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# An Examination of the Phylogenetic Diversity of Green Algae (Chlorophyceae) That Symbiose with Spotted Salamanders (Ambystoma maculatum) in the Egg Stage.

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**TABLE OF CONTENTS**

**Acknowledgements . . . . . 3**

**Abstract . . . . . 4**

**Introduction . . . . . 6**

**Materials and Methods . . . . . 9**

**Results . . . . . 12**

**Discussion . . . . . 15**

**Figures . . . . . 19**

**Tables . . . . . 23**

**References . . . . . 29**

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## ABSTRACT

Green algae form symbioses with a variety of organisms, including eggs of the spotted salamander *Ambystoma maculatum*. In 1909, the species *Oophila amblystomatis* Lambert ex Wille was described for green algae that symbiose with salamanders in the egg stage (Wille). The source of the algae is under debate, and has focused around two hypotheses: 1) that algae enter the eggs from the surrounding water once the egg clutch is laid in a pond, and 2) that the eggs acquire the algae from the maternal reproductive tract, as there is an intimate association between the tissues and even cells of the developing salamander embryo. This study tests both these hypotheses, as well as a third hypothesis developed to account for the salamander reproductive cycle. Male salamanders lay spermatophores, which are protein-filled capsules, on plant matter in and around ponds. Spermatophores are exposed to the environment before use by females in internal fertilization. Thus, we investigated possible sources of the algae in Quarry pond in Connecticut, U.S.A., by comparing *rbcL* chloroplast sequences of algae cultured from spermatophores, cloacal swabs of male and female salamanders, egg jelly samples, and multiple eggs across clutches.

Sequences of algae from eggs were distributed into five phylogenetic lineages; those from jelly, cloacal swabs, and spermatophores were found in three lineages, two of which were shared with those from eggs. In addition, all sequences from cloacal swabs aligned with sequences of free-living algae from the class Trebouxiophyceae, which are known to be heterotrophic. The majority of sequences from eggs aligned with free-living algae from class Chlorophyceae, order Volvocales, which was previously shown to symbiose with eggs of the Northwestern salamander *Ambystoma gracile* (Goff & Stein 1978). We also examined levels of genetic variation within and among egg clutches, and found variation in some clutches but not others. With one

exception, multiple sequences from a single clutch tended to align to two or fewer related lineages. We cannot exclude either the environmental acquisition or the maternal transmission hypothesis, suggesting that both sources may contribute to the presence of algae in salamander eggs.

## INTRODUCTION

Green algae form symbioses with many organisms in various stages of life. Symbiosis is a long-term interaction between two or more biological species in which one or more individuals are affected by the interaction (Moran 2006). In mutualistic symbiosis, also known as mutualism, all participants benefit, such as the symbiosis of green alga *Elliptochloris marina* with temperate Pacific sea anemones (Letsch et al. 2009). In commensalism, only one participant benefits, such as algae that live inside hairs of captive polar bears (Lewin and Robinson 1979); as opposed to amensalism, in which only one participant is harmed. Finally, in parasitism, one participant benefits at the expense of harming the other, such as infections in humans and other mammals involving the green alga *Prototheca* sp. (Lee et al. 1975).

The symbiosis involving unicellular, free-living green algae that occur within eggs of the spotted salamander *Ambystoma maculatum,* is one in which the algae provide oxygen that enhances embryonic development (Gilbert 1944, Hutchinson and Hammen 1958), and the developing embryos of *Ambystoma gracile* produce nitrogen that is transferred to algae in the form of ammonia (Goff and Stein 1978). Evidence of carbon exchange between the embryo and algae has also been found, which opens up the possibility of the algae having either a beneficial or a parasitic nature (Marco and Blaustein 2000, Graham et al. 2013).

It is unknown how green algae enter *A. maculatum* eggs. Female salamanders lay eggs, which have envelopes that are assumed to be impermeable to living organisms, embedded in jelly masses (Gilbert 1942). Multiple eggs within a jelly mass are referred to as a clutch, and clutches are laid in ponds or other watery environments. When the clutch comes into contact with water, the jelly takes up the surrounding water and expands. Gilbert (1942) proposed two avenues for algae acquisition into *A. maculatum* eggs. In the first hypothesis, algae existing in

the female salamander's reproductive tract are incorporated into eggs while the eggs are still inside the mother's body. This hypothesis is supported by the discovery of algae living inside all three envelopes of the egg. In the second hypothesis, the algae are taken up from surrounding pond water (Gilbert 1942). This hypothesis is favored by the lack of algae cultured from swabs of female salamanders' cloacal tissue (Gilbert 1942). However, the salamander egg membrane is thought to be impermeable to algae, which contradicts the possibility of the second hypothesis.

Subsequent studies were unable to conclusively determine the source of the symbiotic green algae. Several studies examined eggs for the presence of green algae under various growth conditions (Gilbert 1942, Goff & Stein 1978, Kerney et al. 2011, Kerney 2011). Over the course of these studies, it was revealed that these algae belonged to the class *Volvocales*. The algae have been discussed as members of the genera *Chlamydomonas* (Goff & Stein 1978) or *Oophila* Lambert ex Wille (Wille 1909).

This study investigates the possibility of a third "hybrid" route for algal transmission into eggs, which combines the environmental source of the algae with the timing of algal acquisition during fertilization. Male salamanders lay proteinaceous capsules called spermatophores, containing thousands of sperm cells, on submerged plant material or rocks in ponds, such as twigs and leaves. Spermatophores are exposed to pond water for undetermined lengths of time before they are picked up by female salamanders and used to fertilize eggs inside the female reproductive tract. The environmental exposure raises the possibility that algae may become associated with spermatophores prior to collection by females, after which they get incorporated into the female reproductive tract. From there, the algae may be incorporated into egg cells prior to the eggs being deposited in the environment.

In this study, we tested the genetic similarity among algae cultured from egg samples from one pond, swabs taken from the reproductive tract of both male and female adult salamanders, spermatophores, and surrounding jelly. Multiple egg clutches were sampled, with multiple eggs taken from each clutch. To isolate and analyze algal DNA only, while excluding DNA from pond bacteria or the salamander embryo, we targeted the chloroplast gene *rbcL* in genetic analyses. The chloroplast is an intracellular structure specific to photosynthesizing organisms such as green algae, and thus avoids non-photosynthetic taxa such as bacteria and oomycetes. Of various chloroplast genes, variation in the *rbcL* gene is appropriate for separating species of microscopic green algae such as those that symbiose with salamander eggs, and is commonly used as a good species-level chloroplast marker (Hall et al. 2011). A sequence alignment was made of the resulting data set, and was then used to make phylogenetic trees. Finally, the data were used to assess degrees of phylogenetic similarity among algae from various samples.

## MATERIALS AND METHODS

*Sample collection, algae isolation, and algae culturing.*

On 1 April – 4 May 2013, samples were collected from Quarry pond in the Yale-Myers forest (41.94440°N, -72.12561°W), a known breeding ground for *A. maculatum*. Five egg clutches were identified and sampled, with five eggs and one jelly sample taken from each clutch. In addition, three spermatophores and one water sample were taken from the surrounding pond water. Swabs were also taken from male and female adult salamanders found in nearby pit traps that had been previously set up. The traps consisted of buckets buried around the bottom of drift fences at the margin of the pond.

In the lab, from the fresh jelly masses that had been collected from each clutch, a total of 25 eggs and 5 jelly samples were isolated. Eggs were removed from inside the egg masses using a spatula, rinsed in sterile water, and ruptured using a sterile pipet tip. The contents and membrane of each egg, as well as a small portion of the jelly, were transferred to sterile 1.5 mL centrifuge tubes containing 500 μL of Bold's Basal Medium (BBM, Bold 1949, Bischoff and Bold 1963). Once positive growth was observed, the samples were put into growth tubes containing agar slants and liquid BBM. Spermatophore and water samples were also placed in growth tubes. Swabs were cultured tip-down in growth tubes containing BBM only. All tubes were capped with excess headspace to ensure adequate oxygen supply. Some samples were additionally plated out onto 1.5"-diameter agarose BBM growth plates. All samples were placed in a growth chamber under a 16:8 light:dark cycle at 18°C and 40 μmol photons * $m^{-2}$ * $s^{-1}$. During the growth period, tubes were opened as necessary to renew the oxygen supply. Table 1 shows the samples that had positive growth.

*Microscopy*

After growth and prior to extraction, samples were put on slides (Fisherbrand® Premium Microscope Slides, Plain, Fisher Scientific Company, L.L.C.) and covered (Fisherbrand® Microscope Cover Glass, 12-542-B, Fisher Scientific Company, L.L.C.). Slides were then examined using differential interference contrast (DIC) light microscopy, via a light microscope (Olympus BX60F, Olympus Optical Co, Ltd., Tokyo, Japan) and attached camera (Olympus DP25, Olympus Optical Co, Ltd.). The digital images were recorded in corresponding file names (cellSens Standard® 1.8.1, Olympus Corporation, Tokyo, Japan).

*DNA extraction, PCR amplification, and sequencing*

From the samples that grew in culture (Table 2), DNA was isolated using a PowerPlant® DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA), according to manufacturer's instructions. For the second centrifugation, Molecular Biology Grade Water (Fisher Scientific Company L.L.C., Waltham, MA) and a 1.5-mL centrifuge tube were substituted for eluent PD6. At each step, samples that were successfully processed were identified for further work by putting 4μL of each sample through gel electrophoresis at 120V, then staining the gel with SYBR®Safe DNA Gel Stain (Invitrogen™ Molecular Probes™, Carlsbad, CA) and visualizing the gel under the transilluminator of a SynGene Bio Imaging System in conjunction with GeneSnap 6.0.5 (Synoptics Ltd, Frederick, MD). Samples that showed up under the transilluminator were then tested for DNA concentration with a NanoDrop® spectrophotometer ND-1000 v.3.8 (Thermo Fisher Scientific Inc., Waltham, MA).

The *rbcL* gene was amplified using primers in Table 1. The PCR reaction mix contained house Taq polymerase prepared by the department from cultured *Thermus aquaticus*, or

commercial Taq and the corresponding buffer (5 Prime®, Gaithersburg, MD); 10x PCR Buffer II (Applied Biosystems®, Carlsbad, CA); $MgCl_2$ solution (Applied Biosystems®); primer stocks (Integrated DNA Technologies®, Coralvilla, IA); and a 1:1:1:1 solution of dATP, dCTP, dGTP and dTTP (EpiCentre®, Madison, WI). A MiniCycler™ was used for all PCR amplification reactions (MJ Research, Inc., St. Bruno, Quebec, Canada) according to the following cycle: 30 cycles of 94°C for 1min15s, annealing at 56°C for 2min00s, 72°C for 2min15s, 29 cycles for 1min00s each, 72°C for 7min00s, and 10°C for 5min00s. Of the resulting PCR products, those that appeared under transillumination were cleaned using a NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Bethlehem, PA) prior to observation with the spectrophotometer.

Cycle sequencing was then performed on cleaned samples using the BigDye® Terminator v1.1 Sequencing Standard Kit (Applied Biosystems®), and the same MiniCycler™ as used for PCR amplification with the following cycle: 96°C for 00min30s, 50°C for 00min15s, 60°C for 04min00s, 27 cycles for 1min00s each, and 10°C for 10min00s. The cycle-sequenced samples were run on an ABI3100 Sequencer 3130x (Applied Biosystems and Hitachi).

*Phylogenetic analyses*

Sequence reads were edited, compared against existing sequences in the National Center for Biotechnology Information online database, and assembled into contigs as appropriate using either Sequencher™ 4.5 (Gene Codes Corporation, Ann Arbor, MI) or Geneious® 6.0.3 (Biomatters Ltd., Auckland, New Zealand). Edited sequences were compared against published sequences in the National Center for Biotechnology Information online database, and together they were used to construct an alignment. Bayesian analysis was done using MrBayes under the GTR_invgamma model of sequence evolution.

## RESULTS

*Microscopy*

Green algae in culture were observed microscopically (Figure 1). Algae were found in various phases of growth; some appeared to be dividing within the algal envelope as they underwent meiosis (Figure 1b, e). On occasion, distinct multiple sections within a single cell were identified prior to DNA extraction and identification, such as those of *Tetracystis aeria* (Table 2, Sample 2013-73). Some algal cells possessed anywhere between one and four flagella (Figure 1a, g, h).

The algae observed also took on ellipsoid, coccoid, or rod-like shapes (Figure 1c, d, f). Some cells were surrounded by gelatinous sheaths (Figure 1c, e). Several displayed the orange eyespot characterizing motile algal cells, which acts as a photoreceptor and is used by motile cells to navigate up a light gradient (Figure 1a, g, h).

*DNA processing and comparison against published sequences*

Various rbcL primers were successfully used, most notably the primer pair M35 and M1161r (Table 1). Forty sequences were obtained using primer M35, 36 sequences using primer M1161r, and 1 sequence using M1390r to replace M1161r (Table 1).

Several algal cultures were found to be mixed when individual sequence reads from the same sample were compared to published sequences in the NCBI database, and also when matching reads were fitted together to make consensus sequences. Some eggs contained more than two taxa of algae (Figure 2).

*Bayesian analysis and distribution of samples*

The Bayesian analysis was performed using *Ulothrix zonata* as an outgroup. The resulting phylogeny was examined to assess phylogenetic variation of algae across clutches and within particular clutches.

Sequences were separated into 13 distinct lineages (Figure 3). Most of the lineages contained sequences of free-living algae found in the NCBI database. Of the two largest lineages in the phylogeny, lineage 1 (containing *Chlorococcum* sp. J7) encompassed algae from eggs, two spermatophores, and the Clutch 1 jelly sample. Lineage 3 (containing *Chlamydomonas pseudogloeogama*) encompassed eggs only. Two of the four spermatophore samples were distributed into lineage 1, and the other two into lineage 9 (containing *Microthamnion kuetzingianum*) and a swab from a male salamander. *M. kuetzingianum* is known to associate with salamander reproductive tracts.

Three of the five sequences obtained from male swabs overlapped with egg-containing lineages. The remaining two sequences were distributed into the *M. kuetzingianum* lineage, and a swabs-only lineage with a female swab and *Stichococcus*. There was no single lineage encompassing overlapping algae from spermatophores, swabs, and eggs.

*Across-clutch variation and within-clutch variation*

Across-clutch variation was relatively high. Some of the sequences collected from both swabs and spermatophores aligned to sequences of free-living algae not previously known to live within the salamander reproductive tract, suggesting that the algae may have been associated with the spermatophores while still inside the male salamanders' reproductive tracts.

While within-clutch was lower than across-clutch variation, degrees of variation within clutches differed widely. Clutch 3 had relatively low within-clutch variation. Of the seven sequence reads from Clutch 3, five were collected into lineage 2, while the other two fell into lineage 1 and lineage 8 (containing *Asterococcus korschikoffii*). However, Clutch 1 had relatively high within-clutch variation. The six sequence reads from eggs in clutch 1 fell into lineages 2, 5, 6, 7, and 13, which were spread out over the tree. Neither lineage 5 nor lineage 7 contained any reference sequences from GenBank. Interestingly, the sequence from the Clutch 1 jelly sample fell into lineage 1, which did not contain any sequences from Clutch 1 egg samples.

## DISCUSSION

Most of the lineages of algae cultured from salamander swabs, spermatophores, and eggs aligned to free-living algae sequences taken from GenBank. Surprisingly, the green algae samples cultured from eggs were distributed across eleven different lineages, with the majority clustering in the two largest lineages of the tree. It was previously thought that *Oophila amblystomatis*, the symbiotic algae of *A. maculatum* eggs, consisted of a single species of green algae rather than multiple species. However, the sequences found to be most closely related to the two largest lineages came from two different but related genera of green algae: *Chlorococcum* and *Chlamydomonas*. Lineages 4, 5, and 7 did not align to any of the GenBank sequences. This most likely means that there are no similar sequences in GenBank, which explains why three of the sequence reads did not make it into the tree because there were no close matches in GenBank. The algae may not yet be sequenced, or they might be of a new lineage.

In several cases, algae samples from different sources were phylogenetically linked. Out of six swab samples, three aligned with algae from eggs, suggesting a link between algae living in the cloaca and those symbiotic with eggs (Figure 4). Of the other three swabs, one aligned with algae from a spermatophore in lineage 9 (containing *Microthamnion kuetzingianum*). As found in other studies, this supports the hypothesis that algae that are capable of forming a symbiosis with the adult salamanders' reproductive tracts can also survive in an external environment (Kerney 2011).

Within-clutch variation was lower than across-clutch variation. Out of the five clutches with samples from at least three eggs, four clutches had at least half of all samples that aligned to the same lineage, indicating a close phylogenetic association within the clutch. For instance, many of the samples from Clutch 3 initially matched to different sequences in BLAST analysis

and thereby suggested that the symbiotic algae were phylogenetically mixed (Figure 2). Yet the Bayesian alignment showed that the majority of the algal sequences from this clutch fell within the same lineage (Figure 3). This demonstrates that the sequences still may be from the same species of algae, and that there is likely to be a common source of algae within a single clutch. On the other hand, the single jelly and five egg samples from Clutch 1 included algae from six different lineages (Figure 3). The significant lack of a common lineage in this clutch suggests that the direct environmental acquisition hypothesis may not be the primary factor in how algae enter the eggs.

Overall, the phylogeny shows cloaca-associated algae being more genetically diverse than egg-associated algae. The cloaca-associated algae aligned to free-living algal sequences such as *Stichococcus* and *M. kuetzingianum*, both of which are members of the Trebouxiophyceae class of algae, as well as sequences from the Chlorophyceae class. For the most part, the egg-associated algae tended to be restricted to chlorophytes, particularly the order Volvocales. Goff and Stein (1978) previously showed that algae found in symbiosis with eggs of the Northwestern salamander, or *Ambystoma gracile*, also belonged to Volvocales. The correlation between egg-associated algae in both *A. maculatum* and *A. gracile*, and Volvocales, suggests that the algae previously labeled *Oophila* are likely to be found in this order. Some free-living algae that the egg-associated algae aligned to came from the genera of *Chlorococcum*, *Chlamydomonas*, and *Asterococcus* (Figure 3; Lineages 1, 2, 3, 6, 8, 11, 12). In particular, *Chlorococcum* sp. J7 and *Chlamydomonas pseudogloeogama* bore significant similarity to fourteen out of the eighteen egg-associated algae sequences assessed in the tree. Some algae from the swabs, such as *M. kuetzingianum*, were not associated with any of the egg samples at all, suggesting that some species of algae which can occur in adult salamander reproductive

tracts may not be capable of surviving in symbiosis with eggs. Because of the marked differences between cloaca- and egg-associated algae, it may be that algae need to have different traits in order to symbiose either with adult salamander reproductive tracts or with salamander embryos. The close alignment of egg-associated algae with free-living algae in the order Volvocales suggests that the traits enabling the symbiosis are characteristic of, and unique to, Volvocales. Whether these particular traits are genetic, metabolic, or otherwise is still up for future investigation.

Unfortunately, it has not been shown in this study whether there is a direct link between swabs, spermatophores, and eggs, as there was no single lineage found to encompass all three eggs. In this experiment, no data were obtained on the diversity of free-living algae in pond water. Also, there were not enough samples within a particular lineage to conduct an analysis of molecular variation on the samples. Future work could increase the sampling size to include more samples in general, as well as collection of samples from the environment. Getting environmental data on the algal community in a pond, as opposed to the diversity of algae living in salamander reproductive tracts, in the same study might help to distinguish between the two previously proposed hypotheses. More data in general would also need to be collected from cloacal swabs, spermatophores, and eggs.

Another possible route to take would be to investigate why certain species of algae can live in eggs, but others have not been found in eggs. In particular, the alignment of cloaca-associated algae primarily to trebouxiophytes and alignment of egg-associated algae to chlorophytes could be further investigated, as this difference suggests that algae might have distinct metabolic profiles in order to benefit from the different symbioses. Environmental temperature, pH, and other factors might also inhibit or promote the survival of a particular

group of algae in one environment or the other, thereby causing the difference in symbioses formed with algae.

## FIGURES

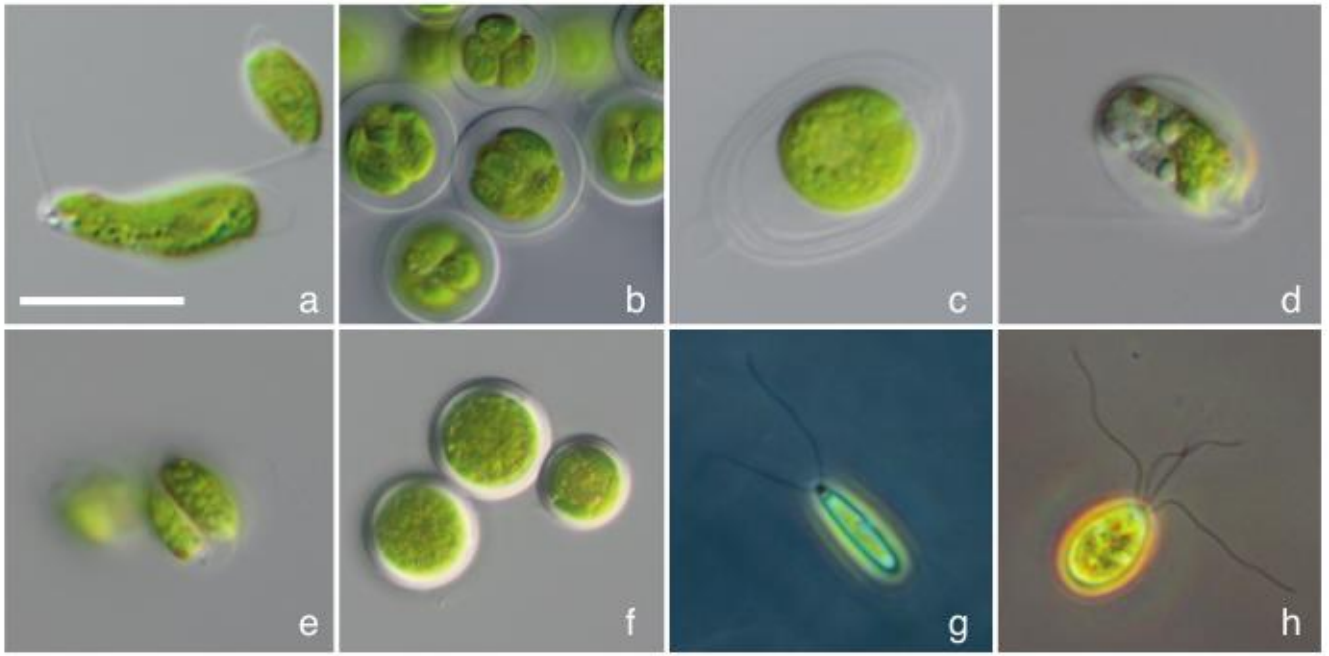


**Figure 1.** Light micrographs of selected algae cultured from spotted salamander eggs. Scale bar = 10 μM.

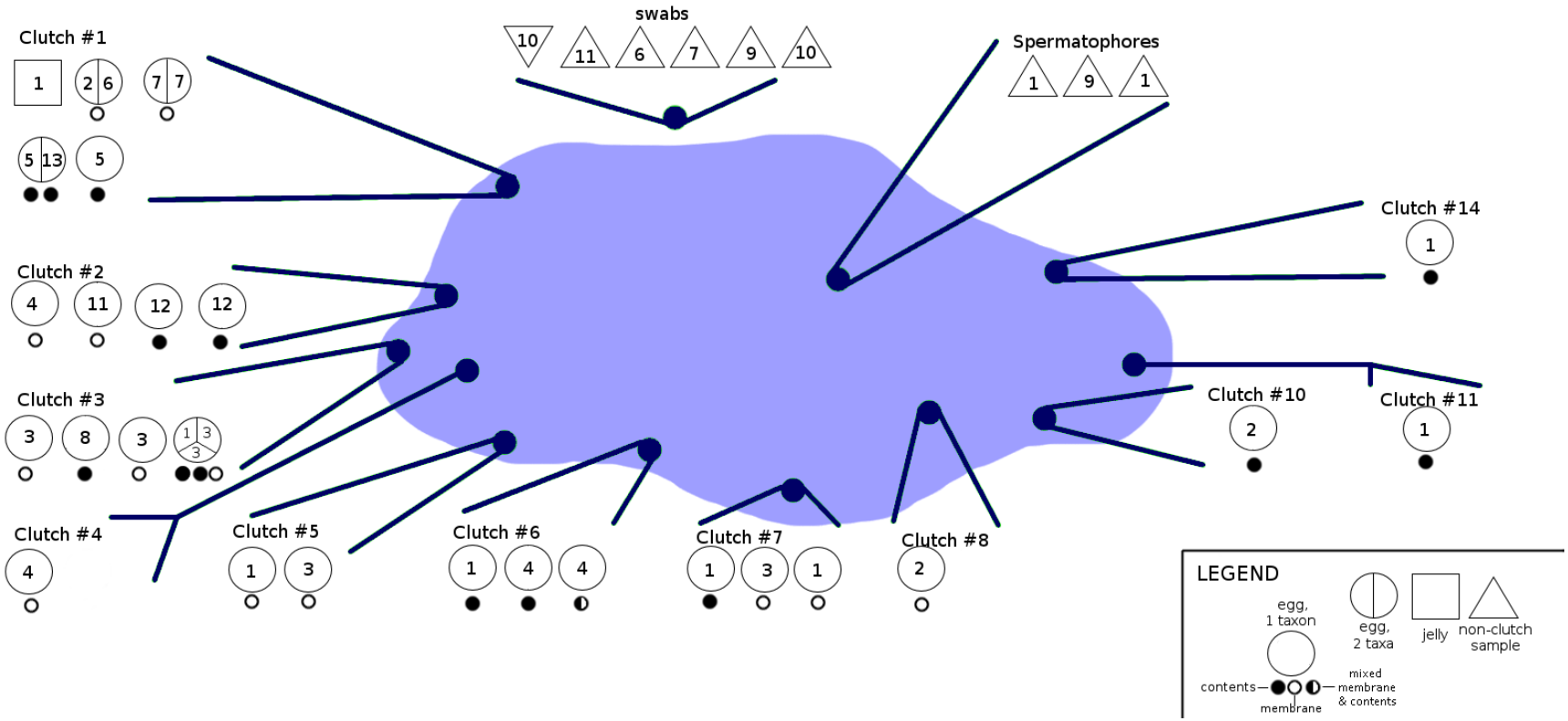


**Figure 2.** Diagram of samples collected from Quarry pond, including the number of published algal sequences in GenBank that the individual sequence reads from each sample matched to. Different geometric shapes indicate different sources of the samples. Numbers inside each demarcated area correspond to a lineage in the Bayesian tree (Figure 3).

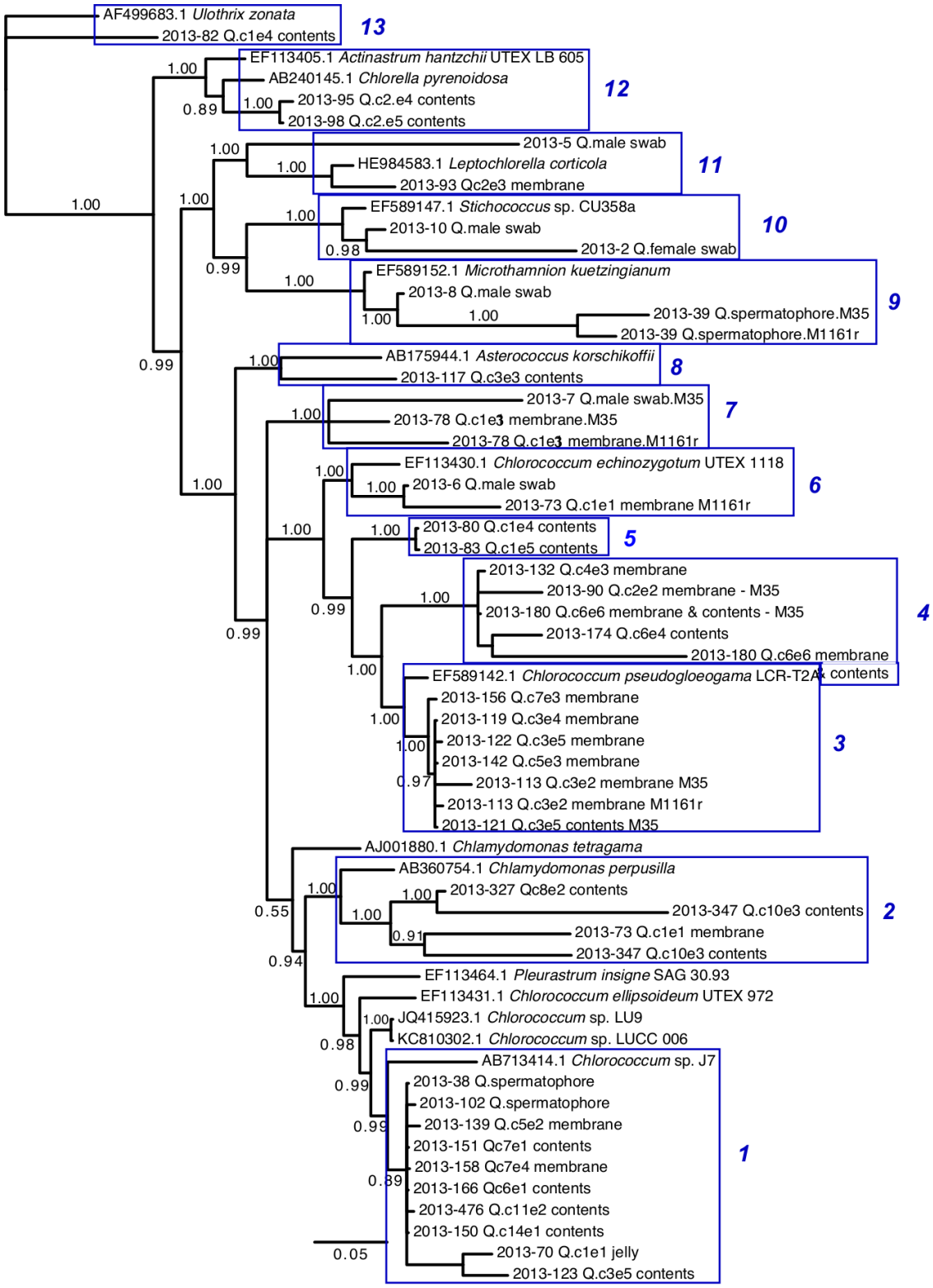


**Figure 3.** Bayesian tree of all sequences collected, plus related published algal sequences from GenBank. *Ulothrix zonata* was used as an outgroup. Numbers above branches indicate posterior probabilities. Scale bar = expected number of substitutions per site.

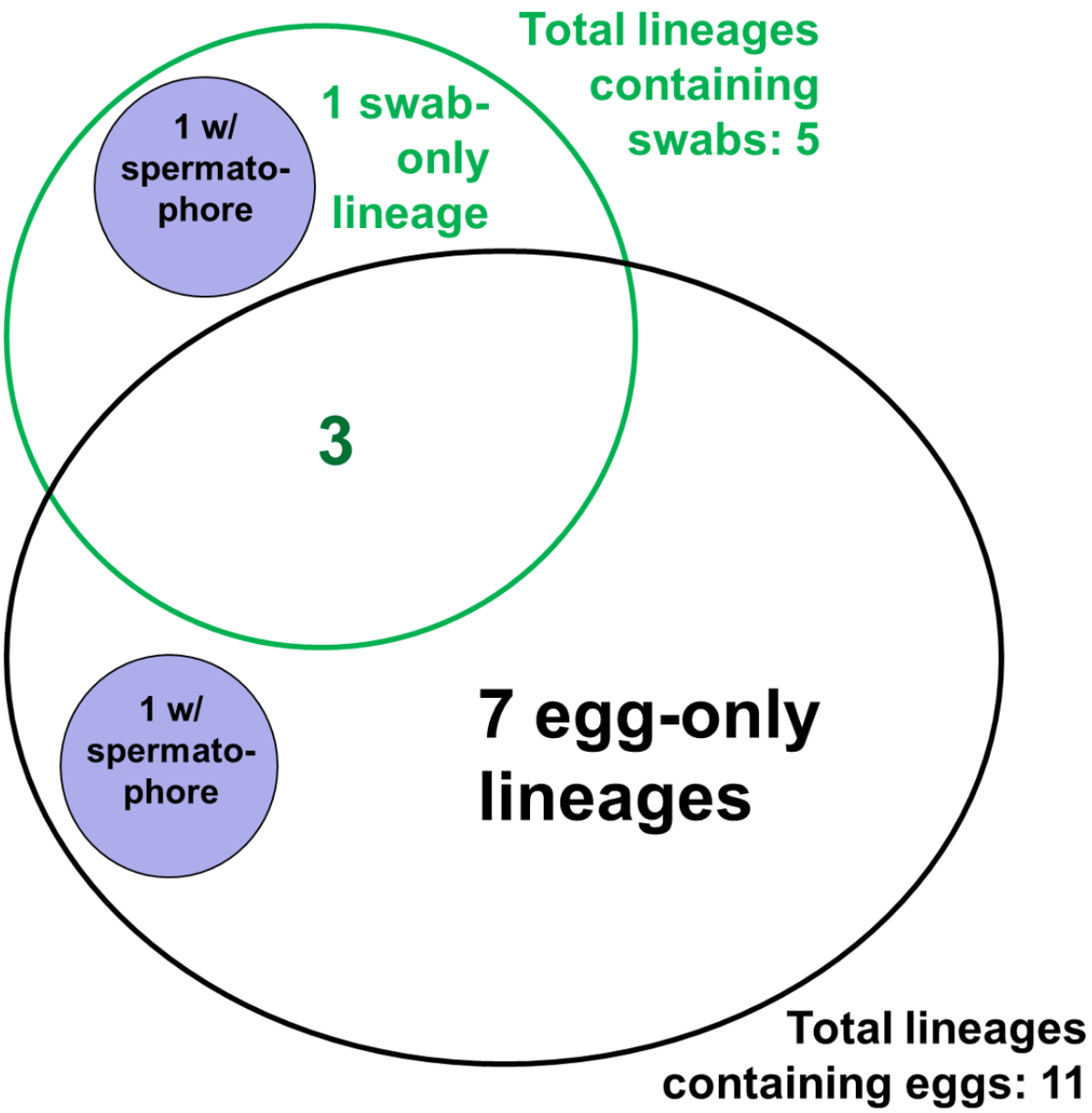


**Figure 4.** Venn diagram of lineages in Bayesian tree. Shows overlap of different sample sources between lineages.

## TABLES

**Table 1.** Table of all *rbcL* PCR and sequencing primers used to attempt to obtain sequences.

| **Primer** | **Sequence (5'--> 3')** | **Citation** |
|---|---|---|
| M35 | GGR WTT AAA GCT GGT GTA AAA GAC T | McManus & Lewis 2011 |
| M636 | GCT TTG GAG AGA TCG TTT CT | Lewis et al. 1997 |
| M650r | CGG TCT CTC CAA CGC ATG A | McManus & Lewis 2011 |
| M955 | CGT WTA TCT GGT GGA GAY C | McManus & Lewis 2011 |
| M1010r | CCT TGA AGT TTA CCT ACA AC | Lewis et al. 1997 |
| M1161r | CAT GTG CCA TAC GTG AAT AC | McManus & Lewis 2011 |
| M1390r | CTT TCC AAA YTT CAC AAG CAG CAG | McManus & Lewis 2011 |

**Table 2.** Collection data and closest GenBank sequence matches of samples taken from Quarry pond.

| Date of Collection (mm/dd/yyyy) | Sample Identity | Sample Number | Sequencing Primer | Closest GenBank match to individual sequence read | Closest GenBank match to consensus read |
|---|---|---|---|---|---|
| 04/01/13 | Female swab, outer bucket | 2013-2 | M35<br>M1161r | *Stichococcus*<br>Short | N/A |
| 04/01/13 | Male swab, outer bucket | 2013-5 | M35<br>M1161r | HQ287482.1 *Coccomyxa*<br>HQ287482.1 *Coccomyxa* | HQ287478.1 *Coccomyxa* sp. PL2-1 |
| 04/01/13 | Male swab, outer bucket | 2013-6 | M35<br>M1161r | EF113430.1 *Chlorococcum*<br>EF113430.1 *Chlorococcum* | EF113430.1 *Chlorococcum echinozygotum* UTEX 1118 |
| 04/01/13 | Male swab, outer bucket | 2013-7 | M35<br>M1161r<br>M35<br>M1161r | HQ246361.1 *Scenedesmus*<br>No match<br>No match<br>No match | N/A<br><br>N/A |
| 04/01/13 | Male swab, outer bucket | 2013-8 | M35<br>M1161r | EF589152.1 *Microthamnion*<br>EF589152.1 *Microthamnion* | EF589152.1 *Microthamnion kuetzingianum* CCAP 450/1b |
| 04/01/13 | Male swab, outer bucket | 2013-9 | M35<br>M1161r<br>M35<br>M1390r | Short, noisy<br>Short, noisy<br>No match<br>No match | N/A<br><br>N/A |
| 04/01/13 | Male swab, outer bucket | 2013-10 | M35<br>M1161r | EF589147.1 *Stichococcus*<br>EF589147.1 *Stichococcus* | EF589147.1 *Stichococcus* sp. CU358a |
| 04/01/13 | Female swab, outer bucket | 2013-22 | M35<br>M1390r | No match<br>No match | N/A |
| 04/01/13 | Spermatophore | 2013-38 | M35<br>M1161r | JQ415923.1 *Chlorococcum*<br>EF113431.1 *Chlorococcum* | JQ415923.1 *Chlorococcum* sp. LU9 |
| 04/01/13 | Spermatophore | 2013-39 | M35<br>M1161r | EF589152.1 *Microthamnion*<br>EF589152.1 *Microthamnion* | N/A |

| Date of Collection (mm/dd/yyyy) | Sample Identity | Sample Number | Sequencing Primer | Closest GenBank match to individual sequence read | Closest GenBank match to consensus read |
|---|---|---|---|---|---|
| 04/08/13 | c1 jelly | 2013-70 | M35<br>M1161r<br>M35<br>M1161r | Short, noisy<br>Short, noisy<br>AB713414.1 *Chlorococcum*<br>KC810302.1 *Chlorococcum* | N/A<br><br>KC810302.1 *Chlorococcum* sp. LUCC 006 |
| 04/08/13 | c1 e1 membrane | 2013-73 | M35<br>M1161r<br>M35<br>M1161r | Short, noisy<br>Short, noisy<br>EF113476.1 *Tetracystis aeria*<br>EF113430.1 *Chlorococcum* | N/A<br><br>N/A |
| 04/08/13 | c1 e2 contents | 2013-74 | M35<br>M1161r<br>M35<br>M1161r | Short, noisy<br>Short, noisy<br>Short, noisy<br>Short, noisy | N/A<br><br>N/A |
| 04/08/13 | c1 e3 contents | 2013-77 | M35<br>M1390r | No match<br>No match | N/A |
| 04/08/13 | c1 e3 membrane | 2013-78 | M35<br>M1161r | U80809.1 *Chloromonas*<br>AJ001881.1 *Chlorogonium* | N/A |
| 04/08/13 | c1 e4 contents | 2013-80 | M35<br>M1161r | EF589142.1 *Chlamydomonas*<br>EF589142.1 *Chlamydomonas* | EF589142.1 *Chlamydomonas pseudogloeogama* LCR-T2A |
| 04/08/13 | c1 e4 contents plated | 2013-82 | M35<br>M1390r | AF499683.1 *Ulothrix*<br>AF499683.1 *Ulothrix* | AF499683.1 *Ulothrix zonata* |
| 04/08/13 | c1 e5 contents plated | 2013-83 | M35<br><br>M1161r | EF589142.1 *Chlamydomonas*<br><br>EF589142.1 *Chlamydomonas* | AJ001880.1 *Chlamydomonas tetragama* ribulose bisphosphate |
| 04/08/13 | c2 e1 membrane | 2013-87 | M35<br>M1390r | No match<br>No match | N/A |

| Date of Collection (mm/dd/yyyy) | Sample Identity | Sample Number | Sequencing Primer | Closest GenBank match to individual sequence read | Closest GenBank match to consensus read |
|---|---|---|---|---|---|
| 04/08/13 | c2 e2 membrane | 2013-90 | M35<br>M1161r | EF589142.1 *Chlamydomonas*<br>No match | N/A |
| 04/08/13 | c2 e3 contents | 2013-92 | N/A | N/A | N/A |
| 04/08/13 | c2 e3 membrane | 2013-93 | M35<br>M1161r | EF589142.1 *Chlamydomonas*<br>HE984583.1 *Leptochlorella* | HE984583.1 *Leptochlorella corticola* |
| 04/08/13 | c2 e4 contents | 2013-95 | M35<br>M1161r | EF113405.1 *Actinastrum*<br>EF113405.1 *Actinastrum* | EF113405.1 *Actinastrum hantzchii* UTEX LB605 |
| 04/08/13 | c2 e5 contents | 2013-98 | M35<br>M1161r | EF113405.1 *Actinastrum*<br>HM101339.1 *Chlorella* | AB240145.1 *Chlorella pyrenoidosa* |
| 04/01/13 | Spermatophore, previously plated 4-1-13 | 2013-102 | M35<br>M1161r<br>M35<br>M1161r | Short, noisy<br>Short, noisy<br>AB713414.1 *Chlorococcum*<br>EF113431.1 *Chlorococcum* | N/A<br><br>EF113431.1 *Chlorococcum ellipsoideum* UTEX 972 |
| 04/08/13 | c3 e2 membrane | 2013-113 | M35<br>M1161r | EF589142.1 *Chlamydomonas*<br>EF589142.1 *Chlamydomonas* | N/A |
| 04/08/13 | c3 e3 membrane | 2013-116 | M35<br>M1161r | Short, noisy<br>EF589142.1 *Chlamydomonas* | N/A |
| 04/08/13 | c3 e3 contents plated | 2013-117 | M35<br>M1161r | EF113450.1 *Lobocharacium*<br>AB 175944.1 *Asterococcus* | AB 175944.1 *Asterococcus korschikoffii* |
| 04/08/13 | c3 e4 membrane | 2013-119 | M35<br>M1161r | EF589142.1 *Chlamydomonas*<br>EF589142.1 *Chlamydomonas* | EF589142.1 *Chlamydomonas pseudogloeogama* LCR-T2A |
| 04/08/13 | c3 e5 contents | 2013-121 | M35 | EF589142.1 *Chlamydomonas* | N/A |
| 04/08/13 | c3 e5 membrane | 2013-122 | M35<br>M1161r | EF589142.1 *Chlamydomonas*<br>EF589142.1 *Chlamydomonas* | EF589142.1 *Chlamydomonas pseudogloeogama* LCR-T2A |
| 04/08/13 | c3 e5 contents plated | 2013-123 | M35<br>M1161r | AB713414.1 *Chlorococcum*<br>EF113464.1 *Pleurastrum* | EF113464.1 *Pleurastrum insigne* SAG 30.93 |

| Date of Collection (mm/dd/yyyy) | Sample Identity | Sample Number | Sequencing Primer | Closest GenBank match to individual sequence read | Closest GenBank match to consensus read |
|---|---|---|---|---|---|
| 04/08/13 | c4 e2 membrane | 2013-129 | M1161r | EF589142.1 *Chlamydomonas* | N/A |
| 04/08/13 | c4 e3 membrane | 2013-132 | M35<br>M1161r | EF589142.1 *Chlamydomonas*<br>EF589142.1 *Chlamydomonas* | EF589142.1 *Chlamydomonas pseudogloeogama* LCR-T2A |
| 04/08/13 | c5 e2 membrane | 2013-139 | M35<br>M1161r | AB713414.1 *Chlorococcum*<br>JQ415923.1 *Chlorococcum* | JQ415923.1 *Chlorococcum* sp. LU9 |
| 04/08/13 | c5 e3 membrane | 2013-142 | M35<br>M1161r | EF589142.1 *Chlamydomonas*<br>EF589142.1 *Chlamydomonas* | EF589142.1 *Chlamydomonas pseudogloeogama* LCR-T2A |
| 04/17/13 | c7 e1 contents | 2013-151 | M35<br>M1161r | AB713414.1 *Chlorococcum*<br>KC810302.1 *Chlorococcum* | EF113431.1 *Chlorococcum ellipsoideum* UTEX 972 |
| 04/17/13 | c7 e3 membrane | 2013-156 | M35<br>M1161r | EF589142.1 *Chlamydomonas*<br>EF589142.1 *Chlamydomonas* | EF589142.1 *Chlamydomonas pseudogloeogama* LCR-T2A |
| 04/17/13 | c7 e4 membrane | 2013-158 | M35<br>M1161r | JQ415923.1 *Chlorococcum*<br>AB713414.1 *Chlorococcum* | AB713414.1 *Chlorococcum* sp. J7 |
| 04/17/13 | c6 e1 contents plated | 2013-166 | M35<br>M1161r<br>M35<br>M1161r | Short, noisy<br>Short, noisy<br>AB713414.1 *Chlorococcum*<br>JQ415923.1 *Chlorococcum* | N/A<br><br>EF113431.1 *Chlorococcum ellipsoideum* UTEX 972 |
| 04/17/13 | c6 e4 contents | 2013-174 | M35<br>M1161r | EF589142.1 *Chlamydomonas*<br>EF589142.1 *Chlamydomonas* | EF589142.1 *Chlamydomonas pseudogloeogama* LCR-T2A |
| 04/17/13 | c6 e6 inner membrane and inner contents | 2013-180 | M35<br>M1161r | EF589142.1 *Chlamydomonas*<br>EF113425.1 *Chlamydomonas* | N/A |
| 04/17/13 | c8 e1 inner membrane | 2013-325 | M35<br>M1161r | EF589142.1 *Chlamydomonas*<br>EF589142.1 *Chlamydomonas* | EF589142.1 *Chlamydomonas pseudogloeogama* LCR-T2A |
| 04/17/13 | c8 e2 contents | 2013-327 | M35<br>M1161r | AB360754.1 *Chlamydomonas*<br>AB360754.1 *Chlamydomonas* | AB360754.1 *Chlamydomonas perpusilla* |
| 04/17/13 | c8 e3 contents | 2013-329 | N/A<br>N/A | N/A<br>N/A | N/A |

| Date of Collection (mm/dd/yyyy) | Sample Identity | Sample Number | Sequencing Primer | Closest GenBank match to individual sequence read | Closest GenBank match to consensus read |
|---|---|---|---|---|---|
| 04/17/13 | c10 e3 contents | 2013-347 | M35<br>M1161r | AB360754.1 *Chlamydomonas*<br>AB360754.1 *Chlamydomonas* | N/A |
| 04/17/13 | c10 e4 contents | 2013-349 | N/A<br>N/A | N/A<br>N/A | N/A |
| 04/17/13 | c10 e5 contents | 2013-351 | N/A<br>N/A | N/A<br>N/A | N/A |
| 05/04/13 | c11 e2 contents | 2013-476 | M35<br>M1161r | EF113431.1 *Chlorococcum*<br>KC810302.1 *Chlorococcum* | KC810302.1 *Chlorococcum* sp. LUCC 006 |
| 05/04/13 | c14 e1 contents plated | 2013-510 | M35<br>M1161r | AB713414.1 *Chlorococcum*<br>KC810302.1 *Chlorococcum* | KC810302.1 *Chlorococcum* sp. LUCC 006 |

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