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SHORT COMMUNICATION

Evaluation of Different Phyllosphere Sample Types for Parallel Metabarcoding of Fungi and Oomycetes in *Vitis vinifera*

Falk H. Behrens† **and Michael Fischer**

Julius Kühn-Institute, Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, 76833 Siebeldingen, Germany

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ABSTRACT

Metabarcoding is an effective and cost-efficient approach to study environmental microbiomes and has become a standard method in studying microbial community structures and relative species abundance. In grapevine research on leaf microbial communities, two kinds of sample types, either leaf wash sediments representing the phyllosphere microbiome from leaf surfaces or leaf tissue samples, e.g., leaf disks, including phyllosphere and endosphere microorganisms, are used to characterize leaf microbiomes. The goal of this study was to analyze the advantages and disadvantages of these sample preparation methods for the characterization of the phyllosphere microbiome by fungal metabarcoding with both sample types being processed from the exact same set of leaves. We used a metabarcoding strategy, which can detect Fungi and Oomycetes, facilitating the parallel analysis of these communities. At each sampling time point, species richness was shown to be higher in leaf wash samples, and differences in the community structure between samples was smaller for this sample type as well. Furthermore, by comparing read count abundance to qPCR measured relative proportions of selected amplicon sequence variants, a higher congruence was observed for leaf wash samples. Therefore, metabarcoding analyses of leaf samples using leaf wash sediments outperform analyses using leaf disks and should be applied to characterize phyllosphere fungal communities. As a second goal, we show that the direct comparison of metabarcoding libraries of both sample types prepared from the exact same set of leaves also provides a new strategy to identify endophytes that may not be culturable.

Keywords: metabarcoding, mycobiome, phyllosphere, *Vitis vinifera*

Studying plant associated microbiomes has become an important approach to gain a better understanding of plants and especially crop plant resilience in challenging environments (Hassani et al. 2019). Since culture-dependent microbiological methods are laborious and systemically underestimate microbial diversity of a given habitat, high-throughput sequencing-based parallel detection of PCR amplified marker gene sequences, called metabarcoding, has evolved to be the preferred method to assess microbiome composition (Bai et al. 2015; Francioli et al. 2021). Metabarcoding is a cost- and time-saving method, but it also comes with challenges and technical issues. Beside issues concerning the biological comparability of independently sampled material, or technical issues concerning DNA extraction (Ruppert et al. 2019), for the correct reconstruction of plant associated microbiomes, sampling strategy and sample preparation before DNA extraction can strongly affect the performance of the experiment, as well as experiment outcomes. In grapevine research, an increasing number of metabarcoding studies investigate the effects of environmental properties and their interactions (Abdelfattah et al. 2019; Castañeda et al. 2018; Fort et al. 2016; Liu and Howell 2021; Pinto et al. 2014; Singh et al. 2018; Zarraonaindia et al. 2015), viticultural practices and plant protection (Barroso-Bergadà et al. 2021; Castañeda et al. 2018; Gobbi et al. 2020; Kernaghan et al. 2017; Nerva et al. 2019; Perazzolli et al. 2014), or cultivars (Singh et al. 2018, 2019) on the bacterial and/or fungal microbiome of leaves. For the analysis of leaf microbiomes, there are two different sample preparation strategies, i.e., using leaf tissues directly or sediments from a leaf washing procedure, of which either one or the other has been used to extract DNA for metabarcoding in these studies. Choosing one of these sample types implies sample characteristics that must be evaluated considering the respective scientific question. If leaf tissue is directly used for DNA extraction, phyllosphere and endosphere organisms will be included. Fully expanded grapevine leaves have a size that makes it hard to use several pooled leaves for a sin-

†Corresponding author: F. H. Behrens; falk.behrens@julius-kuehn.de

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gle DNA extraction. To circumvent this, leaf disks can be cut (Barroso-Bergadà et al. 2021; Fort et al. 2016; Kernaghan et al. 2017), but leaf community composition might be biased by using only a small part of each leaf. By using sediments from leaf washing for DNA extraction (Castañeda et al. 2018; Singh et al. 2018), microbial material from the whole leaf surface is included. Therefore, this sample type can be considered to represent phyllosphere microbes. In this study, we employed a metabarcoding strategy that, by the choice of PCR primers, is capable of simultaneously detecting Fungi and Oomycetes while avoiding the amplification of plant host DNA. By preparing each sample type from the same set of leaves, we comparatively analyzed the characteristics and performance of leaf disk and leaf wash samples from grapevine leaves taken at two different sampling time points from three different organic plant protection regimes. We aim to emphasize advantages and disadvantages of these sample types as well as potentially new insights on endophyte identification that arise from the combination of both sample types.

MATERIALS AND METHODS

Eighteen fully expanded *Vitis vinifera* 'Müller-Thurgau' leaves per sample were collected at two sampling time points (02.06.2020 and 30.06.2020) at an experimental vineyard located near Neustadt an der Weinstraße, Germany (49.368088, 8.185871). At each time point, leaves of untreated (untreated control), sulphur (Stulln; Agrostulln GmbH, Germany) treated, or sulphur and copper (Funguran progress; Spiess-Urania Chemicals GmbH, Germany) treated vines were sampled in three repetitions from randomized blocks, resulting in 18 leaf pool samples. Samples from different time points and plant protection regimes were chosen to ensure diverging microbial communities. Sampling was performed at least 1 week after the last plant protection measure. Leaf samples were collected in sterile plastic bags (200 mm × 300 mm; Carl Roth, Germany), stored at 4°C, and processed on the next day.

Two sample types, leaf disk samples and leaf wash samples, were prepared from each leaf pool. First, a cork borer (1 cm) was used to cut one disk from the middle part of each leaf including a major vein. Sample wise pooled leaf disks were ground in liquid nitrogen using a mortar and pestle. About 100 mg of leaf tissue was transferred to a 2-ml tube and supplemented with 250 mg of Zirkonia/glass beads (0.5 mm, Carl Roth). Second, in the original plastic bags, the same leaves were supplemented with 300 ml of sterile leaf wash buffer (0.9% NaCl, 0.01% Tween 80; both Carl Roth) and incubated on an orbital shaker at 150 rpm for 1 h. From each sample, in total 150 ml of leaf wash solution was centrifuged in 50-ml tubes (Greiner Bio-One, Austria) (10,000 × *g*; 4°C; 20 min). After removing the supernatant, the remaining sediments from all three tubes were pooled in 2 ml of sterile double distilled H_2O and transferred into a 2-ml tube (Eppendorf, Germany) for a second centrifugation (max. g; 4°C; 20 min). The supernatant was discarded, and 250 mg of Zirkonia/glass beads (0.5 mm, Carl Roth) was added to each tube. For DNA extraction of both sample types, the Plant DNA Mini Kit (VWR, U.S.A.) was used according to the manufacturer's instructions using a starting amount of 600 µl of SP1 buffer. After adding SP1 buffer, all samples were homogenized using a TissueLyser II (Qiagen, Germany) at 30 Hz for 2 × 3 min before proceeding with the protocol.

Library preparation and sequencing were carried out by All Genetics & Biology SL (www.allgenetics.eu) according to the following protocol. The fungal internal transcribed spacer (ITS1) region was amplified using the ITS1catta (5′-ACCWGCGGARGGATCATTA-3′)/ITS2ngs (5′-TTYRCKRCGTTCTTCATCG-3′) primer combination (Tedersoo et al. 2018; Tedersoo and Anslan 2019) which, due to the specific 3′-end of the forward primer, preferably amplifies fungal and oomycete rather than plant ITS1 regions. To these primers, Illumina sequencing primers were attached at their 5′ ends. PCRs (95°C/5 min; 95°C/30 s, 48°C/45 s, 72°C/45 s for 35 cycles; 72°C/7 min) were performed with 2.5 µl of template DNA, 0.5 µM of the primers, 12.5 µl of Supreme NZYTaq 2× Green Master Mix (NZYTech, Portugal) and ultrapure water up to 25 µl. For multiplexing, oligonucleotide indices were attached in a second PCR round with identical conditions but using only five cycles and 60°C as the annealing temperature. The libraries were run on a 2% agarose gel stained with GreenSafe (NZYTech) and imaged under UV light to verify the amplicon size (100 to 600 bp). Libraries were purified using Mag-Bind RXNPure Plus magnetic beads (Omega Biotek, U.S.A.) following the manufacturer's instructions. Then, these were pooled in equimolar amounts according to the quantification data provided by the Qubit dsDNA HS Assay (Thermo Fisher Scientific, U.S.A.). The pool was sequenced in a MiSeq PE300 run (Illumina, U.S.A.). Negative controls for DNA extraction (K) and template free library preparation (BPCR) were included to check for contamination. To assess the overall quality of sequencing and data processing, an artificial mock community containing equal amounts of DNA from 12 different *in vitro* cultivated fungal species (*Fomitiporia mediterranea*, *Stereum hirsutum*, *Bjerkandera adusta*, *Botrytis cinerea*, *Aureobasidium pullulans*, *Gibellulopsis nigrescens*, *Phaeoacremonium angustius*, *Fusarium avenaceum*, *Fusarium culmorum*, *Aspergillus ochraceous*, *Phaeomoniella clamydospora*, and *Pichia kluyveri*) was sequenced (Mock).

A metabarcoding analysis pipeline processing only forward read sequences was implemented (Pauvert et al. 2019). Data preparation and analysis was performed with R v3.6.3 (R core Team 2020), and *tidyverse* v1.3.1 packages (Wickham et al. 2019) were used for general data processing. Primers were clipped using Cutadapt v3.4 (Martin 2011) and *ShortRead* v1.44.3 (Morgan et al. 2009). Quality filtering, denoising with independent sample inference, removing of chimeras and taxonomic assignment of amplicon sequence variants (ASVs) against the UNITE v8.2 database (Abarenkov et al. 2010) were conducted with the *dada2* v1.14.1 package (Callahan et al. 2016). Raw sequence data have been submitted to the European Nucleotide Archive (https://www.ebi.ac.uk/ena/browser/home) with accession number PRJEB48638.

Sample type specific occurrence of individual ASVs, ASV richness, β-diversity, and abundance of specific ASVs were analyzed and plotted with the R packages *phyloseq* v1.30.0 (McMurdie and Holmes 2013), *vegan* v2.5.7 (Oksanen et al. 2020), *cowplot* v1.1.1 (Wilke 2019), *ggpubr* v0.4.0 (Kassambara 2020), *ranacapa* v0.1.0 (Kandlikar 2021), *lme4* v1.1.27.1 (Bates et al. 2015), *emmeans* v1.6.2.1 (Length 2021), *multcomp* v1.4.17 (Hothorn et al. 2008), *biostat* v0.0.20 (Gegzna 2020), *ggpmisc* v0.4.3 (Aphalo 2021), and *DESeq2* v1.26.0 (Love et al. 2014). The R script for filtering out contaminants with negative control samples (Galan et al. 2016) was adapted from C. Pauvert (https://gist.github.com/cpauvert). All community analyses were performed with ASVs restricted to the kingdoms of Fungi and Stramenopila with individual ASVs present in at least two samples. Sample wise ASV richness was analyzed by plotting rarefaction curves and a generalized linear mixed effect model assuming negative binomial distribution and a logarithmic link function. Fixed factors were sample type, sampling time point, treatment, and the interaction of sample type with the other two factors and block was considered as a random factor. Based on this model, an analysis of variance (type II Wald chi-square tests) was calculated. A post hoc Tukey test based on estimated marginal means was conducted to directly compare significant factor combinations. β-diversity is

displayed by non-metric multidimensional scaling (NMDS) of Jaccard dissimilarities (binary). Permutational analyses of variance (PERMANOVA) with 999 permutations, constrained within blocks, were conducted to evaluate the influence of sample type, sampling time point, treatment, and the interaction of sample type with the other two factors on the community composition. Within-group Jaccard distances were plotted in dependence on sample type and sampling time point and pairwise Wilcoxon rank sum tests for paired data assuming heteroscedasticity were performed. *P* values were adjusted by the Benjamini-Hochberg procedure.

Read count normalization by median-of-ratios from DESeq2 and $\log_2$ transformation was performed for the abundance estimation of single ASVs. For comparisons to qPCR analyses, only samples with more than 10 normalized counts for the respective ASV were considered.

Sample wise qPCR quantification of gDNA was performed with specific primers amplifying ASV/species specific ITS regions (*A. pullulans* (ASV 2)–GATCATTAAAGAGTAAGGGTGCT/AAATTTTAACTCAGACGACCG; *Epicoccum dendrobii* (ASV 4)–CTTCGGTCTGCTACCTCTTAC/AACTGCAAAGGGTTTGAATG). ITS primers from the metabarcoding library preparation were used to quantify overall fungal/oomycete abundance. Dilution series of external gDNA standards prepared from pure culture derived gDNA of the respective species and a fungal/oomycete gDNA mixture were used to calculate target gDNA amounts. Dilution series started between 0.85 and 1.9 ng/µl gDNA and covered six or seven 1:10 dilution steps. Further dilutions lead to cycle threshold values with high standard deviations and were considered to be not reliable. Samples with gDNA concentrations that were not covered by the range of the standard dilution series were removed from the analysis. Non-template controls were included to detect contaminations. All sample qPCR reactions were run in triplicates and averaged threshold cycle (Ct) values were used for the calculation of DNA concentrations. A two-step PCR protocol was run on an Applied Biosystems 7500 fast Real-Time PCR System (U.S.A.) (50°C/2 min; 95°C/10 min; 95°C/15 s, 58°C/30 s, for 40 cycles) followed by melt curve analyses. Reaction mixtures consisted of 5 µl of Luna Universal qPCR Master Mix (NEB, U.S.A.) including ROX, 0.25 µl of a 10 mM primer solution for the forward and reverse primer, respectively, 1 µl of template gDNA, and 3.5 µl of DEPC-treated water. Measured DNA concentrations were normalized by the overall fungal/oomycete DNA amount derived from the ITS-qPCR and $\log_2$ transformed resulting in species-specific relative abundances. To compare metabarcoding and qPCR derived abundances, both results were plotted against each other for each sample type, respectively. Regression equations and Spearman's rank correlation coefficients between metabarcoding and qPCR were calculated.

Scripts for data processing and analysis as well as raw qPCR data can be accessed via https://github.com/fhbw/phyllo_meta_fungi.

RESULTS

To directly compare different types of leaf sample preparation procedures for metabarcoding analyses of Fungi and Oomycetes, we generated leaf disk and leaf wash samples from 18 grapevine leaf pools. To ensure varying community compositions, samples were taken from plots with three different organic plant protection strategies at two different time points. After filtering, across all samples in total 518 ASVs could be assigned to the kingdoms of Fungi and Stramenopila. For leaf disk and leaf wash samples, these were 341 and 485 ASVs, respectively. Checking for unintended host sequences showed that only 0.29% leaf disk reads and 0% leaf wash reads were assigned to the order Vitales. Directly comparing both sample types for the specific occurrence of individual ASVs at each time point shows that considering all Fungi and Stramenopila, 56 and 44 ASVs as well as 204 and 130 ASVs were restricted to leaf disk and leaf wash samples, respectively, whereas 216 and 145 ASV were detected in both sample types, at the given time points (Fig. 1). Overall, most sequences were assigned to the phylum Ascomycetes (350) and Basidiomycetes (117), but also 37 ASVs from the phylum Oomycetes were identified.

Testing if the observed number of ASVs is differing between sample types, we compared ASV richness between leaf disk and leaf wash samples. Rarefaction curves indicate a saturation in ASV detection for all samples, even for those with a relatively low amount of reads (Fig. 2A). The effects of sample type (Wald $\chi^2 = 216.848$, $P < 0.001$), sampling time point (Wald $\chi^2 = 26.822$, $P < 0.001$), and their interaction (Wald $\chi^2 = 17.136$, $P < 0.001$) were found to affect ASV richness and at both time points significantly more ASVs were detected in leaf wash samples (Fig. 2B). Using (binary) Jaccard dissimilarities, visualized by NMDS (2D stress = 0.164; linear $R^2 = 0.869$), to analyze β-diversity between samples, sample type (PERMANOVA: $F_{1,35} = 5.465$, $P \leq 0.001$), sampling time point (PERMANOVA: $F_{1,35} = 6.883$, $P \leq 0.001$) as well as the interaction of these factors (PERMANOVA: $F_{1,35} = 2.098$, $P = 0.015$) were found to significantly influence community

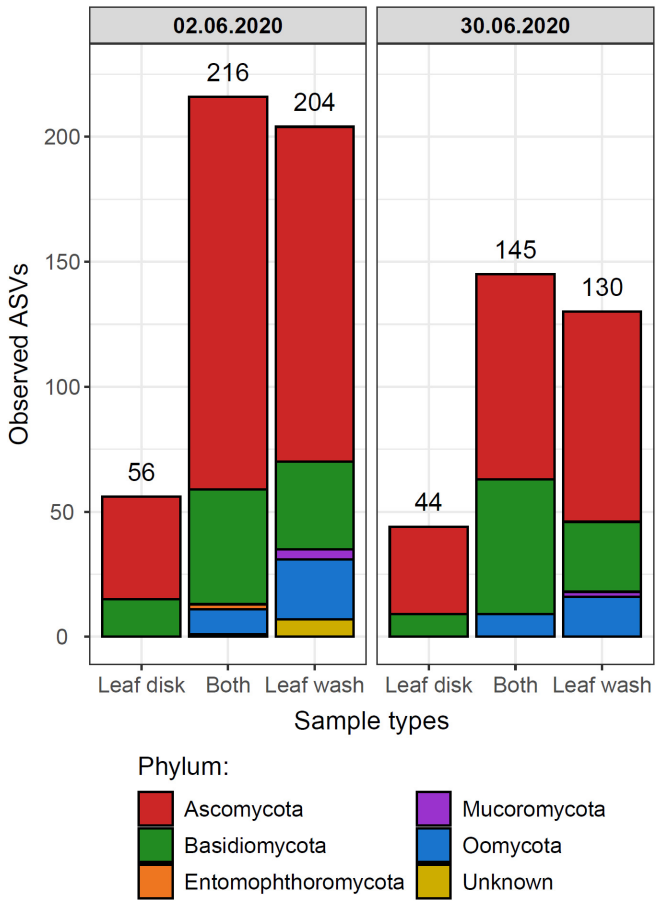

Fig. 1. Specific and shared occurrence of individual amplicon sequence variants (ASVs) between leaf disk and leaf wash samples. All ASVs assigned to the kingdoms Fungi and Stramenopila being detected in at least two samples are grouped for their occurrence in only one of the respective, or both sample types for both sampling time points, respectively. The total number of individual ASVs in each group is indicated above the respective bar, and different colors indicate phylum level classification.

composition (Fig. 3A). Furthermore, the within-group distances of these factor combinations are higher and/or more scattered for leaf disk samples indicating a higher congruence of leaf wash samples (Fig. 3B).

To evaluate the capability of sample types for correctly analyzing the relative abundance of individual ASVs, we directly compared metabarcoding derived read count abundances to qPCR measured amounts of ASV abundance relative to the total fungal biomass. For

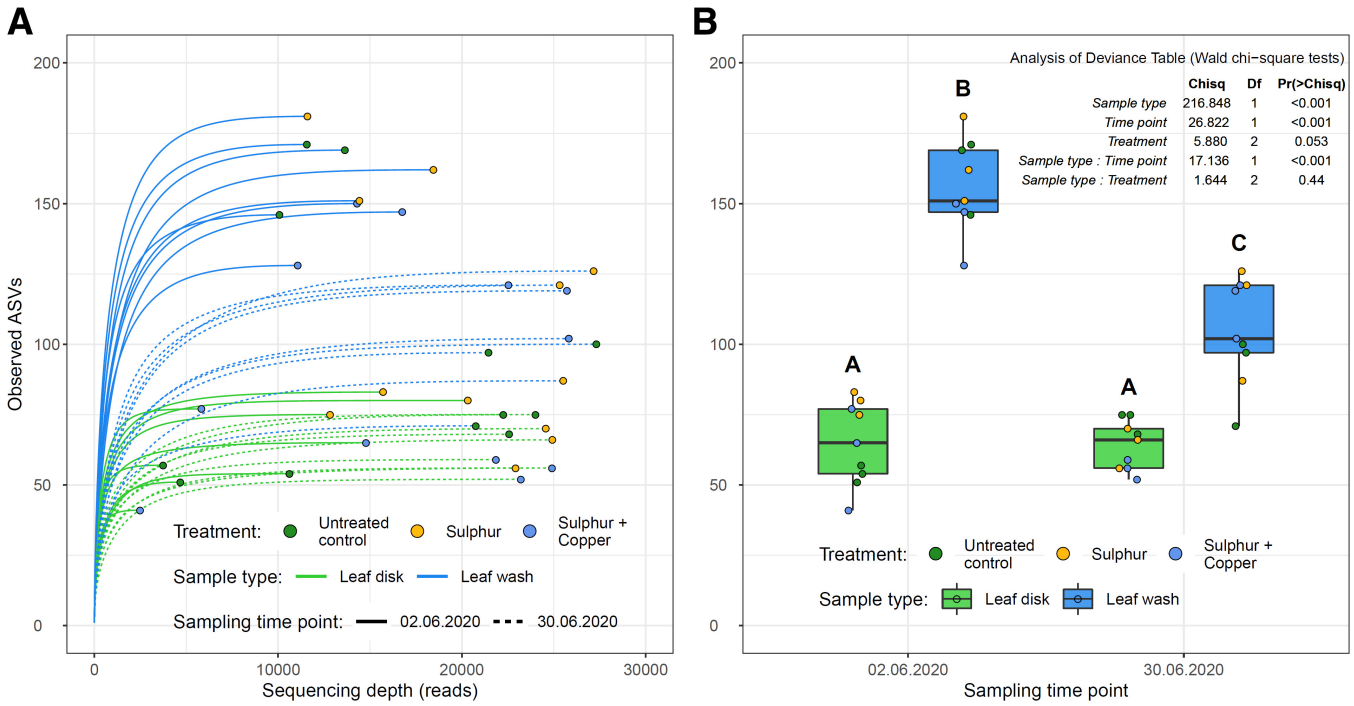

Fig. 2. Alpha diversity metrics to compare leaf disk and leaf wash samples. **A,** Rarefaction curves show the saturation level of amplicon sequence variants (ASVs) detection for each sample. **B,** ASV richness is plotted in dependence on sample type, sampling time point, and treatment. The analysis of variance table (Type II Wald chi-square tests) displays significant factors and interaction terms. Significant differences between groups of factor combinations determined by the post hoc Tukey test based on estimated marginal means are indicated by differing letters ($P \leq 0.05$).

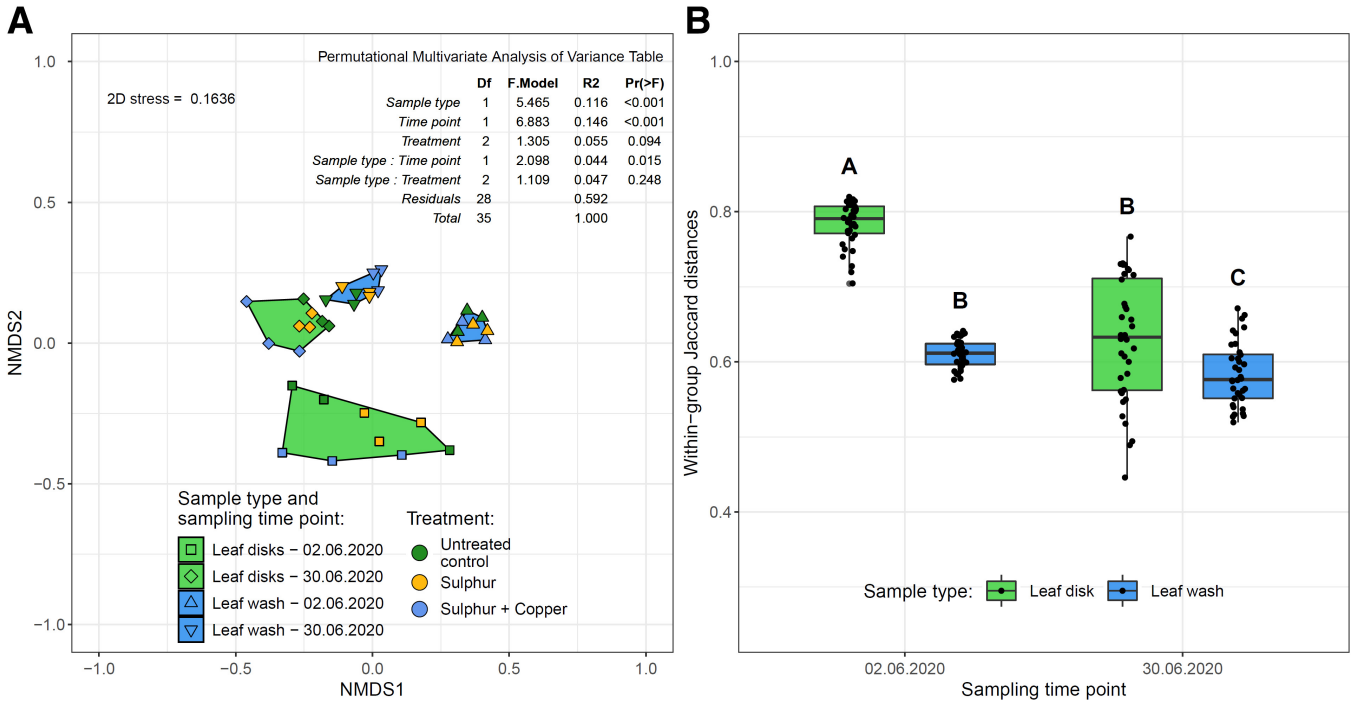

Fig. 3. β-diversity metrics to compare leaf disk and leaf wash samples. **A,** Non-metric dimensional scaling (NMDS) ordinations from (binary) Jaccard distances show differences in the amplicon sequence variants community composition of samples. The permutational analyses of variance table (999 permutations) displays significance ($P(>F)$) and variation explained (R^2) by factors and interaction terms. **B,** Within-group Jaccard distances ($n = 36$) for the same factor combinations are displayed by boxplots, and pairwise Wilcoxon rank sum tests were conducted. *P* values were adjusted by the Benjamini-Hochberg procedure, and differing letters indicate significant differences between groups ($P \leq 0.05$).

that, two ASVs (ASV 2, *A. pullulans*; and ASV 4, *E. dendrobii*) with mutually changing read count abundances across the time points were chosen for an exemplarily analysis. In Figure 4, for both ASVs a higher correlation between metabarcoding and qPCR-Analysis can be observed for leaf wash samples ($\rho = 0.89$ and 0.87) compared with leaf disks ($\rho = 0.67$ and 0.6). In addition, for leaf wash samples the slope of the regression equations is 1.3 and 1.2, respectively, while it is only 0.86 and 0.54 for leaf disk samples, indicating a biased relationship between both types of analyses for the latter sample type.

DISCUSSION

Parallel detection of Fungi and Oomycetes by metabarcoding can be of interest, e.g., when oomycete pathogens play a central role for the organism or tissue that is studied by mycobiome analyses. Most commonly used primer combinations do not cover Oomycete detection and/or do not prevent host DNA amplification. As observed for the ITS1catta primer by Tedersoo and Anslan (2019), choosing the ITS1catta/ITS2ngs primer combination successfully led to the detection of many oomycete ASVs and efficiently avoided

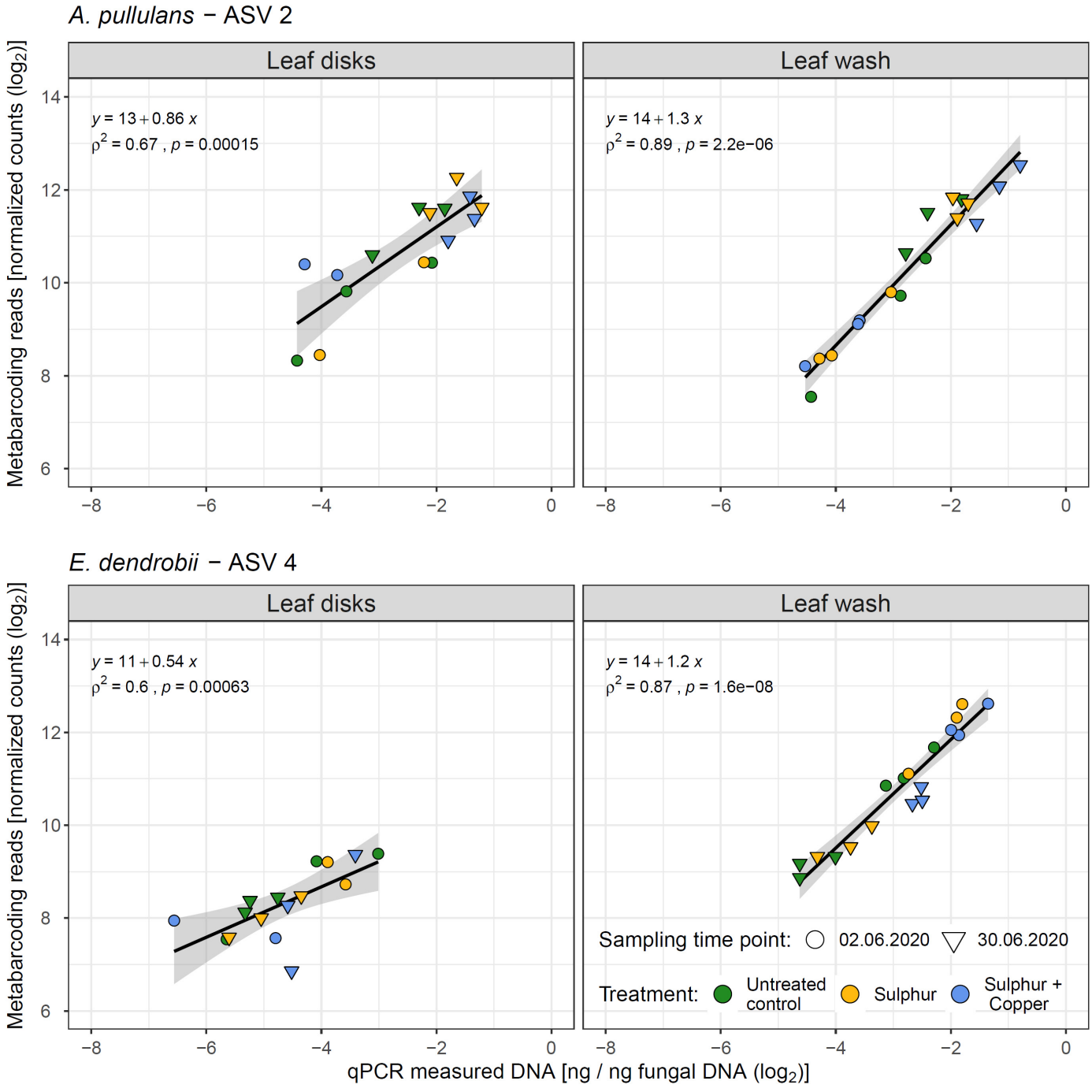

Fig. 4. Relation between metabarcoding and qPCR abundance of individual amplicon sequence variants (ASVs) for leaf disk and leaf wash samples. Normalized $\log_2$ metabarcoding read counts are plotted against qPCR measured $\log_2$ proportional amounts of ASV DNA. The analyzed ASVs, ASV 2 and ASV 4, are assigned to the species *Aureobasidium pullulans* and *Epicoccum dendrobii*, respectively. Plots and data are displayed and calculated for each sample type/ASV combination separately. Regression equation is calculated using a linear model. Gray shaded region represents the 95% confidence region, and Spearman's rank correlation coefficients are displayed with their respective *P* values.

host amplicon amplification, even in leaf disk samples in which most of the DNA comes from grapevine. Hence, as the oomycete *Plasmopara viticola* is one of the most devastating pathogens in grapevine (Gessler et al. 2011), this primer combination proved to be very advantageous for metabarcoding experiments that are related to the phytosanitary situation of grapevine leaves.

The choice of an appropriate sample preparation method largely affects the success of metabarcoding experiments. In this study, we have comparatively analyzed the properties of pooled leaf disk or leaf wash samples, two methods that are frequently used in metabarcoding experiments from grapevine leaves. Considering all samples at each sampling time point, we observed many more sequences being specific for leaf wash samples compared with leaf disks. Therefore, by choosing leaf disks, which represent only a small part of each leaf, many phyllosphere ASVs can be overlooked. Analyses on endophytes are typically performed by the incubation of surface-sterilized leaf segments on culture media followed by the isolation of microbes (Bruisson et al. 2019; Gomes et al. 2018). Also, some metabarcoding studies on endophytes work with leaf sterilizing procedures (Milazzo et al. 2021; Rojas et al. 2020), which is likely to increase the relative abundance of endophyte DNA but cannot eliminate all foliar traces. In this case, community data analyses on endophytes can be conducted, as traces of epiphytic DNA are negligible in many calculations, but the identified ASVs/operational taxonomic units (OTUs) cannot per se be considered to be endophytes. By preparing leaf disk and leaf wash samples from the same set of leaves, we identified endophytic ASVs by their occurrence in solely leaf disk samples. Due to our filtering and analysis parameters, these ASVs were detected in at least two independent leaf disk samples and no leaf wash sample at the given time point. By this procedure, new and potentially not cultivable endophytes can be identified. In this study, the order of cutting leaf disks followed by the preparation of leaf wash samples from the same leaf pools was chosen, because the main goal was to compare the fungal communities of both sample types with emphasis on the phyllosphere. However, changing the order of preparation of sample types would be advantageous for leaf wash samples and the identification of endophytes. By this, for leaf wash samples the area of cut leaf disks is not withdrawn before washing, and for leaf disk samples the detection of endophytes will be more pronounced with epiphytic microorganisms being washed off before sampling.

To point out differences in the observed community structures, we compared ASV richness and β-diversity between both sample types. Likely, due to the larger leaf area that is comprised in leaf wash samples, at both sampling time points ASV richness was significantly higher in this sample type. In addition, the differences between the samples, as displayed by NMDS and within-group Jaccard distances, are much smaller for this sample type, as well. Hence, leaf wash samples give a deeper insight into phyllosphere communities, while at the same time comprise less variation between samples. This can be particularly advantageous when leaf mycobiome compositions are not fixed and more complex, as observed in several studies for earlier time points of the growing season (Fort et al. 2016; Gobbi et al. 2020; Liu and Howell 2021).

Apart from analyzing community structures, reliable differential abundance analyses are an important criterion for microbiome analyses. In this regard, much attention was drawn to optimizing read count normalization procedures (Weiss et al. 2017), but a methodical independent evaluation of ASV/OTU-specific read count abundances is rarely reported. Albaina et al. (2016) and Murray et al. (2011) have observed a good correlation of high-throughput amplicon sequencing data and qPCR measurements of the proportional amount of prey species sequences in the diets of fish and penguins, respectively. Here we assessed the performance of leaf disk and leaf wash samples for differential abundance analyses by comparing metabarcoding read counts to qPCR measured relative proportion of ASV DNA for two frequently detected ASVs. Strikingly, for both ASVs the correlation between metabarcoding and qPCR measurements are considerably higher for leaf wash samples. Hence, the much higher congruence of both analyses emphasizes the accuracy of metabarcoding differential abundance analyses using leaf wash samples.

In conclusion, for analyses of phyllosphere communities and ASV abundance, leaf wash samples were shown to be superior to leaf disks and should be the favored sample type, if endophytes do not have to be included in the analysis. Further studies covering a higher number of observations and more sampling time points could solidify these findings. The data presented in this study originate from grapevine, but the obtained conclusions might be transferable to other plant species, especially those with large leaves.

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LITERATURE CITED

Abarenkov, K., Nilsson, R. H., Larsson, K-H., Alexander, I. J., Eberhardt, U., Erland, S., Høiland, K., Kjøller, R., Larsson, E., Pennanen, T., Sen, R., Taylor, A. F. S., Tedersoo, L., Ursing, B. M., Vrålstad, T., Liimatainen, K., Peintner, U., and Kõljalg, U. 2020. The UNITE database for molecular identification of fungi-Recent updates and future perspectives. New Phytol. 186:281-285.

Abdelfattah, A., Sanzani, S. M., Wisniewski, M., Berg, G., Cacciola, S. O., and Schena, L. 2019. Revealing cues for fungal interplay in the plant-air interface in vineyards. Front. Plant Sci. 10:922.

Albaina, A., Aguirre, M., Abad, D., Santos, M., and Estonba, A. 2016. 18S rRNA V9 metabarcoding for diet characterization: A critical evaluation with two sympatric zooplanktivorous fish species. Ecol. Evol. 6:1809-1824.

Aphalo, P. J. 2021. ggpmisc: Miscellaneous Extensions to 'ggplot2'. R package version 0.4.3. https://cran.r-project.org/web/packages/ggpmisc/ggpmisc.pdf

Bai, Y., Müller, D. B., Srinivas, G., Garrido-Oter, R., Potthoff, E., Rott, M., Dombrowski, N., Münch, P. C., Spaepen, S., Remus-Emsermann, M., Hüttel, B., McHardy, A. C., Vorholt, J. A., and Schulze-Lefert, P. 2015. Functional overlap of the Arabidopsis leaf and root microbiota. Nature 528: 364-369.

Barroso-Bergadà, D., Pauvert, C., Vallance, J., Delière, L., Bohan, D. A., Buée, M., and Vacher, C. 2021. Microbial networks inferred from environmental DNA data for biomonitoring ecosystem change: Strengths and pitfalls. Mol. Ecol. Resour. 21:762-780.

Bates, D., Mächler, M., Bolker, B., and Walker, S. 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67:1-48.

Bruisson, S., Zufferey, M., L'Haridon, F., Trutmann, E., Anand, A., Dutartre, A., Vrieze, M., and Weisskopf, L. 2019. Endophytes and epiphytes from the grapevine leaf microbiome as potential biocontrol agents against phytopathogens. Front. Microbiol. 10:2726.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods 13:581-583.

Castañeda, L. E., Miura, T., Sánchez, R., and Barbosa, O. 2018. Effects of agricultural management on phyllosphere fungal diversity in vineyards and the association with adjacent native forests. PeerJ 6:e5715.

Fort, T., Robin, C., Capdevielle, X., Delière, L., and Vacher, C. 2016. Foliar fungal communities strongly differ between habitat patches in a landscape mosaic. PeerJ 4:e2656.

Francioli, D., Lentendu, G., Lewin, S., and Kolb, S. 2021. DNA metabarcoding for the characterization of terrestrial microbiota-pitfalls and solutions. Microorganisms 9:361.

Galan, M., Razzauti, M., Bard, E., Bernard, M., Brouat, C., Charbonnel, N., Dehne-Garcia, A., Loiseau, A., Tatard, C., Tamisier, L., Vayssier-Taussat, M.,

Vignes, H., Cosson, J.-F., and Bik, H. 2016. 16S rRNA amplicon sequencing for epidemiological surveys of bacteria in wildlife. MSystems 1: e00032-16.
Gegzna, V. 2020. biostat: Routines for Basic (Bio)Statistics. R package version 0.0.20. https://gegznav.github.io/biostat/
Gessler, C., Pertot, I., and Perazzolli, M. 2011. *Plasmopara viticola*: A review of knowledge on downy mildew of grapevine and effective disease management. Phytopathol. Mediterr. 50:3-44.
Gobbi, A., Kyrkou, I., Filippi, E., Ellegaard-Jensen, L., and Hansen, L. H. 2020. Seasonal epiphytic microbial dynamics on grapevine leaves under biocontrol and copper fungicide treatments. Sci. Rep. 10:681.
Gomes, T., Pereira, J. A., Benhadi, J., Lino-Neto, T., and Baptista, P. 2018. Endophytic and epiphytic phyllosphere fungal communities are shaped by different environmental factors in a Mediterranean ecosystem. Microb. Ecol. 76:668-679.
Hassani, M. A., Özkurt, E., Seybold, H., Dagan, T., and Stukenbrock, E. H. 2019. Interactions and coadaptation in plant metaorganisms. Annu. Rev. Phytopathol. 57:483-503.
Hothorn, T., Bretz, F., and Westfall, P. 2008. Simultaneous inference in general parametric models. Biom. J. 50:346-363.
Kandlikar, G. 2021. ranacapa: Utility Functions and 'shiny' App for Simple Environmental DNA. R package version 0.1.0. https://rdrr.io/github/gauravsk/ranacapa/
Kassambara, A. 2020. ggpubr: 'ggplot2' Based Publication Ready Plots. R package version 0.4.0. https://rpkgs.datanovia.com/ggpubr/
Kernaghan, G., Mayerhofer, M., and Griffin, A. 2017. Fungal endophytes of wild and hybrid Vitis leaves and their potential for vineyard biocontrol. Can. J. Microbiol. 63:583-595.
Lenth, R. V. 2021. emmeans: Estimated marginal means, aka least-squares means.: R package version 1.6.1. https://cran.r-project.org/web/packages/emmeans/index.html
Love, M. I., Huber, W., and Anders, S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15: 550.
Liu, D., and Howell, K. 2021. Community succession of the grapevine fungal microbiome in the annual growth cycle. Environ. Microbiol. 23: 1842-1857.
Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal 17:10-12.
McMurdie, P. J., and Holmes, S. 2013. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8:e61217.
Milazzo, C., Zulak, K. G., Muria-Gonzalez, M. J., Jones, D., Power, M., Bransgrove, K., Bunce, M., and Lopez-Ruiz, F. J. 2021. High-throughput metabarcoding characterizes fungal endophyte diversity in the phyllosphere of a barley crop. Phytobiomes J. 5:316-325.
Morgan, M., Anders, S., Lawrence, M., Aboyoun, P., Pagès, H., and Gentleman, R. 2009. ShortRead: A bioconductor package for input, quality assessment and exploration of high-throughput sequence data. Bioinformatics 25:2607-2608.
Murray, D. C., Bunce, M., Cannell, B. L., Oliver, R., Houston, J., White, N. E., Barrero, R. A., Bellgard, M. I., and Haile, J. 2011. DNA-based faecal dietary analysis: A comparison of qPCR and high throughput sequencing approaches. PLoS One 6:e25776.
Nerva, L., Pagliarani, C., Pugliese, M., Monchiero, M., Gonthier, S., Gullino, M. L., Gambino, G., and Chitarra, W. 2019. Grapevine phyllosphere community analysis in response to elicitor application against powdery mildew. Microorganisms 7:662.
Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E., and Wagner, H. 2020. vegan: Community Ecology Package. R package version 2.5-7. https://cran.r-project.org/web/packages/vegan/index.html
Pauvert, C., Buée, M., Laval, V., Edel-Hermann, V., Fauchery, L., Gautier, A., Lesur, I., Vallance, J., and Vacher, C. 2019. Bioinformatics matters: The accuracy of plant and soil fungal community data is highly dependent on the metabarcoding pipeline. Fungal Ecol. 41:23-33.
Perazzolli, M., Antonielli, L., Storari, M., Puopolo, G., Pancher, M., Giovannini, O., Pindo, M., and Pertot, I. 2014. Resilience of the natural phyllosphere microbiota of the grapevine to chemical and biological pesticides. Appl. Environ. Microbiol. 80:3585-3596.
Pinto, C., Pinho, D., Sousa, S., Pinheiro, M., Egas, C., and Gomes, A. C. 2014. Unravelling the diversity of grapevine microbiome. PLoS One 9:e85622.
R Core Team. 2020. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
Rojas, E. C., Sapkota, R., Jensen, B., Jørgensen, H. J. L., Henriksson, T., Jørgensen, L. N., Nicolaisen, M., and Collinge, D. B. 2020. Fusarium head blight modifies fungal endophytic communities during infection of wheat spikes. Microb. Ecol. 79:397-408.
Ruppert, K. M., Kline, R. J., and Rahman, M. S. 2019. Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. Glob. Ecol. Conserv. 17:e00547.
Singh, P., Santoni, S., This, P., and Péros, J.-P. 2018. Genotype-environment interaction shapes the microbial assemblage in grapevine's phyllosphere and carposphere: An NGS approach. Microorganisms 6:96.
Singh, P., Santoni, S., Weber, A., This, P., and Péros, J.-P. 2019. Understanding the phyllosphere microbiome assemblage in grape species (*Vitaceae*) with amplicon sequence data structures. Sci. Rep. 9:14294.
Tedersoo, L., and Anslan, S. 2019. Towards PacBio-based pan-eukaryote metabarcoding using full-length ITS sequences. Environ. Microbiol. Rep. 11:659-668.
Tedersoo, L., Tooming-Klunderud, A., and Anslan, S. 2018. PacBio metabarcoding of Fungi and other eukaryotes: Errors, biases and perspectives. New Phytol. 217:1370-1385.
Weiss, S., Xu, Z. Z., Peddada, S., Amir, A., Bittinger, K., Gonzalez, A., Lozupone, C., Zaneveld, J. R., Vázquez-Baeza, Y., Birmingham, A., Hyde, E. R., and Knight, R. 2017. Normalization and microbial differential abundance strategies depend upon data characteristics. Microbiome 5:27.
Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J., Kuhn, M., Pedersen, T., Miller, E., Bache, S., Müller, K., Ooms, J., Robinson, D., Seidel, D., Spinu, V., Takahashi, K., Vaughan, D., Wilke, C., Woo, K., and Yutani, H. 2019. Welcome to the tidyverse. J. Open Source Software 4:1686.
Wilke, C. O. 2019. cowplot: Streamlined Plot Theme and Plot Annotations for 'ggplot2'. R package version 1.1.1. https://cran.r-project.org/web/packages/cowplot/index.html
Zarraonaindia, I., Owens, S. M., Weisenhorn, P., West, K., Hampton-Marcell, J., Lax, S., Bokulich, N. A., Mills, D. A., Martin, G., Taghavi, S., van der Lelie, D., and Gilbert, J. A. 2015. The soil microbiome influences grapevine-associated microbiota. mBio 6:e02527-14.