Emergence and Molecular Characterization of Protist *Amphibiothecum penneri* in American Toads (*Anaxyrus americanus*) in Northern New England

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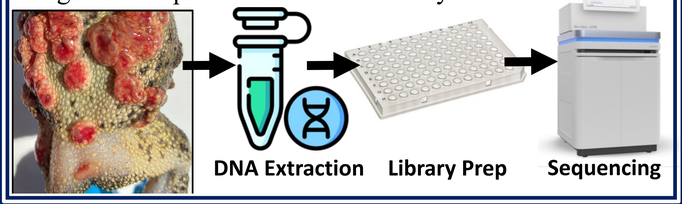
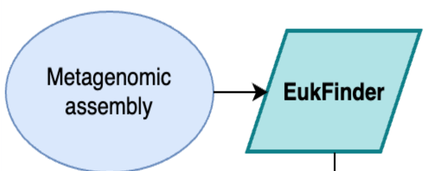
Introduction

- Protist are a growing, but poorly characterized threat to amphibian health
- *Amphibiothecum penneri* is a unicellular holozoan protist pathogen of American toads, that does not have a published genome.
- From 2022-2025 increasing numbers of toads in the Lamprey River Watershed were diagnosed with *A. penneri* infection by the MEED lab and HCGS.
- Our aim was to assemble the *A. penneri* genome from a clinical sample.
- Since host and pathogen are eukaryotes, it is challenging to parse out their genomes in a mixed sample.
- Targeted filtering and validation analyses were used to generate a protist-enriched assembly.



Methodology

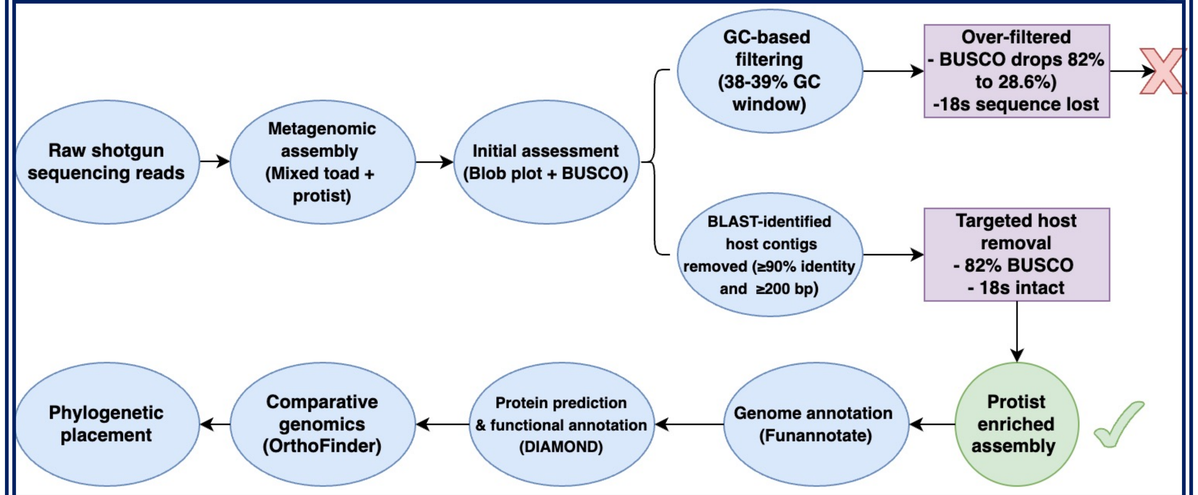
- Metagenomic contigs were classified using EukFinder, a taxonomic binning tool that assigns contigs based on sequence similarity and composition.
- Contigs were partitioned into Euk (known eukaryotic), EUnk (eukaryotic unknown), and Pro (prokaryotic) bins, with the target protist genome recovered from the EUnk bin.
- Assembly composition and completeness were evaluated pre- and post-filtering using BUSCO with eukaryota and tetrapoda lineage datasets to distinguish protist signal from host contamination.



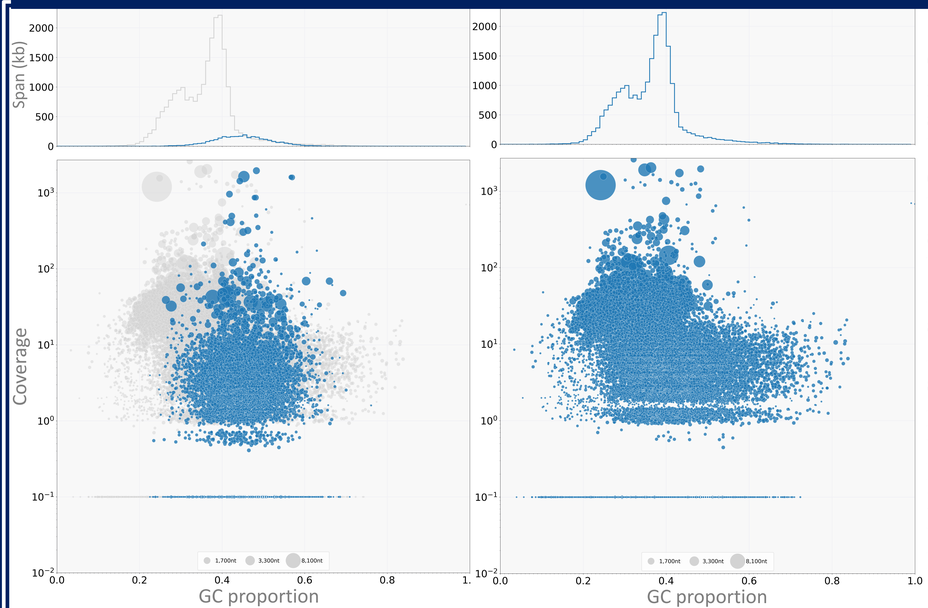
Bins

Euk	*EUnk*	Prok
➢Contigs that match known eukaryotic genomes ➢Mostly host ➢Possible conserved euk sequences	➢Contigs defined as eukaryotic with no close reference genome ➢Target protist ➢ Residual host contamination	➢Contigs matching bacterial/archaeal sequences ➢Not relevant to target DNA

Workflow



Evaluation



Blob plots show GC content versus coverage for contigs before (left) and after (right) BLAST-based host removal (≥90% identity, ≥200 bp). The unfiltered assembly forms a single broad cluster representing mixed host and protist sequences. After filtering, the assembly is enriched for protist-derived contigs with reduced host signal. Remaining clustering likely reflects GC variation and/or residual host DNA.

Fig 1 (above): Blob plot comparison before and after BLAST-based host removal.

BUSCO analysis (Euk and Tetrapod datasets) was used to assess genome completeness and host contamination. GC-based filtering reduced eukaryotic completeness, indicating over-filtering, whereas BLAST-based filtering preserved completeness while maintaining low tetrapod signal. The similarity between Initial and BLAST-filtered Euk results suggests that removed sequences may primarily represent conserved eukaryotic genes shared between host and protist.

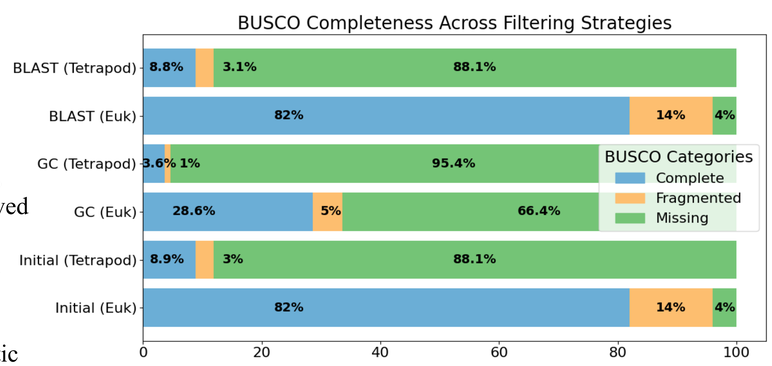
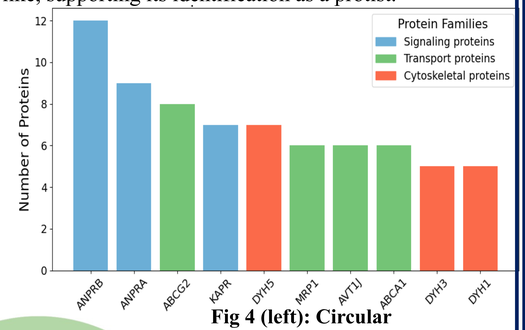


Fig 2 (above). BUSCO completeness between filtering strategies.

Results

- Protein homology searches using DIAMOND, a sequence alignment tool for comparing predicted proteins to reference databases, found matches across diverse eukaryotes. This reflects conservation of core eukaryotic genes and database bias toward well-annotated model organisms.
- Functional annotation revealed conserved protein families involved in transport, cytoskeletal dynamics, and signaling, consistent with a eukaryotic genome.
- Phylogenetic analysis using OrthoFinder, a tool that infers evolutionary relationships based on shared orthologous proteins, places *A. penneri* within holozoan protists, consistent with prior 18S rRNA-based identification. The well-annotated model organism *Xenopus laevis* (African clawed frog) does not cluster with the *A. penneri* proteome, confirming that the recovered genome is not amphibian-like, supporting its identification as a protist.

Fig 3 (right): Top annotated protein families identified by DIAMOND, color coded by protein function.



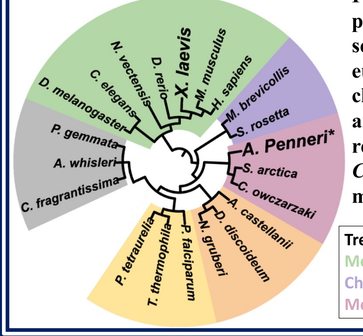


Fig 4 (left): Circular phylogenetic tree with colored sectors denoting major eukaryotic clades. *A. penneri* clusters within holozoan protists and groups with unicellular relatives such as *S. arctica* and *C. owczarzaki*, distinct from metazoan lineages.

Conclusions

- Separation of a co-extracted host and protist genome using EukFinder-based binning and targeted filtering recovered a high-quality draft genome of *A. penneri*.
- While sufficient for phylogenomic placement, the assembly remains below reference quality due to fragmentation and residual contamination.
- The recovered genome supports its classification as a holozoan protist and enables further analysis of pathogenic mechanisms, diagnostics, and mitigation strategies.

References

BUSCO: Manni et al., 2021, *Mol. Biol. Evol.,* DIAMOND: Buchfink et al., 2021, *Nat. Methods,* OrthoFinder*:* Emms & Kelly, 2019, *Genome Biol.,* GeneMark: Lomsadze et al., 2005, *Nucleic Acids Res,* Funnotate: Palmer & Stajich, 2020, *Zenodo,* Eukfinder (Roger Lab), available at: https://github.com/RogerLab/Eukfinder

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