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Digging Out the Devils: Molecular Examination of Amoeba-like Cells from Cranial Tissue of the Endangered Rio Grande Silvery Minnow

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Molecular Examination of Amoeba-like Cells from Cranial Tissue of the Endangered Rio Grande Silvery Minnow

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The Plight of the Rio Grande Silvery Minnow
(*Hybognathus amarus*)

- Fish species endemic to southwestern United States
- Critically Endangered
  - Human alteration of Rio Grande
- Now occupies ~5% of its original range.
- Aquaculture efforts started in 2000 through U.S. Fish and Wildlife Service
Trouble in Paradise

- Unusual symptoms in the summer of 2012
  - Spinning behavior
  - Chronic low-level mortalities
- Viability of restocking efforts questioned
- Investigation undertaken by VIMS at the College of William and Mary
  - Water and habitat quality
  - Pathogenic microbe screens
  - Gross necropsies of affected specimens
- All came back negative, leaving the cause a mystery
The Plot Thickens

- A second investigation in 2013 discovered an unusual cell in the connective tissue of the cranial floor
  - Amoeba-like cell
  - Possible infectious organism
  - Identification a priority

Photo Credit:
Dr. Wolfgang K. Vogelbein, The Virginia Institute of Marine Science, College of William and Mary
Amoebic Infectious Agents – The Suspects

- Pathogenic Amoebae
  - *Acanthamoeba, Balmuthia, Neoparamoeba* spp.
    - Amoebic Gill Disease

- Parasites with Amoeboid Life Stages
  - Myxosoans
    - *Myxobolus cerebralis* – causative agent of whirling disease
      - Infection causing whirling behavior similar to observed symptoms
    - *Henneguya, Kudoa, Buddenbrockia, Tetrascapuloides* spp.
      - Many infect gills or other organs, but new or related species a possibility
Preliminary Investigation

- PCR – Polymerase Chain Reaction
  - Molecular technique used to amplify and visualize a target DNA sequence in a sample
  - General, nonspecific primers designed to amplify a wide range of myxosoan and amoebic species
  - Testing performed on digested samples from affected fish

- No amplification of DNA targets

- Identity of amoebic organism still a mystery
Going Deeper With Illumina Sequencing

- Taking a DNA ‘snapshot’
- Generic Eukaryotic primer sets would amplify all 18S sequences present in sample
- Sequences would be analyzed using Illumina MiSeq platform
  - Uses NGS to collect up to 8 Gb of sequencing data
    - For reference, older sequencing methods collect up to 10 Kb/day
  - Allows sequencing of multiple loci at once
    - Two loci examined
~450,000 sequences retrieved from each of the two eukaryotic 18S loci amplified

- A program known as cdhit was used to cluster similar sequences together
- Reduced number of different sequences to ~1000 per loci

Clusters representing more than 0.01% of the total number of sequences were analyzed using BLAST in Geneious® software
Results - 1st Locus (EUK 1136)

Distribution of BLAST Results from Illumina Sequencing of EUK 1136 Locus

- Fish: 98.9356%
- Bacteria: 0.74416%
- Unknown: 0.1293%
- Plants: 0.0708%
- Reptile: 0.05%
- Mammal: 0.02162%
- Fungus: 0.012%

Percentage of Total Sequences Recovered
Results – 1\textsuperscript{st} Locus (EUK 1136)

BLAST Results from Illumina Sequencing of EUK F\_R Locus with Background Removed (no fish)

- **Bacteria**: 3500 sequences
- **Unknown**: 500 sequences
- **Plants**: 1000 sequences
- **Reptile**: 1500 sequences
- **Mammal**: 2000 sequences
- **Fungus**: 2500 sequences

Malassezia spp.
Results – 2nd Locus (EUK F_R)

Distribution of BLAST Results from Illumina Sequencing of EUK F_R Locus

- Fish spp.: 95.773%
- Plant: 1.1088%
- Bacteria: 0.9032%
- Unknown: 0.5576%
- Fungi: 0.501851%
- Nematode: 0.3513%
- Algae: 0.282066%
- Crustacean: 0.184437%
- Human: 0.0870485%

Percentage of Total Sequences Recovered
Results - 2nd Locus (EUK F_R)

BLAST Results from Illumina Sequencing of EUK F_R Locus with Background Removed (no fish)

Total Number of Sequences

- Plant: 4500
- Bacteria: 3600
- Unknown: 2400
- Fungi: 2200
- Nematode: 1800
- Algae: 1200
- Crustacean: 800
- Human: 500

Groupings:
- Candida spp.
- Malassezia spp.
- Z. bailii
Discussion/Conclusion

- No Amoeba or related species detected from either locus
  - Unknown Sequences?

- Possible alternatives
  - Pathological host cell
  - Primer sets not specific for organism
  - Detection threshold of Illumina not tested

- Results of this study unable to provide identification for mysterious cell.


Questions?