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The Protistan Microbiome of Grassland Soil: Diversity in the Mesoscale



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Genomic data for less than one quarter of ~1.8 million named species on earth exist in public databases like GenBank. Little information exists on the estimated one million small sized (1–100 μm) heterotrophic nanoflagellates and ciliates and their taxa-area relationship. We analyzed environmental DNA from 150 geo-referenced grassland plots representing topographical and land-use ranges typical for Central Europe. High through-put barcoding allowed the identification of operational taxonomic units (OTUs) at species level, with high pairwise identity to reference sequences ($\geq 99.7\%$), but also the identification of sequences at the genus ($\geq 97\%$) and class ($\geq 80\%$) taxonomic level. Species richness analyses revealed, on average, 100 genus level OTUs (332 unique individual read (UIR) and 56 class level OTUs per gram of soil sample in the mesoscale (1–1 000 km). Database shortfalls were highlighted by increased uncertain taxonomic lineages at lower resolution ($\geq 80\%$ sequence identity). No single barcode occurred ubiquitously across all sites. Taxa-area relationships indicated that OTUs spread over the entire mesoscale were more similar than in the local scale and increased land-use (fertilization, mowing and grazing) promoted taxa-area separation. Only a small fraction of sequences strictly matched reference library sequences, suggesting a large protistan “dark matter” in soil which warrants further research.

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Introduction

Molecular surveys in planktonic marine systems covering two thirds of the Earth surface have unveiled a large diversity of small protists (de Vargas et al. 2015). Conversely, much less information exists on the remaining one third of the earth surface covered by soil. Studies on soil

ecosystems have typically focused on the smallest (prokaryotes) and larger organism size classes (nematodes, insects) (Allan et al. 2014). Recent global surveys of organismal diversity in the ocean revealed that small eukaryotes comprise by far the major part of eukaryotic genotype diversity (de Vargas et al. 2015; Sunagawa et al. 2015) indicating an enormous functional diversity within this size class (Lima-Mendez et al. 2015). During the last 5 years, soil protist biodiversity has increasingly been studied using high through-put next-generation sequencing (NGS) techniques

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(e.g., Bates et al. 2013; Geisen et al. 2015; Lara et al. 2011; Lentendu et al. 2014). Although NGS studies covered large areas in some cases, often few samples were taken and scaling aspects were not taken into consideration.

The issue of spatial scaling and distribution patterns, i.e. taxa-area relationships, is a central topic in ecology (Green and Bohannan 2006). Martinus Beijerinck (1898) hypothesized that microbial life is uniformly distributed due to its small size (<2 mm) and that the 'environment selects' its inhabitants, implying high turnover rates (Fenchel and Finlay 2006). According to this hypothesis, local sites are expected to harbor 10^4 to 10^7 active individuals of live protist species per gram of soil (Bates et al. 2013) and a huge number of encysted species in their "seedbank", representing a significant proportion of global diversity (Finlay 2002). This implies a moderate slope in the species-area curve resulting from low global species richness and the absence of endemic species for geographically distant sites. It remains a matter of sampling intensity to prove this high local/global species ratio (Woodcock et al. 2006). In contrast to this a moderate endemicity model for protists was recently proposed by Foissner (2006). Apart from that, many rare taxa and cryptic species may exist as a result of both diversification and endemism of dispersed organisms across spatial scales, prompted by their short generation times and rapid genetic divergence (Horner-Devine et al. 2004). Because no taxa-area curve is available for protists, we investigated aspects of biogeographical scaling in soil protist communities by analyzing sampling sites in distances of one up to 1 000 km.

Here, we present a comprehensive study of protist diversity in soils, using a large sample size ($n=150$) within a defined mesoscale. We would like to study whether local to global ratios of soil protists was comparable to records for marine systems (de Vargas et al. 2015) including new or seldom recorded lineages. In order to obtain optimal resolution of most protistan supergroups, we used the most widely studied SSU (small subunit) rRNA gene as a marker gene in metabarcoding approaches together with a curated database for the SSU rRNA gene (Guillou et al. 2013). We focused on the common soil protistan supergroups Rhizaria, Alveolata, Stramenopiles, Excavata and Opisthokonta, which are well-covered by general SSU primers (Pawlowski et al. 2012). We aimed to obtain high taxonomic resolution and hence possible unknown protistan diversity by using long sequence read lengths (>500 bp). This increased the reliability of taxonomic assignment (Hadziavdic

et al. 2014; Lentendu et al. 2014; Wang et al. 2014) and resolution at protistan supergroup level (de Vargas et al. 2015; Taib et al. 2013).

Results and Discussion

NGS and Barcoding of Protists

Environmental sequences from ecological studies using next-generation sequencing (NGS), usually bin these query sequences into meaningful operational taxonomic units (OTUs). In these approaches many cleaned sequences are lost (Caron et al. 2009). To optimize our analyses and include, if possible, all of the unknown diversity of soil protists, we had to modify existing pipelines (Supplementary Material Fig. S1). This is because sequences that bin into OTUs are thought to share similar ecological affiliation and hence are suitable to model ecological consistency (Koeppel and Wu 2013; Preheim et al. 2013). In this closed-reference based approach, trimmed NGS sequences (maximum length 530 bp) were filtered and dereplicated to produce unique individual reads (UIRs). UIRs identified singleton reads (abundance = 1) which were removed. During de novo sequencing a singleton is an OTU represented by a single NGS sequence, and the sequence could have an abundance of >1. This singleton OTU could be informative of a species and an indicator of rare biosphere lineages (Zahn et al. 2013). In our study, singletons were also retained, because we identified it differently. We defined singletons as dereplicated reads with an initial abundance of 1. These singletons with an initial abundance of 1 were removed to circumvent the dangers of pyrosequencing related artifacts (Tedesoo et al. 2010). UIRs were classified directly to the closest reference sequences (accession number) in the curated Protist Ribosomal Reference (PR²) database (based on GenBank, version 203, downloaded June 2016) (Guillou et al. 2013; Supplementary Material Figs S2, S3), the largest database for 18S rDNA sequences. This form of closed-reference-clustering to an accession number also identifies operational taxonomic units (OTUs), because more than one UIR can be linked to a single accession number. In order to retain the most accurate information in complex communities, compensate for errors and artifacts of pyrosequencing and to get an upper and lower estimate of the real species richness of protists, the dereplicated query sequences or UIRs (upper estimate) were analyzed alongside the number of OTUs (conservative estimate).

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