Closed-Tube DNA Barcoding Analysis of the Species and Global Distribution of Naegleria: A Worldwide Genus of Single-celled Amoeboflagellates
Heather Schiller , John Deng , Elaine Lai , Chandler Fulton , Lawrence J. Wangh
Department of Biology, Brandeis University, Waltham Massachusetts, USA

Abstract
Background: Naegleria are abundant, free-living, freshwater amoebae with a worldwide distribution that are able to differentiate into swimming flagellates. Species within this genus are quite diverse, probably because the genus evolved over a billion years ago. Neotropical species like N. gruberi live on the five temperate continents, but N. antarctica is thermophile, and N. fowleri is thermophilic opportunistically human pathogens (the brain-eating amoebae). Previous analysis of short ribosomal ITS sequences cataloged roughly 40 geographically separated species. Our analysis, using a combination of Closed-Tube Barcoding of the CO1 gene target plus DNA sequencing suggests better defines the number of species. Analysis of about 75 isolates from around the world using both CO1 and ITS sequences show agreement in the degree of relatedness among isolates in most cases.

Results: Closed-Tube DNA Barcoding is an efficient, cost-effective method for amplifying the CO1 barcoding target sequence from large numbers of isolates and then scanning the resulting single-stranded DNA for sequence variations using Lights-On/Lights-Off PCR. We are using a common set of probes, comprised of these subsets of probes to resolve all species within this genus. Subsets are labeled in different fluorescent colors. This experimental design allows us to compare the fluorescent signatures of different isolates and immediately observe whether sequence differences are clustered in one region of the CO1 target, or distributed throughout. These predictions can be confirmed byBLITHE-N-tetra sequencing.

Significance: This ground-breaking study of Naegleria points the way to using Closed-Tube Barcoding as a method for detection of species variation in microscopic eukaryotes—a world that is largely unknown. Using this approach we will be able to map the species variation in small or large ecosystems. We can also use our approach to selectively test for N. fowleri, or virtually any pathogen in a water sample.

Introduction
Naegleria: • Single-celled, freshwater, eukaryotic • Differentiate from limax amoebae to streamlined flagellates

Cyst Amoeba Flagellate
• Once thought to be a genus with one species, Naegleria gruberi • The genus has many species, including the human pathogen, N. fowleri. • The genus has more diversity than mammals. • Challenging to define species of Naegleria because of their propensity to reproduce asexually, despite their capacity for sexual reproduction

Species identification:
• The mitochondrial ITS (internal transcribed spacer) sequence in the rDNA plastid (Ref. 1)

Species identification:
• The barcoding sequence of the mitochondrial cytochrome c oxidase I CO1 gene (Ref. 2)

Diversity Analysis via Closed-Tube Barcoding
We utilized LATE-PCR in order to amplify the CO1 region and coated the resulting single-stranded DNA with a set of nine Lights-On Lights-Off probes. When these probes bind and melt off, they increase or decrease their fluorescence, which is detected by a PCR machine. As these probes will bind to different sequences of DNA at different temperatures, each species has a unique fluorescent signature.

Conclusions from Sequencing Analysis
1. Construction of phylogenetic trees based on ITS and CO1 sequences in this genus is a work in progress. While the trees are broadly similar, certain ambiguities remain to be examined more closely.
2. The ITS tree shows thick branches, while the CO1 tree shows finer arborization.
3. In both trees, N. fowleri species (- -') group together and cluster on a branch of their own.

Conclusions from Closed-Tube Barcoding
1. Every strain or species of Naegleria analyzed thus far by Closed-Tube Barcoding has its own fluorescent signature, reflecting its unique CO1 sequence.
2. N. fowleri has a unique signature which is easily recognized compared to other N. species and may be useful for rapid analysis of clinical samples.

Variation & Coexistence of Naegleria in a Birdbath
Birds, robots, and insects use the birdbath and carry in water and soil. Protocol: Disinfect the birdbath, wait for it to become “dirty,“ and then sample the water.
• Would the same species of Naegleria be reintroduced after each cleaning?
  ▪ 10 isolates of Naegleria were taken from successive water samples
  ▪ At least 3 different species were reintroduced
• How many Naegleria species can coexist in the birdbath?
  ▪ 13 isolates of Naegleria were taken from one water sample
  ▪ At least 3 different species coexisted in this one sample
• We conclude that Naegleria species coexistence is possible, even between distinctly related species
  ▪ The same species are not always reintroduced
• Future experiments will evaluate congruence of species defined by CO1 versus ITS gene sequences

Acknowledgments
Funded in part by Brandeis University. Thank you to Ronit Kaufman, Adam Osborne, Jackie Jean-Chapman, and Jessica Chow, Aquiles Sanchez.

Contact Information:
Wangh@brandeis.edu

References

Figure captions:
Fluorescent signatures of FBB #1-8 and NEG-H
N. Fowleri vs. FBB4 Fluorescent Signature
N. Fowleri vs. BBH19 Fluorescent Signature
Location of the birdbath
42°22'10" N 71°16'56" W