Diversity of Microbial Eukaryotes in Undersampled Environments

John Christian Gaby

Cornell University

Department of Microbiology

Laboratory of Dr. Daniel Buckley

Abstract

The study of microbial eukaryotes has not traditionally been the realm of microbiologists, who focus mainly on bacteria. Molecular methods and an attempt at enrichment of protists were undertaken in this study. DGGE of timepoints from soil enrichments revealed a change in community members as well as some members' relative abundances. However, complications with faint or absent bands due to low PCR yield hinder confidence in these conclusions. Single cell PCR yielded product for 8 of 12 cells isolated from a sediment sample of Trunk River, Woods Hole, MA. However, a sequencing attempt failed for all 8 of these. The 18S clone library of Sippewissett Salt Marsh sediment DNA near Woods Hole, MA yielded only 1 sequence type, identical to *Pleurosigma intermedium*. Examination by light microscopy of anaerobic enrichment attempts revealed no morphologically distinct microbial eukaryotic cells.

Introduction

Historically microbiologists have sequestered themselves to the realm of Eubacteria and Archaea while microbial eukaryotes have remained the province of protistologists. Thus, cultivation-independent molecular techniques that have been applied extensively by microbiologists have taken longer to be applied to microbial eukaryotes, and when applied these techniques have revealed extensive diversity in much the same manner as for Bacteria (Moreira, 2002)

There are an estimated 200,000 described protist morphospecies, but only a few thousand protists have been described by SSU rRNA sequence data. The ability to differentiate so many species based upon morphology alone is not possible with Bacteria because of their generally smaller size and the limited morphological features that may be distinguished with a light microscope. At present, the vast majority of described eukaryotic morphospecies do not have a corresponding SSU rRNA sequence. Also, many unique sequences have not been associated with already described morphospecies where they may exist. There are means for associating these and methods have been worked out previously (Massana, 2002; Takano, 2006). These techniques involve the use of FISH probes and single-cell manipulation and PCR.

A survey of existing literature reveals aquatic environments are the most sampled. Though extreme environments are generally thought to harbor less eukaryotic diversity, the highly acidic Rio Tinto in Spain has been shown to hold unique eukaryotic diveristy (Zettler, 2002), challenging this notion. Wetlands such as bogs are acidic and anaerobic, and perhaps could harbor as yet undiscovered diversity in the eukaryotic realm. Furthermore, anaerobic environments, though less sampled, have proven to hold diversity not found elsewhere(Dawson, 2002; Stoeck, 2003a; Stoeck, 2003b).

In this study, molecular techniques and a cultivation approach were applied in the exploration of the microbial eukaryotic diversity from environments around Woods Hole, MA.

Materials and Methods

Soil Enrichments and DGGE Analysis

Soil samples were collected from a woodlot at the corner of Millfield St. and Quisset Ave. in Woods Hole, MA. Two soil samples were taken within the woodlot approximately 3 m apart. The soils were friable and had a definite aggregate structure at the time of sampling. One sample was from within a depression, and the other from the bank overlying the depression. Four types of enrichment were set-up using each of the two soil samples for a total of 8 enrichments. 10 g of soil was added to a 50 mL Erlenmeyer flask for each enrichment, and then 10 mL of one of the following was added for each soil type: distilled water, a 1:10 dilution of LB broth, a 1:100 dilution of LB broth, or a 1:100 (w/w) dilution of casein. These were left at room temperature on the benchtop during the time course.

A T_0 DNA was extracted from the two water-moistened enrichment samples within several hours of collection using a MoBio Soil DNA Extraction kit. 1 g of soil was extracted with the kit. A T_1 DNA extract was taken two days after the start in the same manner as before. A T_2 DNA extract was taken 6 days after the start, but this time 0.5 g was removed for extraction, and this amount was spun in a centrifuge and any water was pipetted off (this modification was to potentially address a low DNA yield suspected to result from overloading the reagents in the kit with the waterlogged soil).

Also extracted using the MoBio Soil DNA extraction kit were DNAs from Sippewissett Salt Marsh (1

g), Trunk River (1g spun down, water removed), Cedar Swamp (1 g spun down, water removed), and School Street (0.5 g) Marsh, all in or around Woods Hole, MA.

DGGE conditions and PCR reactions were performed according to Gast et al., 2004. The DNAs were used in a PCR reaction to generate products for DGGE analysis. The primers 960FbGC 5' CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCCGGCTTAATTTGACTCTAAC 3' (GC clamped), and 1200R 5' GGGCATCACAGACCTG 3' (universal) were used in a PCR reaction with the DNAs to generate a an ~250 bp fragment. The primer pair amplifies the V7 region of the srDNA. 1 µL of DNA extract was used as template in a 50 µL PCR reaction. A touchdown PCR thermocycler program was used with the following specifications: an initial annealing temperature of 65 ºC was followed for two cycles with 45 s at 95 ºC, 45 s at 65 ºC, and 45 s at 72 ºC per these cycles. Then, the annealing temperature was decreased 2 ºC every two cycles until the final annealing temperature reached 55 ºC, followed by 25 cycles at the 55 ºC annealing temperature with 45 s at 95 ºC, 45 s at 55 ºC, and 45 s at 72 ºC. PCR products for each of the DNA extracts were visualized on a 1.3% agarose gel stained with ethidium bromide to ensure the presence of product. To the remaining PCR products, 5 µL of 0.3 M Sodium Acetate and then 0.6 volumes of isopropanol were added then left at -20 ºC overnight to precipitate. The products were transferred to a centrifuge tube and spun for 15 minutes at high speed in a centrifuge to pellet the DNA. Liquid was pipetted off and the pellet dried for a few minutes in a 37 ºC heating block. 4 µL nuclease free water was added to the DNA and the tube was again placed in the 37 ºC heating block for the DNA to dissolve. Samples were mixed with 6 µL loading dye and loaded onto the DGGE gel. Standard markers were added to the gel to enable comparison within and between gels.

A denaturing gradient of 35 to 85% urea-formamide was used and the DGGE gel was run at 60 ºC at 95 v overnight (16h). Gel was stained with ethidium bromide to visualize bands.

Single Cell PCR

Individual microbial eukaryotic cells were picked using pulled 25 µL glass capillary tubes (VWR). Cells were picked from a film of microbes on a glass coverslip that was "seeded" in the following way: sediment from Trunk River (near Woods Hole, MA) was placed in a plastic bin, pooling water was removed, then lens paper was placed on top to form a microbe permeable barrier upon which the glass coverslips were placed and left for about 1 week. This prevented the sediment from adhering to the coverslips. Using pulled capillary tubes and a "Schnoz" mouth suction tube and capillary receptacle, individual cells were isolated from the incubated glass coverslips, rinsed in 200 µL sterile water in a 96-well plate once, then transferred to a PCR tube. PCR amplification was carried out by way of a protocol used in the laboratory of Dr. Rebecca Gast (personal communication). First, cells are subjected to 3 to 5 cycles of freeze thaw at -80 ºC, then 5 µL of Lyse-n-go™ (Pierce) reagent was was added and the manufacturer's protocol was followed for lysing the cells.

Next, a series of three nested PCR reactions were performed. The first reaction used EukA/EukB primer pairs, the second used 373f/EukB, and the third used 373f/1200r. The sequence of primer EukA is 5' ATCTGGTTGATYCTGCCAG 3'. The sequence of primer EukB is 3' CACTTGGACGRCMWCCTAGT 5'. The sequence for primer 373f is 5' GATTCCGGAGAGGGAGCCT 3'. The sequence for primer 1200r is already described in the section on DGGE. The first PCR reaction is 100 µL to dilute the ~10 µL of template and lysing reagent. The latter two PCR reactions use 50 µL. The standard PCR reaction for 50 µL is 1 µL of 100 ng/µL of both primers, 4 µL of 2.5 mM nucleotides, 5 µL of $MgCl_2$, 10 µL of 5x buffer, 0.25 µL of Taq DNA polymerase, and then the reaction volume is brought to 50 µL with nuclease free water. The

thermocycler was programmed as follows: 5 minute initial denaturation and hot start cycle at 95 ºC followed by 40 cycles of 95 ºC for 30 s, 62 ºC for 30 s, and 72 ºC for 2 minutes, then a final extension at 72 ºC for 7 minutes. PCR products were ran on a 1% agarose gel and stained with ethidium bromide after each PCR stage to assess presence of amplification products.

PCR products from the third and final PCR were exo-SAP treated per manufacturer protocol and sent for sequencing using the 373f primer. 8 of 12 attempts yielded product in the final PCR, and these were submitted for sequencing.

Clone Library

A clone library was constructed from PCR products from Sippewissett DNA. Primers EukA/EukB were used to amplify eukaryotic 18S sequences from the sample, and the resulting ~800bp product (the expected product should be ~1800 bp) was cloned using TOPO TA cloning with TOPO 2.1 vector, a blue-white screening vector, per the manufacturer's protocol.

24 clones were picked from the plates and submitted for plasmid sequencing.

Anaerobic Enrichments

Anaerobic enrichments were set-up. 50 mL serum vials were set-up with either a grain of rice, 1:10 LB, 1:100 casein, or live yeast, all prepared in autoclaved trunk river porewater. Sterilized serum vials containing 25mL of media were purged of oxygen and filled with nitrogen, then were inoculated with a glass coverslip seeded with organisms from Trunk River sediment as already described in an anaerobic glovebox chamber. Vials were then resealed and incubated at room temperature in the dark for about 5 days, then a subsample extracted with a syringe was viewed microscopically.

Results

Soil Enrichments and DGGE Analysis

Figure 1 shows the DGGE gel for the T_0, T_1, and T_2 soil enrichment timepoints. Some of the PCR products were faint when assessed on an ethidium bromide stained agarose gel, and these inevitably resulted in faint bands on the DGGE gel as well because of the low amount of product present. Figure 2 shows a DGGE gel with the T_0 timepoints again and the four environmental samples. Both figures show images that were altered in contrast and brightness to make the faint bands better visible. In the original image (not included) the marker lanes are distinguishable.

Single Cell PCR

The single cell PCR did not yield an amount of product visualizable on the first gel (figure 3). The second round yielded product for two of the isolated cells as well as for the positive control. The third PCR yielded product for 8 of 12 cells isolated. Some show more than one band, and it should be noted that the negative control, using nuclease free water as template, yielded a faint band.

Clone Library

The PCR yielded a band at about 800bp, much shorter than the 1800 bp fragment that the primers amplify. 24 clone colonies were picked from the plates and submitted for sequencing. 4 returned

vector sequence, and the remaining 20 were all identical to *Pleurosigma intermedium* as indicated by both a BLAST search and phylogenetic analysis using the software package ARB (Ludwig et al., 2004).

Anaerobic Enrichments

As revealed by examination with a light microscope, no morphologically distinct microbial eukaryotes were witnessed in the anaerobic enrichments.

Discussion

Once samples such as soil are removed from the native conditions of their environment, then the community of microbes may be expected to immediately begin to change. Further alteration of the environment by addition of enriching media may further cause alteration and even result in a decrease in diversity. DNA samples taken from the enrichments after the initial T_0 measurement revealed less DNA was present in the enrichment as seen on an ethidium bromide stained gel and as assessed spectrophotometrically using a nanodrop device (data not shown), perhaps indicative of a decrease in the overall population size. The DGGE further revealed that there appeared to be a decrease in diversity over time for some enrichments, especially for soil 2, for which many bands were not present in the enrichments. It appears that the enrichment selects for certain species, but the species present in the enrichments were not identified by the usual process of band excision, DNA extraction, and sequencing due to time constraints. Some lanes show faint or no bands, and this could be due to low yield from the PCR. Further refinement of this analysis would involve optimizing the PCR so that adequate product is loaded to ensure that organisms present are represented by their respective bands in the DGGE.

The single cell PCR was successful in yielding product for 66% of the cells isolated. However, sequencing yielded noisy electroferograms. This could be perhaps due to multiple eukaryotic species being placed into the PCR tube after picking, as there are known to exist pico-planktonic eukaryotes that would not be seen under the dissecting microscope. Further rinsing to dilute out these would remedy this. Also, the negative control had a faint band present, and this contamination could lead to the noisy sequence.

The clone library was not diverse, with only one sequence type occurring out of 20 clones. Since the band generated in the PCR was not of the expected size, a different primer set should be selected for amplification that generates a shorter product more likely to be amplified under usual PCR reaction conditions.

No morphologically distinct eukaryotic cells were witnessed in samples from the anaerobic enrichments. The anaerobic enrichments could contain smaller eukaryotic cells not distinctly identifiable by light microscopy. PCR amplification of enrichment DNA extracts, followed by cloning and sequencing could reveal their presence. DNA of microbial eukaryotes has been identified in anaerobic environments (Dawson and Pace, 2002), and further exploration of enrichment and cultivation strategies may bring these organisms into culture.

No new microbial eukaryotes were identified in this study, however the application of techniques such as DGGE and single-cell PCR were successful in identifying changes in the community of microbial eukaryotes within enrichments over time and in amplifying a portion of the 18s of isolated cells,

respectively. These techniques in concert with other molecular ones such as clone library construction may be applied to explore undersampled environments with different enrichment strategies in further searches to expand the known diversity of protists.

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Figure 1: DGGE gel of the soil enrichment timepoints.

Figure 2: DGGE gel of environmental samples and T_0 again for the soil enrichments.

Figure 3: The 3 agarose gels showing the products of the consecutive nested PCR series after each reaction. The order is from left to right, with the initial PCR products on the far left, the second PCR products in the middle, and the final products on the far right.