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The Symbiotic Green Algae, Oophila (Chlamydomonadales, Chlorophyceae): A Heterotrophic Growth Study and Taxonomic History

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ABSTRACT

The green alga *Oophila ambystomatus* (Chlamydomonadales, Chlorophyceae) is well known as the symbiotic partner of *Ambystoma maculatum*, the yellow-spotted salamander. Recent molecular work has revealed phylogenetic diversity within isolates of *Oophila* from New England. Here I investigate physiological properties and genetic diversity of the algal symbionts representing different evolutionary lineages. In other well-studied symbiotic systems involving algae, e.g., *Chlorella* and *Hydra*, algae and lichen, or *Symbiodinium* and Scleractinian corals, the algal symbiont is capable of multiple trophic modes, thus influencing its capacity as a partner. In this study I investigated whether genetically differentiated strains of *Oophila* are capable of absorbing organic carbon compounds (heterotrophic growth) and hypothesize how this might play into its symbiotic relationship. A growth study examined each strain's ability to grow in light (16:8 L:D; 41 μmol/m^2) and in dark (0:24 L:D; 0 μmol/m^2) in three different media types (BBM, BBM+glu, BBM+gal). I determined there is metabolic variability among the strains that indicates each may provide differential benefits to their partner.

I also reviewed the taxonomic history of *Oophila* to resolve confusion over its validity. After a brief recount of its history, I determined that the name is valid, and suggest a recent collection from the type locality of Middlesex Fells Reservation be designated as an epitype given that the aged type material lacks diagnostic features and its DNA is degraded. An epitype will provide future researchers an unambiguous anchoring specimen and sequence for future taxonomic, molecular and physiological studies.

INTRODUCTION

The group of green algae referred to as *Oophila* (Chlamydomonadales, Chlorophyceae) is best known for the symbiotic partnerships they share with the yellow spotted salamander, *Ambystoma maculatum*. Algae are found to inhabit eggs from an early developmental stage and eventually form a bloom in most eggs within a clutch. Much research has focused on defining the interaction between the symbionts and has discovered that the algae and salamander embryos provide a benefit to each other. Oxygen and fixed carbon produced by the photosynthetic algae are used by the embryo (Bachmann *et al.*, 1985; Graham *et al.*, 2013; Graham *et al*., 2014; Hutchinson & Hammen, 1958; Pinder & Friet, 1994; Valls & Mills, 2007), and the embryo excretes nitrogenous wastes that are used by the algae (Goff & Stein, 1978).

Recent work has examined genetic diversity of the algal symbionts in the hopes of better understanding their variability as symbiotic partners and how algae first enter salamander eggs. In other symbiotic systems involving algae, including *Chlorella* and *Hydra*, and *Symbiodinium* and Scleractinian corals, algae are selected by their hosts for particular metabolic traits. Current molecular phylogenetic work on *Oophila* has revealed that the symbiotic algae are not monophyletic, and I hypothesize that genetically distinct algae are not physiologically equivalent. This matter is addressed in Chapter 1.

Unexpected genetic diversity among the symbiotic algae isolates from eggs of *Ambystoma maculatum*, has led to the designation of a large and diverse *Oophila* clade (Kim *et al*., 2014), but the specific- and generic-level taxonomy of the genus has not been reviewed in any comprehensive way with modern tools and data. This is due in part to the taxonomic uncertainty of the type species and additional lack of genetic material to

represent the type. Some workers insinuate that *Oophila amblystomatis* is an invalid combination, but my preliminary assessment of its taxonomic history (detailed below) indicates the name and description are valid. Given the molecular disparity in *Oophila ambystomatis* collections from the Northeast, *O. ambystoma* is a good candidate for epitypification, where collection of a new specimen from the type locality could be used to represent the holotype (Chapter 2). Formal taxonomic changes will be presented in a publication resulting from this chapter.

CHAPTER I

TESTING HETEROTROPHY AND MIXOTROPHY OF GREEN ALGAE (CHLAMYDOMONADALES, CHLOROPHYCEAE) ASSOCIATED WITH THE EGGS OF THE YELLOW-SPOTTED SALAMANDER, *AMBYSTOMA MACULATUM*

INTRODUCTION

Photosynthetic organisms use ATP and NADPH, produced during the light reactions of photosynthesis, to build organic sugars from CO_2 (Graham *et al.,* 2009). Some photosynthetic species of algae, including diverse green algae (Neilson & Lewin, 1974; Liu *et al.,* 2014), are considered mixotrophic, having a mixture of trophic modes. These lineages regularly employ photosynthesis but under certain environmental conditions also obtain organic compounds from their surroundings, thus augmenting their photo-dependent carbon-fixation cycle. This ability, called heterotrophy, would enable algae to use external carbon sources for growth, when photosynthesis is limited. Liu *et al.* (2014) evaluated the heterotrophic abilities of a small number of diverse green algae using a range of organic carbon compounds including acetate, fructose, glycerin, glycine, glucose, starch and sucrose, and showed variation among and even within green algal lineages. This ability might prove to be important for the interaction between the symbiotic algae and their partners.

There is growing evidence that the algae forming symbiotic relationships with fungi and animals commonly have capacity for heterotrophic growth. The green alga *Chlorella* that forms a symbiosis with the green hydra, *Hydra viridissima* (Kovacević *et*

al., 2007) has an ability to grow under both heterotrophic and mixotrophic conditions (Liang *et al.,* 2009). The dinoflagellate *Symbiodinium*, that forms a symbiosis with Scleractinian corals, can grow under heterotrophic conditions by feeding on bacteria (Jeong *et al.,* 2012) and by absorbing carbon under heterotrophic and mixotrophic conditions (Xiang *et al.,* 2013).

In the mixotrophic partnership that exists between green algae and the yellow spotted salamander, *Ambystoma maculatum*, the green algae are primarily found within salamander eggs and quickly multiply until the developing embryo is almost obscured by dense algae (Gilbert, 1942). The algae photosynthesize inside the egg (Bishop & Miller, 2014) and supply the developing embryo with oxygen (Bachman *et al.,* 1985; Hutchinson & Hammen, 1958). The provision of dissolved oxygen inside the egg increases body mass and decreases development time of *A. maculatum* embryos when compared to embryos that lack a symbiotic partner (Graham *et al.,* 2013). The embryo may confer nutritional benefits to the algae as embryonic respiration and metabolism in the form of dissolved carbon dioxide and nitrogenous waste are hypothesized to enhance algal growth (Gilbert, 1942; Gilbert, 1944).

Recent molecular work on *Oophila* indicates there are multiple lineages of symbiotic algae involved in this symbiosis (Kim *et al.,* 2014; Lin & Bishop, 2015). Given the non-monophyly, each species, or even strain of a given species, may differ in its capacity for photosynthesis and growth through different metabolic pathways. Besides variation among strains, the physiological optima for photosynthesis may also vary with environmental conditions. Thus, across diverse environmental conditions, certain algae may be more or less beneficial to their symbiotic partners.

The objective of the current study is to test the hypothesis that different strains of green algae associated with eggs of the spotted salamander vary in their capacity for heterotrophy and mixotrophy. I examine the heterotrophic growth capability of different strains of symbiotic algae and discuss what impact this could have in their symbiotic relationship with *Ambystoma maculatum*. First, I determined that eight strains of symbiotic algae form three distinct phylogroups. Then, I examined the ability of these eight strains to survive under a full factorial design of two photoperiods and three liquid media treatments. Chlorophyll fluorescence of experimental cultures was measured over the course of a twenty-eight-day period to evaluated growth of each strain. Results were analyzed to determine if these algae perform heterotrophy and mixotrophy and if these capabilities vary among strains.

MATERIALS AND METHODS

Collection and Isolation

As part of a larger study on algal diversity, salamander eggs from a pond in the Yale-Myers Forest, Tolland, CT ("Quarry" 41.94440°N, -72.12561°W) were collected during the salamander's breeding season and transported to the University of Connecticut. Algae were isolated from salamander eggs in a sterile environment and cultured in 1.5 mL centrifuge tubes containing 500 µL Bold's Basal Medium (BBM; Bold, 1949; Bischoff & Bold, 1963) by Xue (2014). Each culture was grown to a high density and verified to be unialgal through morphological observation and with molecular investigation of the chloroplast gene *rbc*L. These strains remain in good health and have been used for subsequent physiological investigation.

Culturing

Algal strains used in this experiment were grown in replicate cultures containing BBM and given an antibiotic treatment consisting of Ampicilin & Cefotaxime (Kan & Pan, 2010) to ensure cultures were free of bacteria and fungi. The axenic cultures were grown to a density of 1 x 10^6 cells/mL and maintained at a high rate of growth. Relative fluorescence units (RFU) were measured and correlated with cell counts for each stock culture to allow cell density estimation of each culture replicate during the experiment (Fig 1).

Media Preparation

Three solutions of BBM were prepared at volumes of 1.5 L each and autoclaved at 121°C for 20 min. Two modifications of standard BBM medium contained 1% D-glucose (BBM+glu) and 1% D-galactose (BBM+gal), respectively, and mixed over a warm plate until completely dissolved. This yielded a final concentration of 1% for each organic substrate. The pH of each medium was verified using a calibrated Orion Star A111 pH meter (Thermo Scientific) and adjusted to pH 6.6. Each medium was then passed through Whatman No. 1 filter paper and then distributed into sterile 25 mL glass culture tubes.

Experimental Design

Algae in the log phase of growth were used to established replicate treatments (n=3). Each algal strain was distributed in glass test tubes at a density of up to 1x10^6 cells/tube, with a final volume of 20 mL (Table 1). Each algal strain (n=8) was tested in a light (2) x medium (3) factorial design. The three media treatments were BBM, BBM+glu, BBM+gal, and the two light conditions 16:8 L:D; 41 μmol/m^2 and 0:24 L:D; 0

μmol/m^2. D arkness was achieved by covering 0:24 L:D photoperiod culture tubes in aluminum foil. All experimental culture tubes were placed in an incubator at 18°C and left to grow for 28 d, with daily agitation.

Cell density of each culture was evaluated at day t=0, and every 2d, for the duration of the 28d experiment. Growth was monitored using a Synergy H1 Hybrid Reader (BioTek) that measures fluorescence at an emission of 675nm, with each being an average of four readings (See Supplementary Material: Spreadsheet S1).

Phylogenetic Tree Construction

Individual sequence reads (n=8) were edited and assembled by Xue (2014) in Genious 9.0.5 to generate consensus sequences. Consensus sequences were run through BLAST (http://blast.ncbi.nlm.nih.gov/, last accessed August 8, 2016) and a 52-taxon *rbc*L dataset was compiled and aligned in Geneious. PAUP* (Swofford, 2002) automated model selection was run using the AICc and BIC criteria, and the GTR+I+G model was chosen for the *rbc*L dataset (rmatrix=(0.76297064 2.199366 4.8115995 0.96031153 7.3429332) basefreq=(0.26355985 0.13955192 0.20767833) rates=gamma shape=0.79950836 pinv=0.20767833) and the TnR+I+G model was chosen for the 18S dataset (rmatrix=(1 2.3729843 1 1 5.0036588) basefreq=(0.24775298 0.20541567 0.27398467) rates=gamma shape=0.55398631 pinv=0.54397673). Bayesian analysis was run in 4 chains of $3x10^6$ generations with three heated chains. Trees were sampled every 5,000 generations and the first 500,000 samples of each run were discarded as burnin. Alignments can be found in Supplementary Material (S2, S3).

RESULTS

Molecular Phylogenetic Analyses

In this study, *rbc*L sequences of eight strains of symbiotic algae were used from Xue (2014). Analysis of the *rbc*L dataset revealed the eight symbiotic sequences were non-monophyletic and formed three well-separated clades in the 52-taxon tree. Strain 2013-80 forms clade I, strains 2013-123, 2013-151 and 2013-476 formed clade II, and strains 2013-119, 2013-129, 2013-142 and 2013-150 form clade III and (Fig 2). Clades I and III fall within the "*Oophila* clade" identified by Kim *et al.* (2014). Two sequences produced by Lewis & Landberg (2011) also fall within the larger "*Oophila* clade" and are distinct from Clades I and III. Members of Clade II fall outside of the larger "*Oophila* clade" into the *Protosiphon* clade. An 18S tree generated for this study highlights the large "*Oophila* clade" identified by Kim *et al.* (2014) containing a diverse range of *Oophila* isolates. Two sequences isolated by Lewis & Landberg (2011) fall within this clade and 2013c-469 forms a clade with the 18S sequence of Kerney *et al.* (2011) (Fig3), which is distinct from our clades I and III (Fig 2.) and represents a clade not examined in this study (Fig 3). Clade II is recognized in the 18S tree by the association with *Protosiphon* (Fig 3).

Growth Experiment

All strains grown under a diurnal (16:8; L:D) photoperiod and in all three media treatments exhibited an increase in cell number, from the start to the end of the experiment (Figs. 4A-H), with the exception of strain 2013-151 grown in BBM and strain 2013-476 grown in BBM+gal (Figs. 4G-H). These cultures exhibited population maintenance but not an increase in cell number. Also, all strains grown under the diurnal

(16:8 L:D) photoperiod condition achieved higher final cell density than cultures grown under the no light (0:24 L:D) photoperiod condition (Figs. 4A-H).

In the dark treatments (0:24 L:D) strains 2013-80, 2013-119, 2013-123 and 2013-151 did not show significant growth in any of the three media treatments and were maintained near initial populations throughout the experiment (Figs. 4A-C,G). Strain 2013-129 experienced negative net growth in BBM alone throughout the experiment, whereas treatments containing glucose and galactose had positive net growth (Fig. 4D). Strain 2013-142 grown in BBM experienced negative net growth throughout the experiment whereas cells in the BBM treatments with glucose and galactose were maintained around their starting cell numbers (Fig. 4E). Strains 2013-150 and 2013-476 experienced negative net growth in all three media treatments in the dark (Figs. 4F,H). Results from this study are summarized in Table 2 with an indication of positive growth, negative growth or net population maintenance (neutral) over the course of the experiment.

The type of media in which the algae grew had a significant effect on the growth of each strain (*ANOVA*, see Table 3) with the exception of 2013-80 (*ANOVA*, $p = 0.184537$). Also, as expected, the photoperiod had a significant effect on the growth of each strain (*ANOVA*, see Table 3). The interaction of the media treatment and the photoperiod was significant in enhancing the growth of each strain (*ANOVA*, See table 3) with the exception of strains 2013-119 and 2013-142 (*ANOVA*, $p = 0.2662375$ and $p = 0.713$, respectively). A summary of the individual effects and interactive effects of the independent variables is presented in Table 4. The final cell density of each strain grown in either BBM+glu or BBM+gal media in the light, was not significantly different from

one another (*ANOVA*, See Table 3) with the exception of strain 2013-129 which performed better in BBM+gal, and 2013-476 which grew better in BBM+glu (*ANOVA*, $p = 0.0004$ and $p = 0.0076$, respectively). In general the final cell density of each strain grown in the dark was not affected by the type of organic compound in the medium (*ANOVA*, See Table 3). Of strains grown in the dark, 2013-129 was the only one to exhibit growth in the BBM+glu and BBM+gal treatments significantly above the BBM media treatment (*ANOVA*, $p = 0.0007$ and $p = 0.0063$). Strain 2013-142 was able to grow significantly better in BBM+glu in the dark than in BBM media treatment (*ANOVA*, $p = 0.0143$). The analysis also indicates that the carbon-supplemented media treatments were effective in enhancing growth when coupled with the diurnal photoperiod (ANOVA, See Table 3) with two exceptions. In 2013-129 the photoperiod did not have a significant impact on the BMM+glu treatment, and in strain 2013-476 the photoperiod did not have a significant impact on the BBM+gal treatment (*ANOVA*, $p = 0.88$ and $p = 0.8517$, respectively).

DISCUSSION

Not unexpectedly for organisms that possess chlorophyll, the eight focal strains of symbiotic algae grew best in the presence of light. More surprising are the results demonstrating that the eight focal strains of symbiotic algae grew faster in the presence of exogenous organic carbon (i.e., glucose and/or galactose) in the light and were maintained their populations numbers in the dark for 28d. All eight algal isolates grew in the light grew better with a supplement of sugars than with BBM alone. The study revealed significant differences in growth across the carbon sources, however there was

no correlation between the clades identified in our phylogenetic analysis and preference for type of sugar, because I noted instances where the most closely related strains showed marked different response to the presence of exogenous carbon sources. The *rbc*L tree highlights three clades of symbiotic algae that display variation in their ability to use glucose and galactose. This was expected because the eight strains represent a diverse set of symbiotic partners that are non-monophyletic. Within clade III, all three strains grew to their greatest densities in the light and in the presence of glucose, however strain 2013-476 did not use galactose in the light with the same success as strains 2013-123 and 2013-151. Within clade II, strain 2013-129 grew to its greatest density in the light and in the presence of galactose, which contrasts strongly with the other strains from this clade that grew to greater densities when in the presence of glucose. These results demonstrate metabolic variation in closely related symbiotic algae and indicate the different isolates are not physiologically equivalent.

The greater density of algae in media containing glucose and galactose indicates that these algae have mechanisms to transport organic carbon into the cells as well as pathways to metabolize the molecules. The discovery of cellular maintenance as opposed to cell death in the dark in certain strains suggests that appreciable cell division is not occurring. In many species of algae, light is a signal for cell division and without this stimulus the strains in this experiment may have simply maintained themselves. This research has set the stage for future workers to uncover why these symbiotic algae do not divide in the dark and which metabolic pathways are active that enable them to survive.

As a whole, microorganisms possess many pathways for aerobic glycolysis, but only two have been shown in the algae: the Embden-Meyerhof Pathway (EMP) and the

Pentose Phosphate Pathway (PPP) (Neilson & Lewin, 1974). In both of these pathways, glucose is first transported across the cell membrane via the *hup1, hup2* channels and then, depending on the light conditions, enters one pathway or the other (Octavio *et al.*, 2011). For example, *Chlorella sorokiniana* (Trebouxiophyceae) uses the PPP pathway for 90% of its glucose metabolism in complete darkness when glucose is its sole carbon source. However, in the light, the PPP pathway is down-regulated in *Chlorella* in favor of the EMP pathway (Yang *et al.,* 2000). The regulation of each pathway may be unique among diverse algae. Our results indicate that sugar transporters are present in the symbiotic algae tested but that there may be differences among the strains for kinds of sugars that are used, as seen in the BBM+gal treatments.

Other factors, both abiotic and biotic, may influence how algae metabolize within the eggs in nature. Preliminary heterotrophic growth studies carried out on the same eight strains (unpublished data), resulted in greater heterotrophic growth in BBM+glucose media treatments under dark conditions. This would suggest that metabolic pathways were upregulated to a greater degree than seen in the present study. In these preliminary studies, cultures were not put through an extensive anti-bacterial treatment as enacted in the present experiment. It is possible that bacterial populations play a significant role in the growth and metabolism of these algae. In nature, bacterial populations have been observed in *Ambystoma maculatum* eggs (Hutchinson & Hammen, 1958) and thus are believed to be a part of the egg microbial biofauna, possibly contributing nutrients such as vitamins or play a role in organic carbon metabolism, which may significantly influence the metabolic pathways of the algae (and salamander embryonic development). The importance of the microbiome to this symbiosis deserves much further investigation.

Researchers have yet to conclusively identify how the symbiotic green algae enter *Ambystoma maculatum* eggs. Multiple hypotheses have been proposed. One includes intergenerational transfer of symbionts through vertical transmission (Kerney *et al*., 2011) and another is direct acquisition from breeding habitat (Gilbert, 1942; Lin & Bishop, 2015). An alternative hypothesis of entry is vertical transmission where female salamanders acquire algae from their environment, perhaps on spermatophores laid by the male salamanders, and passively nourish the algal cells until she is ready to lay eggs. Specific organic carbon compounds were chosen for this experiment because these were shown to be produced by female salamanders to nourish stored sperm prior to fertilization (Sever & Kloepfer, 1993). The results of this experiment demonstrate the ability of the focal algae to survive without light for extended periods, supporting the idea that algae would be maintained using exogenous carbon sources if they were within the female salamander.

In this study, I examined a range of symbiotic algae strains isolated from *Ambystoma maculatum* eggs and have demonstrated that three distinct lineages of *Oophila* algae are capable of using external carbon sources for growth in the light (mixotrophic) and in the dark (heterotrophic) to varying degrees. Our findings indicate there is differential use of these carbon sources among and between the groups of algae that I isolated from salamander eggs at one locality in Connecticut. I expect the range of metabolic variability to increase as more algae are examined across the geographic range of *Ambystoma maculatum*, which extends through the eastern United States and into Canada, spanning a wide range of environmental habitats and conditions. This presence of different metabolisms of each alga could have an impact on the benefits that each alga

imparts to its symbiotic partner. Certainly other environmental factors such as light levels, temperature, pH, and nitrogen availability, are expected to influence the metabolism of the photobiotic partners. An investigation across these conditions would be a large study, but it is required if we are to understand the symbiosis of algae with *A. maculatum* eggs, and serve to answer questions researchers have asked since this intimate association was discovered.

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autotrophic/dark-heterotrophic conditions. *Biochemical Engineering Journal* 6: 87-102.

Table 1. Stock strain and initial replicate cell densities.

***Oophila* Strain**	**Density of Stock Culture (cells/mL)**	**Starting Cell Density of Each Replicate**
2013.80	150,000	375,000
2013.119	400,000	1,000,000
2013.123	425,000	1,000,000
2013.129	350,000	875,000
2013.142	200,000	500,000
2013.150	700,000	1,000,000
2013.151	350,000	875,000
2013.476	900,000	1,000,000

Table 2. Summary of the experimental results, comparing initial to final cell densities, for each treatment and strain. Shading indicates positive growth (White), no growth or maintenance (=Grey), and negative growth (Black).

	Diurnal Light (16:8, L:D)			**No Light (0:24, L:D)**		
	BBM	**BBM + glu**	**BBM + gal**	**BBM**	**BBM + glu**	**BBM + gal**
2013-80						
2013-119						
2013-129						
2013-142						
2013-150						
2013-123						
2013-151						
2013-476						

Table 3. Tukey-adjusted mean separations with *aov* and *TukeyHSD*. P-values shown for mean comparisons for the interaction effect. Shaded cells have p-values less than 0.05 indicate statistically significant interaction between independent variables.

	Diurnal (16:8, L:D)			**Dark (0:24, L:D)**			**Across Photoperiod**		
Strain	**BBM+glu : BBM+gal**	**BBM+glu : BBM**	**BBM+gal : BBM**	**BBM+glu : BBM+gal**	**BBM+glu : BBM**	**BBM+gal : BBM**	**BBM Light: BBM Dark**	**BBM+glu Light: BBM+glu Dark**	**BBM+gal Light: BBM+gal Dark**
2013-80	0.8993	0.0154	0.0862	0.9995	0.7975	0.6261	0.2583	0.0001	0.0002
2013-119	0.2305	0.0026	0.1394	0.5677	0.1339	0.8817	0.0168	0.0004	0.0011
2013-123	0.9811	0.0000	0.0000	0.0698	0.1786	0.9899	0.0105	0.0000	0.0000
2013-129	0.0004	0.4143	0.0000	0.7154	0.0007	0.0063	0.0026	0.8800	0.0000
2013-142	0.4715	0.0022	0.0480	0.9056	0.0143	0.0784	0.0042	0.0007	0.0026
2013-150	0.2032	0.0003	0.0133	0.7189	0.7803	0.1432	0.3347	0.0000	0.0000
2013-151	0.6623	0.0021	0.0250	0.9867	0.9989	0.9998	0.4029	0.0001	0.0015
2013-476	0.0076	0.2460	0.3365	0.9999	0.9998	0.9959	0.0254	0.0008	0.8517

Table 4: Independent and combined effect of independent variables on each strain. P-values shown for Anova Type-II test. Shaded cells have p-values less than 0.05 and indicate statistically significant interaction between independent variables.

Strain	Media	Photoperiod	Media:Photoperiod
2013-80	0.1845	0.0001	0.0053
2013-119	0.0005	0.0001	0.2662
2013-123	0.0001	0.0001	0.0001
2013-129	0.0001	0.0001	0.0005
2013-142	0.0001	0.0001	0.7130
2013-150	0.0073	0.0001	0.0002
2013-151	0.0103	0.0001	0.0058
2013-476	0.0328	0.0001	0.0196

Figure 1. Correlation of relative fluorescence units and cell counts for each stock culture of symbiotic algae. Linear trendlines intersect 0,0 and slope is shown above each graph. Insets A-H represent individual strains of symbiotic algae.

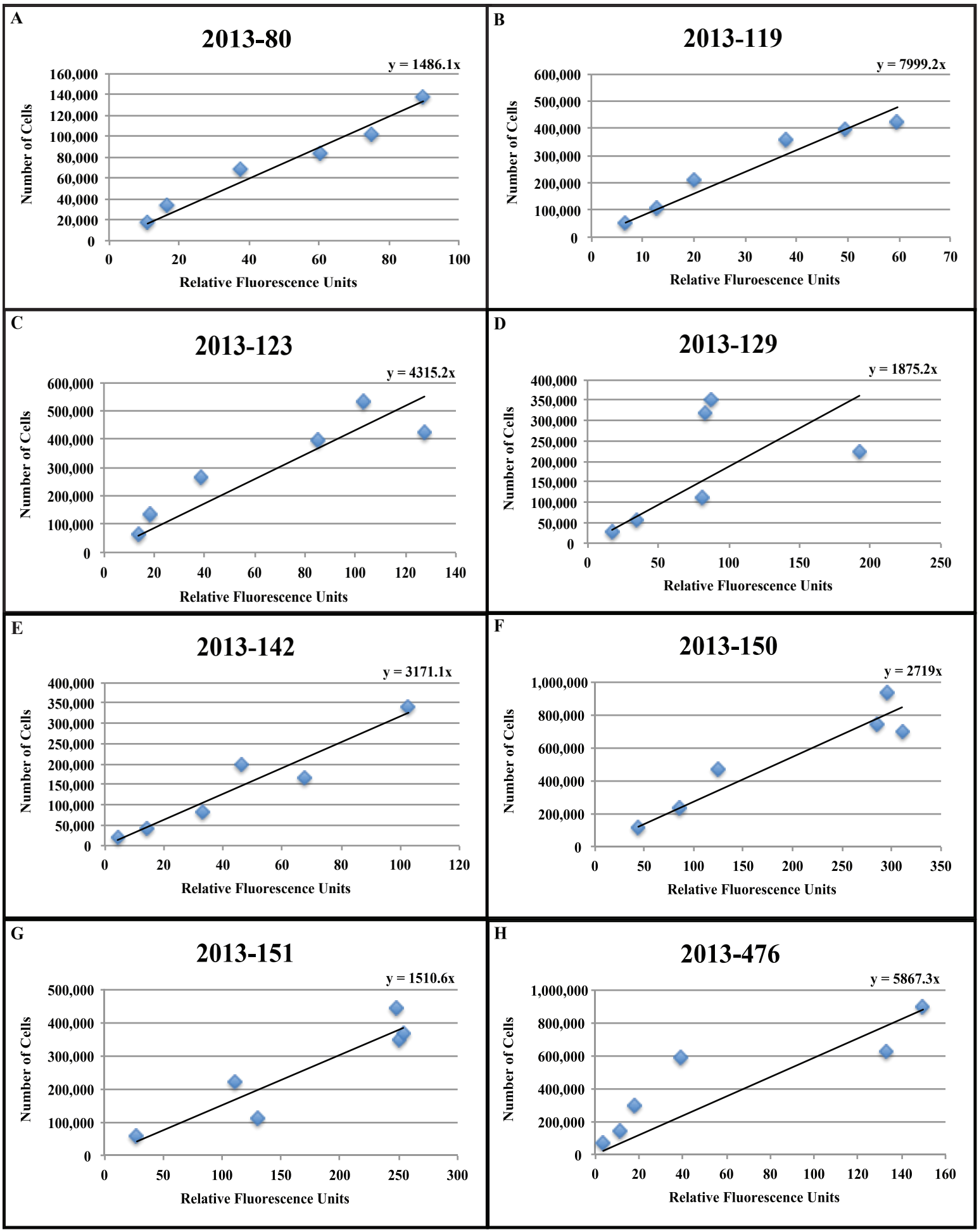


Figure 2. Maximum Likelihood (ML) phylogeny showing the position of symbiotic Oophila strains within the Chlamydomonadales (Chlorophyta), based on analysis of the rbcL gene from 52 green alga. Clades I and III fall within the larger "Oophila clade" designated by Kim et al., (2014) along with two Oophila strains isolated in another study (Lewis & Landberg, 2011). Lewis & Landberg strains are distinct from Clades I and III. Clade II falls outside of the "Oophila clade" and is distinct from our other symbiotic strains. Tree inferred under the GTR+I+G model. Bayesian posterior probabilities shown along branches.

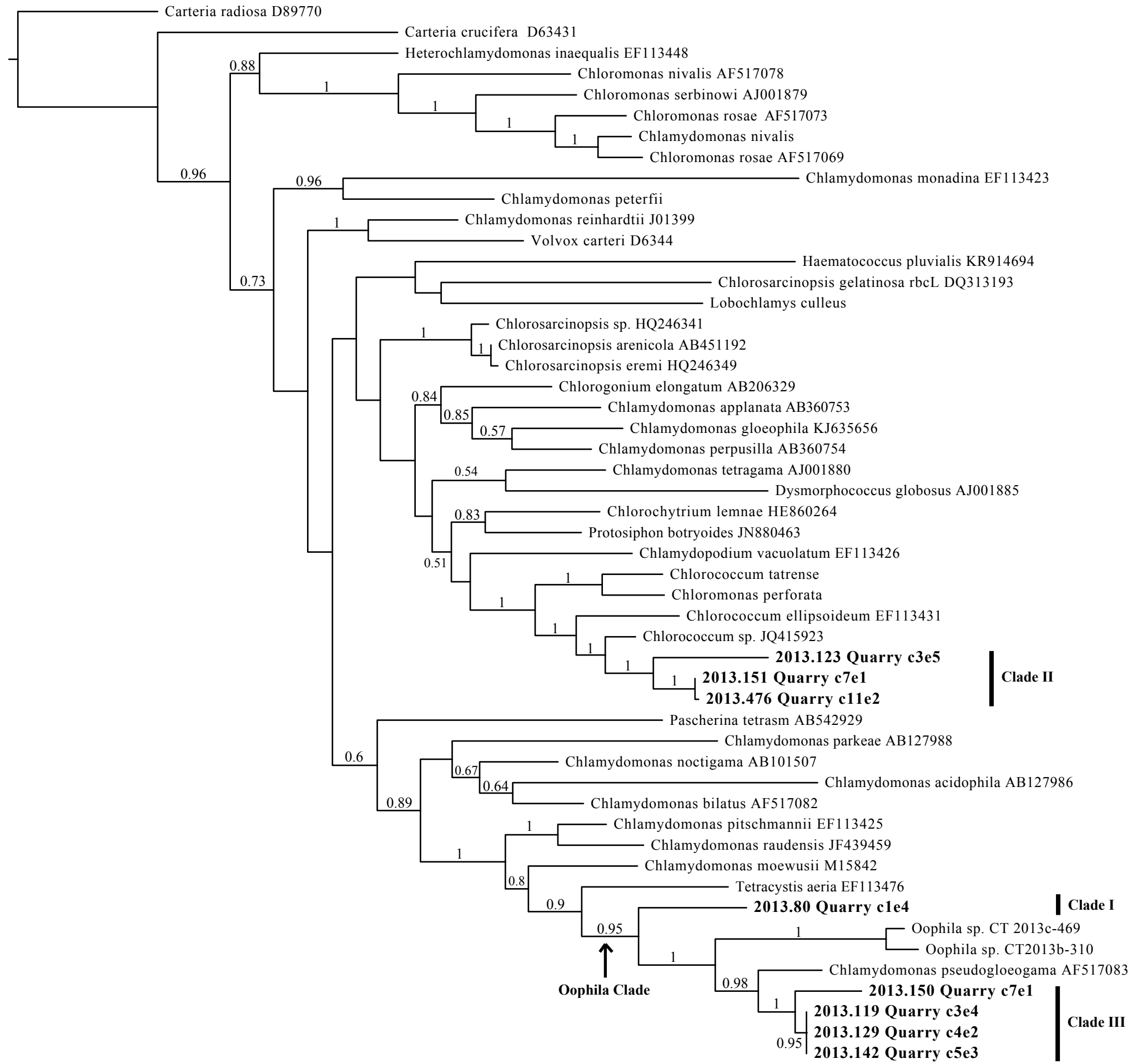


Figure 3. Maximum Likelihood (ML) phylogeny of 18S rDNA data showing the position of symbiotic Oophila strains within the Chlamydomonadales (Chlorophyta). "Oophila clade" designated by Kim et al., (2014) contains diverse range of symbiotic isolates including 18S sequence isolated by Kerney et al., (2011), which forms a clade with Lewis & Landberg (2011) isolate and is distinct from our Clades I and III (see Fig. 2). Clade II represents position of strains 2013-23, 2013-151, 2013-476 and falls outside of the "Oophila clade" designated by Kim et al., (2014). Green stars denote symbiotic Oophila sequences. Tree inferred under the TrN+I+G model. Bayesian posterior probabilities shown along branches.

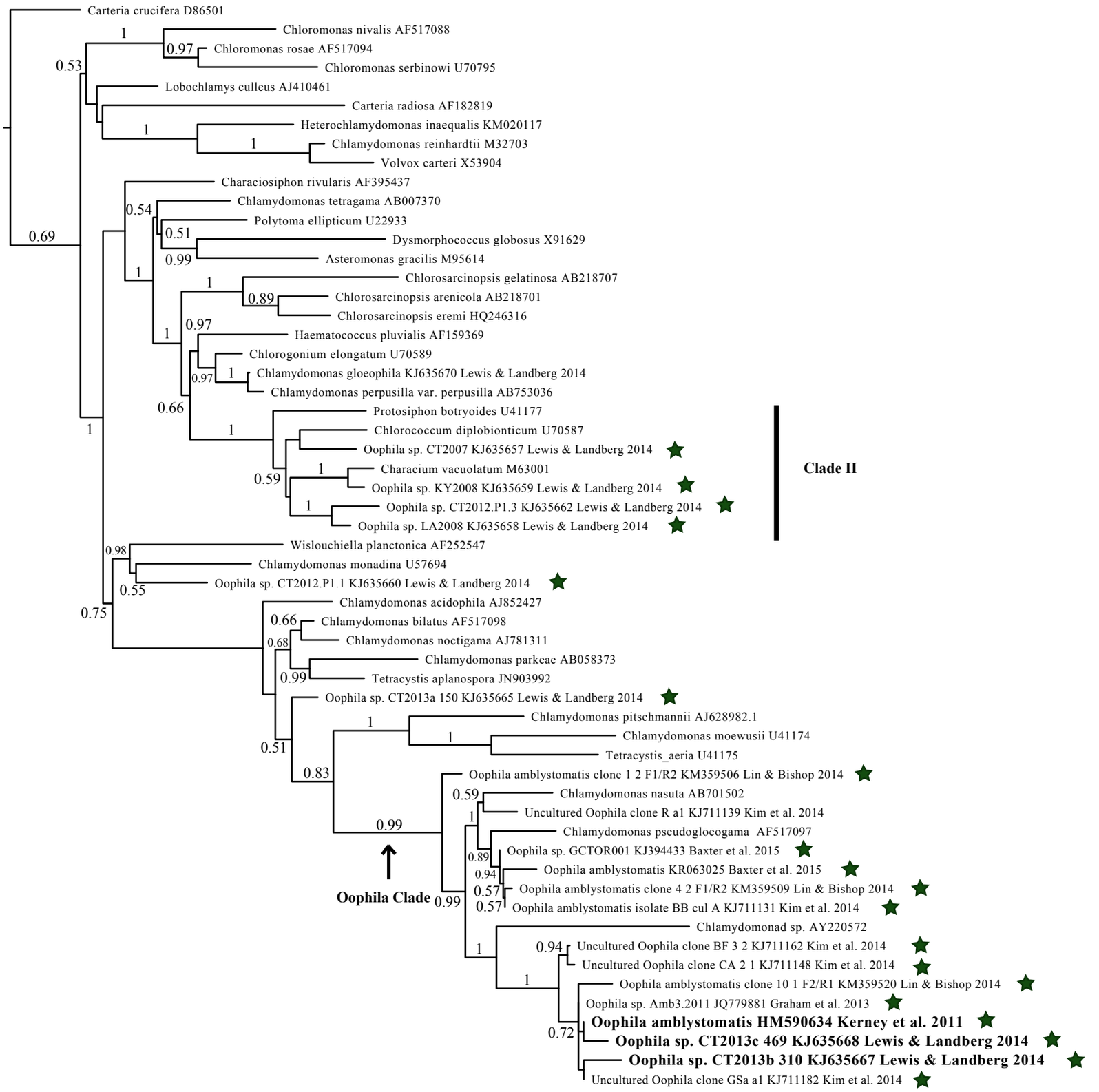
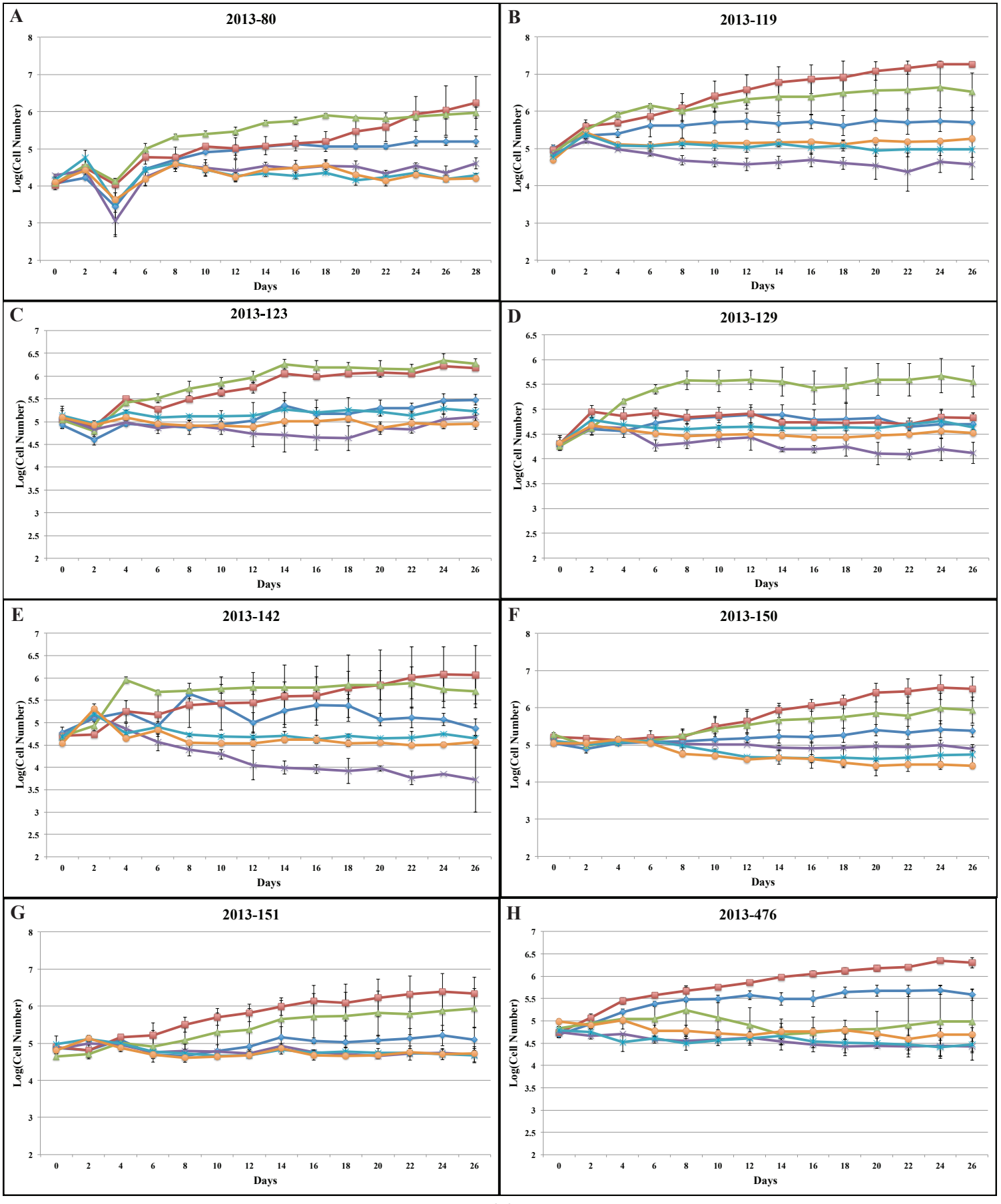


Figure 4. Growth (log cell number) of each focal strain of algae over the course of the 28d experiment in two light and three media treatments. Panels A-H represent individual strains of symbiotic algae.



CHAPTER II

TAXONOMIC HISTORY AND EPITYPIFICATION OF *OOPHILA AMBLYSTOMATIS*

INTRODUCTION

The group of green algae referred to as *Oophila* (Chlamydomonadales, Chlorophyceae) are best known for the symbiotic partnership they share with the yellow spotted salamander, *Ambystoma maculatum*. Currently, *Oophila* Lambert represents a monotypic genus of green algae that, by definition, live within salamander eggs (Lambert, 1910). Like many other genera of microalgae within the Chlorophyta (Pröschold *et al*., 2001), molecular data indicates *Oophila* is polyphyletic (Kim *et al*., 2014; Lin & Bishop, 2015). However, without a molecular barcode to represent the type species, this limits our ability to connect knowledge of the biology of the symbionts to particular species. The taxonomic validity of *Oophila* has been called into question ever since its designation because Lambert's name originates from an ambiguous and short generic description provided in the early 1900s, in a specimen exsiccata that some workers felt to be an invalid form of publication. In the interest of clearing up confusion surrounding this genus, I describe the taxonomic history of *Oophila*.

Oophila amblystomatis first appeared as dried specimens on mica in Fascicle XXVI of the *Phycotheca Boreali-Americana* (*P.B.-A*.) exsiccata in 1905 (Collins *et al*., 1905) submitted by F.D. Lambert, a graduate student of Tufts College at the time. Eighty copies of this fascicle were produced and distributed to inform the scientific community of novel taxa described in recent publications. Accompanying each specimen was a printed label that contained information on the habitat and type locality (Fig. 1). The

label associated with the specimen of *Oophila amblystomatis* reads, "In egg membrane of *Amblystoma punctatum*, ponds in Middlesex Fells, Massachusetts, May, 1905. Descriptions of this species…are to be published in Rhodora." Lambert's written intent to formally describe his alga in the New England Botanical Club journal, *Rhodora*, would have validated the name of his new alga if he had followed through; however he did not subsequently publish in *Rhodora*. It is important to note that *Amblystoma punctatum*, the salamander species Lambert collected algae from in 1905, was subsequently synonymized with *Ambystoma maculatum*. Following his contribution to the *P.B.-A.* exsiccata, Lambert published only a vague reference to algae growing within salamander eggs, in his Tufts College (University) work on *Coleochæte* (1910). Lambert wrote, "The eggs…showed a characteristic green color. This was due to the presence of certain unicellular green algæ, familiar to me by reason of several seasons' observations." Under the International Code of Nomenclature (ICN) for Algae, Fungi and Plants (Chapter IV, Section 1, Article 30.6-7), Lambert's binomial would have been valid as it was published in printed matter with accompanied specimens prior to 1953; however, because he stated a subsequent publication would appear in Rhodora, the *P.B.-A.* publication was not sufficient.

Reference to *Oophila* appeared next in the revisions of the Chlorophyceae by Wille (1909) and Printz (1927) where they listed *Oophila* under the heading of 'Doubtful Genera.' The absence of a detailed description in the *P.B.-A.* exsiccata was noted; however, they correctly recorded the *P.B.-A.* specimens as types. Translating the text from German, some authors may have interpreted the 'Doubtful Genera' designation as Wille and Printz doubting the validity of this taxon; however, the desire was to question

Oophila's placement within the Chlorotheciaceae (Protococcaceae) at the time. According to the ICN for Algae, Fungi, and Plants (2012), Wille's publication with the description of *Oophila amblystomatis* validated the binomial. However, despite their efforts (Wille, 1909; Printz, 1927), other authors publishing on *Oophila* have continued to question the validity of the name (Gilbert, 1942; Kerney, 2011; Kerney *et al*. 2011).

Further complicating matters, algae extracted from *A. maculatum* eggs have been shown to contain multiple lineages (Kim *et al*., 2014; Lin & Bishop, 2015). Currently, we do not have a unique molecular barcode for the type species. Producing one would provide a foundation for further taxonomic work and taxonomic context for the many physiological and molecular studies on this important symbiotic system. The absence of diagnosable type material has made it impossible for researchers to understanding which of the many clades of "*Oophila"* (see Chapter 1) best represents the genus and species.

Although we attempted to extract DNA from paratype material, gathered from *P.B.-A.* fascicles held in private collections, repeated efforts were unsuccessful. This result is unsurprising give the specimens were prepared in 1905 and the DNA has likely degraded beyond recovery with our current molecular techniques.

In this study, eggs of *A. maculatum* were collected from the type locality of Middlesex Fells, Massachusetts, U.S.A. The green algae within the eggs were isolated and their DNA extracted, sequenced and analyzed phylogenetically with published sequences obtained from algae of yellow spotted salamander eggs in the United States and Canada. The new information allows a connection to be made between the type locality material and a particular phylogenetic lineage, for which we have designated an epitype for *Oophila amblystomatis.*

MATERIALS AND METHODS

Sampling from the Type Locality

Ambystoma maculatum eggs containing green algae were collected from the Middlesex Fells Reservation, the type locality of *Oophila*, on March 11, 2015. Two vernal pools were selected within the Winchester boundary of the Fells for their abundant egg masses and distance from walking paths ("Pond 1" 42.45448°N, -71.12601°W; "Pond 2" 42.45456°N, -71.12589°W). Yellow spotted salamander eggs were identified from the stage 36-38 embryos (Harrison, 1969) with their characteristic green algal bloom. Five eggs were removed from three clutches in each pool (n=30) and the remaining eggs were left undisturbed.

Isolation and Culturing

In a Clean Bench (Labconco), individual eggs were removed from the jelly using a spatula, rinsed in sterile water and their membranes broken with sterile pipet tips. The intra-capsular fluids and algae were transferred to sterile 1.5 mL centrifuge tubes containing 500 µL Bold's Basal Medium (BBM; Bold 1949, Bischoff and Bold 1963). Once growth was observed, the samples were transferred into glass tubes containing agar slants and liquid BBM and grown under indirect light (16L/8D; 40 µmol/m^2) at a constant temperature of 18°C. Tubes were capped with excess headspace, and opened periodically during the growth period, to ensure adequate gas exchange. Each culture was grown to a high density and verified to be unialgal through morphological observation and later with DNA sequencing.

DNA Extraction, PCR Amplification, and Sequencing

DNA was isolated from each culture using a PowerPlant® DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA), following the 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 PD7. 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 with products were quantified with a NanoDrop® spectrophotometer ND-1000 v.3.8 (Thermo Fisher Scientific Inc., Waltham, MA).

Sequences were generated from the chloroplast gene *rbc*L in single fragments using multiple primers combinations (Table 1). The PCR-reaction mix contained house Taq polymerase prepared from cultured *Thermus aquaticus*, or 11 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®, Coralville, 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 1m15s, annealing at 48°C for 2m00s, extension at 72°C for 2m15s, repeated for 34 cycles for 1m00s each, with a final extension period at 72°C for 7m00s, and samples held at 10°C for 5m00s. Of the resulting PCR products, those that appeared under transillumination were cleaned using ExoProStar (GE Healthcare Bio-Sciences, Pittsburgh, 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 0m15s, 60°C for 4m00s, 27 cycles for 1m00s each, and 10°C for 10m00s. The cycle-sequenced samples were run on an ABI3100 Sequencer 3130x (Applied Biosystems and Hitachi).

Phylogenetic Tree Construction

Individual *rbc*L sequence reads (n=9) were edited and assembled in Genious 9.0.5 (Biomatters Ltd., Auckland, New Zealand) to generate consensus sequences. Consensus sequences were run through BLAST (http://blast.ncbi.nlm.nih.gov/, last accessed August 8, 2016) and a 55-taxon *rbc*L dataset and a 57-taxon 18S rDNA dataset were compiled (largely from publically accessible data in GenBank) and aligned in Geneious. PAUP* (Swofford, 2002) automated model selection was run using the AICc and BIC criteria, and the GTR+I+G model was chosen for the *rbc*L dataset (rmatrix=(0.49899132 2.5071757 5.0897849 1.1835005 7.9845356) basefreq=(0.28202283 0.13495197 0.19834177) rates=gamma shape=1.0314272 pinv=0.49366328) and the TnR+I+G model was chosen for the 18S dataset (rmatrix=(1 2.3729843 1 1 5.0036588) basefreq=(0.24775298 0.20541567 0.27398467) rates=gamma shape=0.55398631 pinv=0.54397673). Bayesian analysis was run in 4 chains of $3x10^6$ generations with three heated chains. Trees were sampled every 5,000 generations and the first 500,000 samples of each run were discarded as burnin. Alignments can be found in Supplementary Material (S4, S5).

RESULTS

Molecular Phylogenetic Analyses

Eight *Oophila rbc*L sequences were generated for this study from *A. maculatum* eggs collected in the Middlesex Fells Reservation. Analysis of the *rbc*L dataset (n=55) revealed the eight *Oophila* sequences form one clade that we have labeled "*Oophila amblystomatis"* (Fig. 2). A second tree was generated for this study using 18S rDNA sequences. The tree includes the 18S *Oophila* sequence generated by Kerney *et al.* (2011) and another sequence generated by Lewis & Landberg (2011) that enabled comparison between *rbc*L and 18S (Fig. 3) regions. The 18S sequence from Kerney *et al*. (2011) aligns with *Oophila sp.* CT 2013c-469 and is distinct from our sequences obtained from the type locality.

DISCUSSION

As originally described, the name *Oophila amblystomatis* could apply to any green algae living within eggs of *Ambystoma maculatum*. The species currently has no molecular barcode representing the holotype. However, the scientific community regards the 18S rDNA sequence of Kerney *et al.* (2011) as representing the species despite its origin being Halifax, Nova Scotia, Canada, not from the type locality.

In this study, we produced sequences from five eggs collected from the type locality. These sequences form a single clade within the Chlamydomonadales (Fig. 2) that is sister to a *Chlamydomonas pseudogloeogama* sequence yet distinct from another egg isolate, 2013c.469, which pairs with the 18S rDNA sequence of Kerney *et al*. in our 18S rDNA tree (Fig. 3). We conclude that the clade of algae from the type locality of Middlesex Fells, MA is distinct from the Halifax, Nova Scotia isolate. The use of *rbc*L as

a species barcode is appropriate here and shows its utility, uncovering genetic diversity that the 18S sequences do not. Multiple loci are now being used for species delineation in this group of green algae such as the coding region of the chloroplast gene *rbc*L and the coding region of ribosomal DNA, 18S. In order to fully resolve species relationships among *Oophila* isolates from *Ambystoma* eggs, additional work will be required to generate *rbc*L barcodes for isolates from other locations as well as *Ambystoma* species.

After tracing the taxonomy of the genus *Oophila* and confirming it to be valid (Wille, 1909), we suggest an epitype be designated to represent *Oophila amblystomatis* on the grounds that existing type material is demonstrably ambiguous and cannot be critically identified for purposes of the precise application of the name to a taxon (ICN for Algae, Fungi and Plants 2012, CH II, Section 2, Article 9.8). Our *Oophila strain* 2015.303, collected and sequenced in this study, is an appropriate epitype candidate. Formal designation will be made in a peer-reviewed publication, a specimen will be deposited in the George Safford Torrey Herbarium and a living culture will be deposited in the UTEX Culture Collection. Upon epitypification, I also suggest the NYBG *P.B.-A.* specimen (ID 02133073, 02348992) be designated as the lectotype, with the remaining *P.B.-A* specimens as isolectotypes (NYBG 02133074; all other *P.B.-A*. specimens).

It is in our collective best interest to anchor this species to a molecular barcode so that taxonomic, molecular and physiological work within this genus may continue unburdened by past confusion. Sequences that have already been produced will have to be re-evaluated to determine whether they are truly *Oophila* or not based on this new definition.

It remains to be determined whether or not the Middlesex Fells isolates are the same species as Kerney's isolate. The intra-species relationships of closely related taxa will need to be evaluated with both *rbc*L and 18S, and perhaps other suitable barcode markers, to determine if the aforementioned *Oophila* isolates will be considered one or more species. Admittedly, this suggested approach does not incorporate morphological or physiological diagnosis of each strain, which is common practice when dealing with unicellular green algae. Interestingly, our *rbc*L tree indicates that *Chlamydomonas pseudogloeogama* (EF589142) is nested between the MSF clade and the Kerney isolate. This alga was isolated as part of an unpublished project labeled, "Testing cosmopolitanism in New Zealand alpine algae" and was most likely the result of improper identification in a next-gen sequencing analysis. Our data suggests that this report represents a free-living strain of *Oophila* from New Zealand and the record need be amended to avoid the appearance of polyphyly within the genus *Oophila*. Importantly, this report provides an instance of *Oophila* living outside salamander eggs (Kim *et al.,* 2014) and further demonstrates the ability of the algae to exist independent of the symbiosis for which it is classically known. This supports the need for an expanded definition of *Oophila* that other authors have yet to provide (Lambert, 1905; Wille, 1909; Printz, 1928). Additional morphological work on the proposed epitype is also needed to expand the definition of *Oophila* and assist future workers in their studies.

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Table 1. List of specimens used in this study with collection sites. Sequences produced for this study are shown in boldface voucher numbers.

Taxon	**Voucher no.**	**Collection Site**	**Collector(s)/Date**
Oophila amblystomatis F.D. Lambert ex N. Wille	**2015.298**	Clutch 2, Egg 1, Pond #1, Middlesex Fells Reservation, MA, USA	N. Schultz/11 Mar. 2015
	2015.299	Clutch 2, Egg 1, Pond #1, Middlesex Fells Reservation, MA, USA	N. Schultz/11 Mar. 2015
	2015.301	Clutch 2, Egg 2, Pond #1, Middlesex Fells Reservation, MA, USA	N. Schultz/11 Mar. 2015
	2015.302	Clutch 2, Egg 2, Pond #1, Middlesex Fells Reservation, MA, USA	N. Schultz/11 Mar. 2015
	2015.303	Clutch 2, Egg 2, Pond #1, Middlesex Fells Reservation, MA, USA	N. Schultz/11 Mar. 2015
	2015.304	Clutch 3, Egg 1, Pond #1, Middlesex Fells Reservation, MA, USA	N. Schultz/11 Mar. 2015
	2015.305	Clutch 3, Egg 1, Pond #1, Middlesex Fells Reservation, MA, USA	N. Schultz/11 Mar. 2015
	2015.306	Clutch 3, Egg 2, Pond #1, Middlesex Fells Reservation, MA, USA	N. Schultz/11 Mar. 2015
	2015.307	Clutch 3, Egg 3, Pond #1, Middlesex Fells Reservation, MA, USA	N. Schultz/11 Mar. 2015
	CT2013b_310	Kealoha, Yale Myers Forest	L. Lewis/17 Apr. 2013
	CT2013c_469	B-9 Pond, Yale Myers Forest, Totoket Mountain, Northford, CT	M. Urban/29 Apr. 2013
*Oophila sp.*1	UTEX 3005	Halifax Regional Municipality, Nova Scotia, Canada	R. Kerney

Figure 1. Oophila amblystomatis holotype label and specimens as presented in F.S. Collin's personal copy of the Phycotheca Boreali-Americana exsiccata. NYBG P.B.-A. specimens (ID 02133073-4, 02348992) are stored on mica plates presented below label.

Figure 2. Maximum Likelihood (ML) phylogeny showing the position of Oophila amblystomatis within the Chlamydomonadales, based on analysis of rbcL data. The larger "Oophila clade" defined by Kim et al., (2014) is marked and includes sequences produced in this study, from the type locality, and by Lewis & Landberg (2011). Eight algae isolates from Middlesex Fells eggs are denoted by "Oophila amblystomatis" clade. Two Oophila isolates produced by Lewis & Landberg form one clade we label "Kerney et al. (2011) that is distinct from our sequences. Tree inferred under the TrN+I+G model. Bayesian posterior probabilities shown along branches.

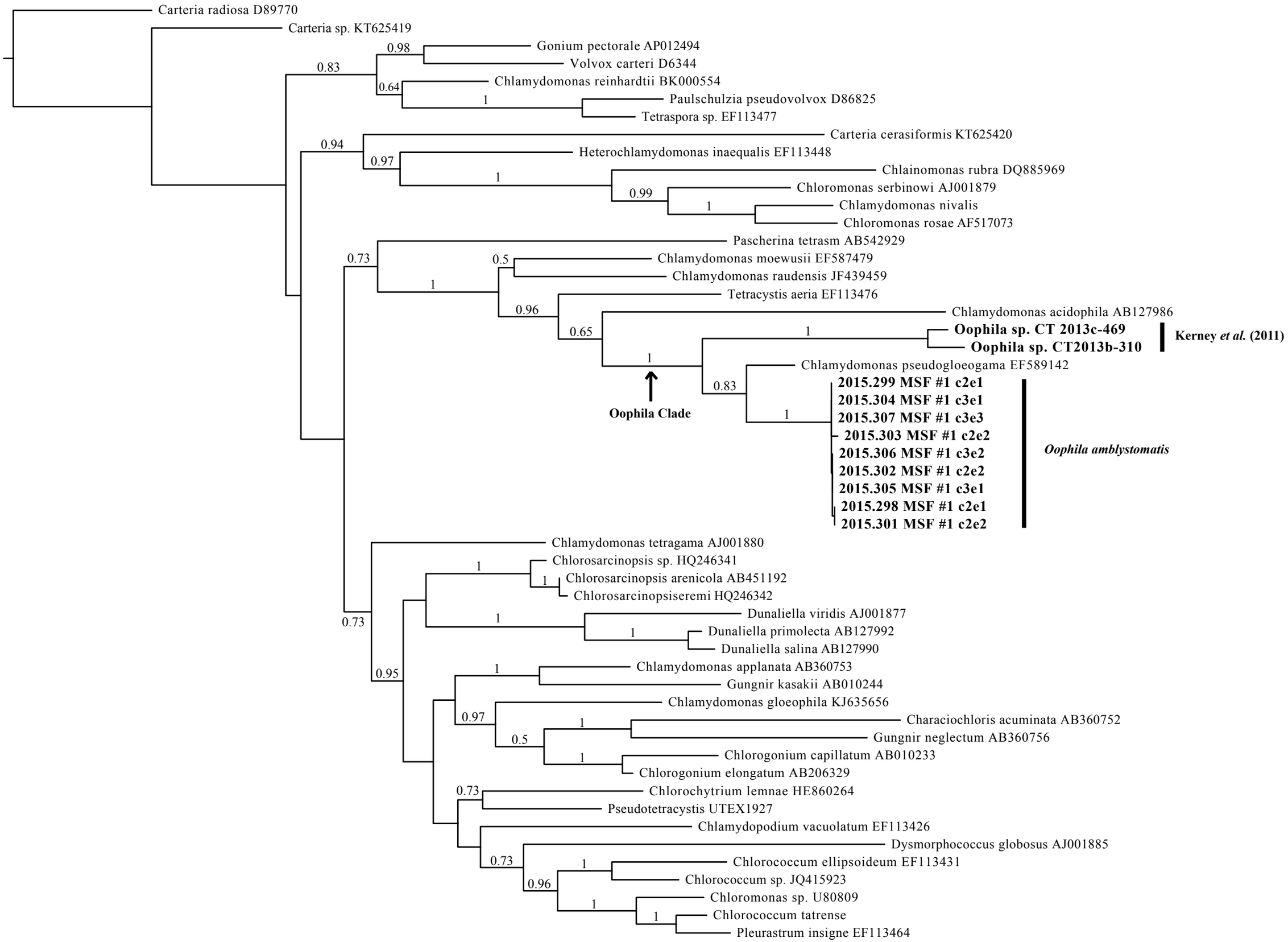

Figure 3. Maximum Likelihood (ML) phylogeny showing the position of symbiotic Oophila strains within the Chlamydomonadales, based on analysis of 18S rDNA data. The "Oophila clade" designated by Kim et al., (2014) contains diverse range of symbiotic isolates including 18S sequence isolated by Kerney et al., (2011), which forms a clade with Lewis & Landberg (2011) isolate 2013c-469 and is distinct from our Oophila amblystomatis clade (see Fig. 2). Green stars denote symbiotic Oophila sequences. Tree inferred under the TrN+I+G model. Bayesian posterior probabilities shown along branches.