Monitoring plant biodiversity in aquatic environmental DNA with low-cost Nanopore Flongle sequencing.

Aquatic Plant eDNA Metabarcoding with Nanopore

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Premise of the study

Cellular and cell-free DNA obtained from environmental samples of soil, water, and air can detect local biodiversity **(Thomsen and Willerslev 2015)**. Environmental DNA (eDNA) sequencing typically relies on standard molecular techniques and resources. Here we present a fully portable workflow designed to work with limited infrastructure for the collection, isolation, extraction, sequencing, and analysis of aquatic eDNA for the monitoring of plant biodiversity.

Methods

We extracted aquatic eDNA from an open pond and a seasonal wetland at Stonehill College in Easton, MA over a span of two weeks in June, 2019. Samples were amplified targeting 350bp to 890bp segments from *psbA3*, *MATK*, *rbcLa*, *ITS2*, *18S*, and *trnL* genomic regions. Amplified DNA was barcoded, pooled up to twelve reactions per run, and sequenced on the Oxford Nanopore MinION sequencer using the low-throughput Flongle flowcells. Amplicon sequences were classified using Kraken2 **(Wood, Lu, and Langmead 2019)** and BLAST **(Altschul et al. 1990)** followed by a lowest common ancestor algorithm using the NCBI taxonomy. Table 1.

Table 1: General Workflow

Step	Description
Sample Collection	Field water filtration with 0.045 µm pore filter
DNA Extraction	DNA Extraction with DNEasy PowerWater Kit
Target Amplification	PCR Target Amplification and 12x Multiplex Barcoding
Sequencing	PCR Barcoding kit (SQK-PBK004) library preparation
	Equimolar Pooled Sequencing
	Flongle R9.4.1 flowcells on MinION
Bioinformatics	Basecalling with Guppy 3.4.5 + high accuracy 'flip-flop' model
	Taxonomic classification with:
	BLAST + Lowest Common Anestor
	Kraken2 classification

For more details on the methods used see **(Callahan and Harbert 2020)**.

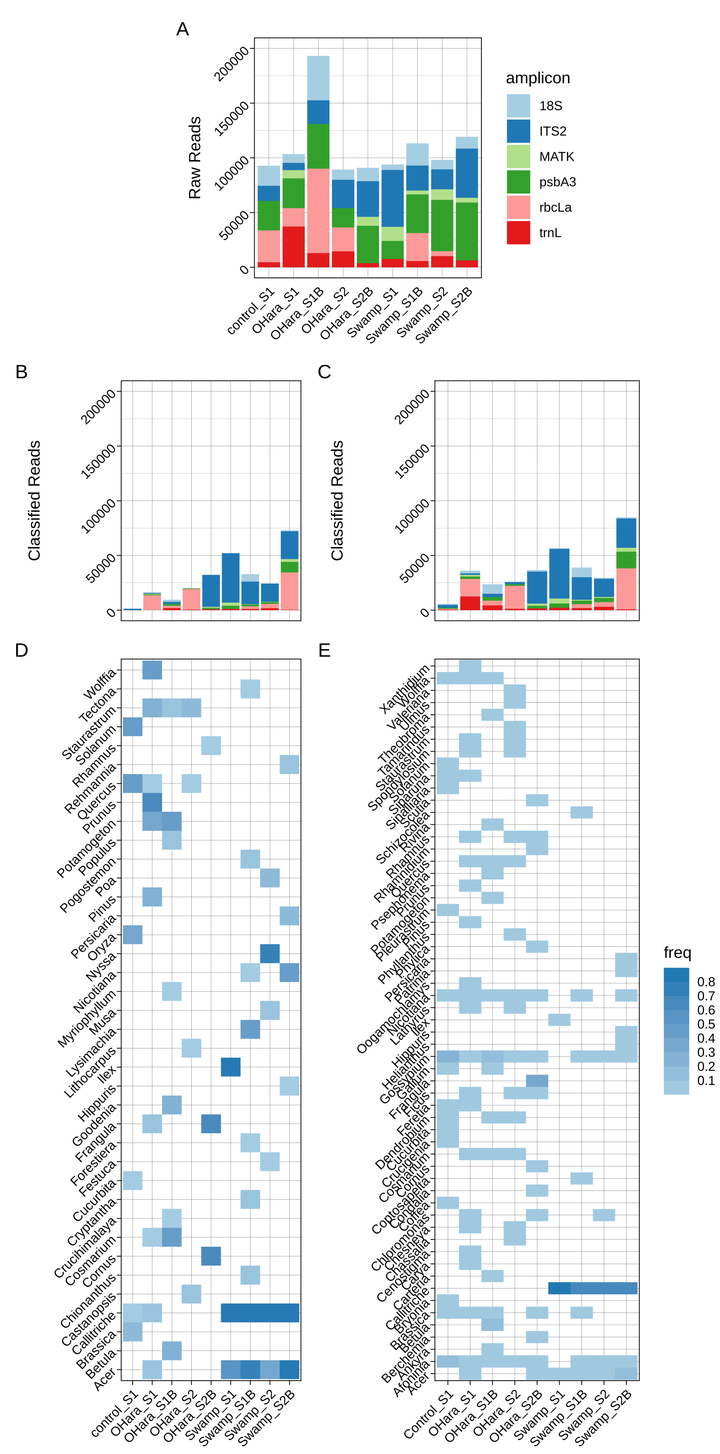

Figure 1: eDNA Classification Results. (A) Total count of reads generated for each sample and amplicon. (B) Count of reads classified by BLAST to genus. (C) Count of reads classified by Kraken2 to genus. (D) Heatmap of read classification to all genera identified by BLAST. (E) Heatmap of read classification of all genera identified by Kraken2.

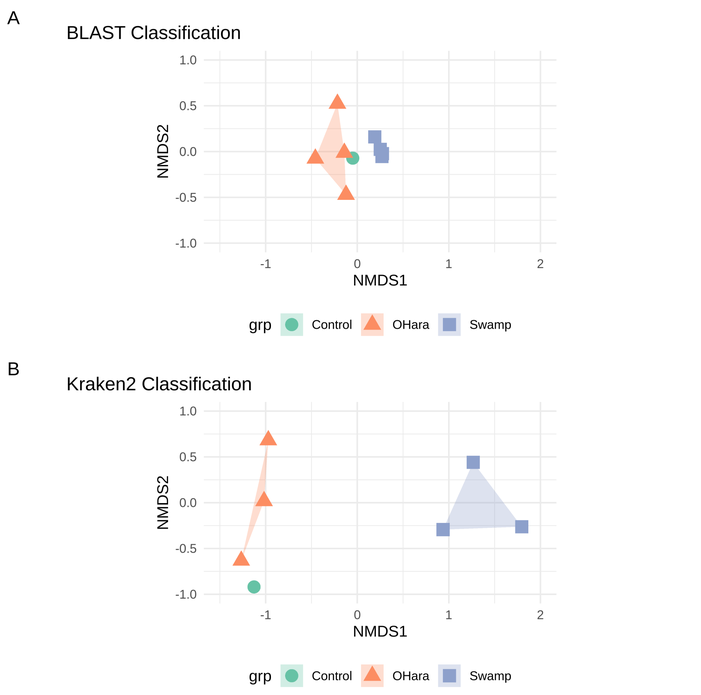

Figure 2: NMDS Ordination plots for (A) BLAST, and (B) Kraken 2 classifications to unambiguous genera.

Results

Metabarcode sequence analysis and taxonomic classification reveals local differences in plant diversity including both aquatic and terrestrial plant taxa Figure 1. Taxonomic composition is reproducible within site and across a two week span between samples Figure 2. All raw data are available at NCBI BioProject: PRJNA488629.

The workflow built around this platform provides end-to-end amplicon-based sequencing that can be used to detect nearby plant diversity. The Oxford Nanopore MinION sequencer and the low throughput Flongle flowcell provides a sequencing platform that combines portability, low cost, and ease of use that are essential features for field applications.

Cost Estimates

Sequencing relatively small datasets on Flongle flowcells provides insights and allows testing of methods at $85 per 6 amplicon sample costs. Table 2. Pooling amplicon primer pools and increased multiplexing could further reduce costs.

Table 2: Consumable Materials Cost Estimates

Component	Total_Cost	Per_Sample_Cost
DNA Extraction: DNEasy PowerWater Kit	$546 / 50 reactions x 5 amplicons each	$2.18
Library Preparation: PCR Barcoding (SQK-PBK004	$650 / 12 Flongle libraries x 12 barcoded amplicons	$4.51
Sequencing: Flongle Flowcell	$90 per flowcell / 12 amplicons	$7.5
Total per Amplicon		$14.19
Total per Sample (6 amplicons)		~$85

Next Steps

- Investigate reproducibility and seasonal variation
- Experiment with primer pools (to increase multiplexing)
- Exchange rapid for ligation chemistry in library prep
- Pipeline publication: https://github.com/developing-bioinformatics/nano_edna
- Bonito Basecalling -> Higher single molecule accuracy will improve taxonomic classification
- Expand to 96 barcodes and MinION flowcells

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References

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Callahan, Jordan, and Robert Harbert. 2020. *Aquatic eDNA Sampling and Plant Community Metabarcoding with Portable Nanopore Flongle Sequencing (V0.0.3) V.3.* https://dx.doi.org/10.17504/protocols.io.bc4wiyxe.

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Wood, Derrick E, Jennifer Lu, and Ben Langmead. 2019. "Improved Metagenomic Analysis with Kraken 2." *Genome Biology* 20 (1): 1–13.

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