Preliminary Study of Kleptoplasty in Foraminifera of South Carolina

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ABSTRACT

Recent studies of living foraminifera, microscopic aquatic protists, indicate that some species have the ability to steal photosynthetic plastids from other microorganism and keep them viable through a process called kleptoplasty. Studying the symbiotic relationships within these diverse protists gives insight not only into evolutionary history, but also their importance to the ecosystem. We determined the presence of these kleptoplastic species and identified presence and origin of sequestered plastids based on morphological identification and molecular data from samples collected at Waties Island, South Carolina. We identified two kleptoplastic genera (Elphidium and Haynesina) and two non-kleptoplastic genera (Ammonia and Quinqueloculina) present in the lagoon. Phylogenomic results indicated that sequestered plastids originated from pennate diatoms from the genus Amphora. However, further research is needed to prevent bias due to environmental impact and corroborate host specificity and plastid origin.

Introduction

Foraminifera are large, unicellular aquatic protists with a shell, known as a test in literature, formed from calcareous, siliceous, or agglutinated organic materials (D'Orbigny, 1826; Cevasco, 2007; Lipps, Finger, & Walker, 2011). A majority of foraminifera are found in marine habitats. However, many species are able to live in estuaries and tidal influenced brackish rivers (Chapmen, 1902). Protists are among the most diverse microorganisms with estimates of 68 modern genera and 1,000 modern species. Foraminiferal fossils date to the Cambrian era (500 million years ago) and are used to reconstruct paleoenvironmental parameters (Sen Gupta, 2002). Modern for aminiferal taxa can be used to determine the ecological, molecular, and phylogenetic importance within habitats. These taxa are used because of their broad ecological adaptability, longstanding geological history, species diversity by the Phanerozoic eon (542 million years ago), diverse taxonomy, utilization of various feeding mechanisms, and role in biogeochemical cycling (Linke & Lutze, 1993; Sen Gupta, 2002; Cevasco, 2007; Wray, Langer, DeSallie, Lee, & Lipps, 1995). For example, a study by Hallock (2000) examines large foraminiferas as bioindicators for global climate change. The ecological importance of large benthic foraminifera and the symbiotic relationships these protists have with other such microorganisms as dinoflagellates and diatoms is currently being actively researched (Ziegler & Uthicke, 2011) as is the incidence of plastid retention (Pillet, de Vargas, & Pawlowski, 2011).

The term kleptoplasty is used to describe a special type of endosymbiosis where a heterotrophic host organism engages in organelle retention (Stoecker, Johnson, de Vargas, & Not, 2009; Stoecker, 1999) sequestering, or stealing, the photosynthetic organelles (plastids) of its prey (Rumpho, Pelletreau, Moustafa, & Bhattacharya, 2011). Sequestered plastids (kleptoplasts) remain functional within the host for extended periods of time, enabling the host to photosynthesize. Due to the kleptoplastic condition, the host becomes mixotrophic, obtaining energy through phototrophy as well as from heterotrophic feeding. Mixotrophy has been shown to have important stabilizing effects on the trophic structure in ecosystems by increasing the total primary and secondary production in planktonic food webs, facilitating carbon transfer from microbial to metazoan trophic levels, and enhancing nutrient cycling (Stoecker, Johnson, de Vargas, & Not, 2009). Moreover, in addition to providing the ability to photosynthesize, kleptoplasty may also contribute to providing oxygen in low-oxygen habitats (Bernhard & Bowser, 1999).

The kleptoplasty phenomenon was first described in the sacoglossan mollusk (sea slug) *Elysia chlorotica*, but it has subsequently been identified in several unicellular marine eukaryotes, including several members of the protist phylum Foraminifera (Corriea & Lee, 2002). Multiple foraminiferal genera are believed to harbor kleptoplasts, including *Bulimina*, *Elphidium*, *Haynesina*, *Nonion*, *Nonionella*, *Reophax*, and *Stainforthia* (Pillet, de Vargas, & Pawlowski, 2011). Initial studies using transmission electron microscopy of the kleptoplastic condition in foraminifera demonstrated that in the genus *Elphidium* as many as $\sim 3.7 \times 10^4$ plastids can be retained per cell. These plastids were found to remain photosynthetically active for up to nine weeks (Correia & Lee, 2002). *Elphidium* and *Haynesina* are common inhabitants of Western Atlantic estuarine and tidal marsh habitats along with multiple non-kleptoplastic genera, e.g. *Ammonia*, *Ammobaculites*, and *Quinqueloculina* (Abbene, Culver, Corbett, Buzas, & Tully, 2006).

Both kleptoplastic genera (*Elphidium* and *Haynesina*) and non-kleptoplastic genera (*Ammonia*) belong to the order Rotaliida and look similar in cellular structure, which makes separating the two genera a challenge. We distinguish between the two genera based on the following morphological characteristics: number of chambers in a single 360 degree turn of spiral test (outer whorl), chamber height, and presence of zipper-like chamber wall connections between previously formed tests (suture bridges) (Buzas, Culver, & Isham, 1985). The genus Elphidium is associated with having numerous distinct suture bridges and long narrow chambers. The genus Haynesina has fewer chambers that are longer and narrower along the outer whorl and no visible suture bridges between consecutively formed chambers. The genus *Ammonia* is associated with having short wide chambers, no suture bridges between chambers, and a large number of chambers around the outer whorl of the test. Foraminiferal diversity within South Carolina coastal habitats has yet to be described. However, during a preliminary survey both kleptoplastic genera (*Elphidium* and *Haynesina*) were present in high abundance at Waties Island, SC. This study aims to identify and examine the potential ecological contributions of the kleptoplastic condition within foraminiferal taxa inhabiting coastal South Carolina through the use of molecular techniques (SSU rDNA extraction and amplification) and phylogenetic analysis.

Materials and Methods

Specimen Collection

Specimens were collected from a tidal lagoon at Waties Island, SC, located adjacent to Hogs Inlet at the southern end of the barrier island. GPS coordinates for the collection site were 33° 50' 35'' N. 78° 35' 44" W. Benthic foraminifera were collected during low tide from the top centimeter of sediment from several locations along the lagoon. The sediment was sieved to remove particles larger than 500 μ m. Once sieved sediment settled out of suspension, the specimens were pipetted from the top layer and observed under a 40x dissecting microscope.

Specimen Preparation

Foraminifera were removed from the sediment and washed three times each with sterile seawater, distilled water, and molecular nuclease-free water to remove contaminants from the specimen. Clean, individual foraminifera were placed into a five microliter droplet of EDTA .25 molar solution and incubated until decalcified. Incubation time ranged between 10 and 20 minutes due to varied individual specimen's calcification level. Incubation facilitated calcium carbonate dissolution of the shell surface (test chelation) and increased accessibility of specimen DNA, while removing the remaining contaminants and microorganisms not consumed by the protist.

In addition to the EDTA decontamination technique, sonication trials were tested using a subset of specimens to test the method as a means of removing microbial and sedimentary contaminations from the outer tests. This sonication method was performed using the Fisher model 100 sonic dismembrator with ultrasonic converter probe. Ultrasonication of six foraminifera was conducted by placing the specimen into a cup of sterile water and then packing ice around the cup. The sonic dismembrator was then set to zero for continuous pulsing, and the probe was placed into the specimen cup for three seconds. The specimens, following sonication, were placed in the EDTA solution and then transferred to sterile molecular grade water. Following debris removal, specimens were transferred to a sterilized glass petri dish and a flamed sterilized needle was used to break open chambers. Then, up to six specimens were transferred to a milliliter Eppendorf Tube® of Buffer RLT Plus lysis buffer (Quiagen AllPrep DNA/RNA Mini Kit) where specimens were homogenized with a pestle.

Fluorescent Microscopy

The naturally occurring ability to emit light when excited by a specific wavelength of visible light (autofluorescence) found in specimens obtained from plant and animal tissues was observed using fluorescent microscopy (Maxwell & Johnson, 2000; Ploem, 2012). The specimens were examined using an Olympus BX51 scope with a TRITC (Tetramethyl Rhodamine Isothloocynate) filter with an excitation/emission of 550/580 nm. This filter cube allows for the detection of autofluorescence originating from photosynthetic pigments characteristic of diatom plastids. Images were taken of living foraminiferal specimens using the 10X and 20X objectives to detect plastid autofluorescence within the host cytoplasm.

DNA Extraction and PCR Amplification

DNA was extracted with Qiagen AllPrep DNA/RNA Mini Kit using the standard "Isolation of Genomic DNA from Tissue" protocol. Following an approach modeled on methods established by Pillet, de Vargas, & Pawlowski (2011) to identify the origin of sequestered plastids within foraminifera from Eastern Atlantic Locations, this research uses a combination of algal 18s ribosomal primers (DiatSSUF and DiatSSUR) and 16S plastid primers (PLA491F and OXY1313R) in PCR amplification (Table 1).

Target	Primer	Primer Sequence
	(forward or	(5'-3')
	reverse)	
Diatom 18S rDNA	DiatSSUF (for)	ACATCCAAGGAAGGCAGCA
	DiatSSUR (rev)	CTCTCAATCTGTCAATCCTCA
Kleptoplast 16S	PLA491F (for)	GAGGAATAAGCATCGGCTAA
rDNA	OXY1313R (rev)	CTTCACGTAGGCGAGTTGCAGC

Table 1. Primers used in amplification and identification of diatom and kleptochloroplast SSU rDNA.

Kleptoplast candidates were determined by the amplification of 16S plastid products and the inability to amplify 18S ribosomal algal products (Figure 1). Candidates that show amplification of the 18S ribosomal algal product and no amplification of the 16S plastid will indicate whole diatom cells present as contamination or food remaining on or within the foraminiferal host.



Figure 1. Flow chart of main primer sets used to amplify 16S kleptoplast DNA and eliminate instances of diatom contamination.

The PCR reaction was established using Ready-To-Go PCR beads (GE Biosciences), 1.5μ l of each forward and reverse primers, 2μ l of extracted DNA, and 20μ l of RNase-free water. The DNA amplification reaction was carried out in the Biorad 100 thermocycler under the following conditions: 2 minute initial denaturing at 94°C, 30 second denaturation at 94°C, 45 second annealing at 50°C, 45 second extension at 72°C, and a final extension for 5 minutes at 72°C for a total of 30 cycles.

The samples were electrophoresed on an agarose gel with a 10μ l mix for each sample consisting of the following components: 4μ l loading dye, 2μ l SYBR Green, and 4μ l amplified DNA. A 10μ l mix of lambda DNA was used for comparison with that of the foraminiferal samples. Electrophoresis condition was as follows: 100 amps for 30 minutes to allow for complete separation of the rDNA SSU. All samples positive for kleptoplasty, which were based on visible bands of amplified 16S plastid rDNA SSU after electrophoresis, were selected for sequencing, cleaned using ExoSAP-IT (Affymetrics), and sent to Genscript DNA sequencing services.

Description of Phylogenetic Methods

The sequence data was then edited using the Geneious® 6.1.7 software package (Biomatters). Whole diatom (18S) and diatom plastid (16S) searches were conducted through the NCBI GenBank through Nucleotide Blast search to determine origin of sequestered plastids. Those GenBank sequences exhibiting sequential similarities to host foraminifera and sequestered plastid were then used in the phylogenomic analyses. Selected plastid 16S sequences recovered from Waties foraminiferal specimens were aligned with the GenBank diatom plastids. Bacterial 16S sequences were included as outgroup taxa. Ribosomal sequence data (18S) from a whole diatom within a foraminiferal host were similarly edited and aligned. Alignments were preformed using MAFFTv7.017 (Katoh, Misawa, Kuma, & Miyata, 2002), which implements a progressive fast Fourier transform (FFT-NS-2) with a gap opening penalty of 1.76. Aligned sequences were then phylogenetically analyzed under the maximum likelihood optimality criterion implemented in PHYML (Guindon & Gascuel, 2003) using the general time reversible model and nearest neighbor interchange topology search. Branch support was determined using 1,000 bootstrap replicates.

Results and Discussion

Specimen Distribution and Seasonality

From August through October, a total of 76 specimens were collected from Waties Island lagoon. Based on the foraminiferal morphological identification, the initial field collection seemed to have a high abundance of non-kleptoplastic genera (*Ammonia* and *Quinqueloculina*). Later collection trips yielded fewer living *Quinqueloculina* specimens; instead, variable numbers of *Elphidium* and *Haynesina* were collected, as well as a stable population of *Ammonia*. During the field season, we observed patchy distribution and seasonal fluctuation in the species. Foraminiferal distributions have variable population sizes within a single environment during periods of rapid growth in population (blooms) occurring at close periods (Morvan et al., 2006). The seasonal variability and distribution within estuaries and intertidal locations have often been linked to environmental factors that have hit a critical threshold. In particular, Murray (2001, 2008) suggests that such factors as sediment disturbances and increased nutrients by larger organisms cause algal population variation, which, in return, affects foraminiferal food supplies. Species were collected over a single field season, which could have affected the yield of kleptoplastic foraminifera. A study conducted in a lagoon in Venice, Italy, used data taken from multiple stations over a two-year span (November 1992 to September 1994), compiling a more complete picture of species seasonality and determining localized species dominance within the lagoon (Murray, 2008).

Fluorescent Microscopy

Of the total number of foraminifera collected, 18 were identified as potentially engaging kleptoplasty based on their generic affiliation and pink/brown pigmentation observed under 20X light microscopy. This microscopic identification was a lower number than the number of specimen initially morphologically identified as *Elphidium* and *Haynesina*. According to a study by Pawlowski and Holzmann (2008), the morphology-based identification of species based on foraminiferal test is often based on limited characteristics and allows for misidentification.

Figure 2 depicts the autofluorescence observed within *Haynesina* specimens using epifluorescent microscopy. This technique was used to confirm the presence of photosynthetic pigments within the cytoplasm of the foraminiferal specimen. Epifluoresce microscopy, however, was unable to resolve the position and taxonomic identity of the autofluorescence owing to the thickness of the specimens (~150 μ m).



Figure 2. Autofluorescence occurring within the cytoplasm located in the chambers of kleptoplastic foraminifera (*Haynesina*) from Waties Island.

Phylogenetic Relationships

The phylogenetic position of the plastids retained within the Waties foraminifera was determined for three foraminiferal specimens as shown in Figure 3. The tree terminals in Figure 3 represent plastid sequences from only those foraminiferal hosts in which diatom ribosomal sequences (18S) were not able to be amplified. The maximum likelihood tree indicates that the plastids retained by Waties foraminifera are diatom in origin similar to the kleptoplast sequences from the European populations of foraminifera recovered by Pillet, de Vargas, & Pawlowski, (2011). Specifically, Figure 3 infers that Waties *Elphidium* plastids are a sister group of *Elphidium* and *Haynesia* plastids samples and *Amphora coffeaeforms*. This relationship indicates that both *Elphidium* and *Haynesia* foraminifera are primarily sequestering plastids from a single genus of diatom prey. These findings correlate with results seen in studies conducted by Correia and Lee (2000) and Pillet, de Vargas, & Pawlowski, (2011), which found species of *Elphidium excavatum* feeding primarily on pennate diatoms such as *Amphora coffeaeforms*. Despite the tree showing evidence of predator-prey specificity toward a single diatom genus, the possibility of environmental bias cannot be ruled out due to sampling a single location.

Phylogenetic positioning of diatom ribosomal (18S) sequences recovered from host foraminifera positive for both diatom ribosomal (18S) and plastid (16S) sequences are represented in Figure 4 at the tree terminals. The placement of these sequences illustrates Waties diatom sequences (18S) recovered from plastid harboring hosts are sister group to three types of *Amphora* diatoms (*Amphora pediculus*, *fogediana*, and *lybica*). However, this relationship could demonstrate prey digestion of multiple diatom species or the harvesting of chloroplasts from more than one species of Amphora living in the lagoon (Pillet, de Vargas, & Pawlowski, 2011).



Figure 3. Maximum likelihood tree showing the phylogenetic position of kleptoplasts from Waties Island foraminifera. Bootstrap support >50% are indicated at the nodes.



Figure 4. Maximum likelihood tree showing the phylogenetic position of diatom 18S sequences recovered from the cytoplasm of Waties Island foraminifera. Bootstrap support >50% are indicated at the nodes.

Conclusion

The study of the kleptoplastic phenomenon at Waties Island, SC, has given a snapshot of the specificity and origins of microbial endosymbiosis occurring between predator and prey that have, until recently, been thought to occur only in higher taxonomical multicellular specimens. However, with regards to fully understanding host specificity and plastid origins, further sampling of *Elphidium* and *Haynesina* populations along the Grand Strand, including inlets in Georgetown, SC, is needed to determine host preference toward pennate diatoms and to exclude possible environmental bias. This research opens a wide range of questions begging to be answered regarding plastid maintenance, retention mechanisms, and localization. Are we seeing horizontal gene transfer between host and prey nucleus resulting in a form of primary, secondary, or even tertiary endosymbiosis? Is the ability to maintain and establish a kleptoplastic relationship being inherited from prehistoric protists? Where is the host storing these active plastids in its cytoplasm? What is keeping these plastids from being fully digested in the host? We can answer these questions further with genetic and cellular investigations into the kleptoplastic condition.

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