Studying evolution in Chlamydomonas yields importance to studying its ecology for which purpose populations in the field must be located. In an effort to do so we modified the traditional method by introducing and optimizing a PCR screen using species–specific and broad-range primers. Primers were designed using *C. reinhardtii* and *C.incerta* coding sequence data. Various regions of CC1690 (*C.reinhardtii)* DNA extract were successfully amplified using *C.reinhardtii*-specific primers and of both CC1690 and CC3871 (*C. Incerta)* extracts using broad-range primers. DNA from algae extracted and cultured from multiple samples of soil was also successfully amplified with amplification occurring in 2/7 samples via *C.reinhardtii*-specific and 5/7 samples via broad-range primers. Sequencing these amplicons to determine their true identity has not yet been completed.

Introduction

The unicellular photosynthetic algae Chlamydomonas and in particular *Chlamydomonas reinhardtii* is a widely used model organism for insight into genetic, molecular, and cytological biology (Harris, 2009) . However, with only a few dozen standard strains used in the lab, little research has been done into the genetic diversity in the genus and its evolution over space and time (Gallaher *et al*, 2015). With recent advances in gene sequencing technology the study of factors involved in evolution such as mutation rate has become feasible (Ness *et al,* 2012). To take advantage and study the population genetics and ecology of Chlamydomonas, it is therefore useful to search for strains of its species currently in the field.

Sack *et al*. devised a method to find and isolate Chlamydomonas from soil samples in 1994. This was done by growing and detecting colonies that resembled those of Chlamydomonas by eye and verification of their identity via mating with known Chlamydomonas individuals (Sack *et al*, 1994). We aim to shorten this procedure and thereby make the hunt efficient by eliminating the need for colony isolation and mating tests unless PCR first detects the organism's genetic material in the sample. Species-specific PCR screens such as that developed by Atkins et al. to detect *Purpureocillium lilacinum* (formerly *Paecilomyces lilacinus*) in soil and clinical isolates have been used to detect a myriad of organisms in their natural environments (Atkins *et al,* 2005; Wilson *et al*, 2000). We will similarly use *C. reinhardtii*-specific primers to detect the species in algal cultures derived from soil. In contrast universal primers are designed using

highly conserved sequence regions such as 23s rRNA, for example in the development of primers able to detect 95 algal and 8 cyanobacterial species by Sherwood and Presting (Sherwood and Presting, 2007). We will similarly design broad-range primers targeting gene sequences conserved across *C. reinhardtii* and *C.incerta* in order to be able to detect both species as well as known and unknown closely related species. In doing so, we hope to find and efficiently detect and, to an extent, differentiate species of Chlamydomonas from soil sampled in the Greater Toronto Area.

Method

Primer Design

Sixteen primers were designed using Primer3 software: i. 9 species-specific pairs explicitly aimed at amplifying regions of the *C. reinhardtii* coding sequence and ii. 7 broad-range pairs targeting the amplification of regions of *C.reinhardtii*, *C.incerta* and closely related species' coding sequence.

- *C.reinhardtii*-specific primers

  These were designed using *C.reinhardtii* coding sequence data and a mispriming library comprised of *C.incerta* coding sequence data. The maximum mispriming score was set to 16 to allow for a minimum of 20% nucleotide mismatches between the primer and *C.incerta* coding sequence to avoid amplifying DNA originating from *C.incerta* and from other species less closely related to *C. reinhardtii*.

- Broad-Range Primers

  C.reinhardtii and *C.incerta* coding sequence data files were aligned using BLAST. *C.incerta* coding sequence data was used to design the primers and the alignment used to ensure a maximum of 20% nucleotide mismatches with the aligned *C. reinhardtii* sequence to ensure primers were capable of annealing with both. Mismatches at the 3' end and those that occurred in rows were specifically avoided to maximize likelihood of successful annealing with the *C. reinhardtii* sequence.

Primer Evaluation

Primers were evaluated with PCR using gDNA extract from lab strains CC1690 and CC3871. Liquid monoalgal cultures were grown in TAP medium before preparation for DNA extraction according to the manufacturer's instructions in the Promega Plant DNA extraction kit. 20 ng DNA was amplified using 0.2 uM of each primer, 200 uM dNTPs, 2.0 mM MgCl, 1 M Betaine, 0.0625 units of Thermo Scientific Taq polymerase and 1X KCl buffer per 25 uL reaction. The Eppendorf Mastercycler was used under the following conditions: 3 min initial denaturation at 95 C, 40 cycles of 1 min at 95 C, 30 s at 5 C below average primer melting temperature, and 1 min at 72 C, followed by 5 min at 72 C for a final extension. PCR products were run on a 2% agarose gel stained with Ethidium Bromide and photographed in a BioRad UV transilluminator. Primer-BLAST and BLAST searches were used to evaluate primer specificity.

Soil Sample Collection and Cell Extraction

7 samples of soil were collected from 2 locations in Mississauga and Milton.

- Riverwood Conservancy in Mississauga, Ontario

2 samples of soil, Upper Culham (UC) and Lower Culham (LC) with UC being higher in moisture content, were collected from the Culham trail near the Credit River in the Conservancy.

- Farmland in Milton, Ontario

1 sample of soil was collected from each of the following: A. Raspberry fields B. Sunflower fields C. freshly ploughed fields D. Blueberry fields E. Fields growing unknown crop

A total of 100 mL of soil was sampled from multiple, random locations from across each of these locations and pooled in plastic ziptop bags. The method described by Sack et al. (1994) was followed for soil sample incubation in water and subsequent cell pellet extraction and resuspension. Pellets from each sample were

separately cultured in TAP medium for 5 days. Centrifugation in 2 mL microtubes at 10,000 rpm for 2 minutes was repeated three times by discarding the supernatant each time to collect sufficiently large cell pellet size. DNA was then extracted using the Promega Plant DNA extraction kit as described in its manual.

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25 uL PCR reactions were prepared and run on a 2% agarose gel as described earlier with DNA extract from each of the seven soil-derived algae cultures.

Results

Primer Evaluation

Four primers, two of each type, were selected based on their success in performing as expected by generating amplicons from either one or both of CC1690 and CC3871 DNA extracts (Table 1).

Table 1: Selected Primers

Primer Pair Name	Forward Primer	Reverse Primer	Gene-of-Origin Information
2R	TCAACTCAGCATACGCATACCG	CCTGGTCGTTGATCTTGTTGGG	1
4R	GCTCAGTCCTCGGTATGGC	CACGTCAATGAAGGTGTTGCC	unknown
3B	CTCAACCGACTGCTACGC	CGGCGTGAACAGGTAGCC	*C.reinhardtii*- 2
8B	GGATGTGCCCATCTACGG	AGCCGCACGTTGTACGC	*C.reinhardtii*- 3

Table 1: Primers successfully tested in PCR with lab strain DNA extract and selected to amplify potential soil algae DNA. 'R' denotes C.reinhardtii-specific type which amplified CC1690 but not C.incerta DNA while 'B' denotes broad-range type which successfully amplified both of the above. 1) matrix metalloproteinase (MMP12), partial mRNA 2) predicted protein (CHLREDRAFT_152728), partial mRNA 3a) GrpE nucleotide release factor (CGE1a|CGE1b), mRNA 3b) glutamine synthetase (GLN1), mRNA-90% coverage 3c) hypothetical protein (CHLREDRAFT_206136), partial mRNA – 90% coverage

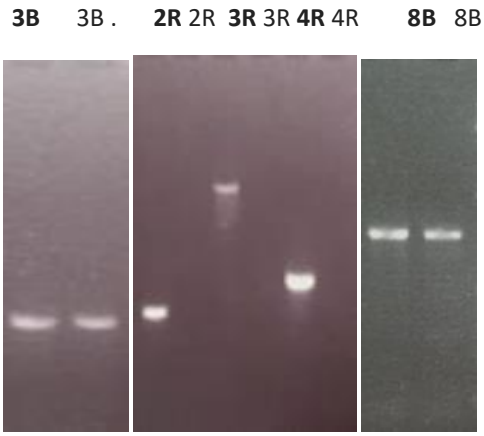

Fig 1: Amplicons generated via PCR with CC1690 (in **bold**) and CC3871 using the chosen primers. 3R was not among those selected.

Products of lab strain DNA amplification larger than expected by Primer3 may be attributable to introns not accounted for by the coding sequences used to design the primers (Table 2).

Table 2: PCR Product Size Evaluation

Primer	**Expected Product Length (PL)/ bp**	**PL with CC1690 or CC3871 DNA/bp**
2R	267	400
4R	617	600
3B	470	500
8B	300	1000

Table 2: PCR product lengths compared with lengths expected by Primer3

BLAST searches reveal some high coverage hits in *Volvox carteri* and non-target hits in *C.reinhardtii.*

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A total of 13 bands, 10 of which were amplified by 8B, 2 by 2R and 1 by 3B are visible. BLAST searches reveal some primer sequence similarity with Volvocine gene sequences. Faint bands may have to be treated skeptically as they could be evidence of amplification resulting from poor annealing with undesired templates in such cases.

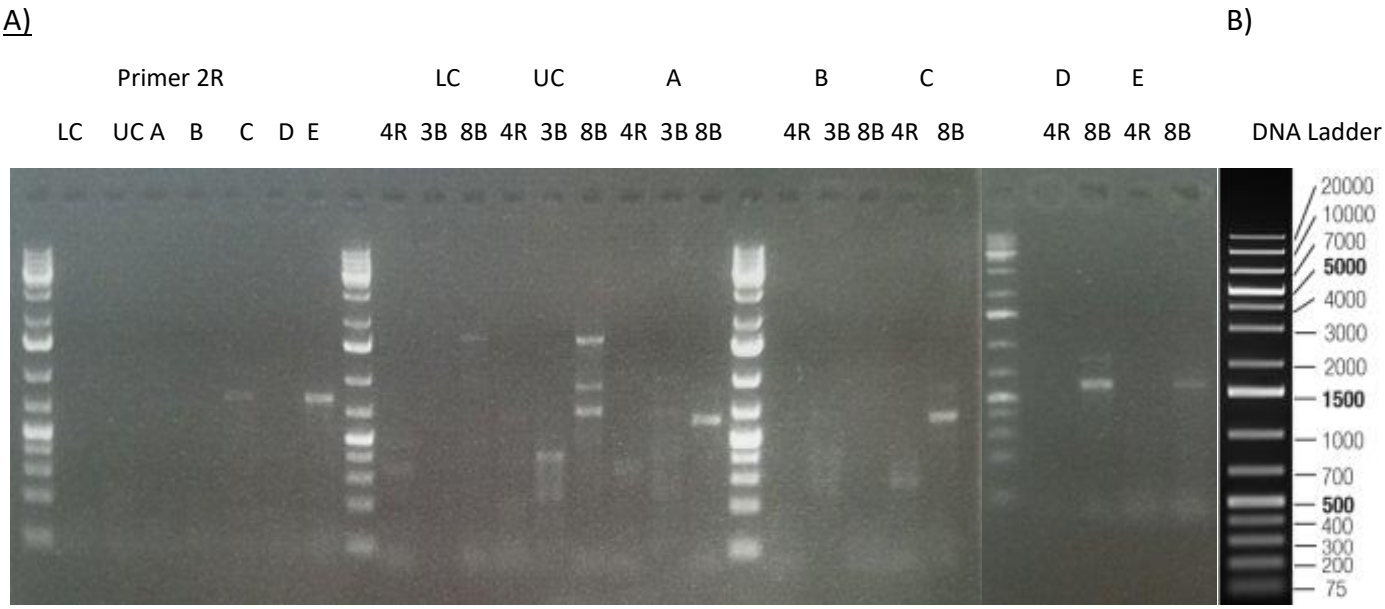

Fig 2: A) Primer 2R was added across the 7 DNA extracts in the first panel with the arrangement changed in the second panel so each extract's amplification using each of primers 4R, 3B, and 8B is grouped in triplets (or pairs where 3B is absent). B) A ThermoScientific 1 kB Plus DNA GeneLadder was used to determine fragment size.

Table 3: PCR Summary

Primer	PL with CC1690 and/or CC3871 DNA /bp	PL- UC/bp	PL- LC/bp	PL-A/bp	PL-B/bp	PL-C/bp	PL-D/bp	PL-E/bp
2R	400					700-1k		700-1k
4R	600							
3B	400	400						
8B	1000	700 1000 1.5-2k	1.5-2k	600		600 700-1k	600 700-1k	600

Table 3: Band results summarized in terms of corresponding estimated PCR product sizes and compared with product sizes obtained with lab strain DNA extract amplification

Future Steps

Primer sequences could be checked for SNPs to ensure species-specific primers are viable across different strains of *C. reinhardtii*. A Volvox genomic sequence mispriming library could be used for both primer

types to improve Chlamydomonas-specific primer design. To improve efficiency and deal with concerns related to culturing the edaphic algal community such as competition and contamination the culturing step may be eliminated and DNA extracted directly from the cell pellet. This may call for modifications to Sack et al's method to increase the frequency of green algal cells that are found in and can be extracted from the water (Sack *et al,* 1994). Although the PCR screen worked successfully, a positive control for primer evaluation with DNA extract from lab strains cultured in soil should still be included as evidence of good scientific technique. Sequencing efforts must be undertaken as follow-up to determine the true identity of these fragments.

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