Molecular Phylogeography and Species Discrimination of Freshwater *Cladophora* (Cladophorales, Chlorophyta) in North America

by

Sara J. Ross

A thesis

presented to the University of Waterloo

in fulfilment of the

thesis requirements for the degree of

Master of Science

in

Biology

Waterloo, Ontario, Canada, 2006

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required finalrevisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

ABSTRACT

Cladophora is a widespread freshwater filamentous cholorophyte genus and is frequently observed in eutrophic waters where it can produce large nuisance blooms. These blooms can have direct impacts on water intake for power generation, irrigation canals and can be aesthetically unpleasant. Much of the ecological and physiological studies on *Cladophora* have assumed that the populations of this genus in North America belong to the species *Cladophora glomerata.* However, this has never been tested despite that it is well-documented that identifying freshwater *Cladophora* to the species level is difficult due morphological variability under different ecological conditions. In addition, the species epithets for freshwater *Cladophora* are based on European collections and it is not clear if these should be applied to North America. This study examines approximately 40 collections of *Cladophora* from the Laurentian Great Lakes and 43 from various locations in North America ranging from the Northwest Territories to Puerto Rico. Initially we determined the nucleotide sequences of the internal transcribed spacer (ITS) region of the nuclear ribosomal cistron and observed sequence divergence to be low (0-3%), demonstrating an inability for this marker to resolve species delineation as divergence of this region was low. Amplification of the inter-simple sequence repeat (ISSR) regions were used to analyze microsatellite motif frequency throughout the genome to evaluate the biogeography relationships, including diversity, of freshwater *Cladophora* sp. five different primers were used on 70 individuals. UPGMA analyses of the presence/absence of bands demonstrate that each of the Great Lake populations separate into groups according to the Lake they were initially sampled from. However, collections from North America are highly variable and do not form well supported biogeographic clades. In addition, these

collections appear to be distinct from type cultures of freshwater *Cladophora* from Europe. Supplementary morphological analysis using suggested taxonomically valid criterion (length and diameter of main axis, ultimate branch, and apical cell) none were able to differentiate Great Lake populations.

ACKNOWLEDGEMENTS

I would sincerely like to thank the following people…

My advisor Dr. Kirsten Müller for her patience, help and support …we'll always have Brad and Angelina!

My committee members, Dr. John Semple, Dr. Robert Hecky, and Dr. Brian Dixon and Dr. Jonathon Witt for their help and guidance

Dr. Robert Sheath and Allison Sherwood and colleagues for all of the *Cladophora* samples.

Dr. Veronique Hiriart-Baer, Sairah Malkin and Troina Shea for introducing me to smelly world of *Cladophora.*

To my very close and supportive friends Andrea Gill and Aline Chhun for each teaching me so much and being my rocks when I needed them.

To Mike Lynch for all of his patience, teaching and guidance, I owe you one!

To Justin Lorentz for always bringing enthusiasm and a smile into the lab.

A most special thank you to my Mom for all the teas, my Dad for all money and my sister Alana for the love and friendship, I could not have gotten here without them. I love you all!

Thank you Mike Sawchuck for supporting me through all the weekends that were given up for this project and most of all making me laugh.

Thank You!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

CHAPTER 1.0: GENERAL INTRODUCTION

1.1 FRESHWATER *CLADOPHORA* BLOOMS

Cladophora (L.) Kützing (Cladophorales) is a filamentous green alga that is globally widespread in both freshwater and marine habitats. In freshwater, *Cladophora* has been reported in pristine streams, eutrophic lakes, and estuaries (Whitton, 1970; Dodds and Gudder, 1992; Sheath and Cole, 1992). It is considered an important component of the ecological community as it provides substrate for epiphyte colonization (Whitton, 1970; Dodds and Gudder, 1992) by *Diatoma* spp., *Cocconeis* spp., *Rhoicosphenia curvata* and *Gomphonema* spp. These diatom species are the most prominent colonizers of Great Lakes *Cladophora* establishing *Cladophora's* role as an important substratum for grazers that feed on these diatom species (Marks and Power, 2000). Although the presence of *Cladophora* may be an indicator of good water-quality due to its inability to prosper in heavily polluted habitats (Liebmann, 1962), most often its appearance has been associated with nuisance bloom conditions. These blooms can be related to anthropogenic sources and eutrophic conditions caused by high phosphorus and nitrate levels (Auer *et al.,* 1982; Dodds and Gudder, 1992; Hiriart *et al.,* 2003). In the 1960's and early 1970's the Laurentian Great Lakes, specifically Lake Ontario and Lake Erie experienced significant incidences of near-shore *Cladophora* blooms (Painter and Kamaitis, 1987). Initially these blooms were attributed to decades of eutrophic conditions associated with excessive inputs of phosphorous into the Lakes through detergents, large amounts of soil run off, municipal waste, and sewage treatment (Jackson *et al.,* 1990, Dodds and Gudder, 1992, John, 2002). As a result, phosphorus inputs were closely regulated and the1972 the Great Lakes Water Quality Agreement (Hiriart *et al.,* 2003) imposed phosphorus abatement strategies. Through legislation,

sewage treatments were upgraded and reductions of allowable levels of phosphates in laundry detergents were established (Stevens and Neilson, 1987). These strategies were initially deemed successful and blooms of filamentous green algae were greatly reduced within the Great Lakes. However, in recent years excessive growths of *Cladophora* have resurged along the shores of the Great Lakes. The factors causing this resurgence are not fully understood; however it may be associated with the invasion of the exotic species of the zebra mussel, *Dreissena polymorpha* in addition to watershed alterations caused by population growth and changing land use (Hecky *et al.,* 2004).

Accumulations of *Cladophora* in aquatic systems have serious implications for water usage. For example, it can create problems in irrigation canals by reducing both flow rate and canal capacity when attached to the sides and base of the canal (John, 2002)**.** Once detached, mats float downstream where they clog pump inlets, irrigation siphons, trash racks and sprinkler heads (Sheath and Wehr, 2003). Clogging of rivers, canals, and drainage ditches has also been cited as the causal factor in flooding events (van den Hoek, 1963, 1984).

Recently, *Cladophora* blooms in Lake Ontario have significantly impacted Ontario Power Plants (OPG) (Personal communication with Keith Garrel, Director OPG). Main water intake pipes to power plants are covered by large travelling screens that filter and remove debris prior to water entering the generator cooling water system. When excessive amounts of *Cladophora* are present there is an increased risk of screens becoming clogged, hindering water intake. This can result in automatic shut down of generators, which occurred in May 2001. Since then, upgrades to screens have been implemented and the OPG has taken a proactive approach by shutting down units when significant levels of

detached *Cladophora* are present. Generator shuts downs are extremely costly, as well as potentially dangerous to the public (all OPG information provided by personal communication with Keith Garrel, Director, OPG).

These blooms may also result in large volumes of *Cladophora* detaching near shore creating dense floating and beached mats that begin to decompose and can create anaerobic conditions leading to fish kills and deaths of other aquatic animals (Millner and Sweeney, 1982). Recently, high concentrations of *E. coli* and enterococci have been documented in beached *Cladophora*, which suggests that this alga has the ability to sustain these bacteria in ambient conditions (Byappanahalli, *et al.,* 2003). Furthermore, decaying *Cladophora* can form malodorous masses and are aesthetically unpleasant with potential to impair recreational activities (Figure 1). This can result in significant economic impacts on local tourism and the values of waterfront properties (John, 2002).

Despite research to discern the ecological, physiological, and biochemical reasons for freshwater *Cladophora* blooms, very little has been done to address the complex taxonomic issues associated with species discrimination within the *Cladophora* genus (Marks and Cummings, 1996). The 'species' is the fundamental unit in taxonomy and different species fill discrete ecological niches through the process of natural selection (Orr and Coyne, 2004). Based on this, different species should display distinctions, such as physiological responses to a particular niche (Pinna, 1999). However, as in the case with many algal groups, *Cladophora* is often not easily delineated to the species level. Therefore the freshwater members of this genus are frequently assumed to be *Cladophora glomerata* (L.) Kützing without significant assessment to verify the taxonomy (Marks and Cummings, 1996). As a result it is uncertain whether researchers are comparing one or many

Figure 1. Beached *Cladophora* mats on the shore of Lake Ontario, Ontario, 2004. Photo by SJR.

genetically distinct species when attempting to understand the impacts and ecological requirements of this macroalga. From a management stance this is significant as many questions remain about the ecological needs and effects of different species or genotypes. For these reasons, being able to determine species (or genotype) is essential in determining the direction of future management of nuisance blooms as different species may react differently to various management techniques.

1.2 Introduction to Species Concepts

If one is to concur that the 'species' is of fundamental importance in biology, then they are inevitably drawn into the debated issues of explaining what a species actually is, and what criteria should be implemented to recognize different species. Currently, there is no consensus on the definition of a species and as a result a number of species concepts exist. Individual researchers tend to choose the species concept that best fits the organism or organisms that they are studying.

1.2.1 Biological Species Concept

The textbook definition of a species utilizes the Biological Species Concept (BSC) established by Ernst Mayr (Mayr, 1942). The criterion for a species under this concept is reproductive isolation. Therefore, if individuals of a particular population do not hybridize, and/or fail to produce fertile offspring when they do, then they are regarded as reproducibly isolated and deemed a good species (Freeman and Herron, 2004). The most arguable problem with the BSC is that it cannot be applied to clonal or fossil species. In addition, hybridization experiments within the algae are a rarity and impossible for many groups (Manhart and McCourt, 1992). Freshwater *Cladophora* species such as *Cl. glomerata,* have not been shown to undergo sexual reproduction in the natural environment, hence the BSC cannot be applied to this genus (Bakker *et al.,* 1992; Manhart and McCourt, 1992).

1.2.2 Morphological Species Concept

In contrast to the BSC, the morphological species concept (MSC) can be applied to asexual and fossil species. The MSC is historically relevant as is has been the central operational criterion employed to delineate species (van den Hoek, 1963; Lomolino *et al.,* 2006). The fundamental assumption made by the MSC is that morphological differences between species have an underlying genetic basis that is heritable (Lomolino *et al.,* 2006). The criterion fails if morphological traits are highly plastic within a species (Manhart and McCourt, 1992). Morphological characters are currently still valid taxonomic criterion to delineate species in the genus *Cladophora*. However, due to the extreme amount of morphological variability induced by varying environment and age, the MSC is nearly impossible to reliably apply to freshwater *Cladophora* (van den Hoek, 1963; Dodds and Gudder, 1992).

1.2.3 Phylogenetic Species Concept

In the phylogenetic species concept (PSC) the purpose of identifying species is to develop a system for naming and classifying the diversity of life that accurately reflects the evolutionary history of organisms (Freeman and Herron, 2004; Avise 2004). To do this, the PSC focuses on identifying monophyletic groups (a group that contain all of the known descendants of a single common ancestor) (Manhart and McCourt, 1992; Freeman and Herron, 2004), thus species are identified by the smallest monophyletic group (Freeeman and Herron, 2004; Avise, 2004). In contrast to monophyletic groups, paraphyletic and polyphyletic groups do not include all descendants of a common ancestor. The existence

of polyphyletic taxa suggests that the current taxonomic structure must be changed if a classification system reflecting phylogeny is desired (Lesk, 2005).

Phylogenetic analyses of marine *Cladophora* using the nuclear 18S rRNA gene suggests that many marine species are paraphyletic (Bakker *et al.,* 1992). These findings have been reinforced by a number of other studies on this genus (van den Hoek *et al.,*1995; van den Hoek and Chihara, 2000). Due to the difficulty in establishing synapomorphic characters (Leliaert *et al.,* 2003) and the lack of stable taxonomic characteristics, there is little arguable basis to revise the sections or otherwise modify its taxonomy. Thus far no taxonomic changes have been made and there is still considerable confusion regarding species identification within the genus.

1.3 TAXONOMY OF FRESHWATER *CLADOPHORA*

Taxonomic confusion within the *Cladophora* genus goes back to 1753, when Linnaeus first established the genus *Conferva.* Nearly a century later Kützing (1843) created the genus *Cladophora,* and moved many species from *Conferva* into the new genus. Kützing's taxonomic revision and the addition of numerous new descriptions of species, subspecies, and varieties by various authors, resulted in over 650 species epithets within the genus *Cladophora* (van den Hoek, 1963). The large number of species was primarily due to the considerable phenotypic plasticity exhibited by *Cladophora*. The most recent revision of the genus was by C. van den Hoek (1963). He established 11 sections based primarily on morphology. Subsequent to this revision, the genus *Cladophora* contained a total of 38 species; 11 freshwater and 27 marine. Freshwater species are found in six of the eleven sections, these are:

Aegagropila
Cl. aegagropila (L.) Rabenh.

Glomeratae
- *Cl. fracta* var. *fracta* (Mull. Ex Vahl) Kutz.
- *Cl. fracta* var. *intRicota* (Mull ex Vahl) Kutz.
- *Cl. glomerata* var. *glomerata* (L.) Kutz.
- *Cl. glomerata* var. *crassior* (L.) Kutz.

Cladophora
- *Cl. rivularis* (L.) v.d. Hoek
- *Cl. surera* Brand

Cornuta
- *Cl. cornuta* Brand

Affines
- *Cl. kosterae* Hoffm. & Tild.

Basicladia
- *Cl. basiramosa* Schmidle
- *Cl. pachyderma* (Kjellm.) Brand

Since van den Hoek's assessment of the genus, an additional freshwater species has been considered, *Cladophora gyrfalconium* Stanford and Prescott, which was identified from Locus Lake Gyrafalcon, Glacier National Park. This is the only known location of this species and it was the only time it was collected (Stanford and Prescott, 1988). Currently, it is unclear which of the six morphological sections *Cl. gyrfalconium* fits into. This study did not examine this species due to the difficulty in obtaining sample material for morphological and molecular analyses.

van den Hoek's placement of freshwater *Cladophora* into the sections and species noted above were based on morphological characteristics of type, field and cultured material. These characteristics included thallus organization, pattern and amount of branching, type of main axes growth (intercalary or acropetal), mode of reproduction, length/width ratio of main axes cell, apical cell diameter, attachment organ, and whether the thallus was attached to a substrate or free-floating. Despite the variety of morphological characters, van den Hoek (1963) noted that many of the morphological characteristics used to separate the sections often overlapped among and within different sections (Marks and Cummings, 1996; Bakker *et al.,* 1992; van den Hoek 1963, 184).

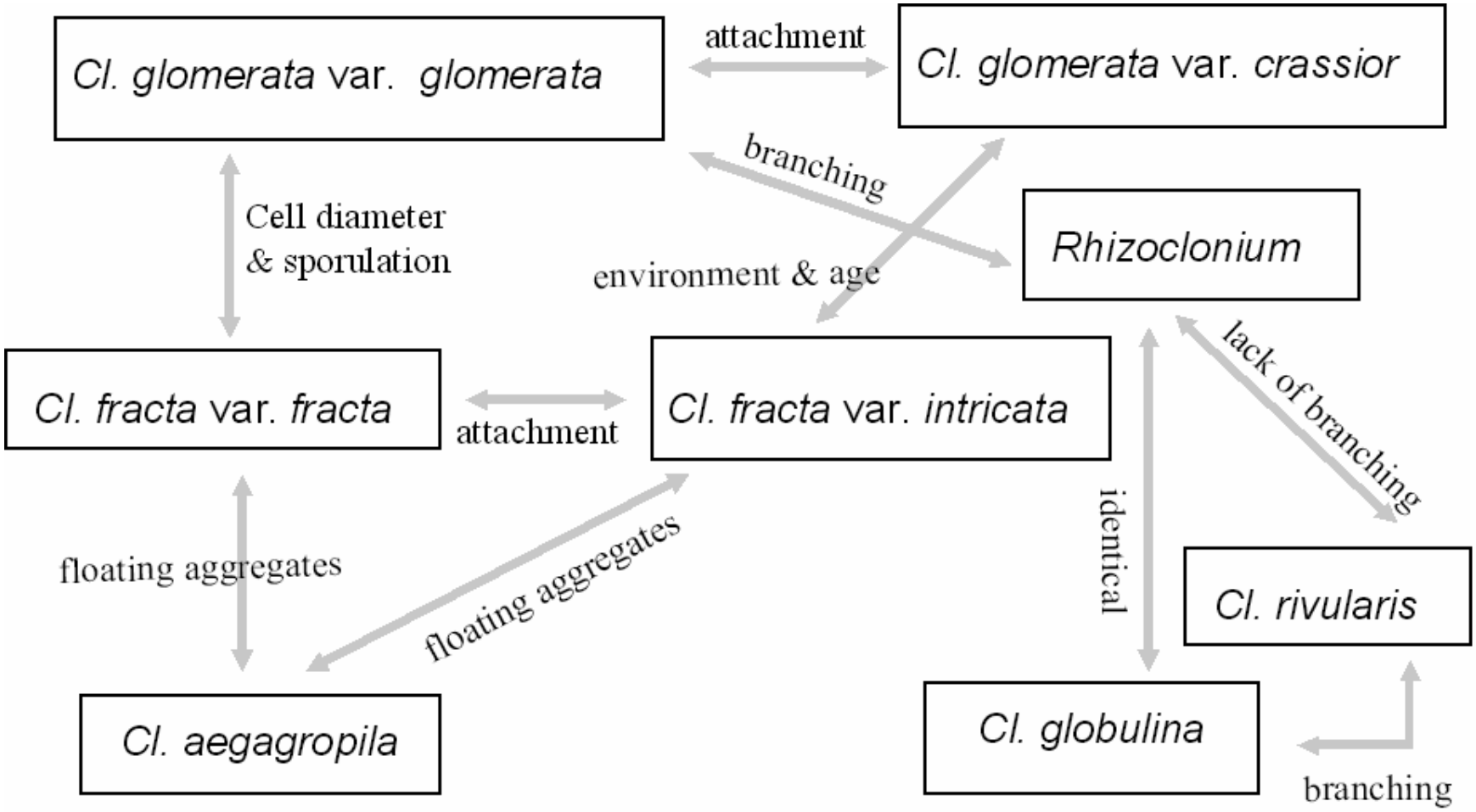
1.3.1 Taxonomic Problems Within Section Glomeratae

Section *Glomeratae* contains four freshwater taxa: *Cl. glomerata* var. *glomerata*, *Cl. glomerata* var. *crassior*, *Cl. fracta* var. *fracta*, and *Cl. fracta* var. *intricata*. It is within this section, with respect to freshwater species, that the greatest overlap among taxonomic boundaries has occurred. For example, distinction between *Cl. glomerata* and *Cl. fracta* var. *fracta* may be possible when considering method of reproduction. It is believed that both varieties of *Cl. glomerata* reproduce asexually through the production of biflagellate zoospores, whereas zoospore formation has not been documented in *Cl. fracta* var. *fracta* (van den Hoek, 1963; Reynolds *et al.,* 2000). However, because zoospore formation by *Cl. glomerata* in the field is rare, its absence does not make it a dependable differentiating characteristic. Unfortunately many of the morphological distinctions overlap depending on the age of the alga or the surrounding environmental conditions (van den Hoek, 1963; Dodds and Gudder, 1992). Figure 2 is a schematic representation of additional morphological criterion that overlap among and within the section *Glomeratae* as well as other sections. Also the genus *Rhizoclonium* has been included as it is often misidentified for *Cladophora* in the field, which will be discussed further in the current paper.

1.4 MORPHOLOGICAL DESCRIPTIONS OF FRESHWATER *CLADOPHORA*

As mentioned above, historically taxonomic delineation of *Cladophora* has been based on various morphological characteristics such as cellular measurements, degree and type of branching, mode of growth, and type of thalli organization. As such, it is important to recognize how morphological characters are affected by external factors. *Cladophora* is often observed attached to substrate in flowing waters, appearing as long

Figure 2. Schematic representation of how the taxonomic characters used for identifying species of genus overlap. Many of these characteristics are dependent on the environmental conditions under which the plant is observed. This also includes the genus, *Rhizoclonium* as it is often misidentified as *Cladophora.*


Cl. glomerata var. glomerata
attachment
Cl. glomerata var. crassior
branching
Cell diameter
& sporulation
environment & age
Rhizoclonium
Cl. fracta var. fracta
attachment
Cl. fracta var. intricata
lack of branching
floating aggregates
floating aggregates
identical
Cl. rivularis
Cl. aegagropila
Cl. globulina
branching

streaming filaments or as free-floating masses on water surface and subsurfaces overlying sediment (Dodds and Gudder, 1992). Morphologically, it is characterized by its multinucleate cells, reticulate chloroplasts, thick cell wall lacking in a mucilaginous sheath, and filamentous-branched thalli (John, 2002). The following sections will discuss some of the morphological characteristics.

1.4.1 Cell size

In his revision of the *Cladophora* genus, van den Hoek (1963) reported that length and width measurements from the main axes, ultimate branch and apical cells were able to aid in discriminating species, even within the highly variable section *Glomeratae*. Suggested cell sizes based on European freshwater specimens from section *Glomeratae* (*Cl. glomerata* var. *glomerata, Cl. glomerata* var. *crassior, Cl. fracta* var. *fracta* and *Cl. fracta* var. *intricata*) are denoted in Table 1. Although the fact that each species is defined by distinct size ranges, these measurements can also display plasticity in both culture and natural environments. For example, cultured *Cladophora* grown in nutrient-depleted media do not produce any branches, but generate very long cells (Wilson *et al.*, 1999). In natural environments, exposure to strong sunlight can decrease the diameter of all filament cells (van den Hoek, 1982). Exposure to high wave action in the littoral region of the north Baltic was reported by Ronnberg and Lax (1980) to reduced cell lengths and increase cell diameters in marine *Cladophora*. Increasing levels of suspended sediments in the Colorado River resulted in a correlative decrease in total filament length (Wilson *et al.*, 1999). This same study reported that drastic morphological changes occurred with filament cells becoming shorter and wider. Even the amount of branching produced by each individual plant can affect cell size. For example in richly branched individuals,

Table 1. Table of morphological parameters used to differentiate European freshwater species of *Cladophora* from the section *Glomeratae*.

Cladophora Species	Diameter of Main Cell (µm) Length : Width	Diameter of Apical Cell (µm) Length : Width	Diameter of Branch Cell (µm) Length : Width
Cl. glomerata* var. *glomerata	100-275 (7-12) : (1.5-5)	(21-31)-(58-91) (6-13)-(1.5-5)	(22-34)-(68-100) (5-10)-(1.5-3.5)
Cl. glomerata* var. *crassior	65-165 (6-12) : (2-5)	(19-24)-(30-55) (9-21) : (2.5-8)	(19-32)-(38-70) (10-15) : (2.5-7)
Cl. fracta* var. *fracta	45-85 1.5-14	16-27 3.5-25	17.5-38 3-17
Cl. fracta* var. *intricata	330-650 10 : 18	(120-160)-(150-200) (7-18) : (2-8)	(120-160)-(170-230) (10-16) : (4-7)

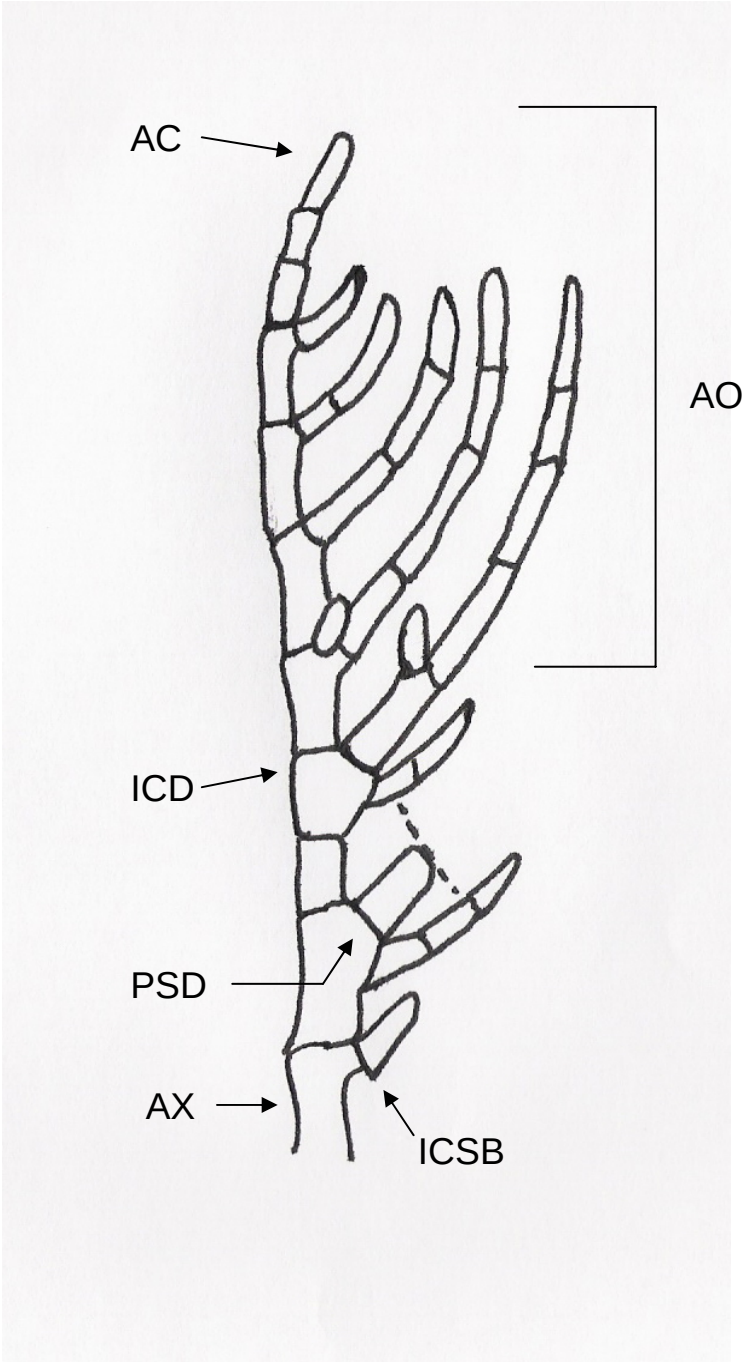
cells of the main axis are reported to be three to six times longer than wide versus sparsely branched individuals in which cells of the main axis tend to be 20-30 times longer than wide (Sheath and Cole, 1992).

1.4.2 Growth and branching

Organization of overall branching is also affected by algal age and habitats. Branching in freshwater *Cladophora* consists of uniseriate filaments inserted at oblique to horizontal angles, and may be sparsely to profusely spaced (van den Hoek, 1995). Studies have shown a trend for increased branching in *Cladophora* with increased water velocity (van den Hoek, 1964, Parodi and Caceres, 1991). As branches grow and age the position of the wall cutting it off from the axis becomes nearly horizontal creating pseudo-dichotomous branching systems (Johnson *et al.,* 1996) (Figure 3). This type of branching often occurs in turbulent waters, which also affects growth and organization of thallus in freshwater *Cladophora* (Parodi and Caceres, 1991).

Development of *Cladophora* is dominated either by apical growth, with acropetal organization or intercalary growth, with an irregular organization (Figure 3). Young *Cladophora* plants initially demonstrate apical growth, where the apical cell itself divides forming daughter cells that then elongate. Apical growth is closely correlated with acropetal organization meaning the most recent formed branch is the one closest to the apex and thus side branches progressively becoming older and longer towards the base (van den Hoek *et al.,* 1963). Acropetal organization often dominates in slow-flowing streams (van den Hoek, 1963). Intercalary growth implies that cells located away from the apex divide and grow which thereby increases the distance between primary branches. Side branches then grow out from

Figure 3. Schematical representation of various types of branching and growth and overall thallus organization experienced by freshwater *Cladophora*. (AC) apical cell, (AX) main axis, (ICD) intercalary cell division, (PSD) pseudodichotomy, (AO) acropetal organization


AC
AO
ICD
PSD
AX
ICSB

below the resulting cross wall making it virtually impossible to differentiate between primary and secondary branches producing irregular organization (van den Hoek *et al.,*1995); (Fig 3 based on a schematic representation by van den Hoek, 1995).
This type of growth often predominates with increased age, in sheltered habitats, rapidly streaming water (van den Hoek, 1963), and following sporulation (Parodi and Caceres, 1991). Intensive sporulation of the terminal plant causes disintegration of side branches reducing the plant to mostly the main axis. Following sporulation, if environmental conditions are favourable, the main axis grows through intercalary divisions and cell stretching with branches sprouting from the recently shortened main axis. These branches tend to be sparse and very scattered (Hoffmann *et al.,* 1984). In less favourable environments intercalary divisions that are no longer followed by stretching of the cells, thus overall filament length does not greatly increase (Hoffmann *et al.,* 1984).

1.4.3 Reproduction

Sporulation is a result of asexual reproduction in freshwater *Cladophora* and is accomplished through the formation of biflagellate zoosporangia. Zoospore formation occurs in the distal cells of side branches through mitotic nuclear divisions (Dodds and Gudder, 1992). Cells at the periphery of the side branches become inflated taking on a barrel-shape, with contents dividing into biflagellate zoospores that escape through the pore at the apical pole of each zooidangium (van den Hoek, 1963, 1984).

1.4.4 Akinete formation

Short photoperiods, low temperatures, desiccation or nutrient depletion, result in cells of the main axis shortening and taking on a club-shaped appearance, and have been reported in both natural and cultured environments (van den Hoek, 1963; Hoffmann *et al.,*

1984). This morphological shift is a result of an increased cell diameter that then further transforms into thick-walled resistant vegetative cells called akinetes (van den Hoek, 1963; Hoffmann *et al.,* 1984). During akinete formation, cell contents become denser, number of chromatophores increase, and starch accumulates, all of which allow *Cladophora* to successfully survive over-wintering through adverse environmental conditions (van den Hoek, 1963). Over-wintering akinetes attach to hard substrates and await more favourable conditions to begin germination and hence are important aspects of *Cladophora*'s perennial growth success (Hoffmann *et al.,* 1984).

Many researchers have recognized that due to the highly variable nature of morphological characteristics, more stable taxonomic characters (those not affected by environment and age) must be uncovered. As a result many researchers have opted for molecular techniques to assess taxonomical identification of species. Molecular techniques provide a new type of data that can be used to reconstruct or verify previously established classifications that were based on morphological or physiological characters (De Bruin *et al.,* 2003).

1.5 Population Genetics Methods for Freshwater *Cladophora*

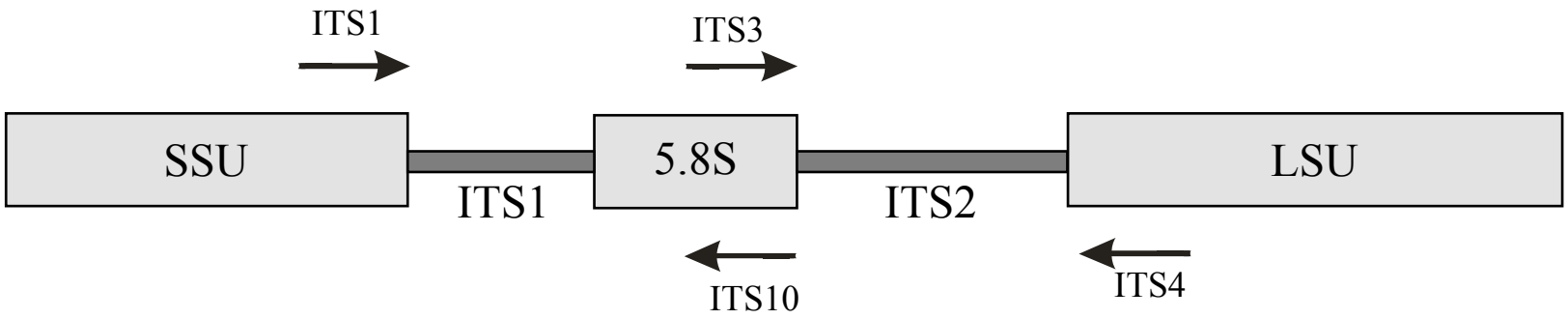
DNA markers have been informative in determination of how populations of given species are distributed, how genetically different populations are, and how much genetic variation is present within and among populations (Karp and Edwards, 1997). Several molecular techniques can be used to examine various population relationships based on sequence divergence of evolutionarily significant regions such as the internal transcribed sequences of the nuclear ribosomal DNA cistron, or polymorphic divergence as in the case

with inter simple sequence repeats (ISSR), each of which will be discussed in more detail in the following sections.

1.6 Internal Transcribed Spacer (ITS) Region

Although ribosomal coding regions of RNA (rRNA), have been extensively used for phylogenetic assessment of algal classes, orders and genera, at or below the species level, rRNA coding regions may reach their limit of resolution due to the highly conserved nature of the genes (Zimmer *et al.,* 1989; Bakker *et al.,* 1992; McManus and Lewis, 2005; Suda *et al.,* 2005). As a result, the regions located between the ribosomal genes, the internal transcribed spacer regions (ITS1 and ITS2) are often preferred for phylogenetic assessment at the species level (Graham and Wilcox, 2000). Although the ITS regions have secondary structural elements that are known to play an important role in the processing of pre-rRNA molecules (Bakker *et al.,* 1995), they are non-coding and thus have less evolutionary constraints. Figure 4 provides a schematical representation of the ITS1 and ITS2 regions of the rRNA cistron and the associated primers used to amplify each of the regions. Fewer constraints permit higher sequence divergence than coding regions and are thus useful for examining variation and resolving taxonomic problems at lower taxonomic levels (van Oppen *et al.,* 1995; Larsen and Medlin, 1997). Comparison of the evolutionary rates in the coding regions or rRNA (18S rRNA gene) and the noncoding region (ITS region) in the marine *Cladophora* species, *Cl. albida* was conducted by Bakker *et al.,* (1995). In comparing the Jukes-Cantor distances within each of the regions, the ITS sequence revealed a 20-50 times higher substitution rate than that observed in 18S rRNA. Based on these characteristics the ITS regions have been used to

Figure 4. Internal transcribed spacer (ITS) regions of the ribosomal gene cistron. Also included are the primers used to amplify each of the regions.



ITS = Internal transcribed spacer
SSU = Rubisco small sub-unit gene
LSU = Rubisco large sub-unit gene

examine genetic differences at the species and subspecies level within various algal groups (Kooistra *et al.,* 1992). Specific examples include: the identification of strains within species of *Scenedesmus* (Chlorophyceae) (van Hannen *et al.,* 2000), the delineation species and species relationships in the red algal agarophytes *Gracilariopis* and *Gracilaria* (Gracilariales); (Goff *et al.,* 1994), migration patterns of *Phycodrys rubens* (van Oppen *et al.,* 1995), as well as for examining relationships among geographically distinct populations marine *Cladophoropsis membranaceae* (Chlorophyta); (Kooistra *et al.,* 1992). This latter study demonstrated that the dispersal of *Cladophoropsis membranaceae* was occurring between the Caribbean and tropical eastern Atlantic (Kooistra *et al.,* 1992).

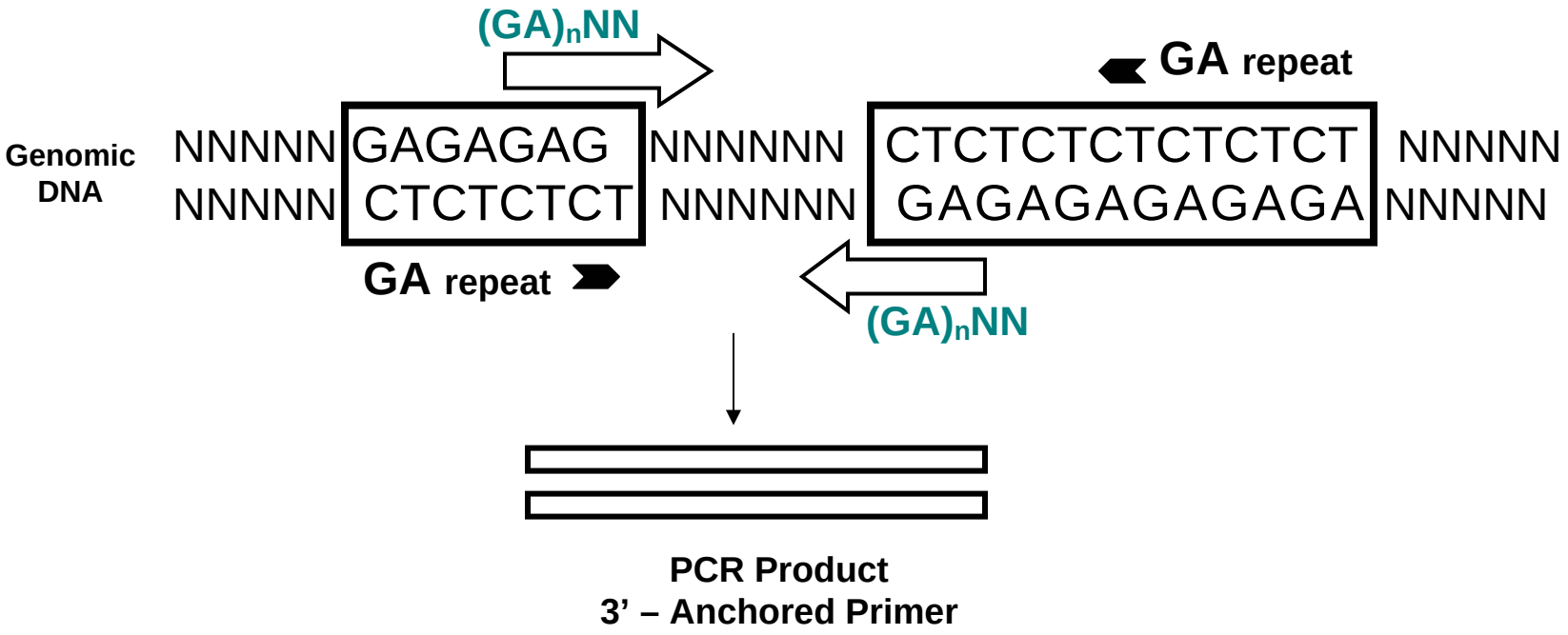
To date there are no published ITS sequence studies on freshwater *Cladophora* from the Great Lakes. One study that included three North American samples of North American freshwater *Cladophora* (Marks and Cummings, 1996), focused on deciphering different morphological or ecological species of freshwater *Cladophora* primarily from Europe. They determined that sequence data of cultured specimens of *Cl. glomerata* and *Cl. fracta* had distinct genotypes, but were still 95% similar. In addition, minimal sequence divergence was noted among all field-collected samples, whether European or North American (Marks and Cummings, 1996). Such findings suggest that the ITS regions may be able to shed some light on interspecific variation within freshwater *Cladophora* but may not be able to fully discern intraspecific variation. As a result multilocus molecular markers were also employed.

1.7 INTER-SIMPLE SEQUENCE REPEATS (ISSR)

The molecular marker inter-simple sequence repeats (ISSR) was chosen to examine population structure and phylogenetic relationships within and between freshwater *Cladophora* populations. ISSR analysis requires no prior knowledge of the genome and entails PCR-amplification of regions between adjacent (within 4000bp), inversely oriented microsatellites using a single SSR containing primer (Zietkiewicz *et al.,* 1994; Karp and Edwards, 1997; Wolfe, 2005) (Figure 5). Gel electrophoresis of the PCR product generates bands of a particular size for that locus representing intervening stretches of DNA between microsatellites (Nagaoka and Ogihara, 1997, Larsen and Medlin, 1997). Usually several paired microsatellites exist, resulting in multiple band generation (Vis, 1999). Bands are then scored as present or absent. ISSRs have been reported as highly reproducible, polymorphic, and informative (Zietkiewicz *et al.,* 1994) because they identify alleles simultaneously at multiple, interspersed loci throughout the genome (Sunnucks, 2000)

Initially this marker was used in higher plants and animals (Zietkiewicz *et al.,* 1994) and has been used to determine genetic variation among closely related individuals and in crop cultivator classifications (Godwin *et al.,* 1997; Blair *et al.,* 1999; Fang and Roose, 1997). The cultivator sector has reported that ISSRs are more reliable and conform more closely to dominant Mendelian inheritance than RAPD markers, rendering it useful for genotype analysis (Tsumura *et al.,* 1996; Nagaoka and Ogihara, 1997). ISSR analysis has also been used to address inter and intraspecific variation for freshwater algae. For example, ISSRs were used to distinguish individual gametophytes of *Batrachospermum boryanum* in an intrapopulation genetic study of a Pennsylvania

Figure 5. Inter-simple sequence repeat PCR reaction schematically represented. A single primer targeting a $(GA)_n$ repeat, anchored at 3'end of repeat, is used to amplify genomic sequence flanked by two inversely oriented $(GA)_n$ elements.


(GA)nNN
GA repeat
Genomic DNA
NNNNN GAGAGAG NNNNNN CTCTCTCTCTCTCT NNNNN
NNNNN CTCTCTCT NNNNNN GAGAGAGAGAGA NNNNN
GA repeat
(GA)nNN
PCR Product
3' – Anchored Primer

stream (Vis, 1999). They noted that there was a large amount of genetic diversity within the stream and that populations located at the upstream and downstream were more closely related than to adjacent populations. Such findings suggested that either high gene flow or dispersal mechanisms were responsible for some of the population structure of the stream. Later Hall and Vis (2002) studied the genetic variation of another freshwater red alga, *Batrachospermum helminthosum* on both an intra- and interpopulation scale. They examined the population structure among and within streams from across eastern North America using the ISSR markers (Vis and Hall, 2001). They observed significant genetic differences *($P<0.05$*) among all streams, with diversity not being reflective of the geographic distances between streams. These findings suggested that long distance dispersal may be an important factor in the distribution and success of *Batrachospermum helminthosum.* Within streams, similar results were noted for *Batrachospermum boryanum* (Vis, 1999). Again genetic similarity and spatial distances were uncorrelated, suggesting that the genetic structure within a stream is more complicated than can be explained by reproduction among closely located individuals. Overall, ISSR analysis revealed that *Batrachospermum helminthosum* had a complex phylogeographical distribution.

Most recently ISSR analysis proved to be a quick and useful molecular tool to solve the problem of morphological identification and strain characterization of the phytoplankton species *Alexandrium* and *Pseudo-nitzshia* (Bornet *et al.,* 2004). Resultant ISSR fingerprints clearly distinguished all tested species and allowed identification from the genus level to geographical species level as well as the separation of toxic and non-toxic strains within the same species. Such findings indicated that amplification of ISSR regions could rapidly differentiate closely related individuals.

ISSR fingerprinting is able to expose differences among closely related individuals than with rRNA coding and non-coding regions (Mindel and Honeycutt, 1990). Additionally, this molecular marker has been shown to produce highly reliable, reproducible, and polymorphic fingerprints (Zietkiewicz *et al.,* 1994), which raises the possibility of using this marker to address the complex taxonomic issues in the genus *Cladophora*. Being able to determine species (or genotype) is essential for interpreting both ecological and physiological studies and for differentiating ecological plasticity from genetic divergence.

1.8 THESIS OBJECTIVES

Determine if the macroalgae identified as *Cladophora* in the Great Lakes is composed of a single or multiple, species or varieties.

Before attempting to establish proper implementation of management strategies for *Cladophora* in the Great Lakes, it is essential to have a well-grounded understanding of the taxonomic composition to ensure that the target organism is being addressed. A molecular study is essential as we will then have the ability to measure differences among genotypes to delineate species as oppose to morphological studies that can only assess the phenotype, which may not be informative enough to distinguish species. At present, there have been no molecular studies conducted on the Great Lakes freshwater *Cladophora* to ascertain if there is truly only one species or if varieties are present. Thus the present study will be focus on the population genetics of *Cladophora* in the Laurentian Great Lakes. This study will also address the utility of using the internal transcribed sequence that could differentiate *Cladophora* populations from each of the Great Lakes. regions

(ITS1 and ITS2) and ISSR's to address taxonomic and biogeographic questions in the genus *Cladophora*.

Examine the genetic relationship and biogeography of the Great Lakes *Cladophora* species with other North America freshwater samples to clarify the distinctiveness of the species and establish their phylogenetic position.

Internal transcribed regions and inter simple sequence repeats have been shown to be divergent between species and thus may be used to reconstruct the biogeographic history of the North American freshwater *Cladophora* species. By increasing the scope of this study and placing Great Lakes *Cladophora* in the context of North America, a better understanding of the taxonomy and biogeography of this genus will be gained.

Correlate morphological characteristics with molecularly delineated species data to probe the existence of stable taxonomical features which could clearly identify different species or varieties of freshwater *Cladophora*.

When discussing species level taxonomic delineation of algae, the morphological species concept often stands out as the most often implemented (Coyne and Orr, 2004). For freshwater *Cladophora*, this concept is clearly not ideal as it is subject to high levels of phenotypic plasticity, based on age, water velocity, sedimentation and other changing environmental conditions limiting usable morphological characteristics (Manhart and McCourt, 1992, John and Maggs, 1997). Based on the premise that neither morphological data nor molecular data sets alone should provide a depiction of the true phylogeny I will first assess the morphological architecture of a *Cladophora* population from a single location over an entire season to observe how morphological characteristics change over a season. I will also use voucher *Cladophora* samples collected from the Laurentian Great Lakes (excluding Lake Superior) to determine if stable morphometric characteristics exist

CHAPTER 2.0: METHODS

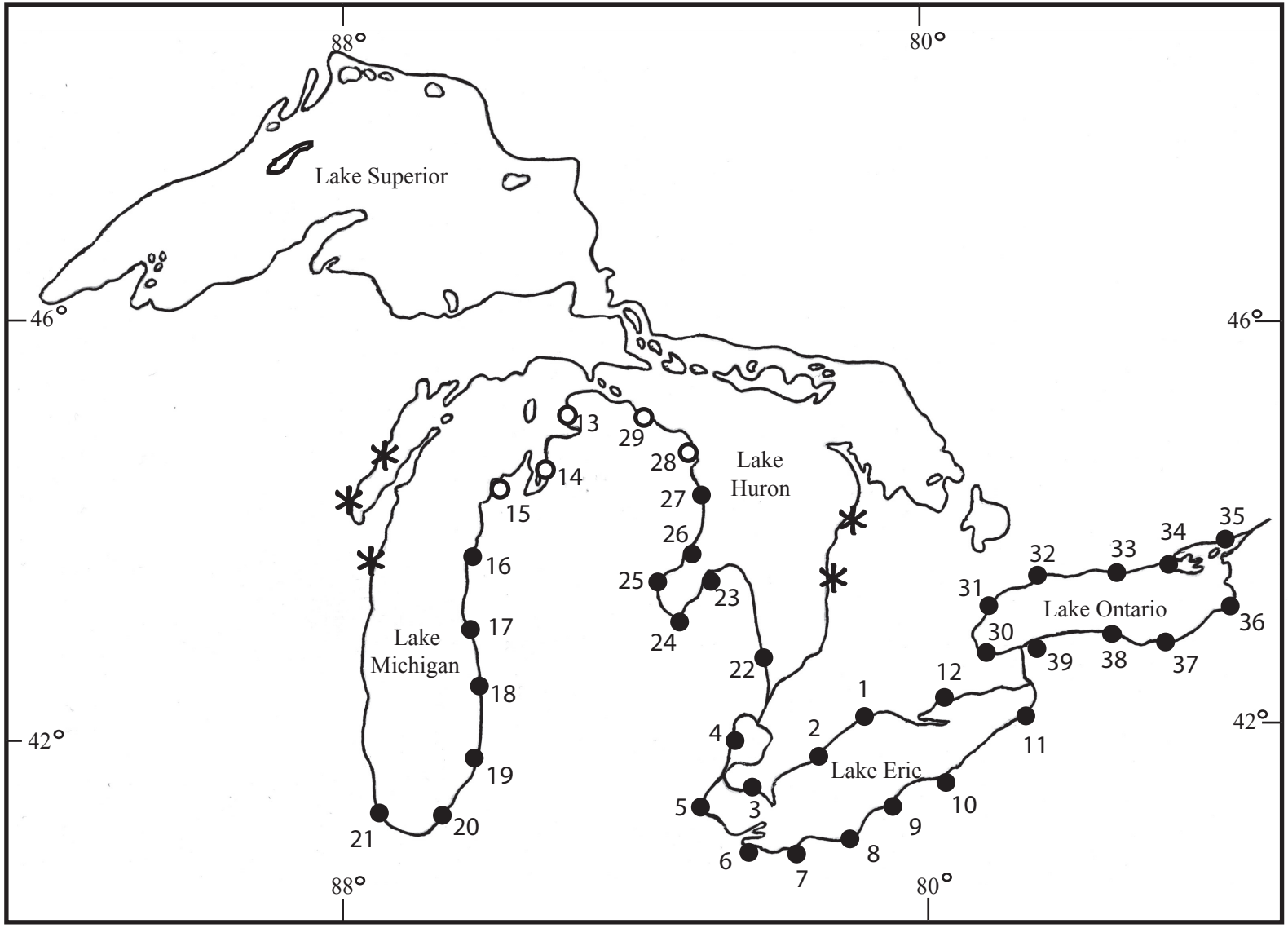
2.1 *CLADOPHORA* COLLECTION METHODS

2.1.1 Collection methods for molecular and morphology analysis

All samples used in molecular and morphological analysis are listed in Appendix A. Freshwater *Cladophora* specimens were collected in the summer of 2005, from all the Great Lakes excluding Lake Superior (Figure 6). Locations for the 2005 survey were a subset of those locations sampled by Sheath (1987), as well as by Troina Shea in 2002 (Appendix B). All samples collected were done so from rocks and substrate located directly along the shoreline. The algal samples collected in 1987 and 2002 were placed on a damp paper towel, stored in a whirl pack, and transported to the lab on ice in a cooler or if not possible, immediately placed in whirl packs filled with silica to desiccate the sample. Sampling was conducted in the same manner, by the author, at each location in order to standardize the sampling effort. Upon return to the lab samples were cleaned of epiphytes, fixed in 2.5% $CaCO_3$-buffered glutaraldehyde for morphological analyses or frozen for later DNA analyses (Samples collected in 1987 and 2002 were processed in the same way). Gluterladehyde was used as it has been shown to be favourable in pigment and cell composition integrity for algae (Jearanaikoo, and Abraham-Peskir, 2005).

In addition, a large number of *Cladophora* samples from different locations, and varying environments from across North America have been collected over a number of years ranging from 1998-2002 by various collaborators. At the time of collection they

Figure 6. Map of the Great Lakes depicting 2005 collection locations. Solid circles indicate locations that *Cladophora* was present and sampled. Hollow circle are locations where no *Cladophora* was present. Star indicate locations not personally sampled, but were samples were attained through external source. Location numbers correspond to locations listed in Appendix C.


88°
80°
46°
46°
42°
42°
88°
80°
Lake Superior
Lake Huron
Lake Michigan
Lake Erie
Lake Ontario
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39

were cleaned and processed following the above-mentioned protocols (personal communication, Kirsten Müller, 2006). Cultures of *Cladophora* were acquired from both the University Of Texas Culturing Center (UTEX), and from the University of Toronto Culturing Center (UTCC). The UTEX collections were samples of *Cl. glomerata* var. *glomerata* (LB1486) *Cl. glomerata* var. *crassior* (LB1488), *Cl. fracta* var. *fracta* (LB473); (Table 2). Although these cultures were collected from European locations, C. van den Hoek deposited each as representative type cultures for each of these species. From the UTTC, two *Cladophora glomerata* samples were acquired (UTCC636 and UTCC13); (Table 2). Samples were maintained in a culturing solution made up of CHU 10 and Lake Ontario water in a 50:50. Samples were subcultured biweekly, and maintained in a 22°C incubator under light/dark periods of 12h/12h.

2.1.2 Collection methods for seasonality morphology study

Throughout the summer of 2004, *Cladophora* samples were collected from a single location for morphological analyses to examine classic taxonomically relevant characteristic and how they change over a growing season. All samples were collected from a single location, on Lake Ontario; Dingle Park located on Oakville, Ontario (Figure 1). The location was very open and thus susceptible to high wave action. The lake bottom was very rocky, providing ample and favourable substrate for *Cladophora* colonization. This location was chosen for a subsequent study being conducted by Sairah Malkin. Sampling occurred from mid-May through to mid-October (13 sampling dates in total) biweekly, ensuring an entire season was accounted for (Appendix C). All samples were collected near shore, at a depth of ~1 meter. Three randomly chosen sites (microsites) within a 10m radius of one another were chosen at each sampling visit for.

Table 2. List of freshwater *Cladophora* cultures used for morphological and molecular study. Cultures were attained from the University of Texas Culture Collection (UTEX), and the University of Toronto Culturing Center (UTCC).

Culture Center	Culture Number	*Cladophora* Species
UTEX	LB 1486	*Cl. glomerata* var. *glomerata*
UTEX	LB 1488	*Cl. glomerata* var. *crassior*
UTEX	LB 473	*Cl. fracta* var. *fracta*
UTCC	UTCC 636	*Cl. glomerata* var. *glomerata*
UTCC	UTCC 13	*Cl. glomerata* var. *glomerata*

collection. All samples were stored on ice and transported back to the lab, where they were placed in 2.5% gluterladehyde for future morphological analysis.

2.2 ITS Analysis Methods

2.2.1 DNA amplification of ITS region

Cladophora filaments used for DNA analyses had their rhizoidal bases removed, cleaned of debris and epiphytes and were then ground in liquid nitrogen. The Qiagen dNeasy Plant Mini Kit™ Qiagen (Qiagen, Canada) was used for DNA extractions. PCR amplification of the internal transcribed spacer 1 (ITS1) region was carried out using the primers listed in Table 3.0 and Figure 4. Amplification was performed using an Eppendorf Master Cycler Gradient PCR machine, with an initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 1 min, 49°C for 1 min, 72°C for 2 min, and a final elongation of 72°C for 3 min. 100µL reaction condition were as follows: 10µL 10X buffer, 2.0mM MgCl2, 200µM each dATP, dCTP, dGTP, dTTP, 0.4µM forward primer, 0.4µM reverse primer, 2 units Taq polymerase and 2µL DNA template. 5µL of the resulting PCR products and a HaeIII digested Lambda phage ladder) were separated on a 1.5% agarose gel (containing 0.0015mg/mL ethidium bromide) run for 30 minutes at 125 V in TBE buffer, followed by visualization on a UV trans-illuminator, using the Syngene BioImaging System (Synoptics Limited© 2000). The PCR products were then cleaned using the Qiaquick PCR Purification Kit, or the Qiaquick Gel Extraction Kit (Qiagen, Canada) if necessary. Sequencing was performed using the University of Waterloo ABI 3730XL sequencer as well as the AB3730 in the University of Guelph Sequencing Facility.

Table 3. Oligonucleotide primers used for the PCR amplification of the internal transcribed spacer (ITS) regions of North American and culture *Cladophora* samples.

Primer Name	Direction	Sequence 5' → 3'	Reference
ITS3	Forward	ACA TCG ATG AAG AAC GTA GC	White et al. 1990
ITS4	Reverse	CTT CCG TCA ATT CCT TTA AG	White et al. 1990
ITS1	Forward	GA AGG AGA AGT CGT ACC AAG G	Variation of ITS5
ITS10	Reverse	GCT GCG TTC TTC ATC GAT	Sherwood et al. 2002
AB28	Reverse	CCC CGG GAT CCA TAT	Sherwood et al. 2002
AB28.4	Reverse	ATA TGC TTA ART TCA GCG GTT	Variation of AB28, designed by TBS.

2.2.2 Sequence alignment

To evaluate the phylogenetic relationships among varieties of two freshwater *Cladophora* spp. genotypes, sequences of the internal transcribed spacer region (ITS1) of the rRNA genes were compared. The resulting sequences were manually edited and aligned with the alignment editor "BioEdit" (www.mbio.ncsu.edu/ BioEdit/bioedit.html). Aligned sequences had approximately 80 base pairs removed from each end to properly align the sequences. The final alignment consisted of 14 sequences used for the phylogenetic analyses.

2.3 ISSR ANALYSIS METHODS

2.3.1 DNA amplification

Cladophora filaments used for DNA analyses were cleaned of debris and epiphytes and were then ground in liquid nitrogen. The Qiagen DNeasy Plant Mini Kit™ (Qiagen, Canada) was used for genomic DNA extractions. PCR amplification of inter-simple sequence repeats were carried out using single primers listed in Table 4. Amplification was performed using an Eppendorf Master Cycler Gradient PCR machine. Initial denaturation occurred at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 44°C for 45 sec, 72°C for 1.5 min, and a final elongation of 72°C for 10 min. 25µL reaction condition were as follows: 2.5µL 10X buffer (100mM), 4µL $MgSO_4$ (25mM), 0.5µM each dATP, dCTP, dGTP, dTTP (10mM), 1µM primer (100 µL), 2 µL Taq polymerase and 2µL DNA template. 20µL of the resulting PCR products (and a O'RangerRuler 100bp ladder) were separated on a 2.0% agarose gel (containing

Table 4. List of ISSR primers used for both analysis of Great Lakes and analysis of additional North American samples and cultures. Amount of banding, percent polymorphic bands, and range of molecular weights. (NA) is North American and (GL) is Great Lakes.

		Total No. Bands		Total No. Polymorphisms		MW[b] size range	
Primer	**Primer Sequence**	**NA**	**GL**	**NA**	**GL**	**NA**	**GL**
ISSR 3	ATTATTATTATTATTGC	-	-	-	-	-	-
ISSR 7	ATTAATTAATTAGCC	-	-	-	-	-	-
ISSR 9	$(GGAAGGCG)_4$	-	-	-	-	-	-
ISSR 13	CTTCTTCTTCTTGGC	-	-	-	-	-	-
***ISSR 10**	GAGAGAGAGACC	385	165	100%	100%	1587-200	1495-196
***ISSR 12**	CACCACCAGCG	388	154	100%	100%	1565-256	1533-195
***ISSR 14**	CTCCTCCTGC	386	163	100%	100%	1500-250	1597-198
***ISSR 15**	GTGGTGGTGGC	385	157	100%	100%	1460-200	1507-161
***ISSR 17**	GAGAGAGAGACC	447	156	100%	100%	1593-256	1599-107

0.0015mg/mL ethidium bromide) and run for 3 hours at 80V in TAE buffer. Visualization of PCR products utilized a UV trans-illuminator, using the Syngene BioImaging System (Synoptics Limited© 2000).

2.3.2 ISSR analysis

To analyze each gel, band sizes were determined by comparing each to a 100bp ladder (Fermentas Canada Inc., Burlington, ON), which served as a molecular weight standard. Due to the large volume of gels run, the analysis required an accurate method of band comparison among each gel for a particular primer. Surprisingly, there is no consensus regarding the best method to score bands comparatively between numerous gels. Hence, the methods used were a combination of those used to score bands of RAPDs (Williams *et al.,* 1990) and microsatellites (Flint *et al.,* 1999).

For each primer, similar bands (those that migrated to the same position) were determined manually by simple visualization and cross-referenced with molecular weights. These bands were obvious for samples collected from each of the Great Lakes, but were difficult to identify when analyzing North American samples due to the large amount of variability in band size over the entire continent. Bands greater than 1550bp and less than 100bp were not scored due to the inconsistency of their appearance in repeated reactions. All bands that had not obviously migrated to the same position in either Great Lakes or North American samples were manually binned based on molecular weights. To achieve this, all bands were ordered by size for each primer (excluding homologous bands that were analyzed beforehand). Manually determined molecular weight ranges were established based on the size of the most common weight groupings. Bands were then placed into each size category based on whether they lay within the pre-determined

thresholds. 94% of the bands for all five primers fell into each of the ranges, with the resultant 6% having more than 1 band within the size threshold. Due to the small percentage of double banding in single thresholds these bands were omitted from the analysis. Following manual binning, molecular weight thresholds were averaged, and that value was given to each bin. A presence (1) or absence (0) binary matrix was constructed from the pooled banding patterns of all primers. Subsequently, a distance matrix was constructed from band presence/absence data using Dice coefficient (Dice, 1945) for pairwise comparisons to produce a similarity matrix. Dice coefficient is as follows:2a / (2a + b + c), where 'a' represents the presence of a given band in both individuals, 'b' represents the presence of the band in the first individual, but not in the second, and 'c' represents the absence of the band in the first individual and the presence in the second. The Dice coefficient employs the 1-1, 1-0 and 0-1 band relationships in similarity calculation, but not the 0-0 relationship. Dice's coefficient has proven to be favorable in genetic studies because it accounts for the possibility that similar bands are not the same (Harris, 1999). The resultant similarity values were used to construct a distance matrix and then subject to Unweighted Pair Group Method with Arithmetic mean (UPGMA) cluster analysis. All dendograms were constructed using PAUP* v.4.0b (Swofford *et al.,* 2001) and trees were visualized using TREEVIEW (Page, 1996).

2.3.3 Multidimensional scaling

The ISSR fingerprint produced for North American *Cladophora* samples were analyzed with the software package SPSS release 9.0 (SPSS, Chicago, IL, USA), to compute Euclidean genetic distances for multidimensional scaling analysis (MDS). Euclidean distances were computed as: $(x,y)=(\sum_I(x_i-y_i)^2)^{1\backslash 2}$ (Al-Barrak *et al.,* 2004), which

is the genomic distance in multidimensional space. MDS was used as an exploratory tool verifying that the interpretation of the data did not depend on the type of analysis used.

2.3.4 Analysis of Molecular Variance (AMOVA)

AMOVA analysis (Excoffier *et al.,* 1992) was executed to analyze genetic distance among populations using the Arlequin v2.0 software package (Schneider *et al.,* 2000). It was used to calculate components of molecular variance at two hierarchical levels: among populations and within populations. Estimation of pairwise F_{ST} values (Flint *et al.,* 1999) was also used to evaluate the genetic structure of the data using Arlequin v2.0 software package (Schneider, 2000). To investigate the genetic structure of the data, each of the Great Lakes were considered as separate populations due to the highly resolved nature of the populations in previously established UPGMA cluster analysis and all additional North American samples were grouped into one population based on the partitioning of samples to a separate group from the North American samples, and due to the lack of resolution of the UPGMA analysis.

2.4 MORPHOLOGY STUDY METHODS

2.4.1 Morphological measurements

Specimens were examined for the following character in replicates of ten: length and diameter of main axis, ultimate branch, and apical cells. To examine morphology, single filaments were spread onto a slide and measurements were performed using an Olympus BX51 light microscope (Olympus America Inc., Ceter Valley, PA, USA) and images were photographed with Spot Insight digital camera (model 3.2.0, Diagnostic

Instruments, Inc, Sterling Heights, MI, USA) and were analyzed using Image-ProExpress software package (Media Cybernetics, Inc., Silver Spring, MD, USA).

For the seasonality study, three individuals from three different locations of sample area (for a total of nine samples) were chosen at random and measured. For morphological analysis among the Great Lakes, ten individuals from each population were measured. Implementing a specific set of criteria for each character ensured consistency in morphological measurements. For example, to remove biases in choosing which cell to measure in the main axis, the cell located two below the first branching point was measured. With respect to the ultimate branch cell, the second cell up from the first branching point was measured. No specific parameters were needed in choosing apical cell for measurement.

2.4.2 Morphological analysis of seasonality study

All statistical analysis was conducted using SYSTAT© Software. Analysis of variance (ANOVA) was conducted to assess significance of variability means between each of the morphological characters over sampling periods. In addition, a cluster tree was created using the hierarchical clustering option, with average linkage and Euclidean distance based on cell measurement means to identify any observable trends in morphology throughout the six-month sampling period. A T-test was conducted to assess the significance of the groupings projected through cluster analysis.

2.4.3 Morphological analysis of Great Lakes study

To examine the taxonomic utility of morphological characters from each population within each of the Great Lakes were then subjected to cluster analysis. The cluster trees were created using the hierarchical clustering option, with average linkage and Euclidean

distance to examine whether populations from each of the Great Lakes will group together based on morphological characters and thus could be used to distinguish *Cladophora* sampled from each lake.

CHAPTER 3.0: RESULTS

3.1 ITS ANALYSIS RESULTS

Alignment of the ITS1 region is presented in Figure 7. Inspection of the alignment showed considerable conservation of this region within North American freshwater *Cladophora* and also the cultures obtained from UTEX. Analysis of sequences showed that 10 sequences were 100% identical; the resultant similarity matrix revealed that all sequences were 98% similar (Table 5). The sequences that showed any divergence [Texas 7, Local Area Waterloo Pond, *Cl. glomerata* var. *crassior* (UTEX 1488), and *Cl. fracta* var. *intricata* (UTEX 1486)], in the sequence were still 98% identical. In total each of the 4 variable sequences had 2 transversions and 2 nucleotide insertions, with the Local Waterloo Area Pond sample as the exception as it had 3 transversions and 3 nucleotide insertions. Based on this considerable similarity, no phylogenetic analyses were carried out on these sequences, as it would have resulted in a large, uninformative polytomy in the resultant cladogram.

3.2 ISSR ANALYSIS RESULTS

3.2.1 Overall banding analysis

Table 4 contains all ISSR primers screened for ISSR analysis. A total of nine primers of various repeat motifs were initially screened on five randomly chosen North American *Cladophora* samples. Two primers (ISSR 8 and ISSR 13) were unable to produce any banding. A combination of stuttering and too few bands prevented primers

Figure 7. Internal transcribed spacer (ITS-1) sequence alignment of freshwater *Cladophora*, produced in BioEdit©. A dot indicates an identical nucleotide.

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   5         15         25         35         45         55
Arizona 200     ACCGACCCTC CTTGGCTAGG GCTGGCCGTC CCAGCGGCGC GCCAGACCCA GGCCTCACCG
Arizona 203     .......... .......... .......... .......... .......... ..........
Guelph Lake     .......... .......... .......... .......... .......... ..........
Hawaii          .......... .......... .......... .......... .......... ..........
Waterloo Pond   .......... .......... .......... .......... .......... ..........
Miami River     .......... .......... .......... .......... .......... ..........
Mexico 18       .......... .......... .......... .......... .......... ..........
Puerto Rico     .......... .......... .......... .......... .......... ..........
LO East Bay     .......... .......... .......... .......... .......... ..........
Texas 7         .......... .......... .......... .......... .......... ..........
UTEX 473        .......... .......... .......... .......... .......... ..........
UTEX 1488       .......... .......... .......... .......... .......... ..........
UTEX 1486       ..A..A.... .......GA. ..AG.C..T. ..T....... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   65         75         85         95        105        115
Arizona 200     CCACGGTACC GTGGTGCCCT GCACCCCCGG GAGAACGTTG TCCCACACGG GGCGCGCAGG
Arizona 203     .......... .......... .......... .......... .......... ..........
Guelph Lake     .......... .......... .......... .......... .......... ..........
Hawaii          .......... .......... .......... .......... .......... ..........
Waterloo Pond   .......... .......... .......... .......... .......... ..........
Miami River     .......... .......... .......... .......... .......... ..........
Mexico 18       .......... .......... .......... .......... .......... ..........
Puerto Rico     .......... .......... .......... .......... .......... ..........
LO East Bay     .......... .......... .......... .......... .......... ..........
Texas 7         .......... .......... .......... .......... .......... ..........
UTEX 473        .......... .......... .......... .......... .......... ..........
UTEX 1488       .......... .......... .......... .......... .......... ..........
UTEX 1486       .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  185        195        205        215        225        235
Arizona 200     ACCCAAGCAC CCGGTACGGG CTTACGGCTG GACGGGCACA CCCAAGCGGG TGGCTCGGCC
Arizona 203     .......... .......... .......... .......... .......... ..........
Guelph Lake     .......... .......... .......... .......... .......... ..........
Hawaii          .......... .......... .......... .......... .......... ..........
Waterloo Pond   .......... ....C..... .......... .......... .......... ..........
Miami River     .......... .......... .......... .......... .......... ..........
Mexico 18       .......... .......... .......... .......... .......... ..........
Puerto Rico     .......... .......... .......... .......... .......... ..........
LO East Bay     .......... .......... .......... .......... .......... ..........
Texas 7         .......... ....C..... .......... .......... .......... ..........
UTEX 473        .......... ....C..... .......... .......... .......... ..........
UTEX 1488       .......... ....C..... .......... .......... .......... ..........
UTEX 1486       .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  245        255        265        275        285        295
Arizona 200     GTGCAGCCGG AAGCTGGGGC CTCCGACCAA GCAGCCATTC GGCGGTGGTC CATTCTCACG
Arizona 203     .......... .......... .......... .......... .......... ..........
Guelph Lake     .......... .......... .......... .......... .......... ..........
Hawaii          .......... .......... .......... .......... .......... ..........
Waterloo Pond   .......... .......... .........C ..C....... .......... ..........
Miami River     .......... .......... .......... .......... .......... ..........
Mexico 18       .......... .......... .......... .......... .......... ..........
Puerto Rico     .......... .......... .......... .......... .......... ..........
LO East Bay     .......... .......... .......... .......... .......... ..........
Texas 7         .......... .......... .......... .......... .......... ..........
UTEX 473        .......... .......... .......... .......... .......... ..........
UTEX 1488       .......... .......... .......... .......... .......... ..........
```

```
UTEX 1486     .......... .......... .......... .......... .......... .........
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                305        315        325        335        345        355
Arizona 200   AGTGGCCACC AACGGGTGGG TGGAGCCCCG CCGCCGCTAT AATTCACAAC AATCATCCTC
Arizona 203   .......... .......... .......... .......... .......... ..........
Guelph Lake   .......... .......... .......... .......... .......... ..........
Hawaii        .......... .......... .......... .......... .......... ..........
Waterloo Pond .A........ .......... .......... .......... .......... ..........
Miami River   .......... .......... .......... .......... .......... ..........
Mexico 18     .......... .......... .......... .......... .......... ..........
Puerto Rico   .......... .......... .......... .......... .......... ..........
LO East Bay   .......... .......... .......... .......... .......... ..........
Texas 7       .......... .......... .......... .......... .......... ..........
UTEX 473      .......... .......... .......... .......... .......... ..........
UTEX 1488     .......... .......... .......... .......... .......... ..........
UTEX 1486     .......... .......... .......... .......... .......... ..........


              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                365        375        385        395        405        415
Arizona 200   AGAATCAACC TGTGTGTGTG CCTTGAGCGT CTAGCAGCCA AGCAAGCTAA CTGAAAGTAA
Arizona 203   .......... .......... .......... .......... .......... ..........
Guelph Lake   .......... .......... .......... .......... .......... ..........
Hawaii        .......... .......... .......... .......... .......... ..........
Waterloo Pond .......... .......... .......... .....A.... .......... ..........
Miami River   .......... .......... .......... .......... .......... ..........
Mexico 18     .......... .......... .......... .......... .......... ..........
Puerto Rico   .......... .......... .......... .......... .......... ..........
LO East Bay   .......... .......... .......... .......... .......... ..........
Texas 7       .......... .......... .......... .......... .......... ..........
UTEX 473      .......... .......... .......... .......... .......... ..........
UTEX 1488     .......... .......... .......... .......... .......... ..........
UTEX 1486     .......... .......... .......... .......... .......... ..........


              ....|....| ....|....| ....|....| ....|....|
                425        435        445        455
Arizona 200   CTCGTACAGC CTTCTATCCA GTCGCCAACC TCGATGCTCT
Arizona 203   .......... .......... .......... ..........
Guelph Lake   .......... .......... .......... ..........
Hawaii        .......... .......... .......... ..........
Waterloo Pond .......... .......... .......... ..........
Miami River   .......... .......... .......... ..........
Mexico 18     .......... .......... .......... ..........
Puerto Rico   .......... .......... .......... ..........
LO East Bay   .......... .......... .......... ..........
Texas 7       .......... .......... .......... ..........
UTEX 473      .......... .......... .......... .......
UTEX 1488     .......... .......... .......... .......
UTEX 1486     .......... .......... .......... .......
```

Table 5. Pairwise similarity index of freshwater *Cladophora* samples for the internal transcribed spacer-1 sequence alignments. (1) *Cl. glomerata* var. *glomerata*, (2) *Cl. glomerata* var. *crassior,* (3) *Cl. fracta* var. *fracta*, (4) Arizona 200 (5) Arizona 203, (6) Guelph Lake, (7) Hawaii, (8) Local Area Pond, (9) Miami River, (10) Mexico 18, (11) Puerto Rico, (12) East Lake Ont. Bay, (13) Texas 7.

.

Cladophora Samples	1	2	3	4	5	6	7	8	9	10	11	12
1												
2	1											
3	1	1										
4	1	1	1									
5	0.98	0.98	0.98	0.98								
6	1	1	1	1	0.98							
7	1	1	1	1	0.98	1						
8	1	1	1	1	0.98	1	1					
9	1	1	1	1	0.98	1	1	1				
10	0.997	0.99	0.99	0.99	0.98	0.99	0.99	0.99	0.99			
11	1	1	1	1	0.98	1	1	1	1	0.99		
12	0.997	0.99	0.99	0.99	0.98	0.99	0.99	0.99	0.99	1	0.99	
13	0.997	0.99	0.99	0.99	0.98	0.99	0.99	0.99	0.99	1	0.99	0.97

amplification (Dow *et al.*, 1995). Finally two primers (ISSR16, ISSR3) were unable to successfully amplify fragments for all five screening samples. A total of five primers appeared to exhibit suitable band variation for analysis. Initially, over 170 North American *Cladophora* extracts (including Great Lake samples) were available for study. Following amplification with all five primers, a number of study samples were removed from the analysis, as they were unable to reliably amplify (very faint banding, or lack of reproducible results). The lack of amplification with certain primers may have been a result of low-quality DNA template. Al-Barrak *et al.,* (2004) found that high-grade DNA extracts were essential to obtain well-resolved (and scorable), fragments. Many of the North American samples (excluding samples collected from the Great Lakes), were extracted over a time period of six years, resulting in potential DNA degradation. Furthermore, the method of DNA extraction has also been stated as a hindrance of PCR amplification. It has been reported that residual phenol from phenol/chloroform DNA extraction methods can inhibit PCR analysis (Wolfe, 1995). A number of North American samples were extracted by an unknown method. It is presumed (personal communication with Dr. Müller), that samples that were unable to produce banding for each of the primers may have been extracted using phenol/chloroform.

Table 4 summarizes banding results for all primer amplifications. All primers chosen for the analysis were either di- or tri- nucleotide repeats anchored at the 3' end. Each primer produced varying numbers of DNA fragments depending on their SSR motif. All primers produced similar numbers of bands (~20) among the 53 individuals. Amplified DNA fragments varied in size from approximately 200bp to over 1550bp. The percent polymorphic bands are accounted for by dividing the number of samples with that

particular band by the total number of samples. Therefore to be considered 100% polymorphic would suggest that only one sample has that particular band. When all ISSR primers were considered together the rate of polymorphism was 100%. It should noted that of the 97 bands, 5 had polymorphic rate greater than 90% and 9 had polymorphic rates less than 10% for the 53 *Cladophora* samples suggesting those bands may not be as informative for species/variety discrimination.

3.3.2 Great Lakes ISSR banding analysis

In considering only samples from the Great Lakes, a total of 68 distinct bands among the 21 individuals were produced (Table 4). When all ISSR primers were considered together the rate of polymorphism was again 100%. Of the 68 total bands, four bands had polymorphic rates greater than 90% and four bands had polymorphic rates less than 10% for the 21 *Cladophora* samples. This is more than was observed when considering all samples together, suggesting a greater internal consistency of bands and thus less differentiation among samples within the Great Lakes. This was also suggested through simple visualization of the banding pattern. For example, primer ISSR10 produced very similar banding patterns for all individuals collected from Lake Ontario

3.2.3 Repeatability of ISSR markers

Not all primers produced the same reproducibility. Primers ISSR14 and ISSR15 had the lowest percent of reproducible bands; 77% and 81%, respectively. The remaining three primers had a repeatability percentage greater than 88%. To ascertain the

reproducibility of resultant ISSR banding pattern within and between gels, a number of precautions were followed. Due to the large volume of samples initially run in addition to the lack of additional DNA extracts, not all samples could be run in duplicates. Instead five randomly chosen samples were run in triplicate with each of the five primers. In addition, each of the samples were run at different times, and results were cross checked to ensure repeatability. In future studies the following precautions should be taken to increase repeatability of reactions: duplicates of each sample should be run in each reaction to confirm internal consistency of PCR reactions. Separate PCR runs should be performed with samples that demonstrated increased amounts of banding, or lower resolution, thus confirming external PCR condition repeatability. Most importantly a positive control with a defined highly resolved banding pattern should be run in every sample is assess consistency and accuracy in band size estimation.

An additional concern is the universal nature of the ISSR primers used to analyze North American *Cladophora*. As all eukaryotes appear to possess microsatellite regions throughout the nuclear genome SSR-primers could amplify DNA from any eukaryote, given they have that specific repeat. This raises the possibility of contamination due to the considerable epiphytic coverage on *Cladophora* filaments, which may insinuate false differences between samples. To test this, a sample from Lake Erie that had a large volume of epiphytic coverage, was run along side a clean sample from Lake Erie. Both were PCR amplified with the primer, both in the same reaction and in two separate reactions. Resultant banding patterns was nearly identical (2 very faint bands were amplified in the "clean" *Cladophora* amplification). This indicated that the amount of DNA extracted from

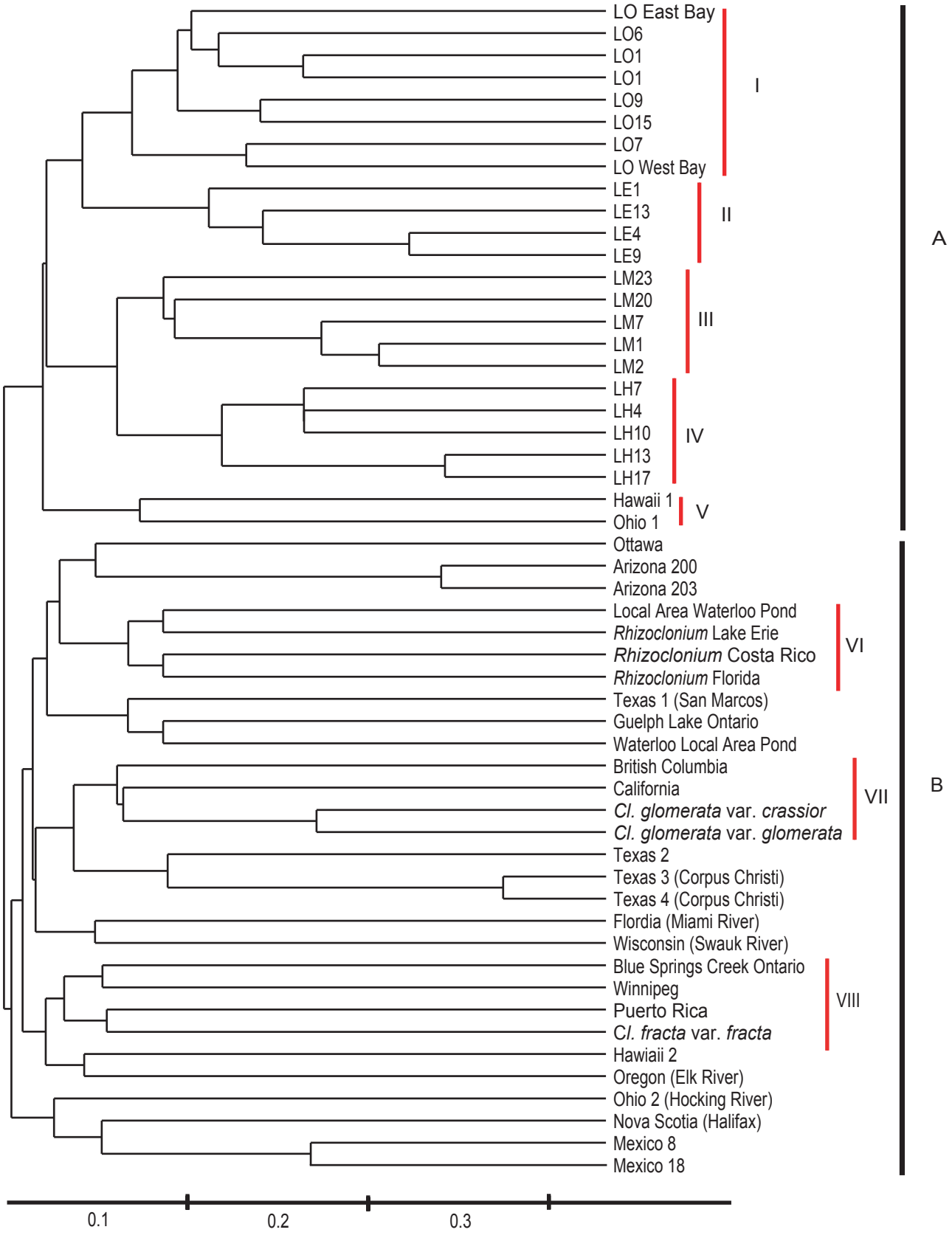
the *Cladophora* filament competitively out-amplified that of any DNA that may have been present as a result of epiphytic coverage.

3.2.4 UPGMA analysis

The binary presence/absence matrix based on all 53 samples and 97 total bands, was evaluated by calculating Dice similarity coefficient. Dice coefficient is a pair-wise comparison based on the proportion of shared bands produced by each of the primers collectively. The subsequent distance matrix was subjected to UPGMA cluster analysis that generated two distinct groups (Figure 8). The uppermost group (A) contained all samples collected from each of the Great Lakes. Within this group were five clusters with four of them representing each of the Great Lakes (I, II, III, IV; Figure 8). The fifth cluster (V) included two samples, Hawaii and Ohio.

The second group (B) contained the remainder of the North American samples, including local creeks and ponds as well as European representative-type cultures. Although group (B) was much less resolved, some trends were visible. For example, cluster (VI) contained all freshwater *Rhizoclonium* samples as well as an additional Minnesota sample and grouped with samples from Arizona and the Ottawa (Ottawa River). Cluster (VII) contained European representative type specimens, *Cl. glomerata* var. *glomerata* and *Cl. glomerata* var. *crassior* along with samples from British Columbia and California. The European representative-type culture specimen of *Cl. fracta* var. *fracta* was in a different cluster (VIII), and grouped with a geographically disperse set of samples including Puerto Rico, Winnipeg, and an Ontario Creek. A number of samples collected from similar locations also grouped together. Specifically, samples from Mexico, Texas, and Arizona.

Figure 8. UPGMA cluster tree of North American (including the Great Lakes) and European freshwater *Cladophora* based on ISSR analysis.


LO East Bay
LO6
LO1
LO1
LO9
LO15
LO7
LO West Bay
LE1
LE13
LE4
LE9
LM23
LM20
LM7
LM1
LM2
LH7
LH4
LH10
LH13
LH17
Hawaii 1
Ohio 1
Ottawa
Arizona 200
Arizona 203
Local Area Waterloo Pond
Rhizoclonium Lake Erie
Rhizoclonium Costa Rico
Rhizoclonium Florida
Texas 1 (San Marcos)
Guelph Lake Ontario
Waterloo Local Area Pond
British Columbia
California
Cl. glomerata var. crassior
Cl. glomerata var. glomerata
Texas 2
Texas 3 (Corpus Christi)
Texas 4 (Corpus Christi)
Flordia (Miami River)
Wisconsin (Swauk River)
Blue Springs Creek Ontario
Winnipeg
Puerto Rica
Cl. fracta var. fracta
Hawiaii 2
Oregon (Elk River)
Ohio 2 (Hocking River)
Nova Scotia (Halifax)
Mexico 8
Mexico 18
I
II
III
IV
V
VI
VII
VIII
A
B
0.1
0.2
0.3

3.2.5 Analysis of molecular variance (AMOVA)

The genetic structure of freshwater *Cladophora* was assessed through hierarchical analysis of molecular variance (AMOVA) (Excoffier *et al.,* 1992) implemented in the program Arlequin ver. 2000 (Schneider *et al.,* 2000). AMOVA analysis permitted the investigation of differentiation among the sampling localities of each of the Great Lakes and North American locations. F_{ST} values represent the proportion of the total genetic variance contained in a subpopulation (the 'S' subscript) relative to the total genetic variance (the 'T' subscript) and are summarized in Table 6. Values can range from 0 to 1, with a high F_{ST} implying a considerable degree of differentiation among or within populations (Flint *et al.,* 1999). Partitioning of genetic variability by AMOVA revealed that most of the ISSR diversity was distributed within each of the populations (78%) with the residual diversity a result of among population variation (21%). This indicated that there was not a great deal of differentiation among the populations, but high differentiation within each group when compared to the amount of variation that separated each population. Comparisons of pairwise F_{ST} indices among each of the Great Lakes indicated that there was moderate differentiation between any of the Lakes. The highest levels of differentiation among lakes were found between Lake Erie and Lake Michigan (F_{ST} = 0.43, P<0.05*)*, Lake Erie and Lake Huron (F_{ST} =0.42, P<0.05),

Table 6. F_{ST} indicies suggesting population structure within populations and among populations established through hierarchical analysis of molecular variance (AMOVA).

Among Population Variation					
	Lake Ontario F_{ST}	**Lake Erie F_{ST}**	**Lake Michigan F_{ST}**	**Lake Huron F_{ST}**	**North American Population F_{ST}**
Lake Ontario	-	-	-	-	-
Lake Erie	0.32	-	-	-	-
Lake Michigan	0.39	0.44	-	-	-
Lake Huron	0.39	0.42	0.33	-	-
North America Populations	0.18	0.17	0.15	0.19	-

Lake Ontario and Lake Michigan (F_{ST} = 0.39, P<0.05) and Lake Ontario and Lake Huron (F_{ST} = 0.39, P<0.05). Lake Ontario and Lake Erie had the lowest amount of genetic variation (F_{ST} = 0.31, P<0.05), followed by Lake Huron and Lake Michigan (F_{ST} = 0.32, P<0.05). Considering each of the Great Lakes against remaining North American populations the overall lowest genetic variation was resolved (F_{ST} > 0.18). Over all loci the average F_{ST} = 0.21, indicating low genetic variation among all individuals.

3.3 MORPHOLOGICAL STUDY RESULTS

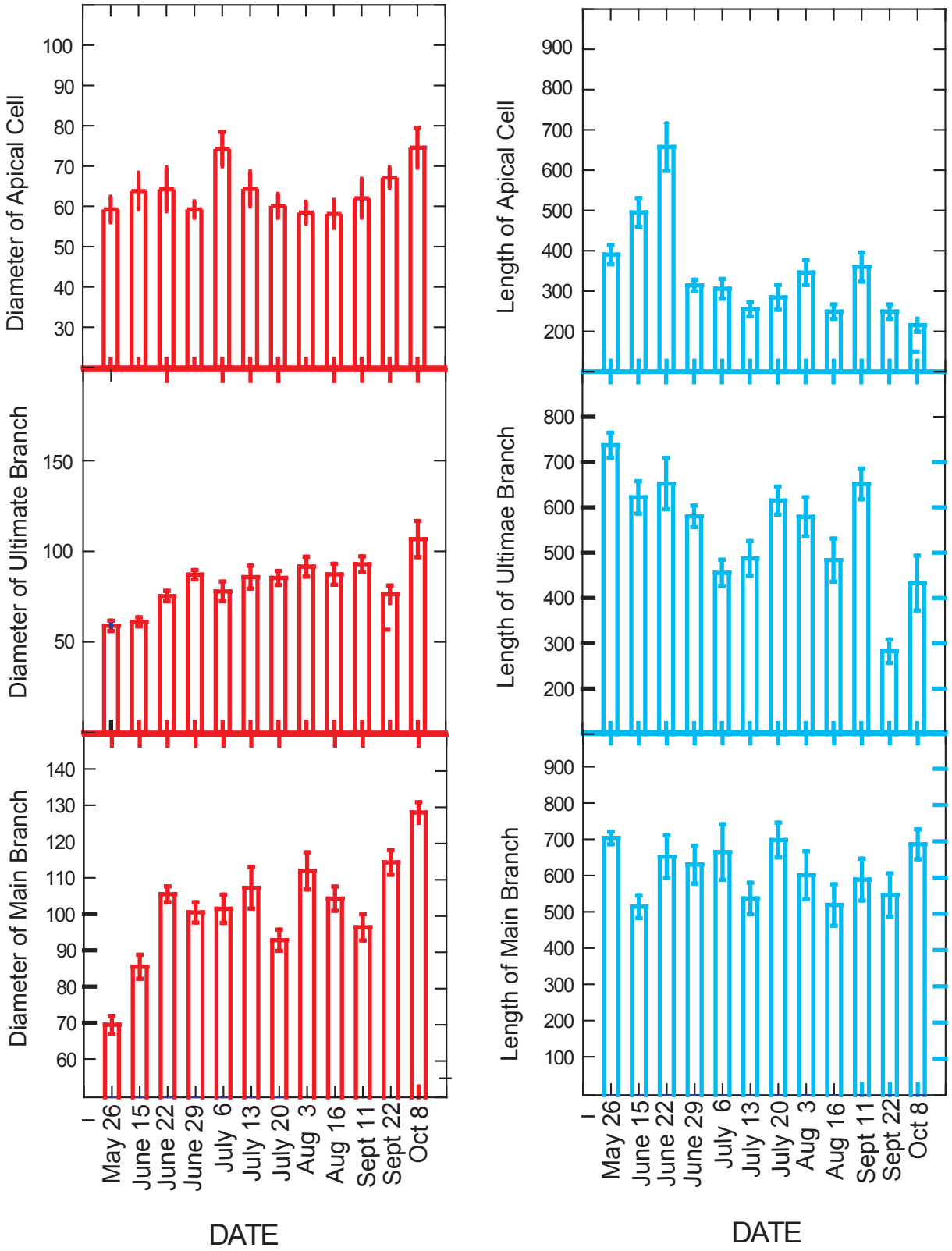
3.3.1 Seasonal morphological analysis

All morphological characteristics studied (the length and diameter of main axis, ultimate branch, and apical cells) over a period ranging from early May to late October displayed a great deal of variation, suggesting their inability to be used alone as valid taxonomical criterion. *Cladophora* collected on June 22, 2004 showed the largest cell size variation, most notably in the lengths of the branch and main axis (Table 7). Overall cell lengths tended to be the most variable throughout the season, with mean lengths of the apical and branch cell ranging between 250-650 µm and 300-750µm, respectively. Analysis of variance (ANOVA) among collection dates indicated that mean cell length and mean cell diameters were almost all significantly different. Figure 9 graphically displays ANOVA mean cell lengths and diameters throughout the season. Results indicate that the diameter of the apical cell was the only morphological character that did not show huge variability over the sampling season, but still not stable enough to be used

Table 7. Summary of mean, minimum, and maximum cell measurements with associated standard deviations for each of morphological characters studied (length and diameter of main axis, ultimate branch, and apical cells). Measurements for both the seasonality study as well as morphological analysis of the Great Lakes are included in the table.

Seasonality Study						
	Diameter of Main Axis Cell (µm)	**Length of Main Axis (µm)**	**Diameter of Branch Cell (µm)**	**Length of Branch Cell (µm)**	**Diameter of Apical Cell (µm)**	**Length of Apical Cell (µm)**
Mean	101.93	617.09	82.19	548.32	63.77	343.27
Maximum	143.00	987.00	154.00	880.00	105.00	987.00
Minimum	56.00	59.00	46.00	167.00	25.00	148
Standard Deviations	17.56	165.50	19.50	163.74	12.43	146.90
Great Lakes Morphology						
	Diameter of Main Axis Cell (µm)	**Length of Main Axis (µm)**	**Diameter of Branch Cell (µm)**	**Length of Branch Cell (µm)**	**Diameter of Apical Cell (µm)**	**Length of Apical Cell (µm)**
Mean	70.00	709.22	58.88	737.11	59.22	390.55
Maximum	79	825.00	73.00	836.00	76.00	526.00
Minimum	56	647	46	595	46	71
Standard Deviations	7.34	51.93	8.50	82.54	9.48	71.82

Figure 9. Histograms produced in SYSTAT © of seasonal sampling morphological averages from the Great Lakes.


Diameter of Apical Cell
Diameter of Ultimate Branch
Diameter of Main Branch
Length of Apical Cell
Length of Ultimae Branch
Length of Main Branch
May 26
June 15
June 22
June 29
July 6
July 13
July 20
Aug 3
Aug 16
Sept 11
Sept 22
Oct 8
DATE
DATE

As a discriminating character *(P < 0.08)*. No apparent growth trends observed over the course of the season in any of the morphological characters.

3.3.2 Cluster analysis

The cluster tree showed some observable trends in the grouping of sampling dates (Figure 10). Most notable is that morphological characters attained June 22, 2004 were the most variable was separate and did not group with any other data. Trends are also apparent in group (I) appear to contain samples from mid-growing season, excluding May 26 sampling date. Group (II) contains is composed of samples from collected late-growing season, excluding July 6 sampling date. Results of the T-test did not indicate that there was a significant difference between the mid and late sampling groups *(P<0.08).* Therefore, though some sampling dates appear to cluster together, a combination of T-test and ANOVA analysis confirmed that certain cell morphological characters were too variable to use as a single taxonomic criterion.

3.3.3 Great Lakes morphology analysis

All morphological measurements varied greatly within and among the Great Lakes. Cell lengths were the most variable with ranges as follows: main branch (267-877 μm), the ultimate branch (215-654 μm), and apical cell (204-675 μm) (Table 7).

3.3.4 Cluster analysis

Hierarchical clustering based on the mean morphological measurements of each population did not resolve all lakes into separate groups (Figure 11). Most notably cluster (I) contained samples from each of the Great Lakes. One significant group was observed (II) as it contained three of the five Lake Huron populations. In addition LM1

Figure 10. SYSTAT © cluster tree created using hierarchical clustering of the average morphological characters (length and diameter of main axis, ultimate branch, and apical cells) for 2005 seasonality study.

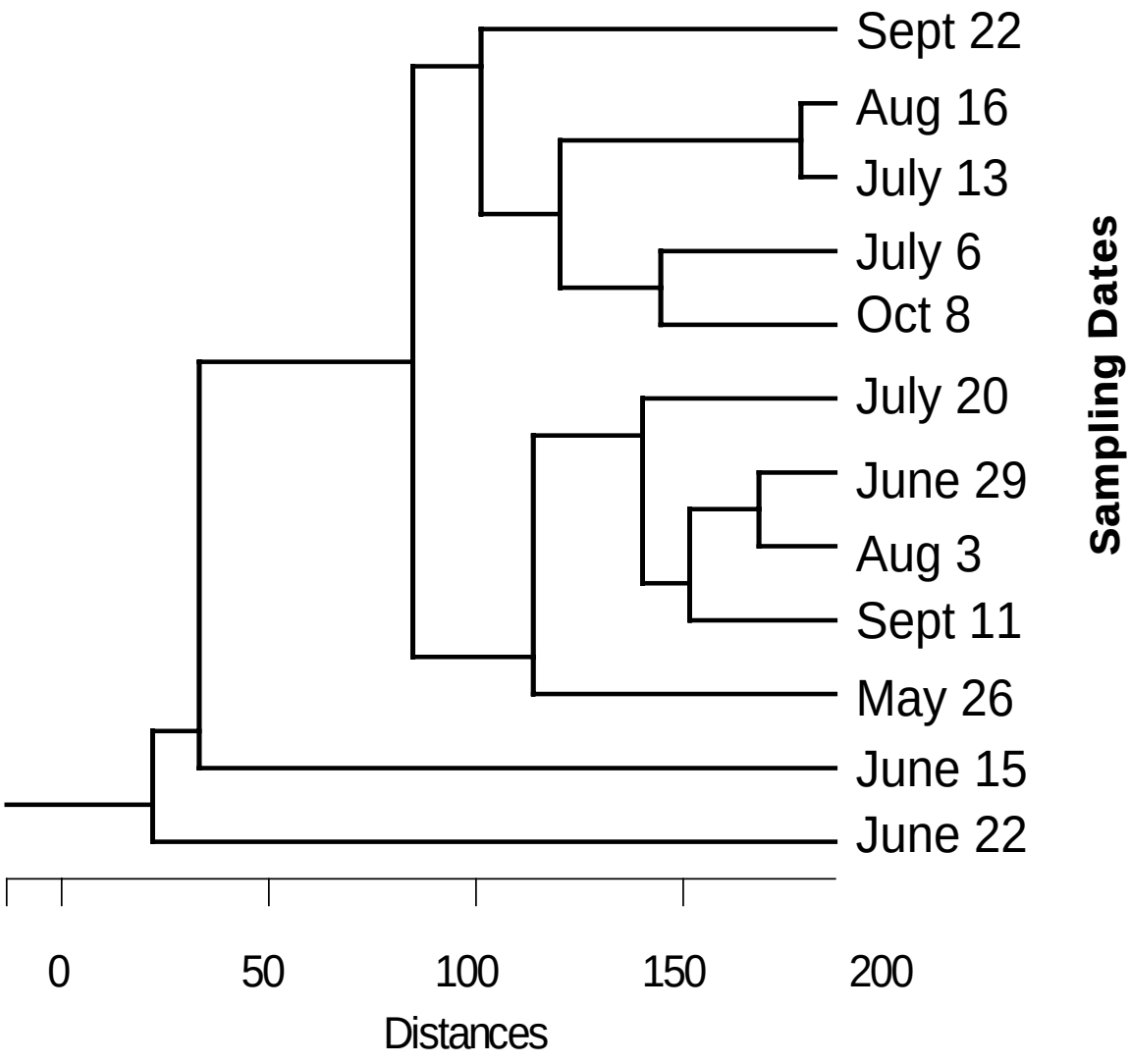
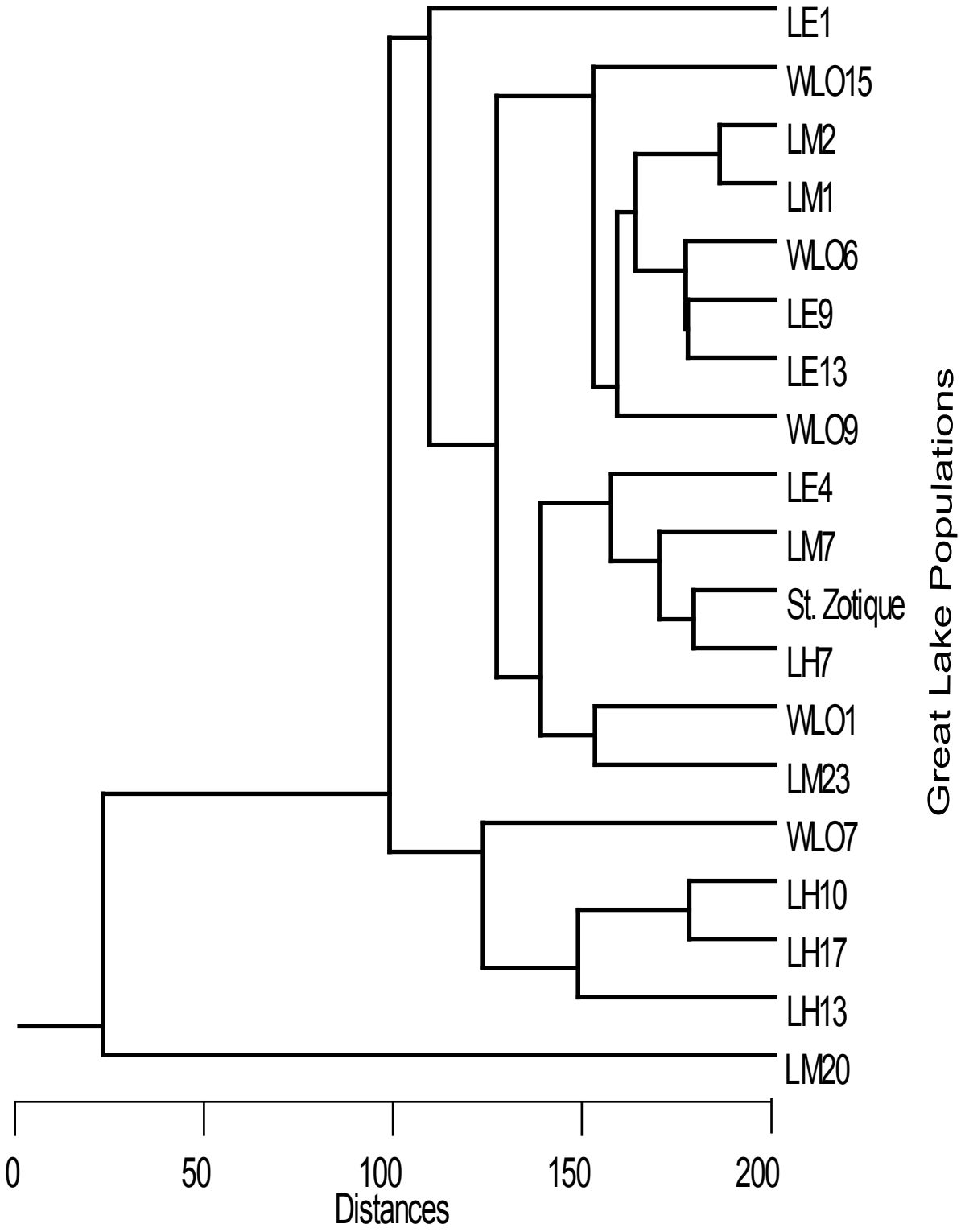

Sept 22
Aug 16
July 13
July 6
Oct 8
July 20
June 29
Aug 3
Sept 11
May 26
June 15
June 22
Sampling Dates
0
50
100
150
200
Distances

Figure 11. SYSTAT© cluster tree created using hierarchical clustering of the average morphological characteristics length and width of main axis, ultimate branch, and apical cells) for morphological analysis of the Great Lakes.


LE1
WLO15
LM2
LM1
WLO6
LE9
LE13
WLO9
LE4
LM7
St. Zotique
LH7
WLO1
LM23
WLO7
LH10
LH17
LH13
LM20
0
50
100
150
200
Distances
Great Lake Populations

and LM2 formed a small group. However the same clustering tree was run with all individuals from each population and Lake Huron individuals were spread throughout the tree (analysis not shown). Cluster analysis was not congruent with the UPGMA tree derived from ISSR analysis (refer back to Figure 8).

4.0 DISCUSSION

4.1 ITS ANALYSIS DISCUSSION

Amplification of the ITS1 region of North American and European freshwater *Cladophora* suggested that there was at least 98% percent similarity among sequences (Table 5). To our knowledge only one other study has utilized the ITS region to study the genetic diversity of freshwater *Cladophora*. This study was based on European freshwater samples, but three North American samples (including one from Lake Huron) were included (Marks and Cummings, 1996). Our results were concurrent with those by Marks and Cummings (1996), who observed sequences to be at least 95% similar indicating the ITS region was highly conserved. Both studies compared field collected *Cladophora* to cultured samples of *Cl. glomerata* var. *glomerata*, *Cl. glomerata* var. *crassior*, *Cl. fracta* var. *fracta*, *Cl. fracta* var. *intricata* (*Cl. fracta* var. *fracta* was not included in the current study as the culture was not longer available). As all cultures were collected and identified by C. van den Hoek, they could be considered representatives of each species. Even when considering the high similarity among samples, Marks and Cummings (1996) determined that each of the cultured samples did display distinct similarities in the ITS sequences, and thus distinct genotypes. This was not observed in the current study, as the sequences of *Cl. glomerata* var. *glomerata* and *Cl. fracta* var. *fracta* were 98% similar (Table 5) and no other sequences displayed nucleotide changes at the positions that separated the two sequences (Figure 7). Interestingly, both studies were conducted on the same strain of cultures, thus theoretically should have produced identical sequences. However,

considerable differences were noted between sequences from Marks and Cummings (1996) study and the present study. In fact, when sequences of the present study were aligned within the published sequences by Marks and Cummings (1996) the similarity between samples was as low as 81%. The majority of the differences appeared at the 3'end of the ITS1 region. Although all sequences in the present study were identical among the first 26bp, the 1996 samples did not correspond at all to our sequences. This raises the possibility that these differences may have been a result of sequencing methods used in 1996 and 2006. However, BLAST searches of our sequences had high e-values with *Cladophora* (though marine) on GenBank. Hence, the sequences from the Marks and Cummings (1996) paper were not included in this study. Regardless of slight variations in nucleotide sequences, both studies revealed very high levels of conservation in the ITS region.

The high conservation of the ITS region across such a vast geographic area may suggest that only one species of freshwater *Cladophora* exists, with local populations representing ecologically and morphologically distinct forms and/or varieties. Single, cosmopolitan species have also been suggested for *Batrachospermum gelatinosum*, a freshwater rhodophyte (Vis and Sheath, 1997), which has been reported across North and South America (Sheath and Cole, 1992). A study of genetic variation among 15 samples collected from across North America revealed between one to three percent variation. This suggested *B. gelatinosum* consisted of a single phenoplastic species. Similarly, *Gonium pectorale* another globally distributed freshwater species from the colonial green flagellate family Volvcaceae (Coleman and Goff, 1994), displayed sequence divergence of three to

five percent among samples collected from across North America, Europe, Japan, and India.

To assume all freshwater *Cladophora* species of Europe and North America are one species would result in the synonymy of each of the varieties within the two species (*Cl. glomerata* var. *glomerata, Cl. glomerata* var. *crassior, Cl. fracta* var. *fracta, Cl. fracta* var. *intricta*) in the section Glomeratae, to one species. This situation has occurred in *Laminaria* (Yotsukura et al., 1999), which like *Cladophora* often displays high degrees of phenotypic plasticity making species identification very difficult. In a study conducted along the coast of Japan, two phylogenetically distinct groups of non-digitate *Laminaria* species groups have been reported: a *L. japonica* group containing six sub-species and a *L. saccarina* group, containing three subspecies. The ITS regions were successful at resolving phylogenetic relationships among the Laminarialean genera, but at the infraspecific level, the ITS regions were 98% similar among all subspecies of both species. Results suggested that only two species of non-digitate *Laminaria* existed along the Japan coast and that recognition of separate subspecies of L. *japonica* and *L. saccarina* should be discontinued and recognized as only *L. japonica* and *L. saccarina*.

An alternative hypothesis, and a more probable one to assuming all freshwater *Cladophora* belongs to a single species based only on the ITS region is that the regions have not evolved at a rate sufficient to resolve the species history. To date there is no ITS molecular clock for freshwater algae, but Bakker *et al.* (1995) estimated that the ITS divergence rate for marine *Cladophora* to be 0.8-2% per Ma. Extrapolating that value to the ITS region of North American freshwater *Cladophora*, and considering the last glaciation event in North America was between 10,000 to 15,000 years ago (Tyler, 1996),

it is likely that the ITS region of freshwater *Cladophora* has not had sufficient time to demonstrate enough divergence to reveal species delineation, evolutionary relationships or biogeographical patterns. Although the ITS region has been successful in determining such relationships in marine *Cladophora* species such as *Cl. vagabunda*, it may be a consequence of the age of the species which has been reported as over twelve million years old (Kooistra *et al.,* 1992).

Low sequence divergence has also been described between marine species of *Enteromorpha*. Amplification of the ITS regions were conducted to determine if molecular information correlated with the morphological separation (Blomster *et al.,* 1999). North American sequences retrieved from Genbank were used in addition to samples attained from across Spain and Ireland for the analysis. Divergence of ITS1 and ITS2 was extremely low (2-4%) among *E. muscoides* and *E. clathrata* suggesting that potentially only one species should be recognized. Even as a single species, the low sequence divergence was surprising given the large geographic area from which samples were collected. Additional studies of *Enteromorpha* species have also revealed low divergence levels. For example, divergence in distantly located Baltic populations of *E. intestinalis* and *E. muscoides* (Leskinen and Pamilo, 1997) diverged by 1-3%, similarly *E. compressa* and *E. intestinalis* from the British Isles reported 2-3% divergence between the two species (Blomster *et al.,* 1998). To assess if *E. compressa* and *E. intestinalis* should be considered as single species, Blomster *et al.,* (1998) employed a more sensitive molecular marker, chloroplast DNA restriction fragment length polymorphisms (RFLP). RFLP analysis resulted in the resolution of two genetically divergent groups representing *E. compressa* and *E. intestinalis* as separate and distant species. Such findings suggested that the two

species may have recently diverged, and thus recognized that the ITS region may have been too conserved to reveal species relationships. These findings suggest that it would be premature to assume all North American freshwater *Cladophora* was a single species based solely on the ITS region.

As a result of the lack of divergence in the ITS region of North American *Cladophora*, it was not possible to determine if one or multiple species are present specifically in the Great Lakes, and more conclusively across North America. Sequencing of the ITS region was also not able to detect differences between European freshwater species *Cl. glomerata* var. *glomerata* and *Cl. fracta* var. *intricata*, of the section Glomeratae within the *Cladophora* genus. To decipher if freshwater *Cladophora* is composed of a single morphologically variable cosmopolitan species, a more sensitive molecular marker such as intersimple sequence repeats must be undertaken.

4.2 ISSR ANALYSIS DISCUSSION

4.2.1 ISSR primer characteristics

To successfully infer population structure hypotheses with inter-simple sequence repeat (ISSR) analysis, it is essential that the resultant banding patterns illustrate a true representation of the genetic structure of the study organism. Currently there are large selections of primers that can be implemented for such analysis. However, it is essential to determine which primers are informative as it is possible that specific primer characteristics may hinder true phylogenetic signal by over- or underestimating the presence of polymorphisms (Blair *et al.,* 1999). Following initial screening for suitable primers, it was

immediately evident that the following primer attributes were advantageous in producing large volumes of resolvable polymorphic fragments in freshwater *Cladophora*.

- Length of primer repeat motif
- Base composition of primer repeat
- Anchoring of primers

4.2.1a Length of repeat motif

Initial screening of primers of varying lengths revealed that di- and tri-nucleotide repeat primers were positively correlated with band resolution and band production (Table 4). Studies of Douglas-fir (Tsumura *et al.,* 1996), and American beech (Morris *et al.,* 2004) each reported optimal band resolution with smaller repeat-motif primers. Screening of longer repeat motifs, such as primer ISSR7 [$(ATTA)_n$], produced background noise, smearing, and stuttering with a maximum production of 4 bands. Primer ISSR9 $(GGAAGG)_n$ is a penta-repeat primer and it failed to produce any banding regardless of the apparent abundance of GA_n repeats throughout the *Cladophora* genome (see discussion below). To ascertain the possibility that differences in amplification of longer repeat primers were not due to annealing conditions, optimization of the protocols was evaluated. This included using a range of annealing temperatures for each individual primer – from 50^o to 65^oC for GC-rich primers (because the predicted melting temperatures (T_m) was 65^o or higher) – without modifying other conditions or times of the PCR program ensuring an optimal environment for ISSR amplification. Such precautions reinforced the theory that the length of the repeat motif may inhibit multiple band production.

4.2.b Base composition of primer repeat

It has been reported that $(AT)_n$ repeats are the most abundant microsatellites found in higher plant nuclear genomes (Wang *et al.,* 1994). This would suggest significant

amounts of amplification should be attained utilizing AT-containing primers. In the current study, two different primers; ISSR7 and ISSR3 based on $(AT)_n$ repeats produced very low or no ISSR fragments, respectively. The lack of amplification suggested that the $(AT)_n$ repeat was potentially lacking, or sparsely located throughout the *Cladophora* genome. Alternatively, a study of *Citrus sinensis* suggested that the lack of amplifiable products may have been a result of the self-complementary nature of $(AT)_n$ primers (Fang and Roose, 1997).

Three of the five primers used in the analysis of North American *Cladophora* were composed of composed of $(GA)_n$ repeats. Indisputably $(GA)_n$ repeats produced the greatest amount of banding (Table 5), and displayed the greatest observable resolution. Similar results were also demonstrated in the freshwater red algae *Batrachospermum helminthosum* which produced between 34-56 bands with primers composed of $(GA)_n$ repeats, versus 23-28 for $(CT)_n$ primers (Hall and Vis, 2001).

4.2.1c 5' Anchored primers versus 3' anchored primers

Theoretically, anchoring avoids amplification from within longer repeat regions, thus reducing the number of false amplifications (Bussell *et al.,* 2005). The consequence of false amplifications, particularly when utilizing a common repeat motif (i.e. $(GA)_n$) primer, is that bands of a particular size may be considered homologus suggesting false relatedness (Lian *et al.,* 2001). In a study of Japanese flounder, a non-anchored $(CT)_7$-primer produced ~36 bands with six appearing homologus across three Japanese flounder populations Lie *et al.,* (2006). Utilization of a 3' anchored $(CT)_7$-primer decreased the number of bands produced to 28, with only two appearing homologus. Cluster analysis of both data sets revealed differences in tree topology, with the unanchored data set

suggesting less genetic variation among populations, suggesting false amplifications occurred. In the current study all primers used were 3' anchored. Only one 5' anchored primer (ISSR13) was initially screened but rejected due to low amounts of banding and excessive smearing.

4.2.2 Genetic structure of North American freshwater Cladophora

4.2.2a UPGMA analysis

Analysis of the UPGMA tree revealed two distinct clusters, a top group (A), hereafter known as the Great Lakes group, and a bottom group (B), hereafter known as the North American group (refer back to Figure 8). The Great Lakes group was clearly resolved as each of the sampled Great Lakes [Lake Ontario (LO), Lake Erie (LE), Lake Huron (LH), and Lake Michigan (LM)] clustered as distinct entities, with the North American group being less resolved. Such groupings suggested four different genotypes or variations representing each of the Great Lakes were present with less resolved genotypes and/or variations present throughout North America. To substantiate the groupings of the cluster analysis, AMOVA analysis of the genetic structure of North American and cultured European samples was conducted. AMOVA analysis indicated that 78% of the total variation of the data occurred within the populations. Partitioning the North American samples into five populations (one population consisting of all samples from across North American and Europe, excluding samples from the Great Lakes which were accounted for as four separate populations) based on the previously established UPGMA clustering, accounted for 21% of the overall variation. These results suggest that though differences among populations were presently concurring with the cluster analysis and the significantly supported F_{ST} values, though low genetic structure supported them.

Analysis of genetic structure for another freshwater alga; *Batrachospermum helminthosum*, reported that genetic variation was partitioned nearly equally within and among stream populations from across North American. They reported that 55% of the variability was due to among stream differences and the remaining 45% due to variation within streams (Hall and Vis, 2002). The high amount of genetic structure suggested by among-stream variation was believed to be the result of sexual reproduction. Similar trends have also been observed for other sexually reproducing algae, for example *Gelidium canariense* (Gelidiales, Rhodophyta) reported 76%-among stream and 23%-within stream variation (Bouza *et al.,* 2006), and 71%-among and 29%-within populations of *Mazzaella laminarioides* (Gigartinales, Rhodophyta) (Faugeron *et al.,* 2001). Importantly, each of these studies also looked at genetic variation between individuals in a population, and noted that variation was a least 80%.

The genetic structure of *Codium fragile* ssp. *tomentosoides*, a clonal marine marcroalgae was investigated to assess genetic diversity between individuals of a single location and among locations of an asexual alga (Kusakina *et al.,* 2006). They determined that only 2% of the total diversity was a result of between individual variation, with the majority of the diversity accounted for by between site variation (98%). Thus the largest contrast between clonal and sexually reproducing algae was the lack of variation between individuals of a single site.

The current study of freshwater *Cladophora* did not analyze genetic variation between individuals of single locations, thus making it impossible to fully compare results to those depicted in either the sexually or asexually reproducing populations. However, the most obvious difference in genetic structure between *Cladophora* and sexually reproducing

algae was *Cladophora's* high overall genetic diversity across all populations, suggesting a lack of genetic structure. These results may indicate that dispersal of *Cladophora* clones has been a significant factor in accounting for the high amount of within-population variation. Clonal growth or asexual reproduction implies each clone remains genetically distinct from one another (Morris *et al.*, 2004; Kusakina *et al.*, 2006). Applying this definition to the study suggest that many different clones may be present and intermixed within each of the Great Lakes and across North America. Birds have been considered the most important transporters of algae (Jorgen, 1996) and the wide geographical distribution of *Cladophora* suggests that dispersal has been extremely effective. This theory would also help explain the lack of differentiation between populations (as suggested by AMOVA analysis) as clones, though genetically distinct, are overall still very similar (Kusakina, 2006). This would suggest that each of the Great Lakes are dominated by different clones, but intermixing of these clones enable genetic variation within each lakes to be maintained.

Additional explanations have been reported to account for higher (then expected) genetic variation in asexually reproducing organisms. It has been suggested that arbitrary molecular markers such as ISSRs can detect relic diversity hidden in clones of a species that in the past reproduced sexually (Esselman et al., 1999). A second explanation suggests that rare sexual events can provide enough variability to keep reasonable levels of genetic variation in asexually reproducing species (Kjolner *et al.* 2004). Although it is impossible to determine if either of these theories has occurred with freshwater *Cladophora*, other techniques, namely microsatellite analysis may be able to elucidate the reproductive history of freshwater *Cladophora.*

4.2.2b Genetic Structure of Great Lake Populations

The grouping pattern within the Great Lakes group (A) were significantly supported *(P<0.05)* when subjected to AMOVA analysis. Among the populations of *Cladophora* in the Great Lakes group relevant biogeographic trends can be noted. Specifically, populations of Lake Ontario were more genetically related to Lake Erie *(F_{ST} = 0.32)* than to either Lake Michigan *(F_{ST} = 0.39)* or Lake Huron *(F_{ST} = 0.39).* Similarly, Lake Huron was genetically related to Lake Michigan *(F_{ST} = 0.33)* than to either Lake Erie *(F_{ST} = 0.43)* or Lake Ontario. As such, levels of genetic differentiation among the Great Lakes appeared to be correlated to the geographic distribution, with lakes in closest proximity to one another being most related, suggesting dispersal of clones within each of the Great Lakes occurs with their closest neighbour. Further support of the biogeography trends of *Cladophora* relatedness was indicated by the highest amount of genetic variation occurring between Lake Erie and Lake Michigan *(F_{ST} = 0.44),* as these two lakes are located furthest from one another.

4.2.2c Genetic structure of North American population

Some hypotheses can be made based on groupings within the North America group of the UPGMA tree, with emphasis on the European representative-types of Cl. *glomerata* and *Cl. fracta*. Cluster (VII) contains both representative-types specimens of *Cl. glomerata* grouping with samples from California and British Columbia. As geographically these samples are not related it is possible to suggest that populations within group (VII) belonged to the European species *Cl. glomerata*. A similar situation was observed in cluster (VIII) as *Cl. fracta* var. *fracta* has grouped with an Ontario Creek, a sample from

Winnipeg, Manitoba, and a sample from Puerto Rico. Due to the extremely large geographical distance between samples, the simplest solution would indicate this group represents the species *Cl. fracta*. Most notable, was that C*l. glomerata* and *Cl. fracta* did not group together, thus concurring with the present delineation of separated species.

Cluster (VI) was represented by each of the *Rhizoclonium* samples along with a sample from Minnesota. The inclusion of the Minnesota sample in the *Rhizoclonium* represented cluster may suggest it was misidentified as *Cladophora* upon collection and is actually *Rhizoclonium.* The possibility of this is great when considering *Cladophora's* aptitude for morphological variation (van den Hoek, 1963). Morphologically, *Rhizoclonium* a long filamentous macroalgae, and is mainly distinguished from *Cladophora* based on it lack of branching. Due to age and sporulation, *Cladophora* filaments tend to display a lesser degree of branching (Whitton, 1970; Dodds and Gudder, 1992), taking on a *Rhizoclonium*-like morphology. Because the Minnesota sample was collected near the end of the growing season (late September), the potential for a simple mistaken identity is highly possible. Furthermore, Power (1992) noted that when studying environment factors affecting *Cladophora* growth, it was often found intermixed with other macroalgae, specifically *Rhizoclonium* increasing the chance misidentification.

Overall, the clustering analysis was supported by AMOVA results, as significant variation was present between populations of the Great Lakes. Potentially the most significant finding of the current study is that of total genetic diversity, less than a quarter of it is suggested to be a result of among population differentiation. This fuels the controversy as to the existence of different species of *Cladophora* present in North America. It is expected that higher levels of genetic diversity should be present among

populations, especially among such geographically disjunctive ones, to suggest the existence of different species. This was not observed in the current study as variation among populations accounted for only 21% of the overall genetic diversity. Assuming *Cladophora* is asexual, future investigations should examine genetic diversity among individuals, to assess the presence of different species or varieties. With respect to the Great Lakes group, ISSR analysis of genetic structure appears most informative at the population and within population level. Thus to further resolve the bottom North America group (Figure 8) and understand the genetic structure of freshwater *Cladophora* on a wide geographic scale, additional sampling of within population (as well as between individuals of each population) must be undertaken for each representative within the tree. It appears analysis of genetic structure at the three hierarchical levels: among groups (i.e., habitat), within groups (i.e., among populations within groups), and within populations (i.e., individuals), may be the only way to assess the presence of different varieties or subspecies of clonal *Cladophora* as assumptions based on genetic diversity between species (as for sexually reproducing organism) will not suffice. The same approach has been proposed to assess variation in the frequency of clonal reproduction among populations of *Fagus grandifolia* with respect to conservation issues. As of yet, it is not possible to conclusively conclude based on molecular data whether one or more species/subspecies exist for freshwater *Cladophora*, but there is preliminary evidence of different genotypes or varieties in the Great Lakes.

4.3 MORPHOLOGICAL STUDY DISCUSSION

4.3.1 Seasonal morphology

Assessment of morphological seasonal variation was conducted to evaluate the limitations in using morphological characters as a taxonomic criterion. The initial goal was to search for stable characteristics that were not influenced by seasonal change, and use those for taxonomic delineation. For such analysis, the diameter and length of the main axis, ultimate branch, and apical cells were measured from a single population of *Cladophora* located on Lake Ontario. Unfortunately all morphometric characteristics showed considerable variation over the six-month sampling season (Table 7). The resultant cluster tree presented in Figure 10, indicates that some general growth trends are present, they were not statically supported, confirming the lack of stability and predictability in the morphometric characters throughout a growth season.

Morphological variability was effectively represented through the graphical results of the ANOVA analysis carried out for each morphological character over the sampling season (Figure 9). Overall, cell lengths were the most variable character ($P<0.005$), with main cell lengths having a 931µm range in size throughout the season. Such results were expected as *Cladophora* growth has been documented as highly variable over a season (van den Hoek, 1963). The start of filament growth is primarily dominated by apical cell divisions and subsequent cell-elongation until a new cell wall is formed and elongation is terminated. van den Hoek (1963) reported that in turbulent and wave beaten waters initial cell elongation can be greatly pronounced; resulting in very long, thin cells. Evidence of this growth trend was observed in the ANOVA results of cell lengths (Figure 9). Both

ultimate and branch cells were longest and had the smallest diameter during the first sampling date (May 22, 2004).

With growth and age, apical growth is often coupled with intercalary growth. Consequently, older/longer cells essentially split by the production of a cross wall, were each cell then starts to stretch at varying rates and varying degrees (van den Hoek, 1963). Intercalary growth starts at some distance from the apex and basipetally increases, being most evident in the oldest portions of the cell filament (van den Hoek, 1963). As previously mentioned, Dingle Park was a very exposed area and thus a combination of apical and intercalary growth may have been the largest contributor to the variability in cells lengths and the lack of observable growth trends.

Figure 10 shows that cell diameters were also quite variable over the season, but appeared largest at the end of the sampling season (Sept 11-Oct 8, 2004). This seems plausible, as by October water temperatures have decreased, making for unfavourable growing conditions. Contributing factors for the diameter increase and cell length decrease may have been two-fold. Evidence of sporulation was seen in many of the filaments appeared degraded at the end of branches. Others were more barrel-shaped, indicating that some plants may have been in the process of zoospore formation. It has been documented in *Cladophora glomerata* that the zooidangium noticeably shorten and swell before and during zooid-formation (van den Hoek, 1963; Dodds and Gudder, 1992). Zoosporogenesis is promoted by a number of factors such as vitamin limitation and shortened photoperiod (Dodds and Gudder, 1992, Hiriart-Baer *et al.,* 2004).

An additional proposition and probably the most likely, is that *Cladophora* filaments were in the process of transforming into vegetative resistant spores called

akinetes. van den Hoek (1963) reported increased cell diameters in late summer, as intercalary cell-division increased and were no longer followed by a stretching of the cells. Consequently, cells begin to take on a club-shaped appearance as they transform into akinetes. Akinete production can be triggered by environmental cues including low temperatures, short photoperiods, nutrient depletion and desiccation (Spender et al., 1980), all of which could have been occurring by late October.

The observed seasonal variation could have a large impact in species discrimination not only in the freshwater genus *Cladophora* but for other alga species also. For example, histoRicolly *Batrachospermum elegans* was differentiated from *B. helminthosum* (a species of the same section within the genus *Batrachospermum*) by the presence of protuberances on trichogynes on *B. elegans*. However, the type specimen of *B. helminthosum* revealed the presence of protuberances, calling this morphological character into question. To determine if protuberances were specific to only *B. elegans*, Vis and Sheath (1998) observed *B. helminthosum* in a stream over a growing season and monitored changes in morphology. It was established that protuberances did appear at certain times of the year, terminating the use of that morphological character as a diagnostic feature of *B. elegans* as it appeared to be a seasonably variable morphological character shared by species.

Similar findings were reported for different species within the chlorophyte genus *Enteromorpha*. Morphologically, *E. muscoides* can be identified by the presence of repeated branching intercalated by short spine-like branches throughout the thallus (Bliding, 1963). However a number of studies have noted that, especially in the summer months, *E. muscoides* does not present obvious spine-like branches. As a result it is easily misidentified as *E. clathrata,* which is also repeatedly branched but lacks spin-like

branching. Thus this morphological feature often fails to clearly differentiate between the two species when the character is influenced by seasonal changes (Blomster *et al.*, 1999; and Burrows, 1959).

Growth parameters are also influenced by seasonal variation. The seasonal growth trends for *Cladophora* in the Great Lakes have shown that it begins growth when water temperatures are approximately 5^{o}C, reaches optimal growth around 18^{o}C and ceases to grow at 25 to 30^{o}C (Dodds and Gudder, 1992; Kirby and Dunford, 1981). Blum (1982) determined that the peak filament length for *Ulothrix zonata* is reached near the end of May, when water temperatures are optimal (Rosemarin, 1980; Kirby and Dunfor, 1981), after which the filaments decreases slightly in length. At such a point filament diameter increased (30-35um), permitting *Ulothrix* to maintain consistent coverage. These results demonstrate the season variation of filament length and diameter for another Great Lakes filamentous alga, which makes morphological characters often hard to use as taxonomic discriminating features.

4.3.2 Problems with Seasonal Morphological Analysis

Unfortunately, many of the samples used for the seasonal analysis were degraded, often making it difficult to consistently follow the abovementioned measurement criterion (i.e. criteria for main axis cell measurement was measuring the cell located 2 cells down (basipetally) from the first branching point). Degradation also increased the fragility of the filaments so breakage became an impeding factor, as it was extremely difficult to untangle and extract a single filament for morphometric analysis. These issues made it virtually impossible to make other observations, such as branching patterns, cell division (which can be deciphered by analyzing cell walls), and amount of branching. In following

studies filaments should be observed and measured immediately. It is also important to be able to extract a single individual to make qualitative observations.

4.3.3 Great Lakes Morphology

To assess the taxonomic utility morphological characteristics have to differentiate *Cladophora* among and within the Great Lakes, identical morphometric measurements implemented in the seasonality study were carried out on populations of the Great Lakes. Although the seasonality study was not able to reveal stable characters that were unaffected by seasonal influence, it was still important to determine if population of *Cladophora* could be differentiated based on morphology. Immediately similar trends as those revealed in the seasonality study begun to emerge. Cluster diagrams based on population means revealed that there were no stable characteristics that could consistently group individuals from populations within each Lake together (Figure 11). For example group (I) consist of populations from each of the Great Lakes. These findings were anticipated for a number of reasons. First, the seasonality study revealed the vast degree of variability over the sampling season; as well Table 7 reveals the large range of cell sizes at any one date. Secondly, *Cladophora* is well known to exhibit a high degree of phenotypic plasticity due to surrounding environments and age (Dodds and Gudder, 1992; van den Hoek 1963). Considering that each of the collecting sites differed in ecological and physical parameters, the variability in morphology from site to site is not unexpected. For example, within Lake Michigan, site LM1 was in a very sheltered Bay with sparse rocks for *Cladophora* attachment (Figure 12). Cladophora collected from site

Figure 12. Photo of 3 sampling locations around Lake Michigan. (A) LM1, is a sheltered Bay, (B) LM2, is a rocky jetty, (C) LM7, is a marina. Photo's by S. Ross and M. Sawchuck, July 3, 2005


A
B
C

LM2 was situated on a rocky jetty that was subject to strong wave action (Figure 12), and site LM7 was from a marina dock which was very sheltered from any light. It is apparent that cell sizes are too variable to be used as stable taxonomic characteristics and are very influenced by environment (Figure 12). Consequently, observational and morphometric analysis of samples from each of the locations did not reveal any correlations nor did they conform to the expected changes induced by each of their environments. For example it would be expected that samples collected from LM2, the rocky jetty would display a decreased length and increased diameter as oppose to samples form LM7, which was situated in a calm environment. Mean measurements for main axes cell lengths for each were nearly identical (607µm for LM2 and 601µm for LM7)

Additional studies focused on phenotypic plasticity of *Cladophora* utilized transplant experiments to assess whether morphological variation observed in freshwater *Cladophora* was a plastic response to the local environment or whether morphology was genetically fixed. A translocation experiment was conducted to reveal the relationship between environment, specifically suspended sediments and morphology in a Colorado river (Wilson *et al.,* 1999). Lengths were first recorded in waters with low suspended sediments, following translocation to water with high sedimentation levels, cell lengths decreased to 401µm. This suggested that although there was a dramatic morphological change in *Cladophora* along the Colorado River, it was one species exhibiting highly variable morphology and not several distinct species.

Morphological variation has been cited as a significant reason species are able to occupy a broad range of physical environments (Sultan, 2001). For instance, variations in

wave exposure are known to have profound effects on morphology of marcroalgae (Hurd, 2000). *Turbinaria ornate,* a marine alga, located in sheltered sites exhibited fronds that tended to be wide, thin and undulate versus exposed sites in which fronds were narrower, thicker and flat with thicker stripes (Fowler-Walker *et al.,* 2006). Levels of morphological plasticity have also been show to occur across exposure gradients in *Egregia menziesii,* a marine kelp (Blanchette *et al.,* 2002). Such morphological differences are thought to enable macroalgae to inhabit wave sheltered environments without compromising their ability to efficiently photosynthesis and grow (Hurd *et al.*1996) and to inhabit wave exposed environments by preventing breakage and/or dislodgement (Fowler-Walker *et al.,*, 2006).

Many freshwater red alga species have been also been documented to show immense morphological variation. The genus *Batrachospermum* contains a number of species separable by minimal distinguishing features (Vis *et al.,* 1995). Sheath *et al*., (1997) noted that *B. gelatinosum* displayed high degrees of thallus plasticity across a wide geographic range in North America. Another red algal genus *Hildenbrandia* has been differentiated on the basis of the conceptacle size and thallus thickness, but this has been argued because studies show that they vary with age and development (Sherwood and Sheath, 1999).

In 1963 van den Hoek, concluded that the genus *Cladophora* exhibits extreme variation in morphology. For this reason he insisted that qualitative analysis of morphology, such as type of growth and amount of branching in conjunction with qualitative measurements, such measurement of cell size are needed to properly identify species. Although cell dimension properties were not able to show any distinction in

Cladophora populations in the current study, a combination of quantitative and qualitative morphological observations, biochemical, and physiological studies must be undertaken before ruling out the use of morphological characters in species discrimination of freshwater *Cladophora*. Unfortunately, due to degradation of voucher samples, additional observations were not possible. As such, based solely on morphometric characters, *Cladophora* showed no consistent growing trends over a season, nor were the same characters able to differentiate *Cladophora* among or within the Great Lakes.

5.0 GENERAL CONCLUSION

The genus *Cladophora* (Cladophorales, Chlorophyta) is cosmopolitan in freshwater, brackish, and marine habitats in both temperate and tropical regions (Marks and Cummings, 1996; John, 2002; Dodds and Gudder, 1992). It is frequently observed in eutrophic waters and can form nuisance algae blooms (van den Hoek, 1963; Reynolds *et al.,* 2000). Despite research to discern the ecological, physiological, and biochemical reasons for freshwater *Cladophora* blooms, very little has been done to address the complex taxonomic issues residing within this genus (Marks and Cummings, 1996).

Species discrimination within the *Cladophora* genus has histoRicolly been based upon a combination of morphological characteristics, but due to the propensity of *Cladophora* to exhibit high degrees of phenotypic plasticity (van den Hoek, 1963; Dodds and Gudder, 1992; Scheiner, 1993) the use of morphology alone, has generated a great deal of frustration among field researchers.

Based on this information, the present study was initiated to address the following objectives:

1. Use molecular data to determine if populations of freshwater *Cladophora* from the Great Lakes were composed of a single or multiple, species or varieties.
2. Examine the genetic relationships of Great Lakes populations to other North America freshwater populations.
3. Determine if there are morphological characters that are stable and do not exhibit plasticity during the growth season of *Cladophora*.

4. Compare molecular and morphological data to determine if there are morphological characters that correspond to the genotypes present in the molecular analyses.

Amplification of the ITS regions of freshwater *Cladophora* consisting of samples from across North America, including each of the Great Lakes, as well as cultures of representative-type specimens of *Cl. glomerata* var. *glomerata, Cl. glomerata* var. *crassior,* and *Cl. fracta* var. *fracta* from Europe exhibited high similarity (over 98%). Due to the lack of variability in this region phylogenetic analyses could not be carried out. The lack of divergence among samples representing such a large geographic range raised two possibilities. The first being, that only one species of freshwater *Cladophora* exists, with local populations representing ecologically and morphologically distinct forms and/or varieties, as have been suggested for *Batrachospermum gelatinosum*, a freshwater rhodophyte also occupying a large geographic range (Vis and Sheath, 1997). The second being that the ITS regions has not had sufficient time to diverge, impeding its ability to resolve genetic relationships of freshwater *Cladophora*. Though no molecular clock exists for freshwater algae, it has been suggested the divergence rate of the ITS region for marine *Cladophora* were 0.8-2% per Ma (Bakker *et al.,* 1995). Recognizing that the last glaciation event in North America was between 10,000 to 15,000 years ago (Tyler, 1996), it is reasonable to assume there has not been adequate time for the ITS region of freshwater *Cladophora* to demonstrate enough divergence to reveal species delineation. As a result it was not possible to determine if one or multiple species are present specifically in the Great Lakes, and more conclusively across North America. Hence a more sensitive molecular marker, intersimple sequence repeats (ISSR) was implemented.

Analysis of North America, including the Great Lakes and European freshwater *Cladophora* resulted in separation of all Great Lake *Cladophora* together, and the residual North America populations together. A high amount of resolution was apparent in the Great Lakes clade each of the sampled Great Lake further separated into distinct entities [Lake Ontario (A), Lake Erie (B), Lake Huron (C), and Lake Michigan (D)]. Within the North America group population trends were less resolved. Additionally, the pattern of grouping within the cluster tree implied that *Cladophora* populations of Lake Ontario and Lake Erie were more closely related to one another than they were to either Lake Michigan or Lake Huron. To test the significance of the cluster analysis, an AMOVA was performed, in which each of the Great Lakes were considered as separate populations due to the highly resolved nature of the top cluster and all additional North America samples were grouped into one population as a result of the lack of resolution in the bottom cluster. F_{ST} values supported this, as less genetic variation was revealed among Lake Ontario and Lake Erie as oppose to Lake Ontario to Lake or to Lake Huron. Similarly, F_{ST} results implied that Lake Michigan was more closely related to Lake Huron than to either Lake Erie or Lake Ontario. These results indicate relevant biogeographic relationships as adjacent lakes (i.e. Lake Ontario and Lake Erie) were more genetically related. Lake Michigan and Lake Erie, were the most differentiated. AMOVA results indicated that within any of the Great Lakes there was less genetic variation than among Lakes. These results implied that different genotypes were present among the Great Lakes (as samples were more similar to one another within each of the lakes than between each of the lakes), though overall genetic variation between all populations was low.

Genetic differentiation among the North America population to each of the Great Lakes, suggesting that all of the North America populations, though separated in the cluster analysis are very genetically similar to any of the Great Lakes. This relationship was supported by MDS analysis of all samples, in which Great Lake population clusters were lost due to the large amount of overlap with North America samples.

Additional trends were also observed in bottom cluster (B) of the UPGMA tree, specifically concerning the existence of separate species for *Cl. glomerata* and *Cl. fracta*. *Cl. glomerata* var. *glomerata* and *Cl. glomerata* var. *crassior* grouped together, and *Cl. fracta* var. *fracta* formed a different group. Based on the separate groupings of each of the two species it appears that there are distinctions between each, suggesting preliminary molecular evidence to support the separation of two European species.

On a molecular basis results of the present study have suggested *Cladophora* of the Great Lakes may be composed of four different genotypes, or varieties. HistoRicolly, European species of *Cladophora* have been differentiated by morphological characters (van den Hoek, 1963). These characteristics included thallus organization, pattern and amount of branching, type of main axes growth (intercalary or acropetal), mode of reproduction, if the organism is attached to a substrate or free-floating, as well as additional morphometric characters including length and widths of main axes, ultimate branch and apical cells (van den Hoek, 1963). Unfortunately, many of the above mentioned taxonomic traits overlap between species due to the high degree of phenotypic plasticity displayed by *Cladophora* making them difficult to implement in species discrimination questions. This confusion has been most evident in the freshwater species of the section *Glomeratae* where morphologically *Cl. glomerata* var. *glomerata* has often been distinguished from *Cl. fracta*

var. *fracta* based on the diameter of main axis cell size (100-275um and 45-85um, respectively), but age and surrounding environment result in overlap of these characters. For example, high sedimentation levels in a Colarado River caused the diameter of Cladophora cells to decrease to *Cl. fracta* ranges (~55um). This was confirmed as phenotypic plasticity, as upon moving *Cladophora* samples, the diameters increased to representative *Cl. glomerata* sizes again (~175um) (Wilson *et al.,* 1999).

Based on the premise that neither morphological data nor molecular data sets alone should provide a depiction of the true phylogeny, morphometric characters were investigated. Before implementing all of the morphological character, a single population located on Lake Ontario was observed over an entire season to assess if any morphological characters were stable throughout the a growing season. ANOVA results of each character separately over the sampling season revealed significant variation over the season, with the most stable being the diameter of the apical cell, though still significantly variable. Analyses of morphological averages over the season did show some seasonal growth trends, specifically at the start and end of the sampling season. Early season (May 22, 2004), cell lengths were longest and diameters were smallest which may be due to *Cladophora* filaments increasing overall length. These findings were congruent with growth trends reported by van den Hoek (1963), who suggested early season filaments put all of their energy into growth, which occurs by stretching of apical cells of the main axis, resulting in a long, thin filament cells. In early October, diameters were recorded at their widest and lengths shortest. Environmental cues including low temperatures, short photoperiods, and nutrient depletion (Spender et al., 1980) have been reported to trigger filament cells of *Cladophora* to transform into akinetes-resistant overwintering spores. As

these environmental factor are all significant in early fall, the increased diameters and shortened lengths may be attributed to akinete formation.

Although a great deal of season variation was noted in each of the morphological characteristics, a large-scale study implementing the same parameters as in the seasonality study were conducted. Due to the lack of voucher samples for many of the North America samples used for molecular analysis, only samples collected from the Great Lakes were used however, populations from the Great Lakes did not form a separate clade containing only these populations.

In his initial revision of the *Cladophora* genus, van den Hoek (1963) reported that *Cladophora* presented a very plastic appearance, so he insisted many morphological measurements should be applied to properly identify species. Thus even though cell dimension properties were not able to show any distinction in *Cladophora* populations, other biochemical, physiological measurements must be undertaken before ruling out the use of morphological characters in species discrimination. Unfortunately, due to degradation of voucher samples, additional observations were not possible for all samples.

A problem that may hinder the delineation of North America freshwater *Cladophora* species is the fact that species epithets for freshwater *Cladophora* are based on European collections. As the European representative-type specimens did not group with the Great Lakes group, it could suggest that different varieties of *Cladophora* may exist in the Great Lakes and that European epithets should not be used to represent Great Lake populations. Adding to the confusion, morphologically, cell sizes for each main axis, ultimate branch and apical cell for Great Lake populations are similar to those recorded for European *Cl. glomerata* var. *glomerata.*

At this point it is not possible to conclusively resolve the question of whether one or more species of freshwater *Cladophora* exist. AMOVA results indicated that four different genotypes (representing each of the lakes sampled in the current study) were present. ITS and ISSR results suggest that only one cosmopolitan species of *Cladophora* is present, with different varieties. This would result in dissolving the two species of within the section *Glomeratae* to a single species, *Cladophora glomerata*.

Future research should first consist of additional sampling of North America streams and rivers, which may help resolve North America samples. Additionally the level of interpretation will be increased if North America populations can be studied among populations, within populations (groups), and within groups (individuals), as assessment of the genetic structure may be the only molecular way to assess the presences of different varieties or subspecies. Great Lake populations should be submitted to additional morphological examination before dismissing its use in species discrimination. Additionally, more informative molecular marker such as microsatellites should be employed. Microsatellites, a multilocus molecular marker are currently reported as the most informative molecular marker as they are: i) polymorphic in nature, ii) randomly distribution throughout the entire genome, iii) codominant in nature and thus heterozygotic individuals can be distinguished from homozygotic individuals, and finally iv) they undergo Mendelian inheritance (Lian *et al.*, 2001). They have successfully been utilized in the marine brown alga *Laminaria digitata* (Billot *et al.,* 1998), to understand reproductive systems and gene flow between populations. They have also helped establish the reproductive live cycle of *Enteromorpha intestinalis* (Alstrom-Rapaport and Leskinen, 2002).

To our knowledge microsatellite analysis has not been conducted on freshwater green algae. Although preliminary attempts to make microsatellite primers were unsuccessful, recent findings have reported a new method to extract large amounts of high quality DNA. Thus we believe microsatellites will provide the most insight into the genetic composition and affirm the type of reproduction in the freshwater *Cladophora* genus as well as resolving other taxonomic problems within the genus.

REFERENCES

Alstrom-Rapaport, C., Leskinen, E. (2002). Development of microsatellite markers in the green algae Enteromorpha intestinalis (Chlorophyta). Molecular Ecolgy Notes (4): 581-583.

Al-Barrak, M., Loxdale, H.D., Brookes, C.P., Dawah, H.A, Biron, D.G., Alsagair, O. (2004). Molecular evidence using enzyme and RAPD markers for sympatric evolution in British species of *Tetramesa* (Hymenoptera: Eurytomidae). Biological Journal of the Linnean Society, (83):
509-525.

Auer, M. S., Canale, R. P. (1982a). Ecological studies and mathematical modeling of *Cladophora* in Lake Huron: 2. Phosphorus uptake kinetics. Journal of Great Lakes Research (8): 93-99.

Avise, J. C. (2004). Molecular Markers, Natural History, and Evolution, 2nd Edition.Sinauer Associates, Inc. MA. Pp 6-10.

Bakker, F. T., Olsen, J. L., Stam, W. T., van den Hoek, C. (1992). Nuclear Ribosomal DNA internal transcribed spacer regions (ITS 1 and ITS 2) define discrete biogeographic groups in *Cladophora albida* (Chlorophtya). Journal of Phycology (28): 839-845.

Bakker, F. T., Olsen, J. L., Stam, W. T. (1995). Global phylogeography in the cosmopolitan species *Cladophora vagabunda* (Chlorophyta) based on nuclear rDNA internal transcribed spacer sequences. European Journal of Phycology (30): 197-208.

Billot, C., Rousvoal, S., Estoup, A., Epplen, J. T., Saumitou-Laprade, P., Valero, M., Kloareg, B. (1998). Isolation and characterization of microsatellite markers in the nuclear genome of the brown alga *Laminaria digitata* (Phaeophyceae). Molecular Ecology (7): 1771-1788.

Blair, M. W., Panaud, O., McCouch, S. R. (1999). Inter-simple sequence repeat (ISSR) amplification for analysis of microsatellite motif frequency and fingerprinting in rice (Oryza sativa L.)

Blanchette, C. A., Miner, B. G., Gaines, S. D. (2002). Geographic variability in form, size and survival of Egregia menziesii. Molecular Ecology Notes (22): 134-137.

Bliding, C. (1963). A critical survey of European taxa in Ulvales. Part I. Operational Botany (8):3 1-160.

Blomster, J., Maggs, C., Stanhope, M. J. (1999). Molecular and Morphological analysis of Enteromorpha intestinalis and E. compressa (Chlorophyta) in the British Isles. Journal of Phycology (34): 319-340.

Blum, J. L., 1982. Colonization and growth of attached algae at the Lake Michigan water line. Journal of Grate Lakes Research (8): 10-15.

Bornet, B., Antoine, E., Bardouil, M., Marcaillou-Le Baut, C. (2004). ISSR as new markers for genetic characterizaion and evlaution of relationships among phytoplankton. Journal of Applied Phycology. (16): 285-290.

Bouza, N., Caujape-Castells, J., Gonzalez-Perez, M.Z, Sosa, P.A. (2006). Genetic Structure of Natural Populations in the Red Algae *Gelidium Canariense* (Gelidiales, Rhodophyta) Investigated by Random Amplified Polymorphic DNA (RAPD) Markers. Journal of Phycology (42): 304-311.

Burrows, E. M. (1959). Growth form and environment in *Enteromorpha.* Journal of Botany (56): 204-206.

Bussell, J.D., Waycott, M., Chappill, J.A. (2005). Arbitrarily amplified DNA markers as characters for phylogenetic inference. Perspectives in Plant Ecology, Evolution and Systematics (7): 3-26.

Byappanahalli, M. N., Shively, D. A., Neverd, M. B., Sadowsky, M. J., Whitman, R. L. (2003). Growth and survival of *Escherichia coli* and enterococci populations in the macro-alga *Cladophora* (Chlorophyta). Microbiology Ecology (46): 2003-211.

Coleman, A. W., Suarez, A., Goff, F. J. (1994). Molecular Delineation of Species and Syngenes in Volvocaceane Green Algae (Chlorophyta). Journal of Phycology (30) 80-90.

Coye, J. A., Orr, H. A. (2004). Speciation. Pp. 109-119. Cambridge: Cambridge University Press.

De Bruin, A., Ibelings, B.W., Van Donk, E.. (2003). Molecular techniques in phytoplankton research: from allozyme electrophoresis to genomics. Hydrobiologia (491) 47-63.

Dice, P., 1945. Nouvelle recherches sur la distribution florale. Bulletin de la Societe Vaudoise des Sciences Naturelles 44, 223-270.

Dodds, W. K., Gudder, D. A. (1992). The ecology of *Cladophora* in freshwaters. Journal of Phycology (28): 415-427.

Dow, B.D., Ashley, M.V., Howe, H.F. (1995). Characterization of highly variable (GA/CT)n microsatellites in burr oak, *Quercus macrocarpa*. Theoretical Applied Genetics (91): 137-141.

Esselman, E.J., Jianqiang, L., Crawfor, D.J., Windus, J.L, Wolfe, A.D. (1996). Clonal diversity in the rare *Calamagrostis porteri* spp. *Insperata* (Poaceae): comparative results

from allozymes and random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers. Molecular Evolution (8): 443-451.

Excoffier, L., Smouse, P.E., Quattro, J.M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application oto human mitochondrial DNA restriction data. Genetics (131): 479-491.

Fang, D. Q., Roose, M. L. (1997). Identification of closely related citrus cultivars with inter-simple sequence repeat markers. Theoretical Applied Genetics. (95): 408-417.

Faugeron, S., Valero, M., Destombe, C., Martinez, E.A., Correa, J.A. (2001). Hierarchical spatial structure and discriminate analysis of genetic diversity in the red alga *Mazzaela laminarioides.* Journal of Phycolgy (37): 705-716.

Flint, J., Bond, J., Rees, D. C., Boyce, A.J., Roberts-Thomson, J.M., Excoffier, L., Clegg, J.B., Beaumont, M.A., Nichols, R.A., Harding, R.M. (1999). Minisatellite mutational processes reduce F_{ST} estimates. Human Genetics (105): 567-576.

Freeman, S., Herron, J. C. (2004). Evolutionary Analysis 3rd Edition. Prentice Hall, Upper Saddle River, NJ. Pp 549-567.

Godwin, I. D., Aitken, E. A. B., Smith, L. W. (1997) Applications of inter simple sequence repeat (ISSR) markers to plant genetics. Electrophoresis. (18): 1524-1528.

Goff, L. J., Moon, D. A., Coleman, A. W. (1994). Molecular delineation of species and species relationships in the red algal agarophytes *Gracilariopsis* and *Gracilaria* (Gracilariales). Journal of Phycology (30): 521-537.

Graham, L. E., Wilcox, L. W. (2000). Algae. Prentice Hall, Upper Saddle River, NJ. 81-85.

Hall, M. M., Vis, M. L. (2002). Genetic variation in Batrachospermum helminthosum (Batrachospermales, Rhodophyta) among and within stream reaches using intersimple sequence repeat molecular markers. Phycological Research. (50): 155-162.

Harris, S.A., (1999). RAPDs in systematics-a useful methodology? In: Hollingsworth, P.M., Bateman, R.M., Gornall, R. J. (Eds.), Molecular Systematics and Plant Evolution. Taylor and Francis, London, pp. 211-228.

Hecky, R.E., Smith, R.E.H., Barton, D.R., Guldford, S.J., Taylor, W.D., Howell, T. (2004). The nearshore phosphorus shunt; a consequence of ecosystem engineering by dreissenids in the Laurentian Great Lakes. Canadian Journal of Fisheries and Aquatic Sciences (61): 1258-1293.

Hiriart-Baer, V.P., Campell, .M, Guildfor, S.J., Hecky, R.E., Moore, L., Charlton, M.N. (2003). *Cladophora* in Lake Ontario.

Hoffman, J. P., Graham, L. E. (1984). Effects of selected physicochemical factors on growth and zoosporogenesis of Cladophora glomerata (Chlorophyta). Journal of Phycology (20): 1-7.

Hurd, C. L. (2000). Water motion, marine macroalgal physiology, morphology, and production. Journal of Phycology (36): 453-472.
In: *Proceedings of the 23rd Conference on Great Lakes Research, International Association for Great Lakes Research.*

Jackson, D. F. (1966). Photosynthetic rates of Cladophora fracta from two sites in Lake Ontario under natural and laboratory conditions. In Proc. 9th Conference on Great Lakes Research, Great Lakes Research Division Publications No. 15, pp. 44-50.

John, D.M. (2002). Order Cladophorales (=Siphonocladales). In: *The Freshwater Algal Flora of the British Isles. An identification guide to freshwater and terrestrial algae*. (John, D.M., Whitton, B.A. & Brook, A.J. Eds), pp. 468-470. Cambridge: Cambridge University Press.

John, D. M., Price, J. H., Maggs, C. A., Lawson, G. W. (1979). Seaweeds of the western coast of tropical AfRico and adjacent islands: A critical assessment. III. Rhodophyta (Baniophyceae). Bulletin of the British Museum of Natural History.

Johnson, M., Shivkumar, L., Berlowitz-Tarrant, L. (1996). Structure and properties of filamentous green algae. Materials Science an Engineering (38): 103-108.

Jorgen, K. (1996). Dispersal of freshwater algae-a review. Hydrobiologia (336): 151-157.

Karp, A. & Edwards, K.J. (1997). DNA markers: a global overview. In: *DNA markers: Protocols, Applications and Overviews* (Caetano-Annolés, G. & Gresshoff, P.M., eds.), 1-14. Wiley-VCH, New York.

Kirby, M.K., Dunfor, W.E. 1981. Attached algae of the Lake Erie shoreline near Nanticoke Generating Station. Journal of Great Lakes Research &: 249-257.

Kooistra, W. H. C. F., Stam, W. T., Olsen, J. L., van den Hoek, C. (1992). Biogeography of *Cladophoropsis membranacea* (Cholorphyta) based on comparisons of nuclear rDNA ITS sequences. Journal of Phycology. (28): 660-668.

Kusakina, J., Snyder, M., Kristie, D.N., Dadswell, M.J. (2006). Morphological and molecular evidence for multiple invasions of *Codium fragile* in Atlantic Canada. Botanica Marina (49): 1-9.

Larsen, A., Medlin, L. K. (1997). Inter- and intraspecific genetic variation in twelve *Prymnesium* (Haptophyceae) clones. Journal of Phycology. (33): 1007-1015.

Lei, Yun-Gun, Chen, Song-Lin, Li, Jing, Li, Ba-Fang. (2006). Genetic diversity in three Japanese flounder (*Paralichthys olivaceus)* populations revealed by ISSR markers. Aquaculture (225) : 565-572.

Leliaert, F., Rousseau, F., Reviers, B., Coppejans, E. (2003). Phylogeny of the Cladophorophyceae (Chlorophyta) inferred from partial LSU rRNA gene sequences : is the recognition of a separate order Siphonocladales justified. Journal of Phycology (38): 233-246.

Lesk, A. M. (2005). Introduction to Bioinformatics 2nd Edition. Oxford University Press, Oxford. Pp 199-201.

Lian, C., Zhou, Z., Hogetsu, T. (2001). A simple method for developing microsatellite markers
using amplified fragments of inter simple sequence repeat (ISSR). Journal of Plant Research. (114): 381-385.

Liebmann, H. (1962). Minimum light requirements for substrate colonization by *Cladophora glomerata*. Journal of the Great Lakes Research (8): 42-53.

Lomolino, M.V., Riddle, B.R., Brown, J.H. (2006). Biogeography 3rd Edition. Sinauer Associates, Inc. Plumtree Road, Sunderland, MA. Pg 320-380.

Manhart, J. R., McCourt, R. M. (1992). Molecular Data and Species Concepts in the Algae. Journal of Phycology. (28): 730-737.

Marks, J. C., Cummings, M. P. (1996). DNA sequences variation in the ribosomal internal transcribed spacer region of freshwater *Cladophora* species (Chlorophyta*). Journal of Phycology* (32): 1035-1042.

Marks, J. C., Power M. E., Parker, M. S. (2000). Flood disturbance, algal productivity and interannual variation in food chain length. Oikos, 90: 20-27.

Mayr, E., (1942). Systematics and the Origin of Species. Columbia University Press, New York.

McManus, H.A., Lewis, L.A. (2005). Molecular phylogenetics, morphological variation and colony-form evolution in the family Hydrodictyaceae (Sphaeropleales, Chlorophyta). Phycologica 44 (6): 582-595.

Millner, G.C., Sweeney, R.A. (1982). Lake Erie *Cladophora* in Perspective. Journal of Great Lakes Research (8) 27-29.

Mindell, D. P., Honeycutt, R. L. (1990). Ribosomal RNA in vertebrates: evolution and phylogenetic applications. Annual Review of Ecological systematics. (21): 541-566.

Morris, A.B., Small, R.L., Cruzan, M.B. (2004). Variation in Frequency of Clonal Reproduction Among populations of *Fagus grandifolia* Ehrh. In Response to Disturbance. Castanea (69) 38-51.

Nagaoka, T., Ogihara, Y. (1997). Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP's and RAPD markers. Theoretical and Applied Genetics. (94): 597-602.

Painter, D. S., Kamaitis, G. (1987). Reduction of Cladophora biomass and tissue phosphorus I Lake Ontario, 1972-1983. Canadian Journal of Fisheries Aquatic Science (44): 2212-2215.

Parodi, E. R., Caceres, E. J. (1991). Variation in number of apical ramifications and vegetative cell length in freshwater populations of *Cladophora* (Ulvophyceae, Chlorophyta). Journal of Phycology (27): 628-633.

Pinna, M. C. C. (1999). Species concepts and phylogenetics. Reviews in Fish Biology and Fisheries (9): 353:373.

Reynolds, C.S., Reynolds, S.N., Munawar, J.F. 2000. The regulation of phytoplankton population dynamics in the world's largest lakes. *Aquatic Ecosystem Health & Management,* (3): 1-21.

Ronnberg, O., Lax, P. (1980). Influence of wave action on morphology and epiphytic diatoms of *Cladophora glomerata* (L.) Kutz. Opheila (1): 209-218.

Rosemarin, A. S. 1980. Aspects of nutrition of two potentially competing filamentous green algae, Cladophora glomerata and Stigeoclonium tenue, from Lake Ontario.
Scheiner, S., M. (1993). Genetics and Evolution of Phenotypic Plasticity. Annual Review of Ecological Systematics. (24): 35-68.

Schneider, S.,Roessli, D., and Excoffier, L. (2000) Arlequin: A software for population genetics data analysis. Ver 2.000. Genetics and Biometry Lab, Dept. of Anthropology, University of Geneva.

Sheath, R G., Cole, K. M., (1992) Biogeography of stream macroalgae in North AmeRico. Journal of Phycology (28): 448-460.

Sherwood, A. R.., Sheath, R. G. (1999). Biogeography and systematics of *Hildenbrandia* (Rhodophyta, Hildenbrandiales) in North AmeRico: inferences from morphometrics and *rbc*L and 18S rRNA gene sequence analyses.
Sinauer Associates, Sunderland, Massachusetts. Pp 15.

Stanford, J. A., Prescott, G. W. (1988). Limnological Features of a Remote Alpine Lake in Montana, including a New Species of *Cladophora* (Chlorophyta).

Stevens, R. J. J., Neilson, M. A. (1987). Environmental control of *Pithophora oedogonia* (Chlorophyceae) akinete germination. Journal of Phycology (16): 424-427.

Suda, S., Nozaki, H., Watanabe, M.M. (2005). Morphology and sexual reproduction of Carteria palmata sp nov belonging to the Carteria group I sensu Lembi (Chlorophyceae, Volvocales). Phycologia 44 (6): 596-607

Swofford, D.L. (2003). *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods).* Version 4. Sinauer Associates, Sunderland, Massachisetts.

Sultan, S.E. (2001). Phenotypic plasticity and ecological breadth in plants. Animal Zoology (41): 1599

Sunnucks, P. (2000). Efficient genetic markers for population biology. Trends in Ecology and Evolution. (15): 199-203.

Tsumura, T., Ohba, K., Strauss, S. H. (1996). Diversity and inheritance of inter-simple sequence repeat polymorphism in Douglas-fir and sugi. Teoretical and Applied Genetics. (92): 40.

Tyler, P. A. (1996). Endemism in freshwater algae. Hydrobiologia (336): 127-135.

van den Hoek, C. (1963). *Revision of the European species of Cladophora. Proefschrift...Rijksuniversiteit te Leiden*. pp. XI + 248, 1 fig, 55 plates, 18 maps. Leiden: E. J. Brill.

van den Hoek, C. (1982). A Taxonomic Revision of te America Species of *Cladophora* (Chlorophyceae) in Norht–Atlantic Ocean and their Geographic Distribution. Norht-Holland Publishng Company, Amersterdam.

van den Hoek, C., Mann, D. G., Jahns, H. M. (1995). Algae: An introduction to phycology. Cambridge University Press415-417. Pp

van Hannen, E. J., Lurling, M., van Donk, E. (2000). Sequence analysis of the ITS-2 region: a tool to identify strains of Scenedesmus (Chlorophyceae). Journal of Phycology(36): 605-607.

Van Oppen, M. J. H., Draisma, S. G. A., Olsen, J. L., Stam, W. T. (1995). Multiple trans-Arctic passages in the red alga *Phycodrys rubens*: evidence from nuclear rDNA ITS sequences. Marine Biology (123): 179-188.

Vis, M. L. (1999). Intersimple sequence repeats (ISSR) molecular markers to distinguish gametophytes of Batrachospermum boryanum (Batrachospermales, Rhodophyta). Phycologia. (38): 70-73.

Vis, M. L., Sheath, R. G. (1997). Biogeography of Batrachospermum gelatinosum

(Batrachospermales, Rhodophyta) in North AmeRico based on Molecular and Morphological Data. Journal of Phycology (33): 520-526.

Vis, M. L., Sheath, R.. G., Entwisle, T. J. (1995). Morphometirc analysis of Batrachospermum section Batrachospermum (Batrachospermales, Rhodophyta) type specimens. European Journal of Phycology (30): 35-55.

Whitton, B. A. (1970). Biology of *Cladophora* in freshwater. Water Research (4): 457-476.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingery, S.V. (1990). Genetic analysis using random amplified polymorphic DNA markers. Methods in Enzymology (218): 704-740.

Wilson, K. P., Shannon, J. P., Blinn, D. W. (1999). Effects of suspended sediment on biomass and cell morphology of *Cladophora glomerata* (Chlorophyta) in the Colorado River, Arizona. Journal of Phycology (35): 35-41.

Wolfe, A. (2005). ISSR Techniques for Evolutionary Biology. Methods in Enzymology (395): 134-144.

Yotsurkura, N., Denboh, T., Motomura, T., Horiguchi, T., Coleman, A. W., Ichimura, T. (1999). Little diergence in ribosomal DNA internal transcribed spacer -1 and -2 sequences among non-digitate species of Laminaria (Phaeophyceae) from Hokkaido, Japan. Phycological Research (47): 71-80.

Zietkiewicz, E., Rafalski, A., Labuda, D. (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplifications. Genomics (20): 176-183.

Zimmer, E. A., Hamby, R. K., Arnold, M. L., Leblanc, D. A., Theriot, E. C. (1989). Ribosomal RNA phylogenies and flower plant evolution. *In* Fernholm, B., Bremer, D., Jornvall, H. [Eds.] The Hierarchy of Life. Proceedings of the Nobel Symposium, Elsevier, Amsterdam, (70): 205-214.

Appendix A

Arizona 200	Collected August 6, 1998 at Montezuma Well by R. Sheath
Arizona 203	Collected August 6, 1998 at Beaver Creek by R. Sheath
Blue Springs Creek Ontario	Collected June5, 2005 by A. Gill and K. Müller
British Columbia	Collected August 12, 2005 by J. Witt
California	Collected July 2004 by R. Sheath
Costa Rico (*Rhizoclonium*)	Collected February 22, 1998 by R. Sheath and K. Müller
Flordia	(Little Miami River) Collected November 24, 1997 by A. Sherwood
Florida (*Rhizoclonium*)	Collected April 13, 2000 by K. Müller and A. Sherwood
Guelph Ontario (Guelph Lake)	Collected July 13, 1999 by A. Sherwood
Hawaii 1	Collected June 3, 2005 by A. Sherwood
Hawaii 2	Collected August 28, 2000 by A. Sherwood
Lake Erie (*Rhizoclonium*)	Collected October 7, 1997 by M. Vis
Michigan (Lions)	Collected June 12, 2002 by T. Shea
Waterloo Local Area Pond	Collected July 2, 2005 by J. Semple
Mexico 18	Collected April 26, 1997 by R. Sheath
Mexico 8	Collected April 26, 1997 by R. Sheath
Nova Scotia	Collected December 26, 1997, by A. Sherwood
Ohio 1	Collected by R. Sheath
Ohio 2	Collected July 15, 1997, by M. Vis
Oregon (Elk River)	Collected June 9, 1998 by R. Sheath
Ottawa (Ottawa River)	Collected by K. Müller
Puerto Rico	Collected October 18, 1997 by A. Sherwood and T. Rintoul
Texas 1 (San Marocos)	Collected May 31, 1997 by R. Sheath
Texas 2 (Corpus Christi)	Collected by K. Müller (Corpus Chrisiti)
Texas 3 (Corpus Christi)	Collected by K. Müller (Corpus Chrisiti)
Texas 4	Collected December 31, 1996 by A. Sherwood
Winnipeg	Collected July 25, 2005 by S. Guildford
Wisconsin (Swauk River)	Collected November 2, 1997 by R. Sheath

Appendix B

Collection Location	**Primer ISSR17**																			
California	0	0	0	1	0	1	1	0	1	0	0	0	0	0	0	0	1	0	0	0
Texas 3 (Corpus Christi 1)	0	0	0	0	0	0	1	0	0	1	0	1	0	0	1	0	1	1	0	0
Texas 4 (Corpus Christi 2)	0	0	0	0	1	1	1	0	0	0	0	1	0	1	0	0	1	1	1	0
Oregon (Elk River)	0	0	0	0	1	0	0	0	0	1	1	0	1	0	0	0	1	1	1	0
Guelph Lake Ontario	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	1	0
Hawaii 1	1	0	0	0	0	0	0	0	0	1	1	0	1	1	0	1	1	0	0	0
Hawaii 2	0	0	0	0	0	0	1	0	1	1	0	0	1	0	1	0	1	1	1	0
Ohio 2 (Hocking River)	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	1
Local Waterloo Pond	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	1	1	0	1	0
Michigan Lions	0	0	0	0	0	0	1	0	0	1	0	0	1	0	1	1	1	0	0	1
Florida (Miami River)	0	1	0	0	0	0	1	0	0	1	0	1	1	1	0	1	1	1	1	0
Mexico 8	0	0	0	1	0	1	1	1	0	1	1	1	0	1	1	1	0	1	1	1
Mexico 18	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	1	1	1
Ohio 1	1	0	0	0	0	0	0	1	1	1	0	0	0	1	0	1	1	1	1	0
Ottawa River	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	1	1	1	1	0
Puerto Rico	0	1	0	1	0	1	0	1	0	1	1	1	1	0	1	1	1	1	1	0
Nova Scotia (Halifax)	0	1	0	0	1	0	1	1	0	0	1	1	0	1	1	0	1	1	1	0
Texas 1 (San Marcos)	0	1	0	0	0	0	1	0	0	0	0	1	0	1	0	1	1	1	1	1
Wisconsin (Swauk River)	0	0	0	0	1	1	1	0	0	1	1	1	1	0	0	1	1	1	1	1
Rhizoclonium Lake Erie	0	1	0	0	0	0	1	1	0	0	0	1	0	0	0	0	1	1	1	1
Rhizoclonium Costa Rico	0	0	0	0	1	0	1	0	1	1	0	0	0	1	0	0	1	1	0	1
Rhizoclonium Florida	0	1	0	0	0	1	1	0	1	0	1	0	1	0	0	1	1	0	1	0
Texas 2	0	1	0	0	1	1	1	1	0	1	1	1	1	0	1	0	1	1	0	0
Cl. fracta var. *fracta*	0	0	0	0	1	1	1	0	0	1	0	0	1	0	1	0	1	1	0	0

Collection Location	Primer ISSR17																			
Cl. glomerata	0	0	0	0	1	0	0	0	1	0	1	0	1	0	0	1	1	1	0	1
Winnipeg	1	0	0	0	0	1	1	0	1	1	0	0	0	1	0	1	1	1	1	0

Collection Location	Primer ISSR12																			
LO1	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	1	1	0	0
LO10	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	1	1	1	0	0
LO6	0	0	0	0	0	1	1	0	0	1	0	1	1	0	0	1	1	0	1	0
LO7	0	0	0	0	1	1	0	1	0	1	0	1	1	0	0	1	0	1	1	0
LO9	0	0	0	0	1	1	1	0	0	1	0	0	1	0	0	1	1	0	1	0
LO15	0	0	0	0	1	1	1	0	0	1	0	0	1	0	0	1	1	1	0	0
LO East Bay	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0	0	1	0	0
LO West Bay	0	0	0	0	1	0	0	0	0	1	0	1	1	0	0	0	0	1	1	0
LE1	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	1	0	1	0
LE4	0	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	1	0	1	0
LE9	0	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	1	0	1	0
LE13	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0
LM1	0	0	0	0	1	0	0	1	0	1	0	0	1	1	0	1	0	1	0	0
LM2	0	0	0	0	1	0	0	1	0	1	0	0	1	1	0	1	0	1	0	0
LM7	0	0	0	0	1	0	0	1	0	1	0	0	1	1	0	1	0	1	0	0
LM20	0	0	1	0	1	0	0	1	0	1	0	0	0	1	0	0	1	1	0	0
LM23	0	0	0	1	1	0	0	1	0	1	0	0	0	1	0	0	1	1	0	0
LH4	0	0	0	0	1	1	1	0	0	0	0	0	1	0	0	0	1	1	0	0
LH7	0	0	0	0	1	1	1	0	0	0	0	0	1	0	0	0	1	1	0	0
LH10	0	0	0	0	1	1	1	0	0	0	0	0	1	0	0	0	1	1	0	0
LH13	0	0	0	1	0	1	1	0	0	0	0	0	1	0	0	0	1	1	0	0
LH17	0	0	0	1	0	1	1	0	0	0	0	0	1	0	0	0	1	1	0	0
Arizona 200	1	0	0	0	1	0	0	1	0	1	0	1	1	0	1	0	1	1	1	0

Collection Location	**Primer ISSR12**																			
California	0	0	0	0	0	0	1	1	0	1	0	1	0	0	1	1	1	1	0	0
Texas 3 (Corpus Christi 1)	0	0	0	0	1	0	1	1	0	1	0	0	0	1	0	1	1	0	1	0
Texas 4 (Corpus Christi 2)	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	1	0	1	0
Oregon (Elk River)	0	0	0	0	1	1	1	1	0	0	1	1	0	1	0	0	1	0	1	0
Guelph Lake Ontario	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	1	1	1	1
Hawaii 1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	0	1	1	1	1
Hawaii 2	0	0	0	0	1	1	1	0	0	1	0	1	0	0	0	0	0	1	1	1
Ohio 2 (Hocking River)	0	0	0	0	1	0	1	1	0	1	1	1	0	1	0	1	1	1	0	1
Local Waterloo Pond	0	0	0	0	1	0	0	1	0	0	0	1	1	0	0	0	0	0	1	0
Michigan Lions	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	0	1	1	1	0
Florida (Miami River)	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	0	1	1	1	1
Mexico 8	0	0	0	0	0	0	0	1	0	1	1	0	1	1	0	0	1	0	0	1
Mexico 18	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	1	1	1	0
Ohio 1	0	0	1	0	1	0	0	0	0	1	1	1	1	1	1	0	1	1	0	1
Ottawa River	0	0	0	0	1	1	1	0	1	0	1	0	1	1	0	1	1	1	1	1
Puerto Rico	0	0	0	0	1	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1
Nova Scotia (Halifax)	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	1	0
Texas 1 (San Marcos)	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	1	1	1	1	0
Wisconsin (Swauk River)	0	0	0	0	1	1	1	0	0	0	0	0	1	0	0	0	1	1	1	1
Rhizoclonium Lake Erie	0	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	1	1	1	0
Rhizoclonium Costa Rico	0	0	0	0	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0
Rhizoclonium Florida	0	0	0	0	1	0	1	1	0	0	0	1	1	0	0	1	1	1	0	1
Texas 2	1	0	0	1	0	0	1	1	0	1	0	1	0	0	0	0	0	1	0	0
Cl. fracta var. *fracta*	0	0	0	0	0	0	1	1	0	1	0	1	0	0	1	1	1	1	0	0
Cl. glomerata	0	0	0	0	1	0	1	1	0	1	0	0	0	1	0	1	1	0	1	0

Collection Location	Primer ISSR12																				
UTEX1486	1	0	0	1	0	0	1	1	0	1	0	1	0	0	0	0	0	1	0	0
Winnipeg	0	0	0	0	1	1	0	1	0	1	0	0	1	1	0	1	1	0	1	0

Collection Location	Primer ISSR14																			
LO1	1	0	0	0	0	0	1	1	0	1	0	1	0	0	0	0	0	1	0	1
LO10	0	0	0	0	0	0	1	1	0	1	0	1	0	0	0	1	0	1	0	1
LO6	1	0	0	0	0	0	1	1	0	1	0	1	0	0	0	0	0	0	0	1
LO7	1	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	1
LO9	1	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	1
LO15	1	0	0	0	0	0	1	1	0	1	0	1	0	0	1	0	0	0	0	1
LO East Bay	1	0	0	0	0	0	0	1	0	1	0	1	0	0	1	0	0	1	0	1
LO West Bay	1	0	0	0	0	0	1	1	0	1	0	1	0	0	0	0	0	1	0	1
LE1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	1	1
LE4	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1
LE9	0	0	0	0	0	0	1	1	0	1	0	1	0	0	1	1	0	1	1	0
LE13	0	0	0	0	1	0	1	1	0	1	0	1	0	0	1	0	0	1	0	1
LM1	0	0	0	0	0	0	0	1	0	1	0	1	0	0	1	0	1	1	1	0
LM2	0	0	0	0	0	0	0	1	0	1	0	1	0	0	1	0	1	1	1	0
LM7	0	0	0	0	0	0	0	1	0	1	0	1	0	0	1	0	1	1	0	0
LM20	0	0	0	0	0	0	1	1	0	1	0	1	0	0	0	0	1	1	1	0
LM23	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0
LH4	0	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	1	0	0
LH7	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0	0	1	0	0
LH10	1	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	1	0	1
LH13	1	0	0	0	0	0	1	1	0	1	0	1	0	0	1	0	1	1	1	0
LH17	1	0	0	0	0	0	1	1	0	1	0	0	0	0	0	1	1	0	1	0
Arizona 200	0	0	0	0	1	0	1	1	0	1	0	1	0	0	0	1	0	0	1	0
Arizona 203	0	0	0	0	1	0	1	1	0	1	0	1	0	0	0	1	0	0	0	1

Collection Location	**Primer ISSR14**																			
California	0	0	0	0	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0	1
Texas 3 (Corpus Christi 1)	0	1	0	0	1	1	0	1	0	1	0	0	1	0	1	0	1	0	1	0
Texas 4 (Corpus Christi 2)	0	0	0	0	0	1	0	0	0	1	1	0	0	0	1	0	1	1	0	1
Oregon (Elk River)	0	1	0	0	1	0	1	0	1	1	0	1	0	1	1	0	1	0	1	0
Guelph Lake Ontario	1	1	0	1	0	0	0	0	0	1	0	1	0	1	0	0	1	0	0	0
Hawaii 1	0	0	0	0	1	1	0	1	0	0	1	0	0	0	0	0	0	1	1	0
Hawaii 2	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	1	1	1	1	1
Ohio 2 (Hocking River)	1	1	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	0	1
Local Waterloo Pond	1	0	1	0	0	1	1	0	0	0	0	1	0	0	0	0	1	0	0	0
Michigan Lions	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	1	1
Florida (Miami River)	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	1	1
Mexico 8	0	0	0	0	0	0	1	0	1	1	0	1	0	0	1	1	1	1	1	1
Mexico 18	0	1	0	0	1	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0
Ohio 1	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	1	1	1	1
Ottawa River	0	0	0	0	1	0	0	1	1	1	1	0	1	0	1	0	1	1	0	1
Puerto Rico	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	1	1	1	0
Nova Scotia (Halifax)	0	1	0	0	1	1	1	0	0	1	0	0	1	0	1	0	1	0	0	0
Texas 1 (San Marcos)	0	0	0	0	1	1	0	1	0	0	0	0	0	0	1	1	1	1	1	0
Wisconsin (Swauk River)	0	0	0	1	1	1	1	0	0	0	0	1	1	0	1	1	1	1	1	1
Rhizoclonium Lake Erie	1	1	0	0	1	1	1	0	0	1	1	0	0	0	0	0	1	1	1	0
Rhizoclonium Costa Rico	0	0	0	0	1	0	1	0	1	0	1	1	0	0	0	0	1	1	0	1
Rhizoclonium Florida	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	1	1	1
Texas 2	0	0	1	0	1	0	1	1	1	1	0	1	1	0	0	0	1	1	0	1
Cl. fracta var. *fracta*	0	0	0	0	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0	1
Cl. glomerata	0	1	0	0	1	1	0	1	0	1	0	0	1	0	1	0	1	0	1	0

Collection Location	Primer ISSR14																			
UTEX1486	0	0	1	0	1	0	1	1	1	1	0	1	1	0	0	0	1	1	0	1
Winnipeg	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	1	0	0	1

Collection Location	Primer ISSR15																			
LO1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1
LO10	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	1
LO6	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	1	0	1
LO7	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	1	1	0	1
LO9	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	1	1	1
LO15	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0	1
LO East Bay	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	1	1	1
LO West Bay	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LE1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	1	1	0
LE4	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	1	0	1
LE9	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	1	0	1
LE13	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	1	1	1	1
LM1	0	0	0	0	1	0	0	1	0	0	1	0	0	1	0	1	1	0	1	1
LM2	0	0	0	0	1	0	0	1	0	0	1	0	0	1	0	1	1	0	0	1
LM7	0	0	0	0	1	0	0	1	0	0	0	1	0	1	0	1	1	1	1	1
LM20	0	0	0	0	1	0	0	1	0	0	1	1	0	0	0	1	1	1	1	1
LM23	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	1	0	1	1	1
LH4	0	0	0	0	0	0	0	1	0	0	1	1	0	1	0	1	0	1	0	0
LH7	0	0	0	0	0	0	0	1	0	0	1	1	0	1	0	1	1	1	1	1
LH10	0	0	0	0	0	0	0	1	0	0	1	1	1	1	0	1	1	1	0	1
LH13	0	0	0	0	1	0	0	0	0	0	1	1	0	1	0	1	1	1	0	1
LH17	0	0	0	0	1	0	0	0	0	0	1	1	0	1	0	1	1	1	0	1
Arizona 200	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	1	1	0	0
Arizona 203	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	1	1	0	0
British Columbia	0	0	0	0	1	0	1	0	0	1	0	0	0	1	0	0	1	1	1	0

Collection Location	**Primer ISSR15**																			
California	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	1	1	1	1	1
Texas 3 (Corpus Christi 1)	0	0	0	0	0	1	0	1	0	1	0	1	1	0	0	0	1	1	0	0
Texas 4 (Corpus Christi 2)	0	0	0	0	0	1	0	1	0	1	0	1	1	0	0	0	1	1	0	1
Oregon (Elk River)	0	0	0	0	0	1	0	0	1	0	1	0	0	1	0	0	1	1	1	0
Guelph Lake Ontario	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	0	1	1	1	0
Hawaii 1	1	0	0	0	1	0	0	0	1	0	1	1	0	0	1	0	1	1	0	1
Hawaii 2	1	0	0	0	1	1	0	1	1	0	1	0	1	0	1	1	1	1	1	1
Ohio 2 (Hocking River)	0	0	0	0	0	0	0	1	0	1	0	1	1	0	0	0	1	1	1	0
Local Waterloo Pond	0	0	0	0	1	1	0	1	0	1	0	1	0	1	0	1	1	1	1	1
Michigan Lions	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	1	1	1	0	1
Florida (Miami River)	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	1	1	1	1	0
Mexico 8	0	0	0	0	0	0	1	0	1	1	0	1	0	1	0	1	1	1	1	0
Mexico 18	0	0	0	0	0	0	1	0	1	1	0	1	0	1	0	0	1	1	1	1
Ohio 1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	1	1	0	1
Ottawa River	0	0	0	0	1	0	1	1	0	1	1	0	1	0	1	1	1	0	1	0
Puerto Rico	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	1	1	1	0	1
Nova Scotia (Halifax)	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	1	1	0	1
Texas 1 (San Marcos)	0	0	0	0	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0
Wisconsin (Swauk River)	0	0	0	0	0	0	0	1	0	1	0	1	0	1	0	0	1	1	0	1
Rhizoclonium Lake Erie	0	0	0	0	1	0	1	1	0	1	0	1	0	0	0	1	1	1	0	1
Rhizoclonium Costa Rico	0	0	0	0	1	0	1	1	0	1	0	1	1	0	0	1	1	1	1	0
Rhizoclonium Florida	0	0	0	0	1	0	1	1	0	0	0	1	1	0	0	1	1	1	1	0
Texas 2	0	0	0	0	0	1	0	1	0	1	0	0	1	0	1	0	1	1	0	0
Cl. fracta var. *fracta*	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	1	1	1	1

Collection Location	Primer ISSR15																			
UTEX1486	0	0	0	0	0	1	0	0	0	1	0	1	0	1	0	1	1	0	0	1
Winnipeg	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0	1	1	1	1

Collection Location	Primer ISSR10																			
LO1	0	0	0	1	0	0	0	0	1	1	0	0	1	1	0	0	1	1	0	0
LO10	0	0	0	1	0	0	0	0	1	1	0	0	1	0	1	0	1	1	0	0
LO6	0	0	0	1	0	0	0	0	1	1	0	0	1	0	1	0	1	1	0	0
LO7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LO9	0	0	0	1	0	0	1	0	1	1	0	0	0	1	1	1	1	1	0	0
LO15	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	1	1	0	0	0
LO East Bay	0	0	0	1	0	0	1	0	0	1	0	1	0	0	1	1	1	1	0	0
LO West Bay	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LE1	1	0	0	0	0	0	0	0	1	0	0	1	1	1	0	0	1	1	0	0
LE4	1	0	0	0	0	1	0	0	1	0	1	1	1	1	0	0	1	1	0	1
LE9	1	0	0	0	1	1	1	0	1	0	1	1	1	1	0	0	1	1	0	1
LE13	1	0	0	0	0	1	0	0	1	0	1	1	0	1	0	0	1	1	0	0
LM1	0	0	0	0	1	0	0	0	1	0	0	1	0	1	0	0	1	1	0	0
LM2	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	1	1	0	1
LM7	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1
LM20	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1	0	0	0
LM23	0	0	0	0	1	0	1	1	0	1	1	0	0	0	0	0	1	1	0	0
LH4	1	0	0	0	1	0	1	1	0	1	1	0	0	1	0	0	1	1	0	0
LH7	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	1	1	0	0
LH10	1	0	0	0	1	0	1	1	0	1	1	0	0	1	1	0	1	0	0	0
LH13	1	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0	1	1	0	1
LH17	1	0	0	0	1	0	1	1	0	0	1	0	0	1	0	0	1	1	0	1

Collection Location	**Primer ISSR10**																			
California	0	1	0	0	0	1	0	1	0	1	0	1	1	1	0	0	1	1	1	1
Texas 3 (Corpus Christi 1)	0	0	1	0	1	1	1	1	0	0	1	0	0	0	1	0	1	1	0	1
Texas 4 (Corpus Christi 2)	0	0	1	0	1	1	1	1	0	0	1	0	0	0	1	0	1	1	0	1
Oregon (Elk River)	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	1	1	0	0
Guelph Lake Ontario	0	0	0	0	1	0	0	0	0	1	0	1	0	1	0	1	1	0	1	1
Hawaii 1	1	0	0	0	1	0	1	0	1	1	0	1	0	1	1	0	1	1	0	0
Hawaii 2	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1	0	1
Ohio 2 (Hocking River)	0	0	1	0	0	1	0	0	0	0	1	0	1	0	1	0	1	1	0	1
Local Waterloo Pond	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1
Michigan Lions	1	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	1	1	0	1
Florida (Miami River)	0	0	0	0	0	0	1	0	1	1	0	1	0	1	0	1	1	1	0	1
Mexico 8	0	0	0	0	1	1	1	0	0	0	1	0	1	0	0	0	1	0	0	0
Mexico 18	1	0	0	0	1	0	1	0	0	0	1	0	1	0	0	0	1	0	0	0
Ohio 1	1	0	0	1	1	0	0	0	0	1	0	0	0	0	1	0	1	0	0	1
Ottawa River	0	0	1	1	1	1	1	1	0	1	0	0	0	1	0	0	1	1	0	1
Puerto Rico	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	1	1	1
Nova Scotia (Halifax)	0	0	0	0	0	1	1	0	1	0	1	0	1	0	0	1	1	0	1	1
Texas 1 (San Marcos)	0	0	0	0	0	1	0	1	0	0	1	1	0	1	0	1	1	0	1	0
Wisconsin (Swauk River)	1	0	0	0	0	0	0	0	0	0	1	0	1	1	0	1	1	1	0	0
Rhizoclonium Lake Erie	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	1	0	1
Rhizoclonium Costa Rico	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	1	1	1	0	0
Rhizoclonium Florida	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	1	1	1	1	1
Texas 2	0	0	0	0	1	0	1	0	0	1	0	1	0	0	1	0	1	1	0	0
Cl. fracta var. *fracta*	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	1	1

Collection Location	Primer ISSR15																			
Cl. glomerata	0	0	0	0	1	1	0	1	0	0	0	1	0	0	0	0	1	1	0	1
Winnipeg	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	1	0	1

Appendix C

Sample Number	Collection Information	Latitude & Longitude
22 LH12	Lexington State Harbor, Huron Ave. off Hwy 25, Lexington, MI, USA. 29 June 05. S. Ross and M. Sawchuck	43° 16’ 7N 82° 32’ 2W
23 LH13	Near Coast Guard station, off Hwy 25, Harbor Beach, MI, USA. 29 June 05. S. Ross and M. Sawchuck	43° 50’ 48N 82° 39’ 18W
24 LH14	Channel breakwall, County Road, off Hwy 25, Caseville, MI, USA. 29 June 05. S. Ross and M. Sawchuck	43° 56’ 32N 83° 16’ 28W
25 LH15	Au Gres River Channel Pier, off Hwy 25, Au Gres, MI, USA. 29 June 05. S. Ross and A. Gill	44° 2’ 39N 83° 41’ 38W
26 LH16	Park behind Police Station, off Hwy 23, Tawas City, MI, USA29 June 05. S. Ross and M. Sawchuck	44° 16’ 2N 83° 31’ 19W
27 LH17	Starlite Beach, off Hwy 23, Alpena, MI, USA. 29 June 05. S. Ross and A. Gill.	45° 4’ 22N 83° 26’ 15W
28 LH18	Harbor and boat Launch, Lake Ave. & Huron Ave., Rogers City, MI, USA. 29 June 05. S. Ross and M. Sawchuck	45° 25’ 7N 83° 48’ 23W
29 LH19	Gordon Turner Park Lighthouse Pier, Huron St., Cheboygan, MI, USA 29 June 05. S. Ross and M. Sawchuck	45° 38’ 30N 84° 28’ 8W
1 LE1	Behind Hoak’s Lakeshore Restaurant, off Hwy 5, Athol Springs, NY, USA. 17 Apr 02. T.B. Shea & K.M. Müller.	42° 53’ 23N 78° 51’ 35W
2 LE2	Small boat park, Lakeshore St., Dunkirk, NY, USA. 17 Apr 02. T.B. Shea & K.M. Müller.	42° 33’ 55N 79° 19’ 27W
3 LE3	Lawrence Park, off Hwy 5, Erie, PA, USA. 17 Apr 02. T.B. Shea & K.M. Müller.	42° 6’ 12N 80° 6’ 15W
4 LE4	Dirt road off marina parking lot, Broad St., Conneaut, OH, USA. 17 Apr 02. T.B. Shea & K.M. Müller.	41° 55’ 35N 80° 34’ 13W
5 LE5	Edgefront State Park, Cleveland, OH, USA. 17 Apr 02. T.B. Shea & K.M. Müller.	41° 28’ 47N 81° 40’ 43W
6 LE6	Battery Park Marina, Sandusky, OH, USA. 17 Apr 02. T.B. Shea & K.M. Müller.	41° 27’ 21N 82° 42’ 52W

Sample Number	Collection Information	Latitude & Longitude
7	Waterfront off Hwy 7, Luna Pier, MI, USA. 17 Apr 02. T.B. Shea & K.M. Müller.	41° 48’ 18N
LE7		83° 26’ 33W
8	Rondeau Provincial Park, Rondeau, ON, Canada. 18 Apr 02. T.B. Shea & K.M. Müller.	42° 19’ 60N
LE9		82° 0’ 0W
9	Harbour, Port Stanley, ON, Canada. 18 Apr 02. T.B. Shea & K.M. Müller.	42° 40’ 0N
LE10		81° 13’ 0W
10	Long Point, ON, Canada. 18 Apr 02. T.B. Shea & K.M. Müller.	42° 34’ 12N
LE11		80° 3’ 0W
11	Marina, Port Dover, ON, Canada. 18 Apr 02. T.B. Shea & K.M. Müller.	42° 46’ 60N
LE12		80° 12’ 0W
12	Port Colborne, ON, Canada. 18 Apr 02. T.B. Shea & K.M. Müller.	42° 52’ 60N
LE13		79° 13’ 60W
13	Silver Beach County Park, Lake St. & Broad St., St. Joseph, MI, USA. 8 May 02. T.B. Shea &	42° 5’ 49N
LM1	A.B. Shea.	86° 29’ 29W
14	Washington Park Marina, Lakeshore Rd. & Brown Basin St., Michigan City, IN, USA. 8 May	41° 42’ 45N
LM2	02. T.B. Shea & A.B. Shea.	86° 52’ 34W
15	Beach at 1st St. & 3rd Ave., Menominee, MI, USA. 10 May 02. T.B. Shea & A.B. Shea.	45° 7’ 16N
LM13		87° 37’ 25W
16	Access at South 2nd St. & Luddington St., Escanaba, MI, USA. 10 May 02. T.B. Shea & A.B.	45° 44’ 46N
LM14	Shea.	87° 4’ 51W
17	Bayfront Park, Petoskey, MI, USA. 11 May 02. T.B. Shea & A.B. Shea.	45° 21’ 58N
LM18		84° 57’ 18W
18	Marina at Clinch Park, Traverse City, MI, USA. 11 May 02. T.B. Shea & A.B. Shea.	44° 45’ 20N
LM19		85° 36’ 10W
19	Pier at 1st Street Beach, Manistee, MI, USA. 11 May 02. T.B. Shea & A.B. Shea.	44° 14’ 41N
LM20		86° 19’ 35W
20	Whitehall Marina, Whitehall, MI, USA. 11 May 02. T.B. Shea & A.B. Shea.	43° 23’ 54N
LM21		86° 20’ 29W
21	South side of channel wall, Grand Haven, MI, USA. 11 May 02. T.B. Shea & A.B. Shea.	43° 3’ 19N
LM22		

Appendix D

Sampling Date	Plant #	Diameter of Main Axis (µm)	Length of Main Axis (µm)	Diameter of Branch (µm)	Length of Branch (µm)	Diameter of Apical Cell (µm)	Length of Apical Cell (µm)
26-May-04	1	56	673	46	836	62	425
	2	68	750	52	674	47	355
	3	68	684	58	780	46	441
	4	78	664	62	595	68	365
	5	79	734	56	824	60	348
	6	72	721	70	784	57	455
	7	79	825	73	735	66	294
	8	64	685	63	621	76	306
	9	66	647	50	785	51	526
15-Jun-04	1	93	569	58	562	51	42
	2	80	598	55	498	64	598
	3	83	489	50	698	70	489
	4	71	368	67	523	32	368
	5	92	496	74	578	74	496
	6	76	559	69	499	76	559
	7	80	672	59	694	77	672
	8	103	549	64	777	69	549
	9	96	375	54	769	61	375
22-Jun-04	1	109	756	82	691	25	756
	2	90	598	81	880	86	598
	3	103	684	81	798	68	684
	4	106	448	88	725	74	448
	5	111	987	71	477	53	987
	6	104	882	74	794	62	882

Appendix E

Sampling Date	Plant #	Diameter of Main Axis (µm)	Length of Main Axis (µm)	Diameter of Branch (µm)	Length of Branch (µm)	Diameter of Apical Cell (µm)	Length of Apical Cell (µm)
	7	109	497	58	339	68	497
	8	108	594	67	684	69	594
	9	114	471	76	485	73	471
29-Jun-04	1	113	1159	85	482	62	294
	2	88	662	98	672	53	301
	3	99	430	97	503	66	419
	4	91	767	93	506	66	354
	5	96	874	79	564	65	299
	6	99	816	76	625	51	276
	7	106	597	93	599	50	306
	8	104	447	81	578	58	299
	9	113	669	82	692	62	275
6-Jul-04	1	106	595	74	483	53	212
	2	111	853	88	373	89	241
	3	113	692	108	305	74	283
	4	123	777	91	396	80	306
	5	98	843	80	489	86	311
	6	99	761	64	477	80	278
	7	94	691	73	420	51	324
	8	84	59	76	598	81	306
	9	90	761	47	558	74	488
13-Jul-04	1	131	328	100	444	45	153
	2	93	505	105	356	75	285
	3	126	599	118	381	75	261

Sampling Date	Plant #	Diameter of Main Axis (µm)	Length of Main Axis (µm)	Diameter of Branch (µm)	Length of Branch (µm)	Diameter of Apical Cell (µm)	Length of Apical Cell (µm)
	4	119	590	80	676	74	247
	5	115	752	63	663	48	218
	6	110	668	90	420	60	275
	7	93	610	55	384	49	232
	8	109	399	80	554	80	362
	9	74	427	81	509	73	258
20-Jul-04	1	89	598	92	549	58	234
	2	103	568	82	678	72	160
	3	82	687	64	623	46	158
	4	94	468	70	591	54	423
	5	89	877	82	469	70	238
	6	81	947	94	661	54	365
	7	92	752	89	509	73	251
	8	104	778	92	667	58	374
	9	106	654	103	788	56	355
3-Aug-04	1	114	730	65	464	66	474
	2	117	453	93	563	58	339
	3	100	386	103	688	77	460
	4	91	341	112	447	49	204
	5	143	625	102	541	59	398
	6	98	516	72	433	51	208
	7	103	588	85	862	54	308
	8	120	935	79	639	53	324
	9	126	881	113	575	59	397

Sampling Date	Plant #	Diameter of Main Axis (µm)	Length of Main Axis (µm)	Diameter of Branch (µm)	Length of Branch (µm)	Diameter of Apical Cell (µm)	Length of Apical Cell (µm)
16-Aug-04	1	101	379	65	470	44	170
	2	102	821	61	568	49	201
	3	91	437	83	329	58	225
	4	111	535	104	782	70	284
	5	108	691	93	371	48	269
	6	105	433	95	326	66	237
	7	111	700	84	489	48	266
	8	124	249	120	406	70	366
	9	90	473	81	613	70	222
11-Sep-04	1	90	530	79	648	59	448
	2	102	4537	100	726	34	396
	3	83	388	73	555	73	588
	4	80	449	102	658	66	199
	5	102	629	101	618	57	294
	6	112	563	114	803	62	256
	7	111	555	81	752	76	379
	8	91	898	102	661	47	321
	9	101	883	84	445	84	356
22-Sep-04	1	124	796	72	319	68	170
	2	131	773	100	167	54	201
	3	118	404	90	322	57	225
	4	107	343	82	334	65	284
	5	96	806	55	202	81	269
	6	107	500	65	235	72	237

Sampling Date	Plant #	Diameter of Main Axis (µm)	Length of Main Axis (µm)	Diameter of Branch (µm)	Length of Branch (µm)	Diameter of Apical Cell (µm)	Length of Apical Cell (µm)
	7	116	364	57	230	68	266
	8	123	491	86	436	73	366
	9	111	489	80	298	66	222
8-Oct-04	1	140	533	100	456	71	173
	2	136	878	88	310	66	232
	3	131	761	71	229	59	205
	4	138	746	114	266	88	193
	5	123	555	54	376	87	273
	6	115	747	154	343	105	312
	7	133	838	127	457	61	148
	8	120	570	191	631	74	178
	9	121	596	132	829	60	22

Sampling Location	Plant #	Diameter of Main Axis (µm)	Length of Main Axis (µm)	Diameter of Branch (µm)	Length of Branch (µm)	Diameter of Apical Cell (µm)	Length of Apical Cell (µm)
LH4	1	126	446	58	340	39	361
	2	85	388	58	294	38	220
	3	80	424	56	524	33	239
	4	94	595	52	297	34	233
	5	88	625	45	333	44	325
	6	79	596	66	596	59	396
	7	82	666	59	685	45	485
	8	91	589	57	547	68	378
	9	99	699	66	512	45	501
	10	106	812	62	412	40	258
LH7	1	87	267	80	287	57	284
	2	100	547	66	335	59	279
	3	81	314	86	252	63	204
	4	76	330	77	215	51	238
	5	61	728	60	347	63	422
	6	88	648	59	574	77	567
	7	78	598	66	499	67	333
	8	68	498	87	654	91	497
	9	71	877	97	485	88	597
	10	78	598	89	580	82	675
		78.8	540.5	76.7	422.8	69.8	409.6

Sampling Location	Plant #	Diameter of Main Axis (µm)	Length of Main Axis (µm)	Diameter of Branch (µm)	Length of Branch (µm)	Diameter of Apical Cell (µm)	Length of Apical Cell (µm)
LH10	1	106	947	81	395	59	299
	2	97	582	56	417	49	203
	3	106	827	68	471	54	350
	4	101	497	64	547	64	335
	5	98	689	58	591	63	252
	6	84	506	48	555	98	215
	7	88	806	44	539	79	398
	8	95	901	89	690	66	408
	9	107	577	77	378	59	332
	10	106	579	68	437	37	353
LH13	1	117	784	61	687	34	272
	2	98	875	77	598	55	359
	3	88	962	64	513	67	346
	4	75	798	59	679	94	321
	5	79	669	88	790	63	485
	6	83	982	80	559	36	501
	7	112	743	74	597	24	487
	8	86	684	86	785	62	377
	9	99	395	59	465	34	202
	10	88	871	72	312	56	201
LH17	1	92	568	66	354	37	184
	2	84	678	75	459	24	365

Sampling Location	Plant #	Diameter of Main Axis (µm)	Length of Main Axis (µm)	Diameter of Branch (µm)	Length of Branch (µm)	Diameter of Apical Cell (µm)	Length of Apical Cell (µm)
	3	92	594	64	610	69	225
	4	61	697	89	452	55	367
	5	37	777	88	349	25	654
	6	84	856	76	555	66	598
	7	90	870	85	380	72	463
	8	118	955	93	316	38	166
	9	101	707	80	769	28	195
	10	87	579	101	662	36	214
LM1	1	85	468	60	263	31	478
	2	94	495	59	346	63	336
	3	89	598	48	578	96	594
	4	79	789	37	169	88	264
	5	92	669	59	444	59	280
	6	89	892	88	678	99	269
	7	72	290	47	246	34	148
	8	86	578	56	551	31	187
	9	95	579	67	192	34	186
	10	89	632	51	247	33	204
LM2	1	80	365	67	198	47	140
	2	92	648	49	597	59	197
	3	84	494	69	647	67	391
	4	77	789	97	598	99	297

Sampling Location	Plant #	Diameter of Main Axis (µm)	Length of Main Axis (µm)	Diameter of Branch (µm)	Length of Branch (µm)	Diameter of Apical Cell (µm)	Length of Apical Cell (µm)
	5	97	881	84	368	59	230
	6	89	676	92	489	79	878
	7	85	878	66	338	43	307
	8	92	307	81	280	34	247
	9	95	454	60	263	39	262
	10	139	579	68	212	54	125
LM7	1	111	406	58	199	52	229
	2	79	214	55	221	38	180
	3	76	631	59	260	40	217
	4	72	366	64	267	41	217
	5	94	495	45	348	87	495
	6	78	679	98	228	69	598
	7	55	598	67	599	98	789
	8	97	679	89	486	75	669
	9	84	849	77	489	78	874
	10	108	727	83	531	39	256
LM20	1	71	999	68	497	31	657
	2	93	846	88	597	96	861
	3	101	562	49	971	64	789
	4	73	664	79	569	91	861
	5	84	861	90	874	66	789
	6	99	789	96	497	39	861

Sampling Location	Plant #	Diameter of Main Axis (µm)	Length of Main Axis (µm)	Diameter of Branch (µm)	Length of Branch (µm)	Diameter of Apical Cell (µm)	Length of Apical Cell (µm)
	7	83	359	61	578	28	789
	8	77	489	73	367	46	461
	9	92	432	76	845	51	369
	10	84	584	54	459	47	423
LM23	1	83	764	63	228	33	353
	2	89	789	87	861	29	452
	3	88	849	74	789	95	524
	4	10	497	69	359	48	581
	5	63	999	67	489	69	495
	6	77	579	59	49	789	659
	7	90	641	83	257	50	277
	8	80	452	78	293	49	221
	9	67	524	77	339	52	230
	10	102	581	84	483	53	249
St. Zotique	1	92	358	73	419	35	237
	2	99	597	97	459	48	664
	3	101	668	87	598	26	861
	4	103	498	88	478	89	789
	5	99	986	59	559	88	598
	6	62	598	96	482	88	601
	7	88	663	51	190	44	146
	8	108	324	84	493	62	302

Sampling Location	Plant #	Diameter of Main Axis (µm)	Length of Main Axis (µm)	Diameter of Branch (µm)	Length of Branch (µm)	Diameter of Apical Cell (µm)	Length of Apical Cell (µm)
	9	85	359	65	232	41	228
	10	87	233	69	269	34	144
LO1	1	96	761	65	301	54	286
	2	94	985	59	329	26	530
	3	89	559	84	510	34	679
	4	59	497	84	603	95	598
	5	109	567	73	459	48	679
	6	84	546	79	603	46	370
	7	73	594	62	267	43	369
	8	78	641	57	721	47	348
	9	76	597	69	382	51	456
	10	70	369	67	310	37	285
LO6	1	95	487	79	261	39	269
	2	89	399	53	278	36	204
	3	99	897	89	302	95	317
	4	73	597	69	165	57	285
	5	64	676	90	540	59	594
	6	55	420	87	459	47	203
	7	37	467	80	579	49	401
	8	94	569	60	367	31	237
	9	88	573	66	244	40	285
	10	82	549	57	198	33	194

Sampling Location	Plant #	Diameter of Main Axis (µm)	Length of Main Axis (µm)	Diameter of Branch (µm)	Length of Branch (µm)	Diameter of Apical Cell (µm)	Length of Apical Cell (µm)
		77.6	563.4	73	339.3	48.6	298.9
LO7	1	74	950	46	180	43	242
	2	82	422	93	268	33	258
	3	77	876	96	226	69	650
	4	59	846	87	364	89	458
	5	97	491	33	261	45	620
	6	74	890	97	594	52	346
	7	88	890	86	222	15	333
	8	94	1053	50	299	36	187
	9	110	378	76	318	38	343
	10	102	536	91	762	40	282
LO9	1	113	872	57	292	72	280
	2	82	351	56	494	40	223
	3	109	676	84	326	48	324
	4	78	234	73	456	59	623
	5	97	597	79	264	67	112
	6	94	366	59	156	61	122
	7	66	461	43	534	29	264
	8	89	420	56	400	37	239
	9	116	422	54	473	43	266
	10	99	647	67	303	58	241
LO15	1	99	497	58	206	34	188

Sampling Location	Plant #	Diameter of Main Axis (µm)	Length of Main Axis (µm)	Diameter of Branch (µm)	Length of Branch (µm)	Diameter of Apical Cell (µm)	Length of Apical Cell (µm)
	2	92	568	52	157	42	116
	3	88	647	15	284	47	226
	4	83	694	39	346	26	360
	5	73	681	79	642	59	256
	6	69	635	88	166	57	225
	7	70	459	69	265	89	194
	8	109	367	76	862	35	174
	9	72	894	64	220	52	112
	10	94	734	55	195	41	237
LE1	1	88	953	75	336	48	295
	2	94	646	88	264	87	221
	3	73	264	49	125	59	309
	4	72	222	59	185	48	345
	5	94	694	85	178	95	278
	6	91	418	70	180	49	217
	7	81	262	71	388	47	267
	8	107	362	70	291	51	267
	9	98	220	82	380	42	103
	10	78	223	65	314	37	112
LE4	1	106	636	83	265	48	355
	2	111	546	94	258	97	441
	3	87	613	68	432	66	365

Sampling Location	Plant #	Diameter of Main Axis (µm)	Length of Main Axis (µm)	Diameter of Branch (µm)	Length of Branch (µm)	Diameter of Apical Cell (µm)	Length of Apical Cell (µm)
	4	99	386	88	264	78	348
	5	91	259	71.6	310.6	45.2	455
	6	85	421	69	534	44	294
	7	102	260	78	485	44	306
	8	68	350	49	205	36	317
	9	100	555	55	212	40	292
	10	84	974	88	320	66	583
LE9	1	97	549	78	348	88	129
	2	90	879	76	450	73	458
	3	93	486	81	463	39	222
	4	103	495	59	212	61	203
	5	100	503	67	265	54	326
	6	95	698	63	237	49	352
	7	102	220	69	245	41	343
	8	65	597	89	235	59	326
	9	78	359	90	452	40	652
	10	93	911	94	123	43	401
LE13	1	77	676	67	156	75	266
	2	106	671	62	235	44	283
	3	107	789	59	233	38	298
	4	104	405	65	375	42	280
	5	75	643	63	157	55	265

Sampling Location	Plant #	Diameter of Main Axis (µm)	Length of Main Axis (µm)	Diameter of Branch (µm)	Length of Branch (µm)	Diameter of Apical Cell (µm)	Length of Apical Cell (µm)
	6	94	573	62	203	46	346
	7	103	382	87	422	35	272
	8	101	695	65	431	40	421
	9	95	433	56	195	49	235
	10	103	407	62	452	43	228