biology letters

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Biol. Lett. 2005 **1**, 243-246
doi: 10.1098/rsbl.2004.0286

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doi:10.1098/rsbl.2004.0286
Published online 26 April 2005

Selective foraging of fungi by collembolans in soil

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Soils contain highly diverse communities of microorganisms and invertebrates. The trophic interactions between these species are largely unknown. Collembolans form an abundant part of the invertebrate community in soils. A prevailing view is that soil collembolans are generalist feeders on fungi, lichens, fragmented litter and bacteria. However, in laboratory food choice experiments, it has been shown that collembolans preferentially select certain taxa of fungi. To examine this apparent contradiction, we developed a molecular technique based on the analysis of 18S ribosomal DNA (rDNA) sequences to explore the diversity of fungi in soils and in the guts of collembolans. We report that the diversity of fungi found in the natural soil was 33 times higher than that in the guts of the collembolan *Protaphorura armata*. The data support the view that collembolan species can be highly selective when foraging on fungi in soils.

Keywords: collembolan; food selection; soil food web; ribosomal DNA; fungi

1. INTRODUCTION

The feeding biology of collembolans has been studied by gut content and stable isotope analyses of field-caught collembolans (Marshall 1978; Scheu & Falca 2000). These results show that most collembolan species are generalists feeding on fungal hyphae, lichens, fragmented litter and bacteria. Studies have also indicated that collembolans have an opportunistic feeding behaviour; available resources in the immediate proximity of the animals rather than the specific distributed resources are ingested (Ponge 2000). On the other hand, most laboratory studies have shown that collembolans are selective in their food choice when given fungi as a food source (Moore *et al.* 1987; Klironomos *et al.* 1999; Jørgensen *et al.* 2003). It has recently been shown in laboratory feeding experiments that the fitness of collembolans can be affected by the selection of certain fungal species in the diet (Scheu & Simmerling 2004). This observation predicts that collembolans should be selective when foraging on fungi in soils.

To investigate whether collembolans have a selective feeding of fungi in natural soils, we developed a method that could identify various taxa of fungi present in the guts of collembolan. The method is based on the amplification and analyses of 18S rDNA sequences (Anderson & Cairney 2004). The method was used to show that the gut of the collembolan *Protaphorura armata* has a significantly lower diversity of fungal species than the surrounding soil. This supports the view that collembolans can preferentially select certain taxa of fungi when feeding in soils.

2. MATERIAL AND METHODS

(a) *Biological material*

The collembolan *P. armata* (Tullberg) originated from laboratory cultures (National Environmental Research Institute, Silkeborg, Denmark). The fungus *Fusarium culmorum* (W. G. Smith) Sacc. originated from a field site at Kalø, Denmark, and was stored in soil at 10 °C (relative humidity below 30%). Prior to the actual experiments, the fungus was inoculated onto malt extract agar (MEA) and incubated for 14 days to induce sporulation. The soil originated from an organically grown field at the agricultural research farm Rugballegård, Denmark (a sandy loam soil, 3% organic content, 13.5% (w/w) water content).

(b) *Experiments in test system*

The soil was dried, sieved (2 mm mesh size) and sterilized (2×25 kGy radiation) and placed on sterilized glass filter paper in Petri dishes. The fungus *F. culmorum* was inoculated onto the soil and incubated for 7 days at 20 °C in darkness. *P. armata* was starved for 5 days before being allowed to feed for 24 h on the fungus. Five individuals of *P. armata* with visible gut content (indication of feeding) were collected and stored at −80 °C.

(c) *Experiments in soil system*

Fresh soil samples were collected (2×20 g (w/w)) before addition of collembolans. *P. armata*, starved for 3 days, were added to the soil that was kept in glass containers with lids allowing gas exchange and allowed to forage for 18 days. On three occasions, collembolans were heat extracted and moved to new fresh soil to provide them with fresh food items. After 18 days, the collembolans were collected by hand, washed in ethanol (70%), and divided into 10 samples (kept on dry ice) each containing five non-dissected animals with visible gut content. Two samples, each containing guts from 20 individuals, were also collected.

(d) *DNA extraction*

DNA was extracted from *F. culmorum*, collembolans or dissected guts using a modified CTAB protocol (van der Wurff *et al.* 2000). DNA extractions from soil were performed using the FastDNA SPIN Kit for Soil (Qbiogene).

(e) *Polymerase chain reaction*

Nested polymerase chain reaction (PCR) was performed by a primary PCR using the fungus-specific primers EF4 and EF3 and a secondary PCR using EF4 and fung5, producing a 550-bp product corresponding to a region in the fungal 18S rDNA (Smit *et al.* 1999). In both PCRs, the following chemistry was used: 2 µl of 10× PCR buffer, 2 mM $MgSO_4$, 0.2 mM of each dNTP, 0.2 µM of each primer, 0.5 U of Platinum *Taq* DNA polymerase (Invitrogen) and 2 µl template DNA (10 ng μl^{-1}) in a final volume of 20 µl. The following cycling condition was used: an initial 3-min denaturation at 94 °C followed by 35 cycles of 10 s at 94 °C, 1 min at 48 °C and 1 min at 72 °C and, finally, an extension at 72 °C for 10 min. After the initial EF4/EF3 amplification, the PCR products were purified by ethanol precipitation and redissolved in 100 µl H_2O, and 2 µl was used in the secondary EF4/fung5 PCR amplification.

(f) *DNA libraries and sequencing*

PCR products from the soil, non-dissected collembolans and gut samples were purified, ligated into the pGEM-T Easy Vector System and transformed into JM109 cells (Promega). From each library, 2×96 randomly collected colonies were transferred into 150 µl 0.1×TE, lysed in a microwave oven for 1–2 min and then stored at −20 °C. The cloned fragments were PCR amplified and sequenced in both directions by using the universal primers T7, SP6, M13 forward or reverse primers and the BigDye kit (Applied Biosystem), and this was followed by analyses on an ABI3100 instrument (Applied Biosystem). The obtained sequences (in total 440) were analysed using the program SEQUENCER (Gene Codes Corporation). The percentage coverage in the three libraries was

Received 30 August 2004
Accepted 15 November 2004

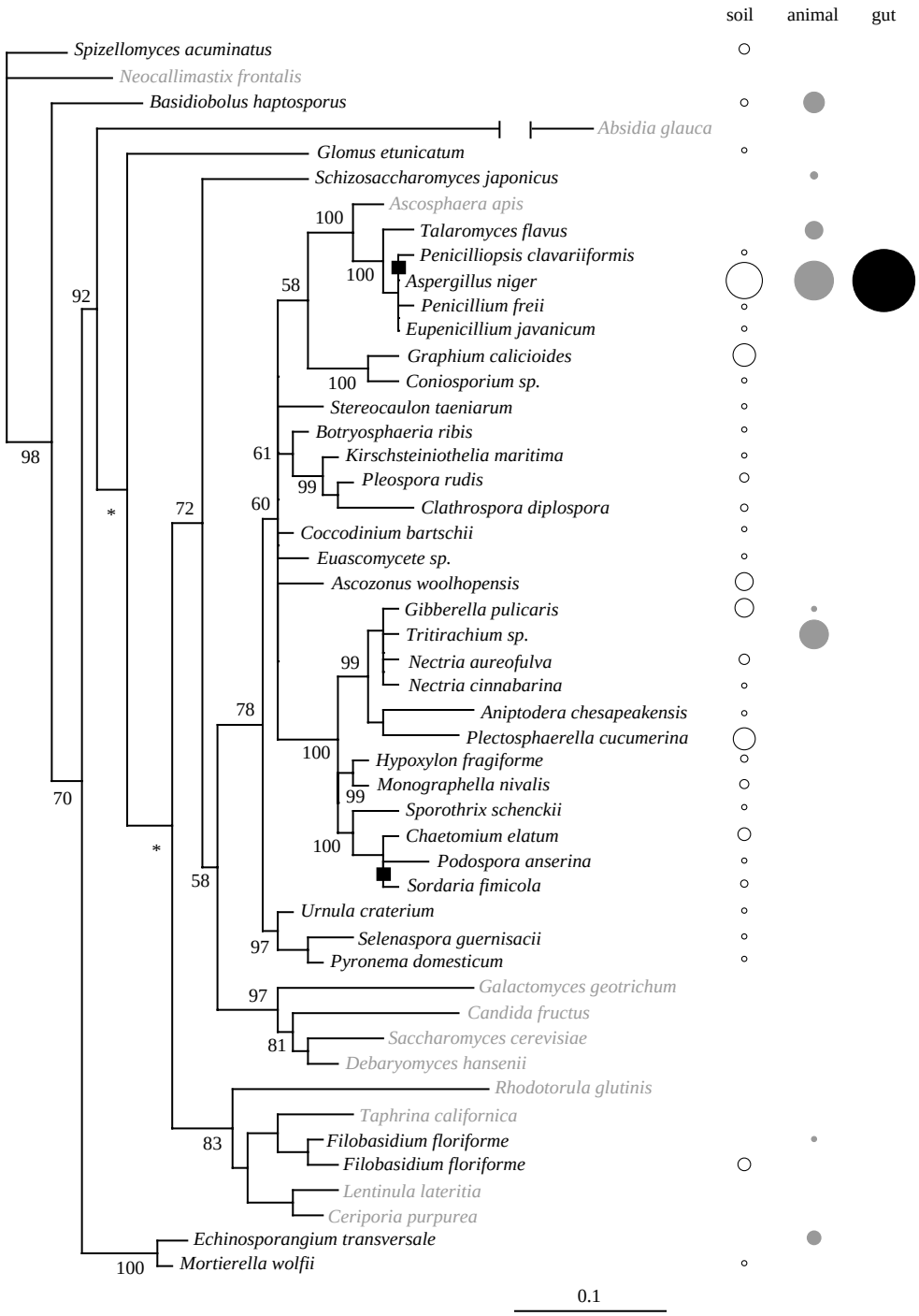

Figure 1. Phylogenetic reconstruction performed by maximum likelihood of sampled 18S rDNA sequences in soil, collembolans (denoted 'animal') and their guts. Numbers correspond to the bootstrap support values of 100 replicates. The scale bar unit is substitutions per site. Sequences from taxa in grey were added to include major fungal groups (as defined by Tehler *et al.* (2003)) that were not sampled. Circle areas are proportional to the number of sampled sequences (in total: 165 from soil, 131 from intact collembolan and 144 from guts). The smallest circle corresponds to one sequence. The percentage coverage of the diversity in the gut, soil and intact collembolan clone libraries were calculated as 99, 80 and 94%, respectively. Asterisks denote branches that were either unresolved or showed a different branching order in the bootstrap consensus tree. The branch to *Absidia glauca* is reduced in the figure. It should be noted that taxon names correspond to the organism names of the closest homologues found by BLAST searches and do not necessarily indicate that the species were identified in the samples.

also calculated (i.e. 1−(number of species/number of clones sampled)×100).

(g) *Sequence analysis*

All fungal small subunit ribosomal DNA sequences (1552 sequences as of 23 January 2004) were downloaded from the European Ribosomal RNA database (http://www.psb.ugent.be/rRNA/). To keep as many sites as possible for phylogenetic analysis, the 277 sequences shorter than 1700 nucleotides were discarded. A database was created of the remaining sequences. The closest homologue for each sampled rDNA sequence from soil, non-dissected collembolans and guts were identified by BLAST searches against the created database (Altschul *et al.* 1990). The identity between the 440 sampled sequences and the best-scoring hit had a minimum value of 91% and a maximum value of 100%. Ninety percent of the sample sequences had an identity of 98% or higher with their best hits. (Unique new sequences have been submitted to EMBL, accession numbers AJ635437-580.) The corresponding aligned sequences, as

retrieved from the ribosomal database, were used for phylogenetic reconstruction. Phylogenetic analysis was conducted using PAUP (Swofford 1998) with the maximum likelihood method. The software MODELTEST was used to evaluate appropriate models and parameter values (Posada & Crandall 1998).

3. RESULTS AND DISCUSSION

Initially, several different PCR primers and conditions were tested for amplifying fungal DNA extracted from the guts of *P. armata* when feeding on the fungus *F. culmorum* incubated in a soil microcosm system. A major problem was finding conditions with specificity for fungal material, while reducing co-amplification of ribosomal sequences from the collembolans. The required specificity and sensitivity, as revealed by amplification and detection of the expected fungal PCR product, were obtained using a nested PCR method. With this method, no non-fungal sequences were amplified.

The developed method was used for analysing fungal DNA in the guts of *P. armata* foraging on a natural community of fungi in soil. The analysis showed that the diversity of fungi in the guts was very low. The maximum difference in sequence identity among the 144 fungal sequences analysed from the guts was only 1%. A BLAST search in the European Ribosomal RNA database revealed that the sequences were most similar to those from *Aspergillus niger* (figure 1).

The diversity of fungal sequences amplified from soil was much higher than that from the guts. The 165 soil sequences matched to 33 different fungal species representing each of the four main fungal phyla *Chytridiomycota*, *Zygomycota*, *Ascomycota* and *Basidiomycota* (figure 1). Such diversity can be expected in a sample from an agricultural soil (Anderson *et al.* 2003). About one-third of the analysed fungal sequences from soil had *A. niger* as the closest homologue. Hence, the gut content of *P. armata* contained only one out of the 33 taxa identified in soil. The frequency of the sequences matching *A. niger* in guts and soils was 144/144 and 51/165, respectively. The probability for such an asymmetry to arise by chance can be estimated as $33\times(51/165)^{144}=1.2\times10^{-72}$.

Analysis of ribosomal sequences in extracts of intact *P. armata* revealed 131 fungal sequences that were associated with sequences in eight different fungal species including *A. niger* (frequency 59/131; figure 1). Apart from fungi in the gut, these samples probably also contained fungal material attached to legs, seta and cuticle (cf. Visser *et al.* 1987).

The fact that all major phyla were amplified in the soil sample demonstrates that the low diversity of fungal species in the gut cannot be explained by a primer bias. A problem that could possibly lead to an underestimation of the diversity of fungi in the gut samples is the fact that the mouthparts and the uppermost part of the gut containing the least digested fungal particles can remain in the dissected animals. A further complicating factor is the potential presence of both hyphae and spores in the analysed samples, which are likely to be co-extracted during the DNA extraction process (Anderson & Cairney 2004). Fungal spores are often found in the guts of collembolans and at least some of these can pass the gut without being degraded (Visser *et al.* 1987). Laboratory experiments have also shown that collembolans can preferentially select actively metabolizing hyphae over senescent hyphae (Moore *et al.* 1987). DNA-based methods cannot separate samples from different tissues of an organism or provide any information on the metabolic activity. Despite these limitations, the data of this study strongly support the view that *P. armata* can preferentially select certain taxa of fungi when foraging in soils. Furthermore, the method developed opens up new possibilities for analysing the trophic interactions at the species level between fungi and invertebrates in soil food webs.

We thank Morten Klamer, Eva Friman and Balaji Rajashekar, Lund University, and Helle Hestbjerg, Danish Institute of Agricultural Science for assistance. The project was funded by the Swedish Research Council, Danish Research Centre for Organic Farming, Danish Environmental Research Institute, Crafoord Foundation and the EU research project T-links. DNA sequencing was performed at SWEGENE Centre of Genomic Ecology at the Ecology Building, Lund, supported by Knut and Alice Wallenberg Foundation through the SWEGENE consortium.

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