White Paper

Characterizing the Microbiome through Targeted Sequencing of Bacterial 16S rRNA and Fungal ITS Regions

Abstract: Using decomposing leaf litter as the model of a dynamic environment, we demonstrate how the QIAGEN Microbial Genomics Pro Suite can be applied to study changes in the complex profiles of environmental bacterial and microeukaryotic communities.

Introduction

Microorganisms play an essential role in the degradation of plant litter. Because this is a dynamic process, the composition of the litter changes continuously and the microbial community dynamically responds to the resulting fluctuations in nutrient availability (1).

While fungi have classically been assumed to be the main decomposers of plant litter, recent studies have indicated that bacteria also contribute significantly to this process. Numerous bacteria have been found able to degrade cellulose, and bacteria from nearly all the major phyla were shown to carry potential cellulase-encoding genes in their genomes (2).

Using the original data of Purahong et al. (3), we demonstrate community profiling through operational taxonomic unit (OTU) clustering analysis of bacterial 16S rRNA and fungal internal transcribed spacer (ITS) amplicons with the tools of the QIAGEN Microbial Genomics Pro Suite.

Study Design

The study was conducted in the Hainich-Dün Biodiversity Exploratory in Central Germany. The three replicate study sites consisted of unmanaged pure beech forest. In October 2009, 18 nylon bags each containing 10 g samples of air-dried freshly fallen beech leaves were prepared for each study site. In November, at the end of the litter fall period, 15 of the bags were placed in the selected study sites, while three additional bags per site were retained for baseline comparisons. The bags were retrieved on five sampling dates: February 10 (89 days), May 12 (180 days), August 24 (284 days) and November 10 (362 days) in 2010, and March 1 in 2011 (473 days). On each sampling date, three bags from each replicate study site were collected.

Materials and Methods

The three bags retrieved from each replicate study site were pooled before analysis. Bacterial V3-V5 16S rRNA gene sequences were amplified using the primers
BAC 341F (5'-CCTACGGGAGGCAGCAG-3') and
BAC 907R (5'-CCGTCAATTCMTTTGAGTTT-3') (4, 5).

Fungal ITS rRNA regions were amplified using the primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3′) (6, 7).
Amplicons were sequenced using 454 technology (Life Sciences). The reads were deposited in the European Nucleotide Archive under the accession numbers PRJEB9175 and PRJEB9300. Bacterial reads were clustered against the SILVA reference database (v128) at 97% identity threshold. Fungal reads were clustered against the dynamic version of the UNITE reference database. We downloaded reads from the NCBI Sequence Read Archive directly through the CLC Genomics Workbench using the tool "Search for Reads in SRA". ▷

Contents

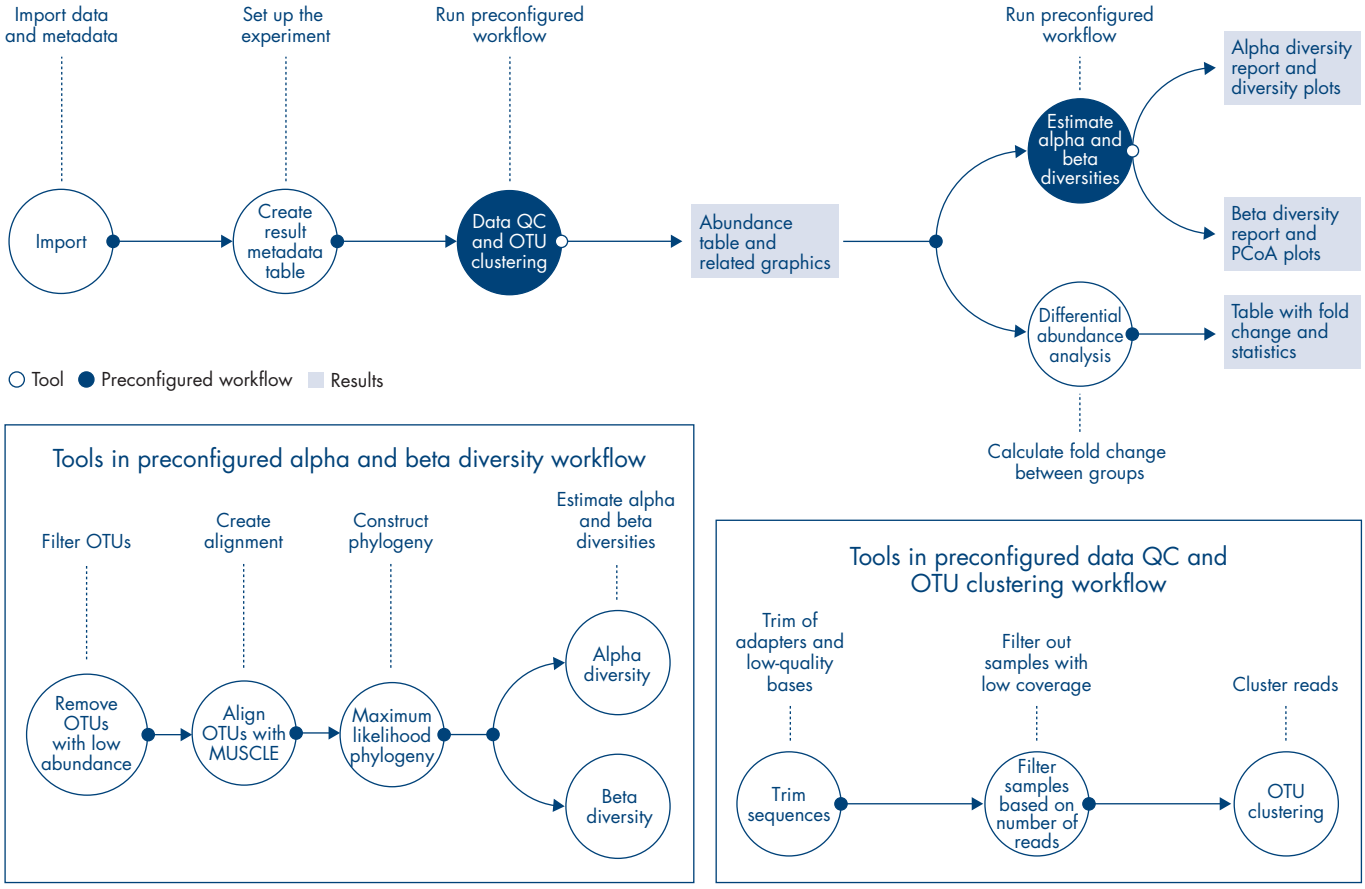

Figure 1. Steps, tools and workflows used for OTU clustering and diversity estimation.

Figure 1 illustrates all tools and preconfigured workflows used for data analysis. All the tools used are available in CLC Genomics Workbench 10.0 and CLC Microbial Genomics Module 2.0 or later.

Results

Bacterial Community Dynamics

The cluster analysis of 16S rRNA data assigned reads to 14 bacterial phyla and 231 genera. The dominant phyla were Proteobacteria (55% of reads), Actinobacteria (25%) and Bacteroidetes (16%). Clustering reads at the family level revealed distinct bacterial communities at early and later stages of decomposition (Figure 2B). In the early stages of decomposition, members of the Microbacteriaceae, Spingomonadaceae, Oxalobacteraceae and Pseudomonadaeae dominated the bacterial community, whereas in the later stages of decomposition, members of the Bradyrhizobiaceae, Micromonosporaceae, Pseudocardiaceae, Streptomycetaceae and Xanthomonadaceae were dominant.

Beta diversity estimates made using the Bray–Curtis dissimilarity support these findings, showing clearly separated bacterial communities on all sampling dates (Figure 3A). We observed the lowest bacterial richness at the early stages of decomposition (Figure 3B).

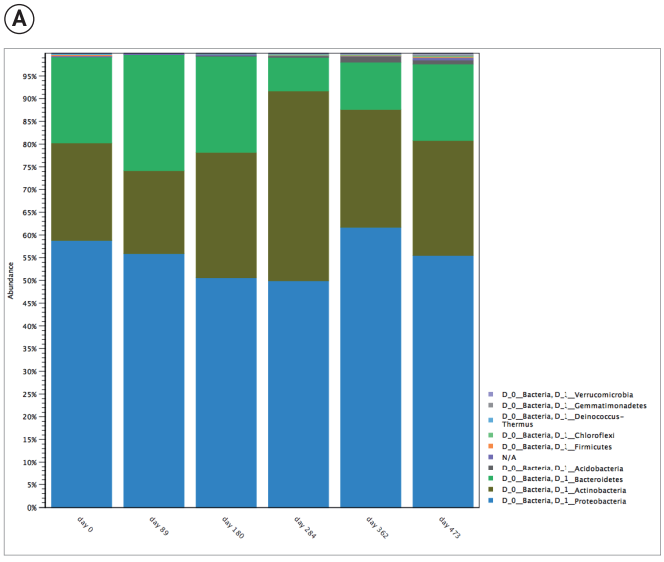

Figure 2. The relative abundance of bacteria in the leaf litter at the A **phylum and** B **family levels.**

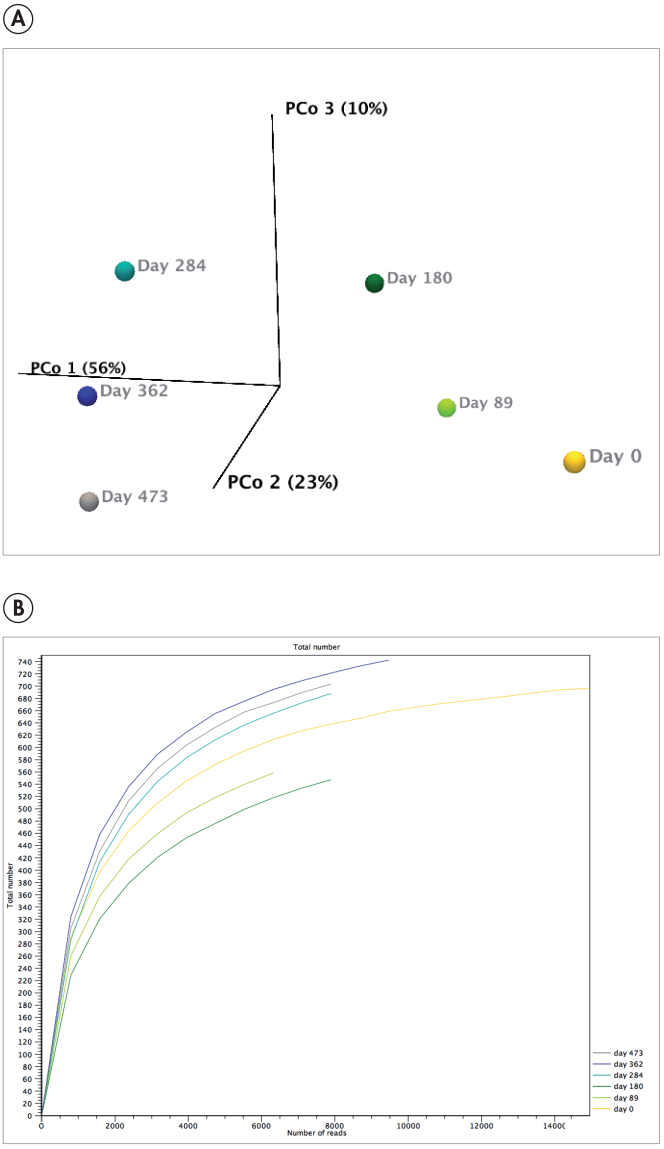

Figure 3. Diversity estimates for bacteria in leaf litter. A Principal coordinates analysis of Bray–Curtis dissimilarities. **B** The number of OTUs as a function of number of analyzed reads (alpha diversity).

Of the 20 most abundant genera (representing more than 1% of the relative abundance), 18 showed a significant change in relative abundance during decomposition (Table 1, Figure 4). A comparison of the relative abundance of the bacterial genera on days 0 and 473 showed alterations in relative abundance ranging from an 847-fold reduction in the relative abundance of *Hymenobacter* to a 1,212-fold increase in the relative abundance of *Streptomyces*.

Table 1. Differential abundance analysis for bacterial genera in the leaf litter on days 0 and 473

	Day 473 vs. Day 0					
Name	**Max. group mean**	**Logs fold change**	**Fold change**	**p value**	**FDR p value**	**Bonferroni**
Hymenobacter	286.00	–9.73	–847.15	2.35E–4	3.53E–4	4.94E–3
Ambiguous taxa 16	261.33	–9.51	–728.71	3.47E–4	4.86E–4	7.28E–3
Janthinobacterium	150.33	–4.28	–19.41	6.02E–7	1.26E–6	1.26E–5
Massilia	273.00	–3.20	–9.18	8.02E–5	1.30E–4	1.68E–3
Pedobacter	322.00	–3.17	–9.03	6.88E–10	2.41E–9	1.45E–8
Pseudomonas	645.00	–3.06	–8.33	1.95E–6	3.73E–6	4.10E–5
Sphigomonas	659.33	–2.36	–5.14	4.19E–11	1.76E–10	8.80E–10
Variovorax	177.00	–1.11	–2.16	4.65E–3	5.14E–3	0.1
Duganella	138.67	–1.06	–2.09	1.50E–3	1.85E–3	0.03
Ambiguous taxa 03	330.67	–0.46	–1.38	0.2	0.21	1
Rhizobium	199.00	–0.11	–1.08	0.7	0.7	1
Flavobacterium	400.00	1.45	2.73	4.64E–3	5.14E–3	0.1
Devosia	81.67	1.59	3.00	5.74E–6	1.00E–5	1.21E–4
Kinesporia	137.67	2.18	4.53	2.21E–9	6.62E–9	4.64E–8
Caulobacter	56.00	2.73	6.62	6.36E–13	3.33E–12	1.33E–11
Chitinophaga	30.33	2.88	7.38	5.70E–4	7.48E–4	0.01
Tardiphaga	119.00	3.70	12.96	0	0	0
Ambiguous taxa 19	153.00	5.61	48.70	0	0	0
Bradyrhizobium	174.33	7.58	191.21	0	0	0
Streptomyces	86.67	10.24	1212.74	6.83E–8	1.79E–8	1.43E–7

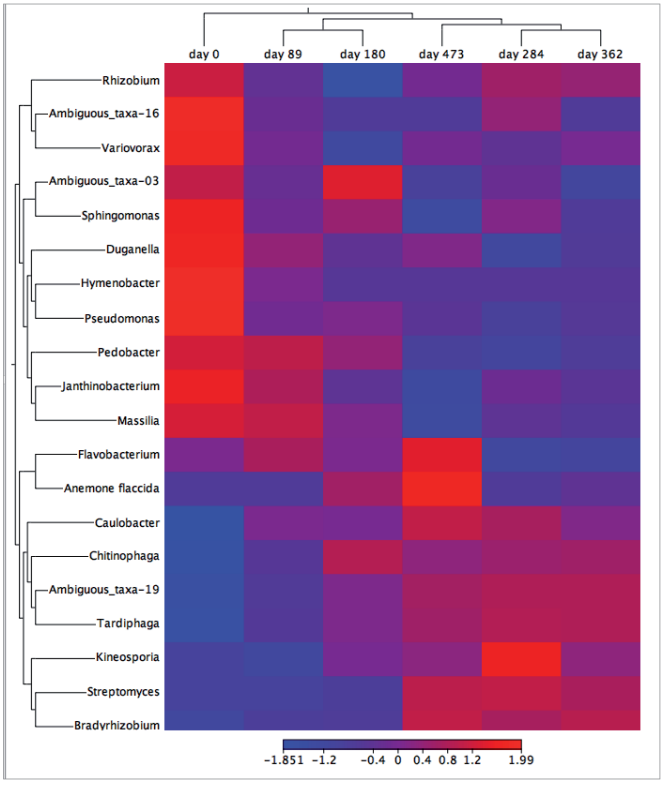

Figure 4. Comparison of relative abundances of bacterial genera across samples over time.

Fungal Community Dynamics

Cluster analysis of fungal ITS amplicon data allocated reads to 3 phyla and 75 genera. Members of the Ascomycota, which accounted for 65% of the reads, dominated during the early stages of decomposition, while members of the Basidiomycota, which accounted for 35% of reads, were found to dominate during the later stages (Figure 5A). Detailed analysis of the relative abundances at the family level revealed that during the early stages of leaf degradation, members of the Helotiales and Mycosphaerellaceae dominated the fungal community. As decomposition progressed members of the order Xylariales became dominant, while during the final stages of decomposition, members of the Casiosphaeriaceae, Agricaceae and Ceratobasidiaceae families and the order Agricales became the dominant players (Figure 5B).

Beta diversity analyses support these findings. Principal coordinate plots of Bray–Curtis dissimilarity show clearly separated fungal communities at all sampling points (Figure 6A). Fungal richness was lowest at the initial stages of decomposition and increased over time, to reach a maximum on day 473 with 83 detected OTUs (Figure 6B).

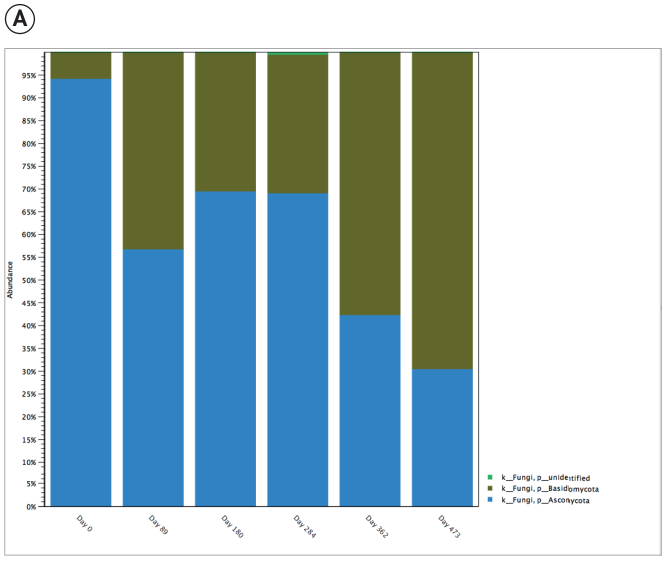

Figure 5. Relative abundance of fungi in the leaf litter at the A **phylum and** B **family levels.**

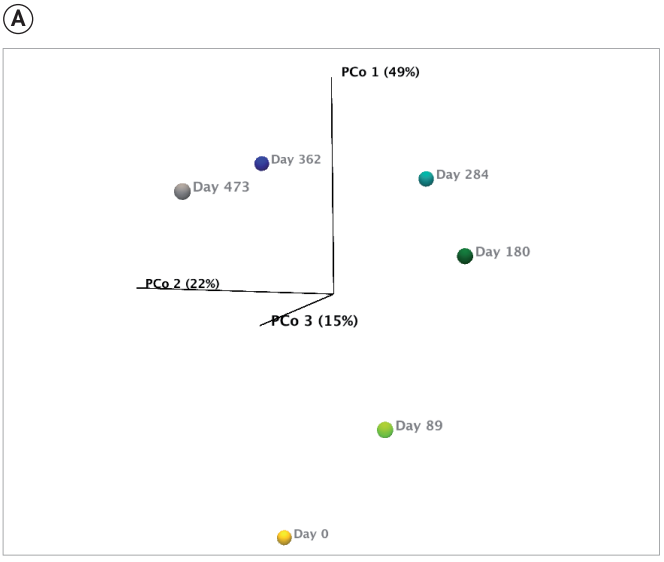

Figure 6. Diversity estimates for the fungi in leaf litter. A Principal coordinate analysis of Bray–Curtis dissimilarities, **B** The number of OTUs as a function of the number of reads.

Differential abundance analysis, comparing the relative abundance across all samples, revealed that of the 13 most abundant genera (representing more than 1% of the relative abundance), 10 showed a significant shift during decomposition (Table 2, Figure 7). Comparison of the relative abundances at the genus level in samples collected on day 0 and 473 showed changes in the range of a 1246-fold reduction in relative abundance to an 8338-fold increase in relative abundance. *Apodus*, *Lepiota* and *Mycena* were the fungal genera with the highest fold increase in relative abundance (6447-fold, 3673-fold and 2598-fold, respectively). ▷

Table 2. Differential abundance analysis for fungal genera in the leaf litter on days 0 and 473

	Day 473 vs. Day 0					
Name	**Max. group mean**	**Logs fold change**	**Fold change**	**p value**	**FDR p value**	**Bonferroni**
Unidentified genus 07	2426.33	–10.28	–1246.58	0	0	0
Unidentified genus 02	400.67	–8.70	–415.50	1.62E–8	5.26E–8	2.10E–7
Efibulabasidium	2.33	–3.54	–11.60	0.23	0.25	1
Flagelloscypha	0.67	1.29	2.45	0.53	0.53	1
Unidentified genus 09	55.00	1.81	3.50	0.08	0.1	1
Ceratobasidium	414.00	4.37	20.71	6.18E–3	8.03E–3	0.08
Unidentified genus 17	113.67	5.41	42.61	2.62E–9	1.70E–8	3.41E–8
Unidentified genus 12	3.67	6.19	72.76	5.88E–3	8.03E–3	0.08
Unidentified genus 06	35.67	8.06	266.48	4.19E–9	1.82E–8	5.45E–8
Mycena	141.00	11.34	2598.16	2.66E–7	4.94E–7	3.46E–6
Lepiota	272.00	11.84	3673.97	2.54E–6	4.14E–6	3.31E–5
Apodus	318.00	12.65	6447.78	4.55E–8	9.85E–8	5.91E–7
Unidentified genus 20	448.00	13.03	8338.80	2.16E–8	5.62E–8	2.81E–7

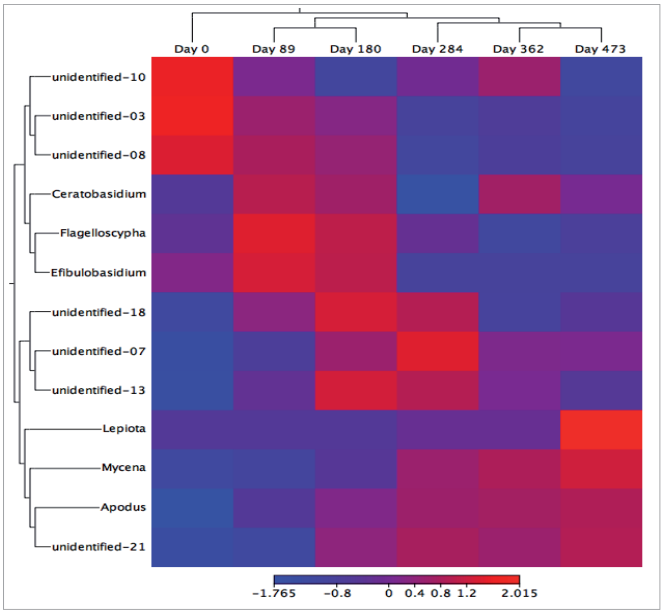

Figure 7. Comparison of relative abundances of fungal genera across samples over time.

Discussion

During leaf senescence, the most labile constituents, such as soluble oligosaccharides, organic acids and amino acids are withdrawn by the tree. As a result, dead leaf material is mainly composed of cellulose, hemicellulose and lignin (2). Degradation of plant material begins with leaching and degradation of soluble and low molecular weight compounds. The next stage is characterized by degradation of the soluble and non-lignified carbohydrates: hemicellulose and cellulose. As decomposition progresses, the concentration of the more recalcitrant lignin increases, and the final stage of decomposition is dominated by degradation of lignin and its derivatives (1). For the microbial community, this creates nutritional fluctuations.

During the early stages of leaf litter degradation (Figure 8), where levels of hemicellulose and cellulose were high, we found ascomycetous species dominated the fungal community. Members of the Ascomycota are well known decomposers of hemicellulose and cellulose. As decomposition progresses and levels of lignin increased, we observed a shift to a fungal community dominated by members of the Basidiomycota. Differential abundance analysis showed a 2500- to 3500-fold increase in the relative abundance of the basidiomycetous fungi *Mycena* and *Lepiota*, which are both well characterized degraders of lignin.

Thus, the change from a substrate rich in hemicellulose, cellulose and lignin to one predominantly composed of lignin and lignified carbohydrates is accompanied by changes in the fungal community from one with members possessing the enzymatic capabilities to degrade more simple compounds to one where members have the required enzymes to feed on more complex and recalcitrant carbohydrates.

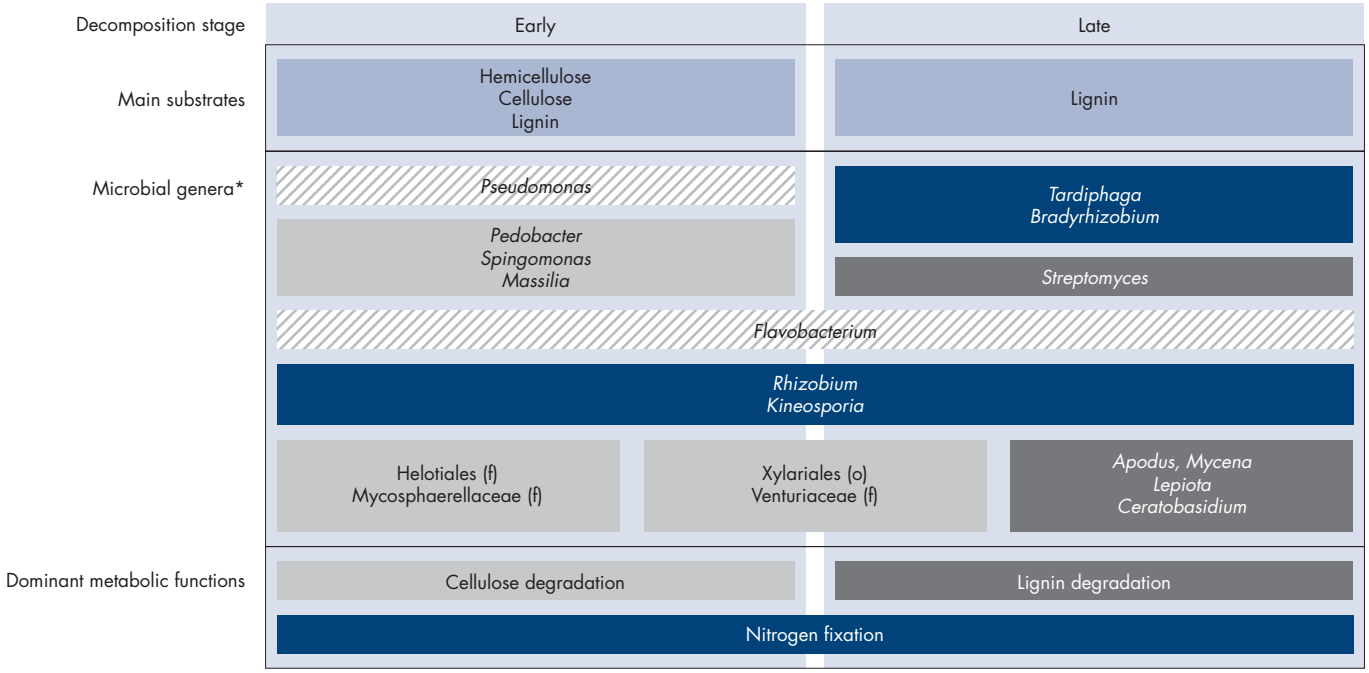

Figure 8. A schematic of leaf degradation showing decomposition stages, available substrates, dominant microorganisms present and metabolic functions. Dark blue: nitrogen-fixing microorganisms. **Light grey:** cellulose-degrading microorganisms. **Dark grey:** lignin-degrading microorganisms. **Striped:** cellulose- and lignin-degrading microorganisms. *Where genus-level identification was not achieved identification to the level of family (f) or order (o) is reported.

The bacterial community was initially dominated by *Spingomonas*, *Pseudomonas*, *Pedobacter*, *Massilia*, *Kineosporia*, *Flavobacterium* and *Rhizobium*. Some of these even persisted into later stages of decomposition (*Flavobacterium*, *Kineosporia* and *Rhizobium*). This is consistent with studies reporting *Sphingomonas* and *Pedobacter* species as cellulose degraders and *Pseudomonas* and *Flavobacterium* as able to degrade both cellulose and lignin (2, 3, 8). In the late stages of decomposition, the bacterial community was dominated by *Tardiphaga*, *Bradyrhizobium* and *Streptomyces*. Filamentous bacteria, such as *Streptomyces,* are highly efficient lignin degraders, consistent with a dominant role in the final stages of leaf litter decomposition (8).

We found the nitrogen-fixing bacteria *Rhizobium* and *Kineosporia* present at all stages of decomposition and *Bradyrhizobium* and *Tardiphaga* present and dominant in the community during the later stages. During decomposition, nitrogen resources decrease and the presence of bacteria able to accumulate atmospheric nitrogen might improve nitrogen resource availability, supporting other members of the microbial community.

Conclusion

Using decomposing leaf litter as a model, we have demonstrated the usability of QIAGEN Microbial Genomics Pro Suite for studying diverse environmental microbial communities with prokaryotic and microeukaryotic members. Our results correlate with those of earlier authors: we found that the observed changes in fungal and bacterial abundances correlate with the predominant metabolic capabilities required at the different stages of leaf decomposition in response to changing nutrient availability.

Further Reading

QIAGEN Microbial Genomics Pro Suite is a complete solution. All the tools required for analyzing amplicon data for metagenomic studies are integrated, and preconfigured workflows get new users started easily. Read more about the CLC Genomics Workbench and CLC Microbial Genomics Module here:

CLC Genomics Workbench
https://www.qiagenbioinformatics.com

CLC Microbial Genomics Module
https://www.qiagenbioinformatics.com

We have also prepared tutorials to illustrate the workflows described in this paper.

OTU Clustering step-by-step:

http://resources.qiagenbioinformatics.com/tutorials/OTU_Clustering_Steps.pdf OTU

Clustering using workflows:

https://www.qiagenbioinformatics.com/clc-microbial-genomics-module-resources/

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