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Temporal changes in fungal communities associated with guts and appendages of Collembola as based on culturing and high-throughput sequencing

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ABSTRACT

Due to high abundance and feeding habits, invertebrates are of great importance for shaping microbial communities at the fine scale. Springtails (Collembola) that feed on fungal spores and mycelia may contribute to dispersal through carrying fungal propagules in their guts or on their appendages. The Collembola–fungal associations are mainly investigated by microscopy or culturing techniques, which allow identify only fungi that have distinctive morphological characteristics or that can be cultured *in vitro*. Here we identified the Collembola-associated fungi on the body surface and in the gut content using both culturing and high-throughput sequencing (HTS) methods. We studied three epigeic Collembola species found on the Norway spruce dominated forest stands throughout the vegetation period – *Entomobrya nivalis*, *Orchesella flavescens* and *Pogonognathellus longicornis*. We discovered over 1200 fungal operational taxonomic units (OTUs), i.e. the proxies for species, based on 97% sequence similarity of the ITS2 subregion of ribosomal DNA. Most of the fungi were saprotrophs, but we detected also mycorrhizal, parasitic and lichenized fungi. Season was the most important factor affecting fungal richness and composition, especially on body surface. Although the data matrix revealed significant effect of substrate, we were unable to detect the significant fungal community differences between body surface and gut samples of conspecifics. There were no significant differences among studied epigeic Collembola species in the preference for fungal diet. Our study demonstrates that collembolans associate with a broader range of fungi than previously observed and thus potentially play an important role in enhancing fungal colonization through dispersal activities.

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1. Introduction

Interactions between soil microbial and animal communities are being increasingly recognized as an essential component of carbon cycling (Crowther et al., 2015). Their associations involve trophic interactions, mutualistic and parasitic relationships as well as dispersal that all affect soil structure and functioning (Wardle, 2002). Most soil animals interact with microbes primarily through direct predator–prey interactions. Such trophic relationships between soil organisms may regulate microbial (Crowther et al., 2013; Caravaca and Ruess, 2014) and faunal activity (Siddiqui and Mahmood, 1996; Klironomos and Hart, 2001). Moreover, as soil invertebrates are regularly in contact with fungal spores and bacteria, they may further shape microbial communities as dispersal agents of viable propagules.

Springtails (Collembola) are one of the most abundant soil micro-arthropods in terrestrial ecosystems. Soil fungi are known to form an important part of their diet. Since the late 1960s, many studies have described the feeding habits of Collembola (e.g. Singh, 1969). Some conclude that collembolans are unspecialized feeders of litter, fungi, bacteria, algae and other soil animals (e.g. Ponge, 1991; Castaño-Meneses et al., 2004), while others have found evidence for selective feeding (e.g. Jorgensen et al., 2005; Ferlian et al., 2015). In the course of feeding and moving among soil mycelia and on fruit-bodies, fungal propagules become attached to the grazers body surface or are ingested that both result in fungal dispersal known as ecto- and endozoochory, respectively. Collembolans may transport fungal propagules that facilitates plant colonization by germinating chlamydospores of arbuscular mycorrhizal fungi (Klironomos and Moutoglis, 1999) and basidiospores of

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ectomycorrhizal fungi (Lilleskov and Bruns, 2005). Their activity may introduce propagules also into uncolonized or disturbed microhabitats that have limited access for wind-dispersed spores. However, fungivores may also reduce fungal fitness by extensive feeding (Lussenhop, 1992) and damaging the consumed spores (Nakamori and Suzuki, 2010). By these means, micro-arthropods may shape the fungal communities by suppressing certain taxa by preferential feeding and promoting other taxa by reduction of competition and transportation of propagules.

Previous research investigating collembolan associations with fungi has been mainly restricted to microscopy and culturing techniques. However, visual analysis of small particles and propagules often lacks sufficient resolution for accurate differentiation between fungal species. Besides, culture-based methods may underestimate the diversity, because many mutualistic and parasitic fungi are very difficult to culture or require specific culturing techniques. The ability to obtain DNA sequence information from environmental samples enables to overcome such biases. Over the last decade, high-throughput sequencing (HTS) has been effectively used to study the structure of soil microbial and animal communities (Hamilton et al., 2009; Tedersoo et al., 2014). Culture-independent approaches such as HTS has established increasing importance for detecting intestine associated microbial communities (Clements et al., 2014; Poulsen et al., 2014; Yun et al., 2014). Molecular methods may provide a finer resolution and overview of the taxonomic composition associated with organisms of interest.

Using both HTS and culturing, we identified the Collembola-associated fungi on the body surface and in the gut content of three epigeic Collembola species. We predicted that HTS outperforms culturing in recovering fungal taxonomic groups. We hypothesized that 1) fungal communities differ on body surface and in gut contents of collembolans due to their selective feeding on fungi; 2) both body surface and gut samples differ by season due to temporal turnover of the fungal community; and 3) gut content samples differ between Collembola species due to different dietary preferences.

2. Materials and methods

2.1. Study sites and sampling methods

The study included three epigeic Collembola species – *Entomobrya nivalis* Linnaeus 1758, *Orchesella flavescens* Bourlet 1839 and *Pogonognathellus longicornis* Tullberg 1871 – that are abundant on the forest floor of mixed and coniferous forests in North Europe. Specimens of Collembola were collected throughout the vegetation period from May to September on 4th of May, 25–26th of June, 14–15th of August and 16–17th of September from two circular 2500 m^2 plots in Estonia (Ilmatsalu and Kardla). The Ilmatsalu site (58.36978° N, 26.55598° E) is comprised of Norway spruce (*Picea abies* (L.) H. Karst). The Kardla site (58.41797° N; 26.58660° E) included ca. 5% of silver birch (*Betula pendula* Roth) in addition to spruce. Both stands were planted in 1970s. At least four specimens of each Collembola species were collected during each sampling period. We used a single-animal aspirator design to minimize cross-contamination between species (Greenstone et al., 2011). The specimens were instantly treated with chloroform to prevent their excretion of gut contents.

2.2. Molecular analyses

Samples were prepared for molecular and culturing analysis on the day of collection. To analyze fungi from the body surface and gut content, we plated particles associated with appendages (legs, antennae and some body hairs or scales) and gut contents on separate Petri dishes with Modified Melin–Norkrans (MMN) culture medium (Marx, 1969; 2% agar, 0.1% glucose, penicillin 200 mg/ml, kanamycin 20 mg/ml). Appendages were placed into 1.5 ml Eppendorf tubes filled with 50 μl sterile water. Tubes were vortexed for 10 s, followed by pipetting the liquid onto Petri dishes and dispersing it with a spatula. For the analysis of gut contents, the same specimen without appendages was surface sterilized with 1.5% sodium hypochlorite (NaOCl) for 15 s and subsequently rinsed in sterilized water for 1 min. Surface sterilization of the body degraded most of the body hairs and scales and thus minimized potential cross-contamination between the body surface and gut content. Further, the head capsule was removed and gut contents were placed into 1.5 ml Eppendorf tubes with 50 μl sterile water. Tubes were vortexed and the contents were plated as described above. The Petri dishes were stored at 20 °C for 30 days. For identification purposes, culturable fungi were subjected to genomic DNA extraction in a lysis buffer (0.8 M Tris–HCl, 0.2 M $(NH_4)2SO_4$, 0.2% w/v Tween-20) (Solis BioDyne, Tartu, Estonia) using the proteinase K method (100 μl lysis buffer and 2.5 μl proteinase K; incubation at 56 °C for 24 h and at 98 °C for 15 min). The Internal Transcribed Spacer 2 (ITS2) was amplified using the primers 5.8SF (5′-ATGCATCGATGAAGAACGC-3′) and ITS4 (White et al., 1990) (Table S1). Thermocycler conditions consisted of an initial 15 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 55 °C, 1 min at 72 °C, and a final 10 min at 72 °C. Amplification success was checked with 1% agarose gel via electrophoresis. PCR products were cleaned using EXO-SAP enzymes (GE Healthcare, Freiburg, Germany). Purified amplicons were sequenced in Macrogen Inc. (Amsterdam, The Netherlands) using the same primers.

The same number of specimens from each species was used for direct molecular analysis. The appendages and gut contents of each specimen were subjected to DNA extraction by use of PowerSoil®DNA Isolation Kit (MoBio, Carlsbad, CA, USA) following manufacturer's instructions. PCR was performed using the forward primer gITS7 (Ihrmark et al., 2012) and reverse primer ITS4ngs (Tedersoo et al., 2014) to target the fungal ITS2 region. The primers were tagged with 10–11 base unique identifiers (MIDs; Table S1). The PCR cocktail comprised 2 μl DNA, 1 μl each of the primers, 5 μl 5× HOT FirePol Blend Master Mix and 41 μl double-distilled water. PCR was carried out in four replications in the following thermocycling conditions: an initial 15 min at 95 °C, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final cycle of 10 min at 72 °C. PCR products were pooled and their relative quantity was estimated during gel electrophoresis of 2 μl DNA sample on 1% agarose gel for 15 min. Negative and positive controls (fruit-bodies of *Peziza whitei* (Gilkey) Trappe 1975, from Australia) were used throughout the experiment (for DNA extraction, PCR and sequencing). Amplicons were purified by use of EXO-SAP enzymes (GE Healthcare, Freiburg, Germany). Purified amplicons were subjected to normalization of quantity by use of SequalPrep Normalization Plate Kit (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions. Pooled PCR products were subjected to ligation of Illumina adapters and 2 × 300 bp paired-end sequencing using Illumina MiSeq technology.

2.3. Bioinformatics

Quality check and manual trimming of sequences obtained from cultured fungi was performed using Sequencher 5.1 (Gene Codes Corporation, Ann Arbor, USA). Quality filtering of the paired-end Illumina data was performed following Tedersoo et al. (2015). Briefly, sequences were trimmed using mothur 1.33.3 (Schloss et al., 2009) (average quality over 25 base pairs ≥ 32 and no ambiguities allowed). Trimmed reads were assembled using PANDAseq Assembler (Masella et al., 2012), with a minimum overlap of

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