Metabarcoding for *Phytophthora*—Benefits and Limitations[1]

Neelam Redekar,[2] Joyce Eberhart,[2] Ebba Peterson,[3] and Jennifer Parke[2,3]

Abstract

The metabarcoding approach has revolutionized the study of microbial ecology with its ability to detect microbial DNA at an unprecedented depth and coverage. This method exploits high-throughput sequencing and DNA barcoding for identification of microbial species, including novel and uncultivable microbes. The metabarcoding approach is gaining more interest by the *Phytophthora* scientific community for monitoring the existence and spread of *Phytophthora* species in diverse habitats. Understanding and mitigating metabarcoding limitations is crucial for achieving better resolution and accurate determination of the diversity of *Phytophthora* species.

Culture-based methods rely on Sanger sequencing for species identification. This approach is limited to detection of culturable species and is a low throughput method that takes a very long time to process a small number of samples. The metabarcoding approach, on the other hand, relies on a next generation high throughput sequencing platform such as Illumina MiSeq that allows for detection of the entire targeted microbial community in the sample at once. Any environmental sample (soil, water, plant, animal) containing a mixture of microbial populations could be processed with the metabarcoding approach in which DNA is first extracted from the sample, amplified with primers specific to markers loci (such as ITS, COX), and the amplified product is then directly sequenced on the Illumina MiSeq sequencer. These 300 bp long Illumina sequences are clustered into operational taxonomic units (or OTUs) based on sequence identity. The OTU sequences are then compared against the sequences of known species for identification. The availability of 384 unique Illumina sequencing barcodes facilitates sample tagging and pooling ('multiplexing') prior to sequencing, and separation of sequences between different samples ('demultiplexing') post sequencing, making metabarcoding a high throughput approach. We have employed this approach to study *Phytophthora* communities in nursery irrigation water, streams and lakes; at multiple native plant restoration sites; and to test the efficacy of disinfestation methods such as soil solarization and chlorination.

Several universal primers targeting genomic or mitochondrial marker regions are available for *Phytophthora* identification, however these primers have not been tested for every known

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[2] Department of Crop and Soil Sciences, Oregon State University, Corvallis, OR 97331.
[3] Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331.
Corresponding author: N. Redekar, Neelam.Redekar@oregonstate.edu.

Phytophthora species (Bilodeau and others 2014, Cooke and others 2000, Robideau and others 2011, White and others 1990). Moreover, any mismatch in the priming site could affect the rate of amplification of the target DNA. For example, ITS6 and ITS7 primers are considered to be universal for oomycete amplification (Cooke and others 2000), however, certain *Phytophthora* species with mismatching ITS7 primer binding sites are less likely to be amplified with this set of primers (Redekar and others 2019, Sapkota and Nicolaisen 2015).

Species identification also relies on the availability of an up-to-date and reliable reference sequence database that includes marker sequences from all isolates of known species. There are four reliable reference sequence databases available for *Phytophthora* species identification (Abad and others 2019, Bilodeau and others 2014, Grünwald and others 2011, Robideau and others 2011). While these databases are accurate, they may not include sequences of newly discovered species or new isolates of well authenticated species. The metabarcoding sequences that do not match any known species are often considered to originate from novel species, and a follow-up isolation effort is required to validate such novel species.

Closely related *Phytophthora* species may have identical DNA marker sequences, and the metabarcoding approach may not be able to differentiate between such closely related species. However, such closely related species are grouped into a species complex, where member species of the complex are indistinguishable with the DNA marker. If sequence identity between closely related *Phytophthora* species is only limited to the amplified region in metabarcoding, then such closely related species are grouped into a species cluster. Metabarcoding with ITS6 and ITS7 primers results in 8 *Phytophthora* species complexes and 15 species clusters.

Metabarcoding can detect rare and abundant *Phytophthora* species within a sample but cannot determine absolute abundance of a single species within a community. Despite this limitation, the metabarcoding approach is the most sensitive method currently available for rare species detection, with an exponentially higher limit of *Phytophthora* detection compared to the quantitative real-time PCR assay. The quantitative PCR limit of detection for the quarantine pathogen *Phytophthora ramorum* was 500 femtogram/µl, whereas it was 0.5 femtogram/µl with metabarcoding.

Metabarcoding cannot differentiate between cellular and non-cellular DNA or relic DNA that could originate from dead organisms. However, we can easily eliminate relic DNA in metabarcoding by treating samples with a high affinity photoreactive DNA binding dye, propidium monoazide (PMA), that tags relic DNA and prevents its amplification in metabarcoding. Effectiveness of PMA in metabarcoding was demonstrated on synthetic samples comprised of a serial dilution series of *Fusarium* spores mixed with relic *Rhizopogon* DNA. *Rhizopogon* DNA was completely eliminated with PMA and only *Fusarium* was detected in metabarcoding.

Metabarcoding could be coupled with *Phytophthora* capture methods such as filtration and baiting. We separately filtered and baited stream water with rhododendron leaves and extracted

DNA from both filters and leaf baits. Metabarcoding showed presence of different *Phytophthora* communities in filters vs. leaf baits. Filtration detected the 'total' *Phytophthora* community, while baiting detected *Phytophthora* species associated with leaf lesions. Filtration captured a greater diversity of oomycete species than did baiting. This was expected because baiting selectively increased the number of active plant pathogens. We, therefore, recommend using a combination of *Phytophthora* capture methods prior to metabarcoding.

In summary, metabarcoding is a high throughput and sensitive method to detect the total microbial community in a sample at once. It relies on next generation sequencing technology such as Illumina MiSeq that facilitates sequencing of 384 samples simultaneously and detection of rare, novel and unculturable species. It also requires universal primers efficient in amplifying the targeted microbial community, and a reliable and complete reference sequence database. Shorter Illumina sequencing reads are sometimes incapable of resolving sequences of closely related species, and in such cases species identification is limited to species complexes or clusters. Despite the challenges, the metabarcoding approach continues to be a promising tool for studying *Phytophthora* ecology.

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