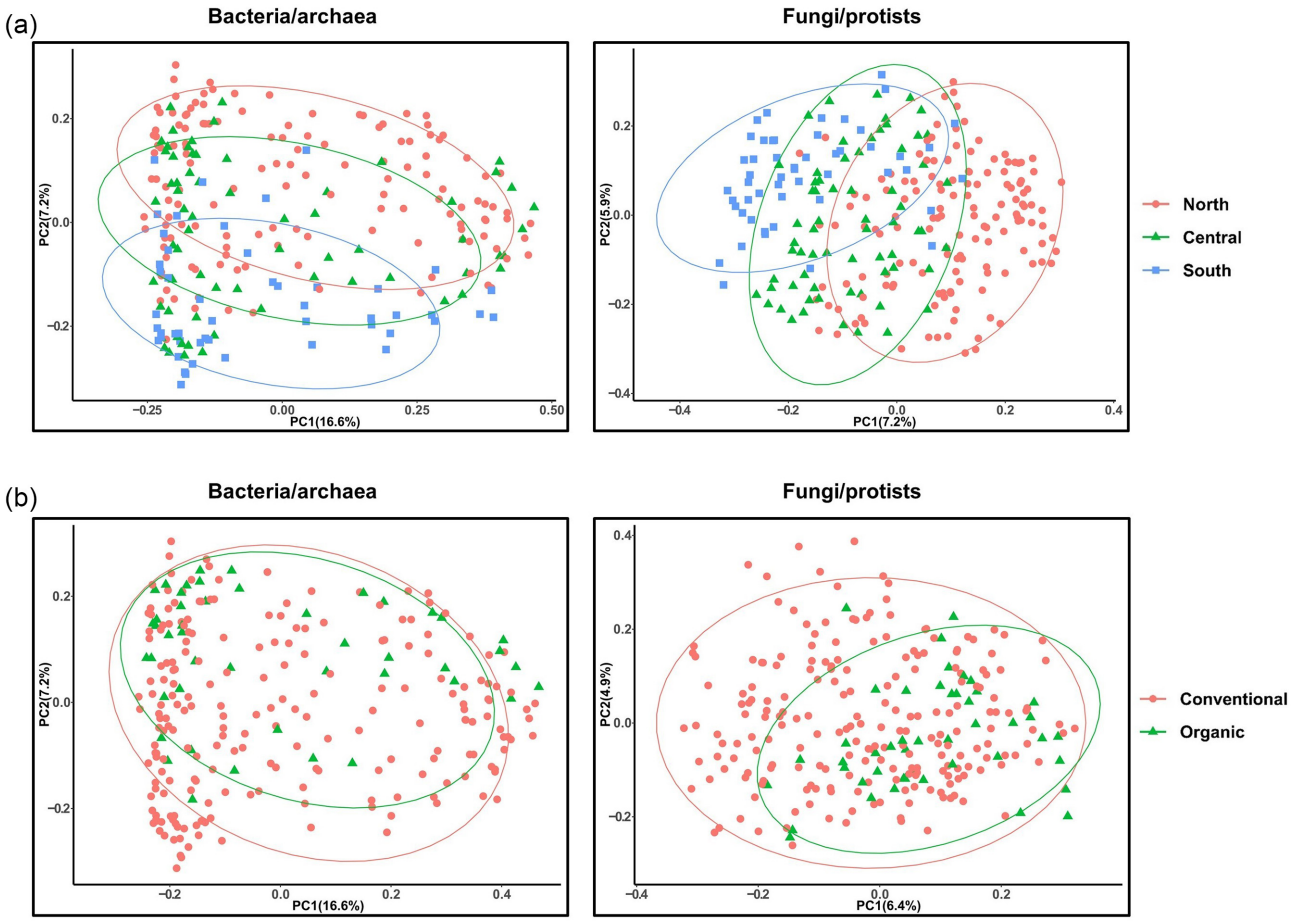

Figure 2. PCoA ordination of beta microbial diversity. PCoA plot based on Bray–Curtis dissimilarity of bacteria/archaea and fungal/protist communities between all samples from different (a) geographic locations and (b) management systems. Significant differences between all samples measured using PERMANOVA (adonis) pairwise comparisons with 999 permutations at *P*-value = 0.001. Benjamini and Hochberg correction method was applied to adjust *P*-values for multiple comparisons.

taxonomic level, *Leptodontidium* sp. and *Cadophora* sp. were highly abundant in the northern region and *Cladosporium cladosporioides* in the central and southern regions (Fig. 4b). In comparison of the farming systems, the incidence of *Ceratobasidiaceae* sp. and *Cadophora* sp. were higher in organic samples, and *Leptodontidium* sp. were more prevalent in samples from conventional farms (Fig. 4d).

We also searched for the presence of pathogenic fungi. A total of 62 pathogenic fungi from different genera were identified in our dataset (Table S7, mean abundance value > 0.0001). A greater number of pathogenic fungi were found less frequently in samples from the northern region and organic farms. Here, a gradient of increasing abundance from north to south was found for *Neonectria* sp., *D. hordeicola*, *Didymella macrostoma*, and *Plectosphaerella paucisepta* (Fig. 4b; Table S7). These taxa were also more prevalent in samples from conventional farms. For example, *Neonectria* sp. and *D. hordeicola* had ∼1.5- and 2-fold higher incidences, respectively (Fig. 4d; Table S7).

Among archaea, *Crenarchaeota*, *Halobacterota*, and *Nanoarchaeota* were predominant phyla in all samples from different regions and farming systems (Fig. S4c). At the lowest taxonomic level, *Nitrososphaeraceae* family that comprise members of *Nitrososphaera* genus was more prevalent in all samples. The unclassified group of *Nitrososphaeraceae* sp. was highly abundant together with *Woesearchaeales* uncultured *euryarchaeote*, *Methanomicrobium* uncultured *Methanolacinia* and Group 1.1c uncultured *Crenarchaeote* in samples from the northern region and organic farms (Fig. S6a). *Cercozoa* sp. were the most prevalent protists representing the Rhizaria supergroup (Fig. S6b). No oomycetes (*Stramenopiles*) were detected, which was unexpected since *Aphanomyces euteiches*, *Phytophthora pisi*, and *Pythium* species, among others, are known as soilborne pathogens in Sweden (Larsson 1994, Hosseini et al. 2015).

To dissect microbial dynamics, our dataset was implemented in the DESeq2 package. A gradient of taxa enrichment was found from south to north (adjusted *P*-value < 0.01, Figs. S7–S11). In organic farms, no bacterial taxa were enriched, whereas only one fungal species (*Solicoccozyma terricola*) appeared in the analysis (log2foldchange = 1.17, adjusted *P*-value < 0.05, Fig. S12). We noticed that the bacterial taxa that were enriched or depleted in our samples mostly had plant growth-promoting or antibacterial properties. For example, *Streptomyces nogalater* was abundant in all geographic regions, particularly in the northern region, compared with the central and southern regions (adjusted *P*-value < 0.01, Table S8). Similar distribution patterns of saprotrophs and endophytes were observed among the fungal communities. A higher number of saprotrophic or endophytic fungi and a

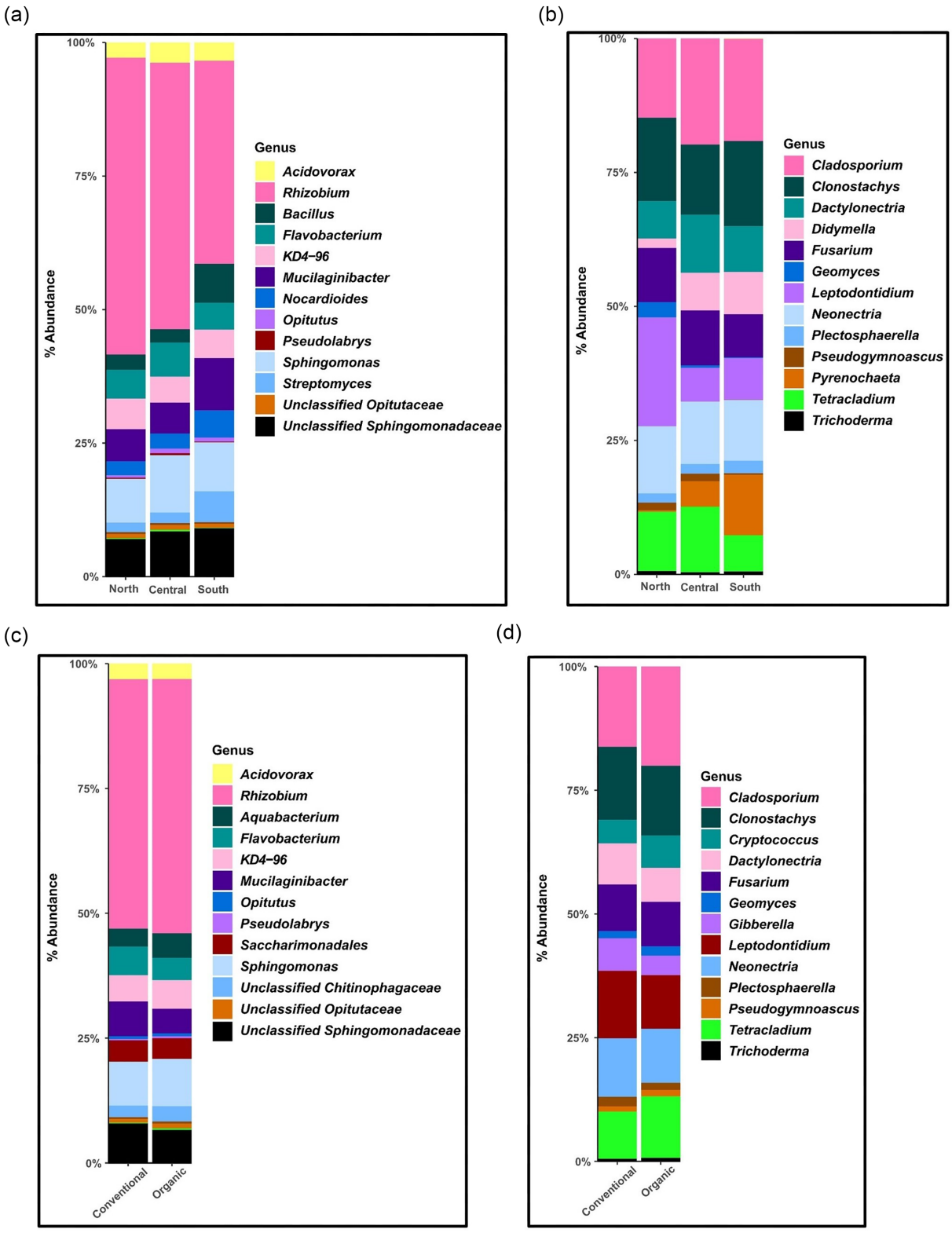

Figure 3. Relative abundances of microbial taxa at the genus level. Distribution of (a, b) bacterial and (c, d) fungal genera in samples from different geographic locations and farming systems. Low-abundance genera (<1%) are not illustrated in the bar graphs.

lower number of pathogenic fungi were enriched in the northern region than in the central and southern regions (adjusted P-value < 0.01, Fig. S10; Table S9). In comparisons between the two farm management systems, no fungal pathogens were enriched in the samples from organic farms (Fig. S12b; Table S9). Taken together, these results highlight two components that greatly impact the population shift between pathogenic and beneficial groups of microbes; geographic location (north) and organic farming are clearly associated with beneficial microbial communities.

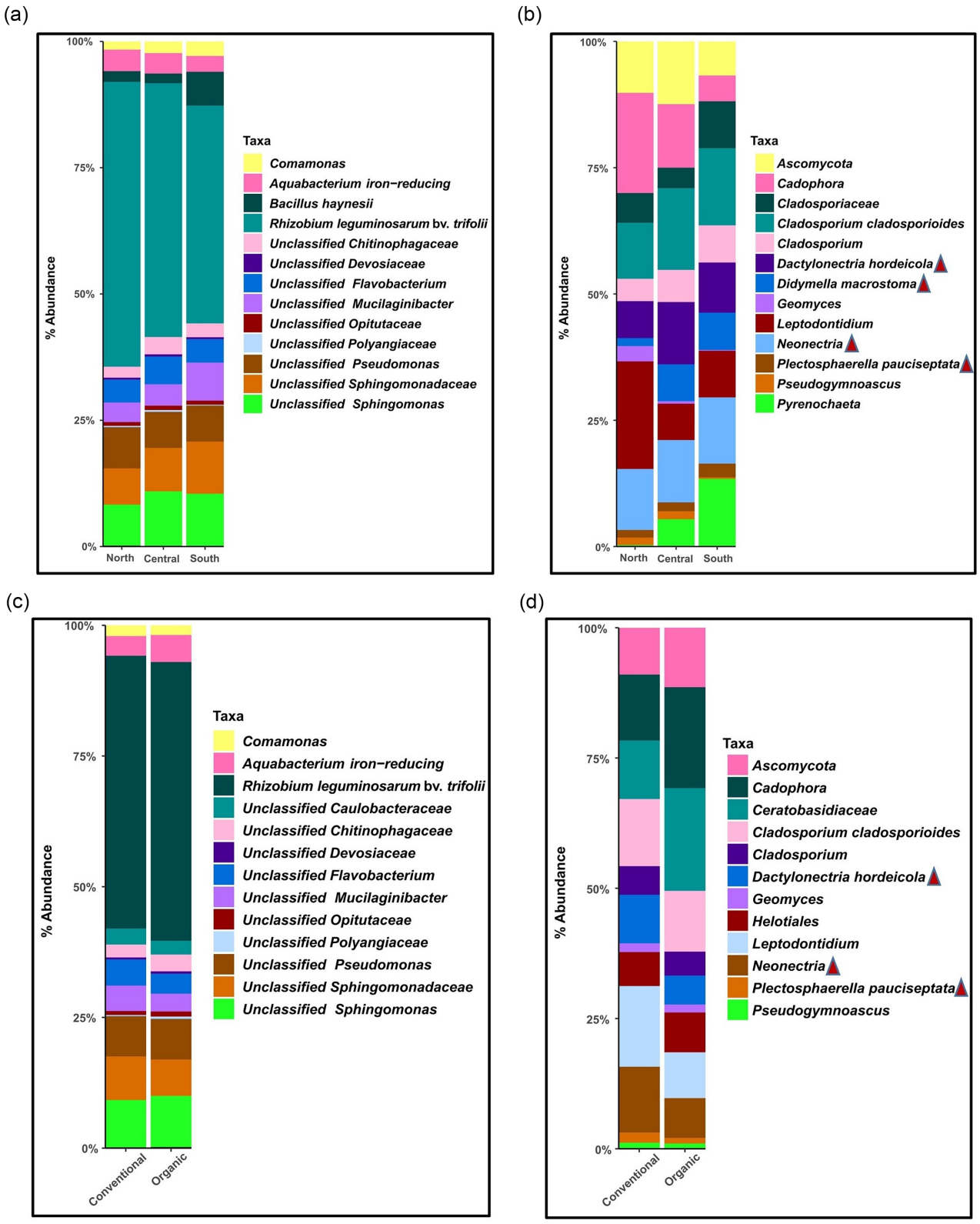

Figure 4. Relative abundances of bacteria and fungi at the lowest taxonomic level. Distribution of (a, b) bacterial and (c, d) fungal taxa in samples from different geographic locations and farming systems. "Triangle" denotes the potential pathogenic group of fungi. Low-abundance taxa (<1%) are not illustrated in the bar graphs.

Rhizobium leguminosarum bv. *trifolii* is a keystone species in the northern region and in organic farms

To gain insights into the co-occurrence patterns of red clover root-associated communities in different regions and farming systems, intrakingdom and interkingdom networks were constructed using the top 50 most abundant taxa to facilitate the visual interpretation of the interactions. Bacterial networks from each region and farming system were more complex and densely connected compared with the fungal networks (Figs. S13–S16; Table S10). Three hub taxa were identified in each network, and they were positively correlated. The clustering coefficient was higher in the bacterial networks, in addition to the number of nodes and edge density. The fungal network based on data from conventional farms deviated somewhat from these features with moderately high-clustering coefficients (Table S10). High modularity indicates more structured communities within a network and a greater number of connections within modules than between networks (Newman 2006). Thus, fungal networks were found to be more modular than bacterial networks, which were particularly prominent in the northern region. Bacterial networks were dominated by beneficial groups of bacterial taxa. The interactions among them were almost 50% positive (co-occurrence). Taxa that are most connected to other taxa are known as central hub taxa or keystone species. When examining the northern and southern bacterial networks, two hub taxa were formed in the south by *Aeromicrobium* sp. and *Serratia* sp., whereas no such hub was detected in the north (Fig. 5a). A major difference between the two regions was the interaction between *R. leguminosarum* bv. *trifolii* and *Aeromicrobium* sp. It was strongly positive in the north but there was no interaction in the south. In a comparison between northern and central bacterial networks, *Actinobacteria* sp. was identified as a hub taxon in the north and was strongly associated with *Microbacteriaceae* and *Micrococcaceae* species but negatively associated with *R. leguminosarum* bv. *trifolii* (Fig. S17a). Furthermore, *R. leguminosarum* bv. *trifolii* was also negatively associated with *Rahnella1* sp. in the south but positively in the central network (Fig. S17b). Strong positive synergy was observed between *Rhizobium* sp. and *Sphingomonas* sp. in the south, an interaction that was lacking in the central network.

No hub taxa were detected in the fungal networks when comparing the northern and southern regions (Fig. 5b). In the north, several fungi were not associated with other fungal taxa, whereas in the south, almost all taxa were connected, except *Gibberella tricincta* (former *F. tricinctum*), a species associated with toxin production on cereals in Europe (Ponts et al. 2020). In the northern network, *G. tricincta* was positively associated with *Cladosporiaceae* sp. (Fig. 5b). When comparing the northern and central networks, *S. tericola* formed a fungal hub taxon in the north and *Tetracladium furcatum* in the central region (Fig. S18a). A strong positive association was observed between *G. tricincta* and the saprotroph *C. cladosporioides* in the north, whereas no such synergy was seen in the central region. Furthermore, the endophytes *Leptodontidium* sp. and *Cadophora* sp. were abundant and positively associated with each other in the fungal network from the north (Fig. S18a). *Cladosporium cladosporioides* was positively associated with the pathogenic group of *G. tricincta* and *D. macrostoma* in the south, and this association was not seen in the central network (Fig. S18b).

In a comparison between conventional and organic networks, no major difference was observed in the percentage of positive association in the bacterial networks. In contrast, the fungal network from conventional farms showed no interaction among most fungal taxa in comparison to the data from organic farms, indicating a higher number of negative interactions among pathogenic or beneficial groups of fungal taxa. Two bacterial hub taxa, *Microbacteriacea* sp. and *Actinobacteria* sp., and two fungal hub taxa, *S. terricola* and *C. cladosporioides*, were detected in the organic networks (Fig. 6a and b).

Comparison of bacterial–fungal interkingdom networks showed that the bacteria formed most of the connections, predominantly with other bacteria. This result is also reflected by the greater abundance of bacterial ASVs compared with fungal OTUs in the samples. Only in the comparison between the northern and southern bacterial–fungal interkingdom networks was one fungal species, *Clonostachys rosea*, associated with bacterial species affiliated with *Rahnella1*, *Curtobacterium*, *Pedobacter*, and *Pseudomonas* (Fig. S19). *Clonostachys rosea* has antifungal properties (Sun et al. 2020), which may influence the surrounding microbes and bacterial clustering. Interactions with *C. roseae* fluctuated in our dataset; they were positively associated with *Enterobacterales* and *Aeromicrobium* species in the north but negatively associated in the south.

Next, co-occurrence networks were constructed to gain insight into the association complexity between *Rhizobium* and potential pathogenic fungi in different regions and farming systems (Table S7). The top 50 most abundant taxa were included in the analysis to facilitate the visual interpretation of the interactions. This approach generated different clusters, one with pathogen candidates and the others on *Rhizobium* species. Unexpectedly, the *Rhizobium* species were negatively associated with all pathogenic groups of fungi, suggesting competition between them. *R. leguminosarum* bv. *trifolii* was detected as the hub taxon in the northern and organic networks, whereas no such hub taxon was found in the central, south, and conventional networks (Fig. 7a and b; Fig. S20a and b). No other legume species were grown in the red clover fields. However, we cannot exclude impact of legume weeds that are commonly in Sweden not least in the central and southern regions. Furthermore, a few fungal pathogens showed positive interactions with each other in the red clover root environment independent of the *Rhizobium* species. These species may play important roles in the persistence of this crop.

Abundance of fungal species based on amplicon sequencing is not correlated with pathogenicity levels

In order to validate the microbiome data in terms of potential new disease-inciting pathogens, we selected nine fungal species based on their mean abundance (Table S11). They were all evaluated on a red clover genotype known to have low levels of resistance. Two of these species, *F. avenaceum* and *S. trifoliorum*, are well-known red clover pathogens (Yli-Mattila et al. 2010). These two pathogens caused high DI; 3.00 by *F. avenaceum* (all the plants assessed were dead 7 days post inoculation) and DI = 2.6 by *S. trifoliorum* (Table S11). *Fusarium culmorum* caused moderate symptoms (DI = 1.4). The other six fungal species generated low DI values.

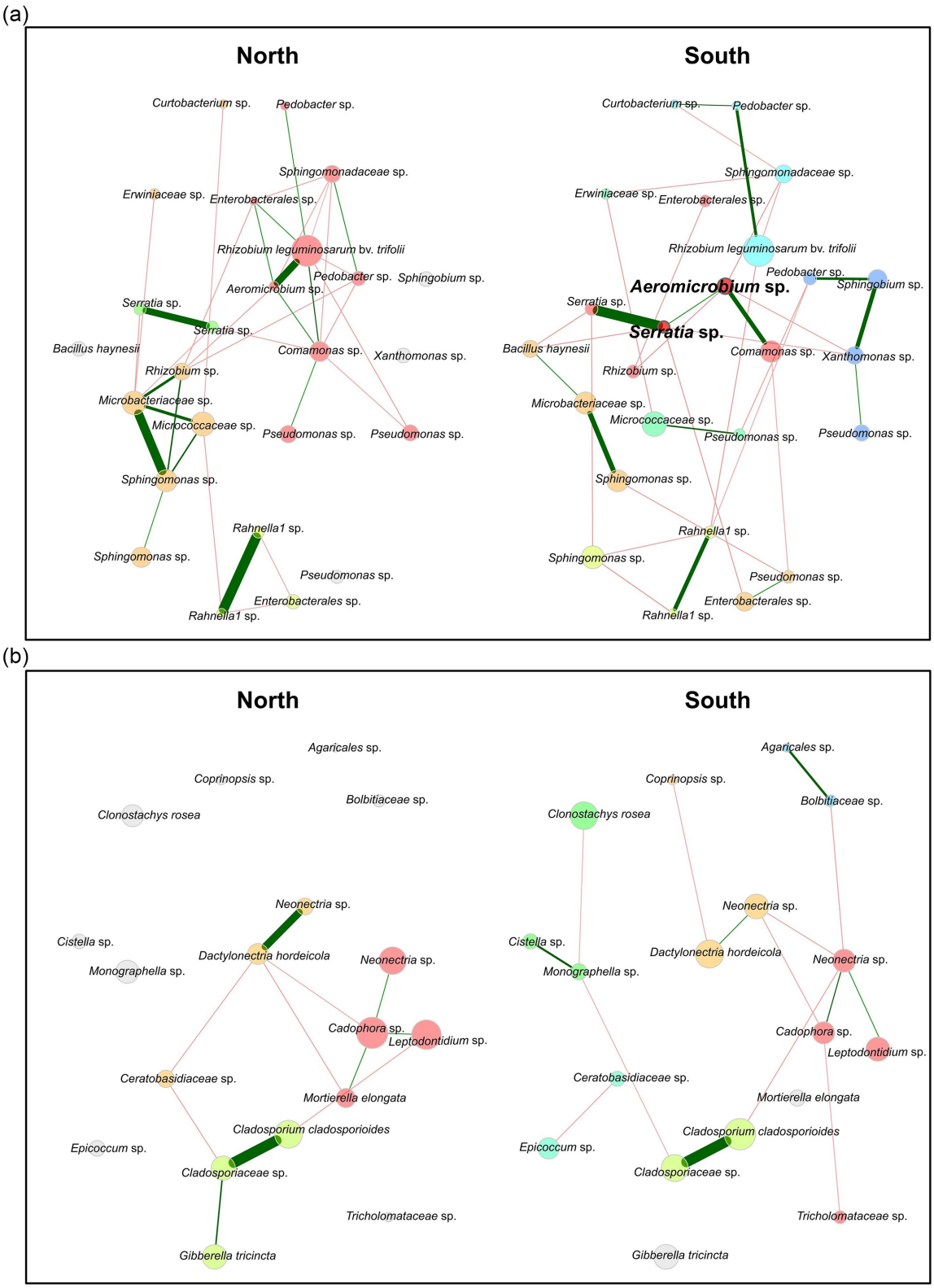

Figure 5. Microbial co-occurrence networks. (a) Bacterial and (b) fungal intrakingdom co-occurrence network based on comparisons between samples from the northern and southern regions. Network comparisons were created based on the Pearson coefficient (Pearson 1909) method using the top 50 most abundant bacterial/fungal taxa. Network properties are described in Table S10. Nodes = taxa and edges = positive associations (green line) or negative associations (red line). Width/color intensity of lines = strength of positive or negative association. Nodes of the same color are clusters. Size of nodes = relative abundance of specific taxa within a cluster. Hubs are nodes with a centrality value above the empirical 90% quantile and are identified by bold text.

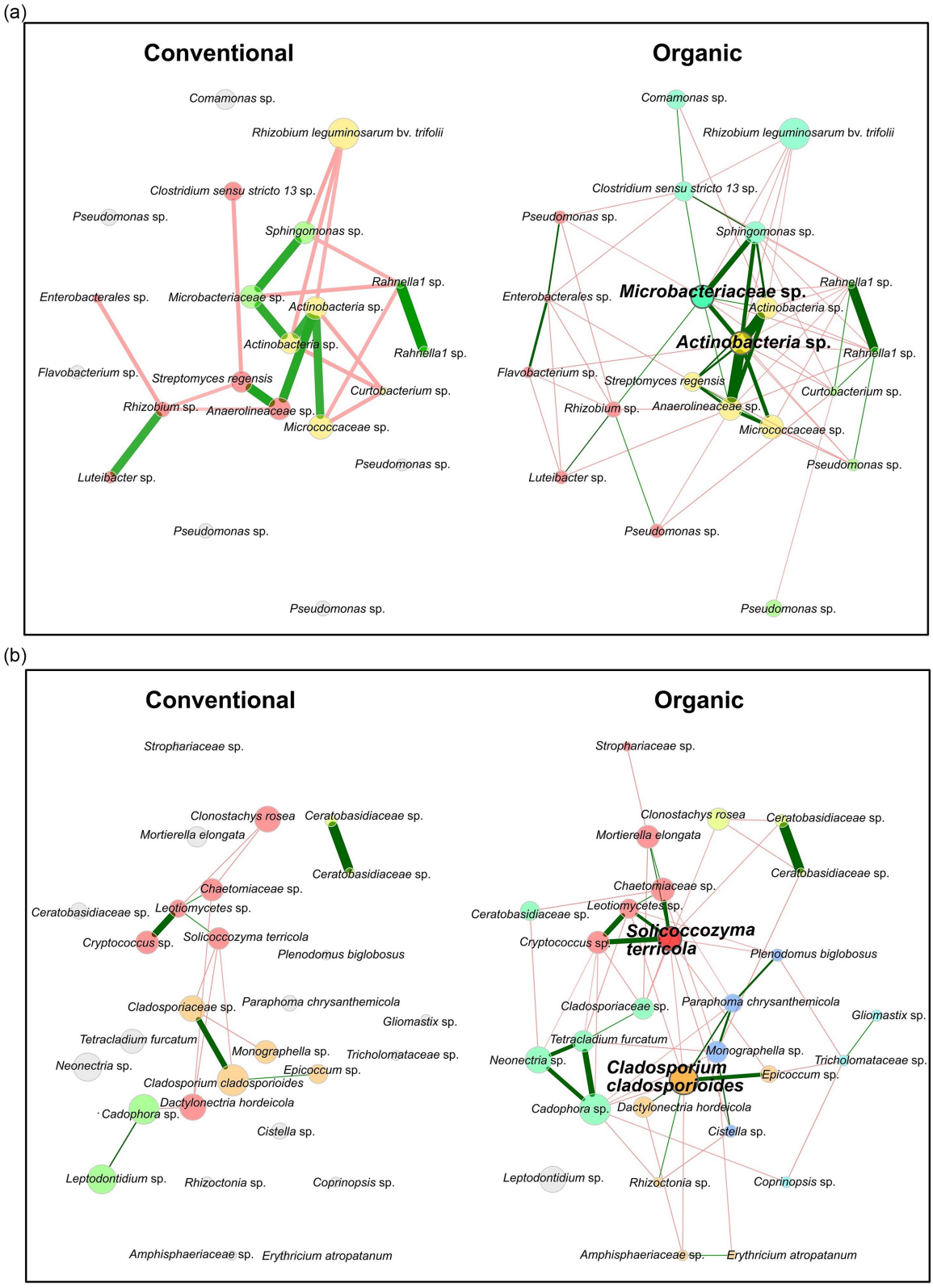

Figure 6. Microbial co-occurrence networks. (a) Bacterial and (b) fungal intrakingdom co-occurrence network comparisons between samples from conventional and organic farming systems. Network comparisons were created based on the Pearson coefficient method (Pearson 1909) using the top 50 most abundant bacterial/fungal taxa. Network properties are described in Table S10. Nodes = taxa and edges = positive associations (green line) or negative associations (red line). Width/color intensity of lines = strength of positive or negative association. Nodes of the same color are clusters. Size of nodes = relative abundance of specific taxa within a cluster. Hubs are nodes with a centrality value above the empirical 90% quantile and are identified by bold text.

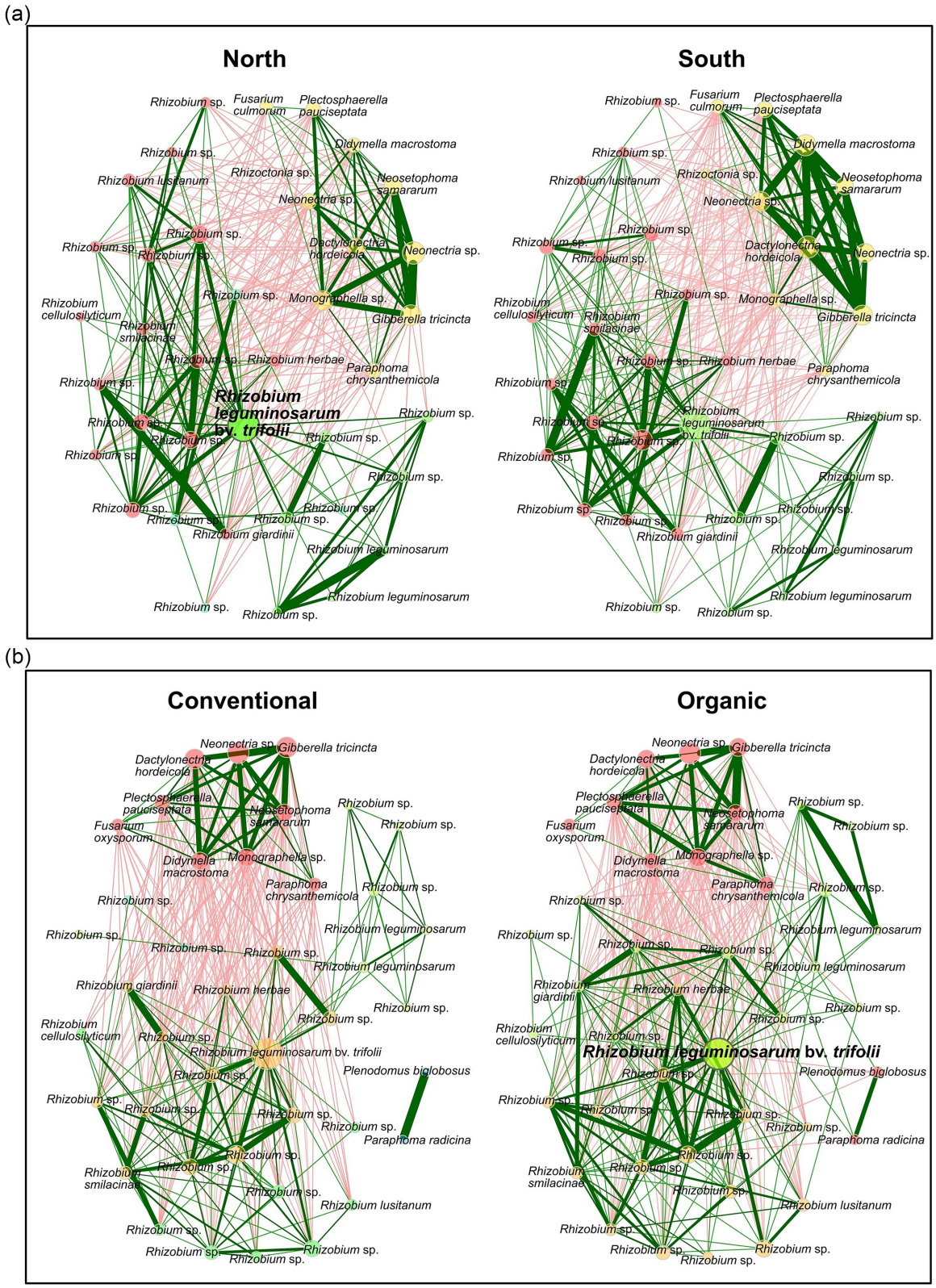

Figure 7. *Rhizobium*—fungal pathogen co-occurrence networks. Comparisons between samples from (a) northern and southern regions and (b) conventional and organic farming systems. Network comparisons were created based on the Pearson coefficient method (Pearson 1909) using the *Rhizobium* genus and potential pathogenic group of fungal genera. Network properties are described in Table S10. Nodes = taxa and edges = positive associations (green line) or negative associations (red line). Width/color intensity of lines = strength of positive or negative association. Nodes of the same color are clusters. Size of nodes = relative abundance of specific taxa within a cluster. Hubs are nodes with a centrality value above the empirical 90% quantile and are identified by bold text.

Discussion

Red clover offers a number of advantages as a perennial forage legume but is constrained by poor persistence. The crop should persist two to three years but rarely does so. One explanation could be that there are unknown pathogens in the soil. This assumption initiated this study where in total 260 samples were collected from farms throughout Sweden. There are few other studies on red clover grown under similar conditions as in Sweden. However, in a study of from field trials located outside Helsinki, Finland, *Proteobacteria* (25%), *Actinobacteria* (20%), and *Bacteroidetes*, *Acidobacteria*, and *Gemmatimonadetes*, each with a relative abundance of ∼10%, were found with minor differences between samples from organic or fertilizer-treated soils of red clover and timothy (Li et al. 2020). Fewer *Proteobacteria* and more species representing *Gemmatimonadetes* and *Acidobacteria* were identified in these field plots than in our data. *Alphaproteobacteria*, a class that includes *Rhizobium* species, had the highest ASV values in all our samples which was expected due to the legume background of our material. Additionally, other potential nitrogen-fixing bacterial genera were found less abundant in our dataset. This is in agreement with the results of previous studies on *T. pratense* (Hartman et al. 2017, Wahdan et al. 2021), *T. repens* (Marilley and Aragno 1999), and *T. fragiferum* (Liu et al. 2007). *Rhizobium* sp. have been identified in the rhizosphere of a wide range of plant species and are considered members of the plant core microbiome (Yeoh et al. 2016). There is competition between various microbes to become attracted to and establish a community surrounding plant roots. Such action includes secretion of metabolites that influence the rhizosphere microbiome (Tkacz et al. 2015, Zgadzaj et al. 2016). For rhizobia, there is also a need to incite nodule formation followed by the transition to a nitrogen-fixing bacteroid (Lardi et al. 2017, Salas et al. 2017, Boivin et al. 2020). The genus *Rhizobium* accommodates 112 species, of which several are nonsymbiotic (Lindström and Mousavi 2020). It is known that nodulating rhizobia harbor genes that are activated by quorum-sensing mechanisms (Sanchez-Contreras et al. 2007). Metabolites involved in quorum sensing and biofilm formation on root systems are also important community factors (Flores-Félix et al. 2021). We can only speculate that the *Rhizobium* species network identified in this work could be a result of a joint population-density action to rapidly colonize red clover roots. Further, previous studies have revealed that many members of the genera *Streptomyces*, *Sphingomonas*, *Pseudomonas*, *Flavobacterium*, and *Mucilaginibacter* play vital roles in producing antibacterial compounds and modulating host performance to stimulate plant growth (Viaene et al. 2016, Duran et al. 2018, Liu et al. 2020, Trivedi et al. 2020, Yin et al. 2021). In the northern region where red clover is grown predominantly and conventional farming is less intense, an abundance of these five bacterial genera associated with plant-beneficial traits was found, which may explain the low abundance of pathogenic fungi in these samples.

Organic farming is dependent on animal manure and organic fertilizers in addition to legume crops as major nitrogen sources. This practice is reflected among genera in archaea that dominate in samples from organic farms. Members of *Nitrososphaera* and *Group 1.1c* are ammonia oxidizers of the superphyla TACK, comprising *Thaumarchaeota*, *Crenarchaeota*, and *Korarchaeota*, which are known to play key roles in the nitrogen cycle (Guy and Ettema 2011, Huang et al. 2021). Other enriched archaea were *Methanomicrobium* in the Euryarchaeota phylum involved in methane production and anaerobic methane oxidation (Evans et al. 2019), and *Woesearchaeales*, a member of another archaeal superphyla (DPANN) present in diverse environments, which are believed to participate in methanogenesis (Liu et al. 2018). Members of the above archaea were also more prevalent in the samples from the northern region. Mitigating methane, a very potent greenhouse gas, is presently prioritized in the agricultural sector worldwide. Biochar amendment in soils and paddies to reduce CH_4 emissions is a promising management practice (Kammann et al. 2017, Nan et al. 2021).

Species-level information in databases and broad ecological information about soil fungi are limited in comparison to information about bacteria and archaea. Although many OTUs were not assigned a trophic mode in the FungalTraits database, a significant number of OTUs were still annotated to a trophic mode at the genus level. Many fungal taxa identified in our study belong to genera known to be endophytic, saprotrophic, and mycoparasitic on species other than red clover (Põlme et al. 2020). This was also the case among the pathogenic group where most species were not previously reported on red clover in Sweden. For example, *Cylindrocarpon*-like species residing in *Neonectria*, *Dactylonectria*, and *Ilyonectria* have commonly been associated with cankers, root rots, and decay on hardwood and coniferous trees (Chaverri et al. 2011, Cabral et al. 2012), and *Plectosphaerella* species are associated with root and collar rots of horticultural crops (Carlucci et al. 2012). The frequently occurring clover rot pathogen *S. trifoliorum* had a very low sequence abundance in our samples. The sexual phase of this fungus occurs aboveground, where apothecia develop from germinating sclerotia shallowly buried in the topsoil. The airborne ascospores initiate infections on stems and leaves, preferably at low temperatures, followed by systemic growth toward the taproot (Öhberg et al. 2008). This life cycle differs from the other pathogens detected in this study, likely explaining the low incidence of this fungus in our samples.

Microbial network analysis provides insight beyond microbial diversity per se, allowing us to reveal co-occurrence interactions among microbial taxa. It has been suggested that complex microbial networks might be beneficial for plants rather than simple ones, because complex networks (high number of interacting microbes) could contribute to a more dynamic environment leading to suppression of soilborne pathogens (Berry and Widder 2014, Yang et al. 2017, Tao et al. 2018). In microbial networks, modularity plays an important role in strengthening the adaptive ability of microorganisms (Kitano 2004). Modules are groups of ASVs/OTUs that interact more closely with one another than with other ASVs/OTUs. Less modules in networks could ensure a faster communication and thereby more efficient regulation between modules in response to environmental stimuli (Tao et al. 2018). A study of the seed microbiome of *Brassica napus* revealed that a high bacterial diversity expressed by tight and complex bacterial networks affects the colonization ability of newcomers, symbionts, and pathogens (Rybakova et al. 2017). In our study, bacterial networks were generally more complex and less modular than fungal networks, which might suggest that these bacterial networks could make the invasion by newcomers more difficult. Keystone or central hub taxa are thought to frequently interact with many other taxa, thereby playing an

important role in maintaining organism structure and function that could be crucial for each biological niche (Banerjee et al. 2018). In this study, several keystone species were identified in the networks from different regions and farming systems. In the constructed networks using lowest taxonomic level data from *Rhizobium* genus and potential pathogenic fungal genera, *R. leguminosarum* bv . *trifolii* was identified as a keystone species as expected. We also identified independent sets of positively interacting fungi. *Fusarium oxysporum* is a common root-colonizing fungus with strains that can be neutral, have endophytic properties or are pathogenic (de Lamo and Takken 2020). Its impact on red clover is, however, unknown. Several grass and small-grain cereal pathogens appeared in the network analysis, such as *G. tricincta* (*F. tricinctum*), *F. culmorum*, and *D. hordeicola* all which could pose threats to several crops. These fungi did not cause any severe symptoms on red clover when inoculated individually. Under field conditions the environment is more dynamic and attenuated plant roots may attract even weak pathogenic species (Tollenaere et al. 2016).

In this study, we aimed to map red clover RAM throughout Sweden to improve understanding of the soil diversity and species dynamics involving *Rhizobium*. The co-occurring network analysis demonstrated clear enrichment of *Rhizobium* sp., which was expected due to the emphasis on red clover in the sample collection. An extensive competition with fungal taxa, not least exerted by *R. leguminosarum* bv . *trifolii* was seen. How such interkingdom interactions affect nodulation capacity under field conditions is unknown. A large share of *Rhizobium* genes is devoted to rhizosphere growth and root colonization, suggesting that successful competition is prioritized to succeed with the next step, symbiosis (Wheatley et al. 2020). Regarding new pathogens on red clover, no such candidates were found in our investigation. Fungi using grass as hosts identified in the study could potentially form a reservoir in the soil as pathogens posing threats to cereals. Enrichment of fungal species with mycotoxin properties (Beccari et al. 2018) is of particular concern requiring follow-up monitoring. That question together with a number of other results generated in this study form the basis for a range of new investigations to understand the impact on microbe populations by contrasting climate conditions, farms with different cultivation regimes and crop rotation schemes, potential fungal pathogens, and nutrient status in the soils. It is notable that pH, organic matter, and nutrients in Swedish arable soils have been very stable during the past two decades (Eriksson 2021). Red clover can promote high carbon sequestering in arable soil (Feiziene et al. 2016) and harbors various beneficial microbes for plant growth and ecosystem sustainability (Wahdan et al. 2021). Increased use of red clover is one among several suggested measures to mitigate negative impact of climate change. This is of particular importance in Sweden where the annual projected temperature rise is higher than the global mean (IPCC: Climate Change 2022; www.ipcc.ch). An estimated increase of up to 0.5°C per 10 years leads to rapid changes in climate zones and new constraints for food and feed production, including increasing challenges from pathogens. We conclude that the microbial diversity is higher in the northern region of Sweden, which is contradictory from our initial hypothesis but we observed patterns consistent with the hypothesis that the longer vegetation period in the southern region of Sweden is associated with the enrichment of potential pathogens. The *Rhizobium* community was found to be associated with negative interactions with pathogenic fungi, it was more pronounced in samples from the north and organic farms. Yet, a few pathogens with low abundance values showed high pathogenicity.

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Supplementary data

Supplementary data is available at *JAMBIO Journal* online.

Conflict of interest

The authors declare no conflicts of interest.

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Author contributions

Shridhar Jambagi (Data curation, Formal analysis, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing), Kristian Persson Hodén (Formal analysis), Linda Öhlund (Investigation, Resources), and Christina Dixelius (Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Software, Writing – original draft, Writing – review & editing)

Data availability

Amplicon sequencing raw datasets have been deposited in the European Nucleotide Archive (ENA) (https://www.ebi.ac.uk/ena/browser/view/PRJEB49635?show=reads) under accession number PRJEB49635. The 16S rRNA sequences of the *Rhizobium leguminosarum* bv. *trifolii* strains have been submitted to GenBank at NCBI (https://www.ncbi.nlm.nih.gov/nuccore/) under accession numbers: MW980046, MW980044, MW980045, and MW980047.

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