Coevolution between lichen mycobionts and photobionts in *Cladonia* section *Cladonia* (Cladoniaceae)

BY

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A Thesis Submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements For the Degree of

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Abstract

In this study coevolution between the Ascomycetous lichen fungi in section *Cladonia* and its chlorophyte algal partner *Trebouxia* was evaluated by comparing phylogenetic trees of each symbiont. Phylogenetic trees were produced from the internal transcribed spacer (ITS) regions and the 5.8S nuclear ribosomal DNA (rDNA) sequences in algal and fungal partners of 17 species of *Cladonia* section *Cladonia* and 21 others. These sequences were obtained using primers designed in conserved regions flanking the ITS regions that are specific to each of the fungal or algal rDNA.

Results revealed that fungal species complexes were polyphyletic. Four *Trebouxia* species (*T. glomerata, T. pyriformis, T. magna* and *T. erici*) are associated with lichens of genus *Cladonia* in Manitoba. There was no evidence of cospeciation in section *Cladonia*. However an isolated event of cospeciation was observed in *Cladonia* section *Cladonia* (in *C. macrophyllodes* and *C. pocillum*) as well as an algal switch by *C. pyxidata.* Algal switching may be very common in lichens. In addition, existence of cryptic and ecological species within section *Cladonia* may obscure cospeciation in this section.

Results from this study indicate that further investigation is required to examine a larger number of individuals within each fungal species. This may reveal existence of cryptic species. In addition, the effect of microenvironmental conditions on algal selection requires more study.

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1. Introduction

Lichens are associations between a mycobiont, usually an ascomycetous fungus, and one or more photobionts, generally a green alga or cyanobacterium. Lichenization is an ancient nutritional strategy for fungi. Lichen-like fossils consist of coccoid cells and thin filaments, preserved in marine phosphorite of the Doushantuo Formation at Weng'an, South China. These fossils are thought to be 551 to 635 million years old (belonging to Neoproterozoic era). Discovery of these fossils suggest that fungi developed symbiotic partnerships with photoautotrophs long before the evolution of vascular plants. *Winfrenatia,* an early zygomycetous lichen symbiosis that may have involved controlled parasitism, is an impression found in Scotland, belonging to early Devonian times (Taylor *et al.* 1997). There are also several examples of fossilized lichens embedded in amber. The fossilized *Anzia* is found in pieces of amber in northern Europe and dates back to approximately 40 million years (Schlee 1990, Poinar 1992). Fossilized *Lobaria* comes from Trinity County in northern California, USA and dates back to the early to middle Miocene (Peterson 1999).

In all lichens the mycobiont forms a unique thallus that is different from a non-lichenized fungal thallus and contains secondary compounds (Ahmadjian 1993) such as compounds derived from acetyl-polymalonyl, mevalonic acid and shikimic acid pathways (Nash 2001). These secondary compounds are unique to lichenized fungi and are the result of the close physiological interactions between photobionts and mycobionts.

Most mycobionts seem to be obligate symbionts, i.e. they cannot survive in nature without the photobiont. Lichenized fungi grow very slowly in pure culture and therefore are unlikely to compete and survive in a free-living state. Lichenization is also a

mechanism by which photobionts may survive harsh environments and thus broaden their geographic distribution. Lichens are distributed over a diverse range of habitats from the tropics to the polar regions that sometimes may not be inhabitable for separate symbionts. Although most lichens occur in terrestrial ecosystems, a few also occur in fresh water and marine intertidal zones. Lichens as the dominant autotrophs also contribute biomass and productivity to many polar and subpolar ecosystems (Nash 2001).

In general, lichenization is a successful symbiotic relationship; however this relationship has been shown to undergo coevolution at higher taxonomic levels. Coevolution can be observed between the order Lecanorales of ascomycetes and the green algal *Trebouxia,* formerly divided into two genera, *Trebouxia* and *Asterochloris* (Tschermak-Woess, 1980). While the genus *Asterochloris* is confined to the suborder *Cladoniineae, Trebouxia* is specific to the suborder *Lecanoriineae*. However, there is not much known about this relationship at the lower taxonomic levels. Therefore this thesis examines coevolution within species of *Cladonia* section *Cladonia*.

Three objectives of this thesis are:

1. To investigate the phylogenetic history of *Cladonia* section *Cladonia;*
2. To investigate the phylogenetic history of the genus *Trebouxia;* and,
3. To compare phylogenetic histories of both partners in *Cladonia* section *Cladonia*.

2. Literature Review

Evidence supporting the mutualistic nature of lichens

In lichens, the heterotrophic mycobiont receives carbon nutrition from the photobiont. This carbon source is in the form of polyols in the case of a phycobiont (green algal partner) or glucose in the case of a cyanobiont (cyanobacterial partner). Cyanobionts also supply the mycobiont with organic nitrogen due to their ability to fix nitrogen. Symbiose cyanobacteria are able to reduce gaseous nitrogen (N_2) to ammonia (NH_3) by the enzyme nitrogenase (Nash 2001) while algal symbioses are incapable of supplying the mycobiont with organic nitrogen. The lichen fungi also receive vitamins necessary for its growth such as thiamine and biotin from the photobiont (Ahmadjian 1964). On the other hand, the alga may receive metabolites such as ascorbic acid from the fungi (Quispel 1943).

Fungal hyphae provide algal cells with a stable and inhabitable microenvironment. Fungal hyphae provide physical protection for algal cells from desiccation and solar radiation, by surrounding the algal cells. Also by producing certain secondary compounds and pigments in the fungal tissue that surround the algae further protection is provided. This allows the algae to extend its geographical distribution and survive in extreme habitats. In some cases algae like *Trebouxia* are so rarely found free-living that lichenization seems to be the only tactic for survival in nature for these algae.

In general, algae that are in symbiotic relationship tend to excrete more sugar than algae that are free-living (Reisser *et al.*, 1991). This loss of CHO could be a major disadvantage while free-living, as they have to compete with non-excreting and hence faster growing, free-living algal species. The formation of a symbiotic relationship can

compensate for the disadvantage of the permanent loss of sugars, by providing a habitat where they can persist without being in competition with free-living, non-excreting forms (Reisser 1991).

Evidence supporting the parasitic nature of lichens

In some literature the symbiotic relationship between algae and fungi in lichens is regarded as controlled parasitism (Ahmadjian 1993). This hypothesis is mainly based on the observation of haustorial penetration of fungi into the algal cells in some lichens in order to facilitate carbohydrate transfer. Haustorial formation is usually seen in parasitic fungi.

The observation of slower growth and a lower reproduction rate in lichenized algae compared with free-living algae also supports the idea of the photobiont being parasitized by the mycobiont. This suppression is partially due to removal of sugars from the algal partner and the lack of nutrients transferred from the mycobiont to the photobiont (Nash 2001).

Degree of lichenization and different growth forms

The degree of lichenization varies from just a few photobiont cells associated with a fungus as it is seen in a crustose thallus of the Caliciales, to more complex thalli in Lecanorales, lichens with a distinct heteromerous morphology different from either of the bionts that form the thallus. Different growth forms show adaptations to variable habitats. Growth forms ranging from pendent thalli to umbliform thalli supply the photobionts with optimum light intensities (minimum exposure to light in case of a pendant thallus

and maximum exposure in case of a foliose or an umbliform thallus) depending on whether the lichen prefers shaded habitats or fully exposed conditions respectively. A stratified thallus structure with a hydrophobic medullary layer and location of photobiont layer in the upper part of the medulla just below the cortex provides optimal carbon dioxide diffusion (under dry or fully hydrate conditions) to the photobiont.

Based on their overall appearance lichens traditionally have been divided into three groups; crustose lichens, foliose lichens and fruiticose lichens (Nash 2001).

Crustose lichens are usually in close contact with their substratum at all points and lack a lower cortex. Crustose lichens can be homoiomerous, a condition in which mycobionts and photobionts are evenly distributed through the thallus; or heteromerous with a stratified thallus structure and different types of tissues.

Foliose lichens are leaf-like, dorsiventral and partly attached to their substrate by various attachment appendages. Foliose lichens can be homiomerous, or heteromerous possessing an upper cortex, a layer of photobiont cells in the upper portion of the medulla, and sometimes a lower cortex.

Fruiticose lichens are heteromerous, pendent or shrubby, and usually with radially symmetric thalli. Some fruitocose lichens such as the genus *Cladonia* differentiate into a horizontal thallus, described as primary squamulose and a stalk or podetium that forms the vertical thallus. This dimorphous structure of thalli is sometimes referred to as "Cladoniiform" thalli (Ahti 1982).

The Cladoniiform morphology in lichen-forming fungi arose multiple times within the ascomycetes (Stenroos and DePriest 1998) and is distributed among five families of Lecanorales, suborder Cladoniineae – Cladoniaceae, Baeomycetaceae, Heterodeaceae,

Cladiaceae and Siphulaceae. The major genera exhibiting this growth form are *Cladonia, Baeomyces* and *Siphula* (Ahti 1982).

2.1. The Mycobiont

One fifth of the kingdom Fungi (Alexopoulos 1996) consists of lichen forming fungi. Most lichenized fungi belong to the class Ascomycetes (phylum Ascomycota), and three known genera belong to the class Basidiomycetes (phylum Basidiomycota).

The phylum Ascomycota is distinguished from other phyla by formation of an ascus, a sac-like cell containing haploid ascospores. Typically eight ascospores are produced within each ascus. Other characters of members of this phylum include septate mycelia with simple pores, Woronian bodies (electron dense spherical bodies found in the hyphae near the septa), a dikaryotic stage in the life cycle, conidia (non-motile asexual spores), and sometimes a complex sexual reproductive structures like apothecia and perithecia (Alexopoulos 1996). For a while, the lichenized ascomycetes were once distinguished from non-lichenized taxa by the presence of concentric bodies (Nash 2001). Concentric bodies consist of two zones of electron-dense material surrounding an electron transparent core (Ahmadjian 1993). They have now been found in fungal plant pathogens as well as saprobes and have been recognized as cell organelles with unknown origin and function. Concentric bodies are found in all types of vegetative cells, but have never been found inside asci. It has also been observed that fungi possessing these organelles show tolerance to desiccation (Nash 2001).

Asexual and sexual cycles in Ascomycetes

In lichenized fungi two types of conidia (conidiospores) are produced in the asexual phase, macroconidia and microconidia. Conidiospores are produced on conidiophores the latter are simple or branched hyphae bearing one or more conidiogenous cells. Microconidia can serve as male gametes (nuclear donors) in the sexual cycle.

The sexual cycle in non-lichenized filamentous ascomycetes consists mostly of an extended haploid phase, a short dikaryotic phase, and a short diploid phase. This is thought to be similar in lichenized ascomycetes.

A trichogyne, which is a long receptive hypha of the female gametangium (ascogonium), develops on the ascogonium and responds to pheromones of the male gametes. The trichogyne grows toward the spermatium until they contact one another. Then plasmogamy occurs which is the fusion of the spermatium with the trichogyne of the ascogonium. The cytoplasm mixes and the male nuclei migrate into the female gametangium. Male and female nuclei pair up on the periphery of the ascogonium and papillae form on the ascogonial walls that become the ascogenous hyphae. The nuclei pairs move into the hyphae and a crozier hook forms around the leading pair of nuclei. A synchronous division of all nuclei pairs is followed by seperation of the crozier hook by a wall to form the ascus mother cell. As the ascus mother cell develops into a young ascus karyogamy occurs, followed immediately by meiosis. Meiosis (reduction division) reduces the number of chromosomes and forms four haploid nuclei with potential genetic recombination and reassortment. Meiosis is typically followed by a mitotic event producing eight haploid nuclei. This is followed by sporogenesis, the packaging of haploid nuclei into spores and formation of mature asci.

Relichenization after sexual reproduction of lichen fungi

After the dispersed spores of the lichen fungi make contact with an alga, an inconspicuous non-stratified crust known as a pre-thallus is formed. The pre-thallus also can be formed with incompatible algae (Ott 1987), but the stimulus that causes the transformation of the pre-thallus into a lichen thallus is provided only by a compatible photobiont. The pre-thallus stage may be very important in the life cycle as it enables the mycobiont to survive until a suitable photobiont becomes available (Beck *et al.* 1998). Fungi may even be able to survive parasitically with incompatible or non-symbiotic algae (Ahmadjian and Jacobs 1981), allowing the lichen fungi to exist in nature before meeting compatible algae.

Classes in phylum Ascomycota

The phylum Ascomycota informally is divided into three classes based on ascocarp morphology (reviewed in Carlile *et al.* 2001). These classes were Discomycetes, Pyrenomycetes and Plectomycetes.

Discomycetes have a disc shaped ascocarp (reproductive structure) called an apothecium. The apothecium consists of a hymenium, subhymenium, hypothecium and exiple. The subhymenium gives rise to the hymenium. The hymenium consists of asci and sterile paraphyses that can be colored or sometimes branched. A hypothecium may develop underneath the subhymenial layer. The exiple is the wall of the apothecium and maybe reduced or surrounded with another wall, which is the countinuation of the thallus. This type of apothecium is known as a lecanorine apothecium. The exiple can also be carbonized and black (lecidine apothecium) or pigmented (biatorine apothecium). The

apothecium structure is ideal for dispersal of spores by wind. This type of ascocarp is the most common fruiting body in lichenized ascomycetes.

Pyrenomycetes have a flask shaped ascocarp called a perithecium. Asci in perithecia are well protected during developmental stages and the spores are discharged from a hole in the neck of the perithecium.

In plectomycetes asci develop inside a spherical ascocarp termed cleistothecium or pseudothecium. In lichens bearing pseudothecia several locules exist close to each other in a mass of fungal tissue and together they appear as a convex aggregation of ascocaps.

All lichenezed fungi belonging to the phylum Ascomycota belong to the Discomycetes and are sometimes referred to as the discolichens. In modern classification of phylum Ascomycota, Discomycetes are included in class Ascomycetes (Alexopoulos 1996).

Lichenized ascomycetes can be assigned to the following 12 orders: Arthoniales, Caliciales, Graphidales, Gyalectales, Lecanorales, Lichinales, Opegraphales, Peltigerales, Pertusariales, Pyrenulales, Teloschistales and Verrucariales (Nash 2001). The order Lecanorales belongs to class Ascomycetes and contains the majority of lichenized Ascomycetes. The order Leconarales is united by features of the ascus structure. Most asci in the Lecanorales have thickened apical regions with rostrate dehiscence in which the inner wall of the ascus elongates at the apex and breaks through the outer wall layers in spore discharge. Asci are bitunicate, possessing two inner walls (endoascus) and two outer walls (exoascus) (Alexopoulos 1996). The order Lecanorales is divided into eight suborders on the basis of ascocarp, ascus apex and ascospore characters (Alexopoulos 1996).

However, based on an SSU rDNA sequence phylogenies, the order Lecanorales is a monophyletic group with five suborders (Cladoniineae, Lecanorineae, Teloschistineae, Agyriineae and Peltigerineae) and the following suborders can be excluded: Acarosporineae, Pertusariineae and Umblicarineae (Stenroos and DePriest 1998). Suborder Cladoniineae is a polyphyletic group (Stenroos and DePriest 1998) and contains fungi with hyaline ascospores (Alexopoulos 1996). This suborder contains 40 families (Hafellner 1994), including Cladoniaceae.

Family Cladoniaceae

The family Cladoniaceae seems to be a polyphyletic group (Stenroos and DePriest 1998). Cladoniaceae with about 500 species is among the largest and most diverse group of lichen-forming fungi (Ahti 2000a), and is distributed among all five continents and is dominant in polar, temperate and tropical areas. Linnaeus (1753, in Ahti 2000a) recognized 12 species of Cladoniaceae and placed all in the genus *Lichen*. Hill (1751, in Ahti 2000a) was the first to place them in the genus *Cladonia*. Taxonomic positions of some of the genera and species in this family are still uncertain. This is becouse many species produce variable morphological features under different environmental conditions; therefore taxonomy cannot be based on morphology alone. Asahina (1950) and Evans (1952) attempted to address the problem in the family by examining the secondary chemistry of the species. Their studies provided the basis for the chemotaxonomy of the Cladoniaceae.

The genus *Cladonia*

The genus *Cladonia* includes more than 400 species worldwide (Ahti 2000a) and 72 species in North America (Brodo *et al.* 2001). In the genus *Cladonia* stalks originate from the primordia of the fruiting bodyand are termed "podetia", as opposed to "pseudopodetia", which originate from the primordia of the horizontal thallus (Nash 2001). Fruiticose structures in the families Baeomycetaceae and Icmadophilaceae are called false podetia (pseudopodetia) based on their development; however, podetia in these families differ morphologically from podetia in Cladoniaceae. In Baeomycetaceae and Icmadophilaceae podetia are not hollow and not always lichenized as seen in Cladoniaceae.

Apothecia in *Cladonia* are usually located at the top of podetia. Podetia are corticate, with a broken or intact cortex, or acorticate, no continious cortex and usually covered with soredia. Soredia are vegetative propagules consisting of algal cells surrounded by fungal filaments and lacking a cortex. Podetia are rarely isidiate in the *Cladonia*. Isidia are corticate outgrowths of the thallus that contain photobionts and can be easily detached and serve as vegetative propagules. Podetia in *Cladonia* often have podetial squamules. Podetia are hollow with a stereome, a tough cylinder of supporting tissue, surrounded by the medulla on the outside. Podetia are usually 0.5-15 cm tall and simple or branched. Pycnidia, flask-shaped bodies containing conidiospores and often resembling a perithecium, are present on the primary thallus or on tips of podetia and produce hyaline or red slime. Pycnidia are dolioliform, pyriform (pear-shaped) or ampullaceous (buttle-like). Conidia are falciform, that is curved and tapering to a point like a sickle. Hymenial disks are shades of brown or red. Spores are fusiform (tapering at both ends like a

spindle), oblong (elongated) or ovid (egg-shaped) and colorless. There are eight, rarely four or six spores per ascus (Ahti 2000a).

Species in this genus are divided into seven taxonomic sections: *Ascyphiferae, Cladonia, Cocciferae, Helopodium, Perviae, Strepsiles* and *Unciales*. This separation is based on gross morphology (such as color of apothecia, primary thallus structure, presence or absence of cortex, thallus perforation and branching pattern) and secondary chemistry (Stenroos *et al.* 2002).

Section *Cladonia*

Section *Cladonia* includes 27 species in North America (Ahti 2000a; Brodo 2001 and Thomson 1984) and 200 species worldwide (Ahti 2000a). Section *Cladonia* is characterized by a persistent, sometimes an evanescent primary thallus, unbranched to slightly branched podetia, with initially closed axils, sometimes perforated with age. Scyphi, cup-shaped structures at the tip of podetia, are closed when present, with central or marginal proliferation. The stereome in podetia has a smooth inner surface. The pycnidia are terminal (located at the top of podetia as apposed to on primary squamules), spherical to turbinate and contain hyaline slime. The hymenial disks are brown. All the major compounds in this section are produced through the acetate-polymalonate pathway and are divided into three goups (Ahti, 2000):

1. depsides or ester-linked polyphenolics such as atranorin (ATR) and homosekikaic acid (HSEK);

2. depsidones or ester- and ether-linked polyphenolics such as fumarprotocetraric acid (FUM), stictic acid (STI) and psoromic acid (PSO); and,
3. dibenzofurans and dibenzofuranoid derivatives such as usnic acid (USN).

Phylogeny of the mycobiont

There is no generally accepted classification for *Cladonia*. A number of genera including *Cladia, Cladina, Gymnoderma, Metus* and *Pycnothelia* have been segregated from *Cladonia*. The taxonomic status of some of these genera is still uncertain. Even the infrageneric taxonomy of *Cladonia* is problematic. Vainio (1894, in Stenroos *et al.* 2002) diveded the genus *Cladonia* into two groups based on the hymenial color. Choisy (1928, in Stenroos *et al.* 2002) also divided this genus into two "Groups", using characters of the podetia and hymenial disk as well as size of the primary thallus. Mattick (1938, in Stenroos *et al.* 2002) classified this genus believing species with closed axils and species with open axils belong to different lineages.

Within the Cladoniaceae the status of the genus *Cladina* is controversial. In America, Asia, Austaralia and Russia *Cladina* was recognized as a separate genus, while in Europe, most authors recognized it as a subgenus of *Cladonia* (Stenroos *et al.* 2002). The main characters distinguishing *Cladina* from *Cladonia* are the crustose primary thallus, highly branched podetia and a lack of cortex and podetial squamules in *Cladina* as opposed to the squamulose primary thallus, non-branched or few-branched podetia and sometimes presence of podetial cortex and squamules seen in *Cladonia*.

Vainino (1880, in Stenroos *et al.* 1997) and Mattick (1938 in Stenroos *et al.* 1997) recognized this group at subsection level. Ahti (1984) accepted *Cladina* as a genus with some hesitations. Ahti (2000b) in a monograph considered this group a genus and divided it into three sections *Cladina* sections *Cladina*, *Impexae* and *Tenues*. However, Stenroos *et al.* (1997, 2002) suggested the monophyly of *Cladina* within *Cladonia*, supporting the inclusion of *Cladina* within *Cladonia*.

A study based on molecular, morphological and chemical data done by Stenroos *et al.* (2002) recognized three subdivisions within the *Cladonia*. The first subdivision is represented by one species *C. wainioi*. The second subdivision includes *Cladonia* sections *Ascyphiferae*, *Helopodium* and *Cladonia*, however, none of these sections seem to be monophyletic. Members of this group all share characters such as brown hymenia and fumarprotocetraric acid as their major secondary compound. The third subdivision consists of *Cladonia* sections *Cocciferae*, *Perviae, Unciales* and the *Cladina* sections *Cladina*, *Impexae* and *Tenues*. Species in this group all have red, ochraceous or brown hymenia and cortical usnic acid.

Cladonia Section *Cladonia*

According to Stenroos *et al.* (2002) based on ITS1, 5.8S, ITS2 and partial sequences of the β-tubulin gene, section *Cladonia* is not monophyletic. Representatives of this section are scattered over the genus *Cladonia* with some groupings representing species complexes in section *Cladonia*. One species complex in this section is *C. verticillata* species complex including *C. verticillata, C. cervicornis, C. macrophyllodes* and *C. rappii*. The second species complex is the *C. gracilis* species complex, represented by *C.*

gracilis, C. cornuta, C. ecmocyna and *C. maxima*. These two species complexes seem to be monophyletic (Stenroos *et al.* 2002). *Cladonia pyxidata* as well as *C. pocillum* do not seem to be monophyletic species and their members are clustered with members of the *C. gracilis* and *C. chlorophaea* species complexes as well as with members of *Cladonia* section *Ascyphiferae*. Members of *C. chlorophaea* species complex such as *C. chlorophaea*, *C. grayi* and *C. merochlorophaea* were also scattered over the genus *Cladonia*, in the phylogeny presented in Stenroos and colleagues (2002), showing the polyphyletic nature of *C. chlorophaea* species complex.

2.2. The Photobiont

Although 20% of all described fungal species are lichenized, their photobionts are understudied in terms of the identity, reproductive mode and degree of specificity in association with the mycobionts. Of the 13,500 species of lichen-forming fungi identified so far, about 12,500 are associated with green algae and approximately 1,000 are associated with cyanobacteria as the photobiont. Of 200 photobionts described to the species level only 100 are green algae (Tschermak-Woes 1988). More current numbers are not available.

Members of *Trebouxia* are the most frequently occurring photobiont of lichens and are present in 20% of all lichens but mainly associated with the order Lecanorales. Lichen fungi belonging to the order Lecanorales are associated with the green algal genera *Asterochloris*, *Trentepohlia* and *Trebouxia*, as well as the cyanobacteria *Nostoc* and *Scytonema* (Tschermak-Woess 1988). All lichens in the genus *Cladonia* are involved

in a symbiotic relationship with green algal species found in the genus *Trebouxia* (Tschermak-Woess 1988).

Taxonomy of the genus *Trebouxia*

Trebouxia belongs to the phylum Chlorophyta. Chlorophyta is subdivided into four distinct lineages, the Chlorophyceae, the Trebouxiophyceae, the Ulvophyceae and Parisinophyceae (Friedl 1996).

The phylogenetic separation between members of the Chlorophyta is demonstrated using differences in their flagellate cells (zoids) ultrastructure such as characteristics of microtubular roots (Fig. 1) (in green algae flagellar basal bodies are anchored in the protoplast by microtubular roots), details of mitosis, and cytokinesis (Melkonian *et al.* 1988).

Based on zoids structure, four lineages are recognized within the Chlorophyta:

1. Zoids with organic body scales;
2. Zoids with four microtubular flagellar roots in a cruciate arrangement and a 1 O'clock - 7 O'clock configuration of the basal bodies;
3. Zoids with four microtubular flagellar roots in a cruciate arrangement and a 11 O'clock – 5 O'clock configuration of the basal bodies; and,
4. Zoids with a unilateral arrangement of the flagellar apparatus or 6 O'clock – 12 O'clock configuration of the basal bodies.

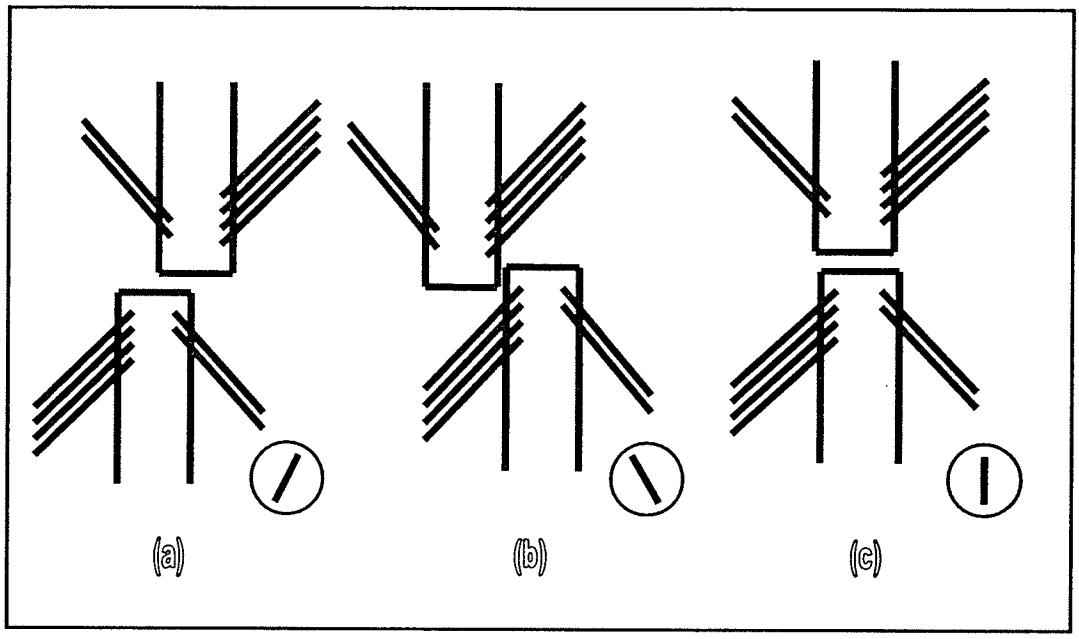

Fig. 1: Variation of microtubular root configuration in green algae. Basal bodies are shown as rectangles and microtubullar roots as bundles of two or four lines. a, 1 O'clock-7 O'clock configuration in Chlorophyceae; b, 5 O'clock-11 O'clock configuration in Ulvophyceae, Cladophorophyceae, Bryopsidophyceae, Dasycladophyceae, Trentepohliaceae and Trebouxiaceae; and c, 6 O'clock-12 O'clock configuration in Zygnematophyceae, Klebsormidiophyceae and Charophyceae as well as to the various classes of land plants (modified from Van den Hoek 1995).

In modern classification the first and the second lineages each have given rise to just one class, Prasinophyceae and Chlorophyceae respectively; the third lineage to six classes, the Ulvophyceae, Cladophorophyceae, Bryopsidophyceae, Dasycladophyceae, Trentepohliaceae and Trebouxiaceae (previously called Pleurastrophycea); and the forth lineage to three classes of green algae, the Zygnematophyceae, Klebsormidiophyceae and Charophyceae as well as to the various classes of mosses, liverworts and vascular plants.

In Trebouxiophyceae, mitosis is semi-closed with non-persistent spindles that collapse after nuclear division. Cytokinesis is brought about by a cleavage furrow, which grows in from only one side (Van Den Hoek 1995).

Trebouxiophyceae was first recognized as a class based on the observation of the metacentric spindle (the positioning of centrioles at the side of the spindles) during cell divisions (Molnar 1975) as opposed to polar positioning of centrioles observed in the majority of green algae. The pair of centrioles in metacentric species is not involved in the formation of the mitotic spindle; instead they are involved in the assembly of the phycoplast, a set of microtubules that lies perpendicular to the original spindles during cytokinesis. Mattox and Stewart (1984) put this group of species in class Pleurastrophyceae. Later on, the name of this class changed to Trebouxiophyceae because *Pleurastrum insigne* the type species of Pleurastrophyceae, was moved to class Chlorophyceae (Friedl 1995 in Graham 2000).

Most green algal photobionts of lichens fall into the class Trebouxiophyceae, suggesting the existence of some clade-specific physiological properties that makes them suitable for becoming lichenized. This group of green algae also contains other symbiotic genera such as *Chlorella*, which is a significant symbiotic alga usually associated with

members of the kingdom Animala. Other well-known members of the Trebouxiacea are also involved in wide range of symbiotic relationships, such as *Stichococcus*, the phycobiont in some lichens and *Prototheca*, an opportunistic pathogen of animals, humans and plants. There are also some free-living examples in this class namely *Microthamnion*, *Eremosphaera* and *Prasiola* (Reviewed in Van Den Hoek 1995 and Graham 2000).

Genus *Trebouxia* as a photobiont

The first time the photobiont was isolated in pure culture it was reported as as *Cystococcus humicola* by Beijerink (1890 in Archibald 1975) and Artari (1902, in Archibald 1975). Today *Cystococcus humicola* is thought to have been a *Trebouxia*. However, the symbiotic alga was known as *Cystococcus* until de Puymaly (1924, in Archibald 1975) proposed the name *Trebouxia* for the algae, isolated and described by Treboux (1912, in Archibald 1975) from the lichen genus *Xanthoria*. Friedl and Gartner (1988) identified 26 species of *Trebouxia* based on cytological characters of vegetative cells such as morphology of plastids, formation of autospores and structure of pyrenoids.

The genus *Trebouxia* is a genus of unicellular, coccoid green algae with a massive axial chloroplast suspended in the centre of the cell. The chloroplast is stellate or wrinkled and contains one pyrenoid surrounded by a continious starch sheath (Archibald 1975). The nucleus lies near the periphery of the cell. Reproduction in *Trebouxia* is primarily by mean of aplanospores with posterior nuclei and lacking stigma or by zoospores possessing two flagella with equal length and sometimes a stigma.

Pyrenoids in *Trebouxia*

Pyrenoids are proteinaceous structures within chloroplasts in both lichenized and free-living green algae. These structures are composed of the enzyme RuBisCo (ribolose 1, 5-bisphosphate carboxylase-oxydase) (Kajikawa *et al.*1988). This enzyme catalyzes the dark reaction of photosynthesis involving the fixation of CO_2 into carbohydrates. The starch produced accumulates near pyrenoids.

Chloroplast thylakoid lamellae entering the pyrenoid matrix may become structurally modified and form tubules. Pyrenoids in the genus *Trebouxia* have diverse ultrastructure features providing useful taxonomic characters. Based on forms and arrangement of thylakoid lamellae within the pyrenoid, Friedl (1989) distinguished eight pyrenoid types of which three are seen in *Cladonia* photobionts (See Fig. 1 in Friedl 1989). The advantage of pyrenoid ultrastructure characters as diagnostic features is that these structures are not influenced by different culture media and are even stable within the lichen thallus (Friedl 1989). This allows a given *Trebouxia* phycobiont to be identified without culturing, by comparing the pyrenoid structure seen within the lichen thallus with that from cultured species.

Reproduction in *Trebouxia*

Reproduction in this algal genus is asexual in the lichenized state, by formation of autospores, asexualy-porduced non-flagellated cells that resemble the parent cells. The occurrence of sexual reproduction of members of *Trebouxia* in the lichen association is controversial. *Trebouxia* is described by Friedl (1995) as colonial with unknown sexual reproduction. Physical evidence of sexual reproduction such as fusion of gametes or

meiotic tetrads have not been observed in any of the green algal photobionts (Friedl and Rokitta 1997). Kroken and Taylor (2000) reported a recombining population structure in a phylogenetic species of *T. jamesii.* This suggests sexual reproduction in *Trebouxia* is present but it has never been observed.

Free-living *Trebouxia*

The existence of free-living *Trebouxia* is a matter of controversy. Some investigators believe there are no free-living population of *Trebouxia* (Ahmadjian 1988) while some reported free-living colonies of *Trebouxia* on bark and soil (Tschermark-Woess 1978, Bubrick *et al.* 1984). Ahmadjian (1988) argues that since free-living *Trebouxia* colonies are never observed in large populations, these free colonies of *Trebouxia* are suspected to be recently separated from lichen thalli or their vegetative propagules such as soredia and isidia. Ahmadjian argues that when these lichenized fragments land on habitats that are not conducive to lichen development, the fungal hyphae of the propagule die and set free the algal cells. These algal cells in turn can separate and form small colonies of free-living *Trebouxia*. However, free-living *Trebouxia* has been found on recently fire-sterilized rocks long before lichens colonize the rock (Mukhtar *et al.* 1994). These free-living colonies of *Trebouxia* were identical to the photobionts of *Xanthoria* and *Buellina* that colonized the rock later (Kroken and Taylor 2000). Finding these free-living colonies of *Trebouxia* rejected the possibility of algae being liberated from lichens.

Intrageneric divisions of *Trebouxia*

Ahmadjian (1960, 1970) divided *Trebouxia* into two groups, *Trebouxia* I and II. *Trebouxia* I group does not divide vegetatively and chloroplast fragments assume a parietal position against the cell wall during chloroplast division. On the other hand the *Trebouxia* II group divides vegetatively and the chloroplast fragments do not assume a parietal position against the cell wall during chloroplast division. Both groups have a large axial chloroplast with one pyrenoid and the nucleus possesses a prominent nucleolus. They both reproduce asexually by aplanospores and wall-less zoospores. Zoospores have two flagella of equal length and may or may not have an observable stigma (Hildreth and Ahmadjian 1981).

Trebouxia is sometimes divided into two genera, *Trebouxia* and *Pseudotrebouxia* based on differences in their mode of reproduction (Archibald 1975). *Pseudotrebouxia* is believed to undergo vegetative cell division (Groover and Bold 1969). In *Trebouxia* species vegetative cells develop directly from zoospores while in *Pseudotrebouxia* zoospores first form autosporangia, which produce autospores and later vegetative cells form from these autospores. Morphogenesis of multilayered cell walls is also different in these two groups. In *Trebouxia* the wall of vegetative cells forms around the zoospores but in *Pseudotrebouxia* the wall of vegetative cells forms around autospores (Archibald 1975, Peveling and Konig 1985).

Ultrastructural and DNA studies however, did not support this generic separation (Ahmadjian 1993, Nash 2001). Pyrenoid ultrastructure is not different between *Trebouxia* and *Pseudotrebouxia* and therefore does not support division of these two groups. The type species of *Trebouxia, T. arboricola* and type species of *Pseudotrebouxia, T.*

aggregata both share arboricola-type pyrenoids (Friedl 1989). In addition, 26S rDNA phylogenetic hypothesis rejected the separation of these two genera and showed that differences in the cell cycle appear to be unreliable in resolving phylogenetic relationships in *Trebouxia* (Friedl and Rokitta 1997).

Ahmadjian (1988) based on ultrastructural evidence found by Mattox and Stewart (1984) suggested that *Trebouxia* had evolved from the filamentous soil alga *Pleurastrum terrestre.* He states that *Trebouxia* and *Pseudotrebouxia* are so similar to *Pleurastrum* that they may even belong to one genus. The main distinguishing character for these genera is the tendency toward non-filamentous growth in *Trebouxia* and *Pseudotrebouxia* and filamentous growth in *Pleurastrum.* However, *Pleurastrum* can grow as filaments or single cells on certain media. A single-celled *Pleurastrum* with axial chloroplast resembles members of *Trebouxia. Pleurastrum* may have an axial or parietal chloroplast and in *Trebouxia,* the chloroplast assumes a parietal position during early stages of cell division. This is believed to be an ancestral trait for *Trebouxia* and supports a link between *Trebouxia* and *Pleurastrum.* On the other hand, pyrenoid ultrastructure does not support this hypothesis since in *Trebouxia* this structure differs morphologically from the pyrenoids seen in species within *Pleurastrum* (Friedl 1989).

Friedl and Rokitta (1997) showed that those *Trebouxia* species with central chloroplasts and true pyrenoids are monophyletic and cluster together in 18S and 26S rDNA tree, forming the *Trebouxia* cluster. *Trebouxia erici* and *T. magna,* representatives of photobionts of *Cladonia* in their study, did not cluster with the *Trebouxia* cluster. They suggested that *Trebouxia* is a paraphyletic genus since *T. magna* was more closely related to some members of the genus *Myrmecia.* This was explained by the absence of a true

pyrenoid in *Myrmecia* as well as *T. magna* and *T. erici,* and the presence of the true pyrenoid in other *Trebouxia* species.

Since *T. arboricola,* the type species of the genus is within the *Trebouxia* cluster; Friedl and Rokitta (1997) suggested that *T. magna* be excluded from this genus. Friedl and Rokitta (1997) reported the distance between *T. erici* and other members of *Trebouxia* to be more than the distance between *T. erici* and a member of the genus *Leptosia* of Trebouxiophyceae.

Of the three pyrenoid-types distinguished in lichen photobionts belonging to section *Cladonia,* two of them (*erici*-type and *magna*-type) had representatives in the Friedl and Rokitta (1997) study. The overall morphology of *T. erici* and species with pyrenoids of *irregularis*-type, the third pyrenoid type in section *Cladonia,* is quite similar in the parietal position of their chloroplasts (Melkonian and Peveling 1988). This could imply that these morphological traits or even non-morphological characters linked to these morphological traits are selected for by *Cladonia* species in section *Cladonia.* This also supports the separation of photobionts of section *Cladonia* and related species from the genus *Trebouxia* and their inclusion in algal genus *Asterochloris* (sensu Friedl).

2.3. Ribosomal DNA as a Molecular Marker

Ribosomal RNA genes (SSU, LSU, 5.8S, ITS1 and ITS2) are some of the most popular macromolecules in phylogenetic studies (Hillis and Dixon 1991).

Phylogenies based on rDNA data can contain both prokaryotic and eukaryotic taxa, since homologous SSU and LSU genes occur in both groups. The mosaic nature of conserved and variable regions in the ribosomal DNA sequence provides information for

examining phylogenies at different ranks. Popularity of this DNA segment in phylogenetic studies provides a great amount of information in the GenBank for comparison. In addition, existence of multiple copies of rDNA makes the gene easy to isolate. Ribosomal RNA exhibits concerted evolution, a phenomenon seen in most repetitive sequences in the genome of organisms ranging from bacteria to mammals (reviewed in Liao *et al.* 1997).

Concerted evolution suggests that members of a repetitive gene family do not evolve independently of each other. Therefore multiple copies of genes in nuclear genomes within a species and population undergo the same kinds of changes at just about the same time, such that their sequences become homogenous and it is less likely to interfere with phylogenetic studies. In addition, concerted evolution homogenizes rDNA repeats within individuals and interbreeding groups while allowing their divergence between species in reproductively isolated groups (Arnheim 1983). Consequently, these tandem repeats are not assumed to differ significantly within individuals or species but to differ between species (Hillis and Davis 1988, Hillis *et al.* 1991). Therefore, when members of a repetitive family are compared, greater sequence similarity is found within a species than between species. This homogeneity is brought by spreading a mutation throughout the first tandem array by a process called "intrachromosomal homogenization". This mutation is then spread further to the second array by interchromosomal gene conversion. Lastly, the mutation is fixed throughout the second array by another round of intrachromosomal homogenization (Liao *et al.* 1997).

Small and large rDNA subunits phylogeny

Nuclear encoded small and large subunits of ribosomal RNA (SSU rRNA and LSU rRNA) genes are widely used to infer phylogenetic relationships at the higher taxonomic rank, due to their conserved structure and function. SSU rRNA and LSU rRNA genes are coding regions; hence, they are more conserved with lower substitution rates relative to the non-coding DNA sequences. Coding regions are assumed to have a lower evolutionary rate of change and are preferred for higher level phylogenetic studies, since diversification events at those levels assumed to have occurred relatively early in their evolution.

The small subunit of ribosomal RNA gene is more conserved than the large subunit; hence it is more useful for evolutionary analysis among major groups. Then again SSU rDNA contains some regions of relatively high variability, such that these molecules can also be used at lower taxonomic levels, including species level investigations.

5.8S ribosomal DNA phylogeny

In recent studies the use of 5.8S ribosomal DNA sequences alone for phylogenetic reconstructions has been abandoned, since there are too few informative sites and the rate of nucleotide base substitutions is too high to study ancient divergences. However, incorporation of this region in ITS sequence data set has not been proven to cause any problem in phylogenetic studies.

Non-coding rDNA regions

Conservation of a DNA sequence is obtained by the integral importance of the DNA region when expressed in some aspect of cell function. The Internal Transcribed Spacer (ITS) region of ribosomal RNA repeat unit is a non-coding region that functions in RNA processing. Considering this function, it has been suggested that the secondary structures of ITS regions are more conserved than the actual nucleotide sequence (Hausner and Wang 2005). Hence ITS sequence is a less conserved region with a high substitution rate relative to the rDNA coding region. Therefore ITS regions are preferred for phylogenetic studies at the species level, since species and sub-species diversification events assumed to have occurred relatively recently.

2.4. Coevolution and Theory of the Geographic Mosaic of Coevolution

The systematic perspective of coevolution, which stresses the degree of congruence and incongruence between host and parasite [symbionts] phylogenies, is termed cospeciation (Brooks 1977). This congruence is a result of speciation of one species in response to the speciation of another (Paterson and Gray 1997). Association by descent or vertical transfer is defined as the association between host and parasite today because their ancestors were associated with each other in the past. This is shown by phylogenetic congruence. On the other hand, association by colonization or horizontal transfer develops from at least one of the species originated in some other context and subsequently became involved in the interaction by host switching. This is shown by phylogenetic incongruence (Hoberg *et al.* 1997).

Thompson's (1999) theory of the geographic mosaic of coevolution, suggests that different genetic and ecological conditions may result in different coevolutionary dynamics over broad geographic scales (Fig. 2). According to this theory, there is a selection mosaic among populations, favoring different evolutionary paths in different populations. There are coevolutionary hot spots, which are the subset of communities in which reciprocal selection is actually occurring. Also there is a continual geographic remixing of the range of coevolving traits, or coevolutionary dynamics resulting from different combinations of the selection mosaics, coevolutionary hot spots, gene flow, random genetic drift, and local extinction of populations. At any moment, coevolutionary hot spots may be few or many. Moreover, reciprocal selection intensity will differ geographically, producing a gradient of warm spots as well as hot spots and cold spots. A local interaction may show no reciprocal selection, or symmetrical but weak reciprocal selection, or asymmetrical (strong on one, weak on other) reciprocal selection, or strong reciprocal selection on both.

Different populations will have different traits as the outcome of different local interactions. These local interactions are usually multispecific and very complex resulting in some well matched and some mismatched traits in interactive species, producing some cases of local maladaptation and species-level coevolution respectively. The outcomes of interactions can vary among communities as well as over time within a community.

The systematic perspective of coevolution is called cospeciation. Cospeciation or parallel cladogenesis is the process of speciation of one species in response to the speciation of another (Paterson and Gray 1997). This leads to creation of a pattern called Fahrenholz' rule, stating that one species phylogeny mirrors another species' phylogeny

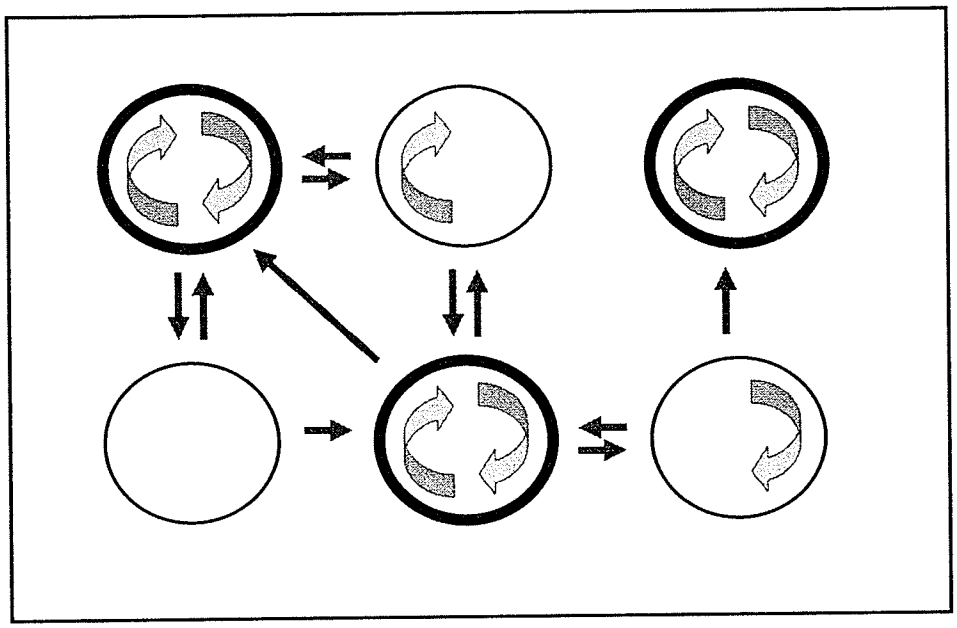

Fig. 2: Geographic mosaic of coevolution between a pair of species. Circles represent communities. Each arrow within circles indicates selection on one species. Arrows between communities indicate gene flow. Coevolutionary hotspots are indicated by bold circles in a matrix of evolutionary cold spots, indicated by fine circles. (Modified from Thompson 1999).

(Brooks 1985). Therefore, cospeciation focuses on the degree of congruence and incongruence between symbionts phylogenies. Congruence in symbiont phylogenies could result from synchronous cospeciation or delayed speciation. Synchronous cospeciation is when equal amount of evolutionary divergence is observed in both phylogenies. Delayed cospeciation is when speciation of one species lags behind speciation in another species and shows a lower amount of divergence (Hoberg *et al.* 1997).

Selectivity and Specificity in lichen association

Selectivity is first defined as "preferential interaction between organisms"; and specificity, as "cell-cell interactions with absolute exclusivity" by Galun and Bubrik (1984). Beck *et al.* (2002) used the term selectivity for "the characterization of interactions between organisms viewed from the perspective of one biont only", and the term specificity for "the symbiotic association as a whole". They suggest that specificity is dependent on the degree of selectivity by other bionts. Therefore, if two bionts always associate with one another, and not with any other genotype, they are assumed to be specific for one another.

In sexually reproducing lichens, where mycobionts reproduce via ascospores, relichenization is thought to be a necessary process in the life cycle of the lichen. In this case, genetic homogeneity of the photobiont depends on several factors, including the selectivity of the mycobiont, the availability of suitable photobiont species and the environmental conditions.

High selectivity is a situation where one species or genotype of algae always associates with a lichen fungus species or genotype even though other algae may be more common in the same habitat. Low selectivity is when a mycobiont associates with a more common alga in the habitat. In the case of low selectivity, more than one algal genotype is expected to occur in populations comprising one mycobiont species or genotypes. Such heterogeneity of the photobionts could be explained by multiple relichenizations due to sexual reproduction (Beck *et al.* 2002).

If both bionts exhibit a high degree of selectivity, the association is considered to be specific. The only way of proving specificity is to investigate different lichen species and to determine whether the partners are exclusively associated with each other or not (Beck *et al.* 2002). High specificity is when one mycobiont taxon is exclusively associated with one algal taxon. In this case, in a lichen association, the phylogeny of the fungal partner will reflect that of the algal partner showing coordinated speciation.

Examples of selectivity and specificity in lichen associations

When a mycobiont associates with photobionts belonging to different families, it exhibits a low degree of selectivity. For example, *Chaenotheca chrysocephala* is found to be associated with either *Trebouxia* or *Stichococcus* (Tibell, 1980). Since other mycobionts can also form lichens with both photobionts, the association is not exclusive, therefore, it is not specific. In another study based on ITS sequence data, Tibell (2001) found that of five clades of *Chaenotheca*, two are associated with *Stichococcus* only, and one with *Trebouxia* only, showing a high selectivity of mycobionts toward photobionts. While in the other two clades of *Chaenotheca*, two or three photobiont genera were

present, showing a low selectivity of mycobionts toward photobionts. At the genus level also *Chaenotheca* is remarkable for association with four genera of photobionts, *Dicttyochloropsis*, *Stichococcus*, *Trebouxia* and *Trentepohlia,* showing a low degree of selectivity. A highly selective photobiont was demonstrated in the genus *Letharia*, always being associated with algae from the *Trebouxia jamesii* species complex (Kroken & Taylor 2000).

Mycobionts at higher taxonomic levels such as suborders and families and genera are strongly selective toward their photobionts. One example of this strong selectivity can be observed in order Lecanorales of ascomycetes and the green alga *Trebouxia. Trebouxia* was formerly divided into two genera, *Trebouxia* and *Asterochloris* (Tschermak-Woess, 1980). While the genus *Asterochloris* is confined to the suborder *Cladoniineae, Trebouxia* is specific to the suborder *Lecanoriineae*. Most families of *Lecanoriineae* are only associated with members of *Trebouxia*, indicating a high selectivity of mycobiont in this suborder; while in Parmeliaceae, *Trebouxia* and *Asterochloris* are co-occurring, and in Biatoraceae and Lecanoraceae, *Trebouxia* and *Dicttyochloropsis* are co-occurring (Rambold *et al.* 1998). This shows a lower selectivity of mycobionts in Parmeliaceae and Lecanoraceae toward their photobionts.

Beck *et al.* (2002) showed that two different strains of *T. asymmetrica*, which were almost identical in their ITS-sequence, are associated with *Fulgensia fulgida*. One of these algal strains has not yet been found in association with other fungi, therefore this association could be a specific association. Palsrud and Lindblad (1998) analyzed four species of cyanolichens using the secondary structure of tRNA introns as population

markers. Only one intron type was found in each lichen thallus indicating a high degree of specificity.

Coevolution was reported in a number of fungal and non-fungal symbionts and their partners; such as ascomycetous fungi, *Epichloë* spp., that are obligate symbionts of grasses and span a continuum from antagonism to mutualism in their symbiotic relationship (Schardl *et al.* 1997), in tripartide symbiotic associations of fungus-growing ants, their basidiomycetous cultivars, and their garden's parasites (Currie *et al.* 2003); and in slave-making ants *Protomognathus americanus* with its hosts *Leptothorax* spp. (Blatrix *et al.* 2003). Coevolution was also reported in a number of previous studies on lichen partners; in some *Nostoc*- containing lichens (Paulsrud and Lindblad 1998) and in green algal lichens with *Trebouxia* and the fungal genus *Letharia* (Kroken and Taylor 2000).

Piercey-Normore and DePriest (2001) did not find any statistical support for the overall cospeciation between fungal and algal species of the genus *Cladonia* on a global scale. However they did find consistent parallel evolution in small groups within the genus. Parallel speciation was traced within a clade containing *C. peltastica, C. pulviniformis* and *C. spinea* as well as in another clade containing section Ascyphiferae, *C. turgida, C. furcata* and *C. farinaceae. Cladonia furcata* and *C. turgida* both lack vegetative propagules such as soredia. Both have squamules and are highly branched that can act as means of vegetative reproduction upon fragmentation. Apothecia are present in *C. furcata* but are rare in *C. turgida* (Thomson 1984, Brodo 2001). Therefore asexual reproduction by means of fragmentation should be more common than sexual

reproduction in these species. This suggests that vertical transfer of algae is more common in these species implying an increased chance for parallel cladogenesis.

Since Piercey-Normore and DePriest (2001) examined representatives from the entire genus *Cladonia* and samples were collected from a worldwide distribution. This thesis will focus on a narrow taxonomic breadth, *Cladonia* section *Cladonia*, and a narrow geographic range, North America. Method of reproduction will also be taken into account in the exaqmination of coevolutionary patterns in *Cladonia* section *Cladonia.*

3. Materials and Methods

Taxon sampling and Identification

A total of 12 species of the genus *Cladonia* section *Cladonia* were collected from Manitoba and Nova Scotia (Table 1).

Species identifications were determined by morphological (Ahti 2000a; Brodo *et al.* 2001; Thomson 1984) and chemical analysis using standardized thin-layer chromatography (Culberson 1972) as well as comparison with existing herbarium material and confirmation by T. Ahti (Helsinki University, Finland). Up to three specimens for each species were examined to assess variation within a species. However, one specimen represented a species when additional specimens were not available.

Standardized Thin Layer Chromatography

Species identifications were supplemented with standard Thin Layer Chromatography (TLC) of secondary compounds and comparison of R_f classes (Culberson 1972). Secondary compounds from a single podetium of each sample were extracted by a hot acetone extraction on microscope slides for two 5-minute and one 10-minute interval, then spotted onto 6.5x20 cm silica coated glass plates (Fisher Sci., Ottawa, ON). Three TLC chromatograms were produced for each sample by placing the plates in three different solvents. Solvent 'A' wascomposed of toluene, dioxane, and glacial acetic acid (180:45:5), solvent 'B' was composed of hexane, methyl-tetra-butyl-ether, and formic acid (140:72:18). Three to five minutes was required for pre-treatment with 60% formic acid. Solvent 'C' was composed of toluene and acetic acid (200:30) and required three to five minutes pre-treatment with glacial acetic acid.

Table 1: List of *Cladonia* species in section *Cladonia* used in this study, their TLC numbers, collection numbers and sampling locations.

Taxon	TLC Number	Collection Number	Collection site	Code in figures
C. chlorophaea (Florke) Sprengel	SB40	Normore1480	MB	*C. chlorophaea*3738
C. coniocrea (Florke) Sprengel	SB25	Normore1384	MB	*C. coniocrea*1112
C. cornuta (Linnaeus) Hoffmann	SB30	Normore1824	MB	*C. cornuta*1314
C. ecmocyna Leight	SB33	Normore972	MB	*C. ecmocyna*2728
C. gracilis (Linnaeus) Willd	SB46	Normore1443	MB	*C. gracilis*8990
C. grayi G. Merrill	SB49	Normore947	MB	*C. grayi*9596
C. grayi Merrill	SB50	Normore971	MB	*C. grayi*9798
C. macrophyllodes Nylander	SB36	Normore1056	MB	*C. macrophyllodes*2930
C. merochlorophaea Asahina	SB52	Normore930	MB	*C. merochlorophaea*4344
C. ochrochlora Florke	SB64	BeiggiNS06	NS	*C. ochrochlora*9394
C. pocillum (Acharius) Grognot	SB12	Normore1784	MB	*C. pocillum*0102
C. pocillum (Acharius) Grognot	SB38	Normore1058	MB	*C. pocillum*2526
C. pocillum (Acharius) Grognot	SB39	Normore1059	MB	*C. pocillum*8788
C. pyxidata (Linnaeus) Hoffmann	SB13	Normore1626	MB	*C. pyxidata*0304
C. verticilata. (Hoffmann) Schaer	SB16	Normore2401	MB	*C. verticilata*0506
C. verticilata. (Hoffmann) Schaer	SB17	Normore2370	MB	*C. verticilata*0708
C. verticilata. (Hoffmann) Schaer	SB19	Normore1624	MB	*C. verticilata*0910

TLC plates were placed in solvents until the solvent front reached 0.5 cm from the top of the glass plate. After the plates dried, they were examined under two UV wavelengths (365 and 254 nm) to indicate quenching or fluorescence, sprayed with 10% sulfuric acid and placed on a slide warming tray to detect presence of any fatty acids in the extract. Next, the plates were placed in 80°C oven for 10 minutes to develop the chromatograms. Spot characteristics and R_f classes were used to determine compounds by comparison with known compound characteristics (C. Culberson, unpublished).

Morphological and Chemical characters

A total of 23 morphological and chemical characters were examined (Tables 2& 3). These characters were investigated using a dissection microscope (Olympus VM-ILA-2) and standardized thin layer chromatography.

DNA isolation

Individual podetia from lichen associations were visually examined and portions that appeared to be in good condition were used for isolation of genomic DNA using a CTAB (cetyltrimethylammonium bromide) extraction protocol modified from Grube *et al.* (1995). In this protocol, lichen tissue was ground in eppendorf tubes using plastic pestles with 500µL of TES buffer (100 mM of Tris-HCl [pH 8.0], 10mM of ethylenediaminetetraacetic (EDTA) and 2% Sodium dodecyl sulphate (SDS). Afterward, 140µl of 5M NaCl and 70µL of 20% CTAB were added to each tube and samples were incubated at 65° C for one hour. Proteins were extracted by adding an equal volume of Chloroform: Isoamyl alcohol (24:1) and centrifuged for five minutes at 5,000 rpm. The

Table 2: Chemical and morphological characters and character states

1: Usnic acid
- 1- Present
- 0- Absent

2: Fumarprotocetraric acid
- 1- Present
- 0- Absent

3: Atranorin
- 1- Present
- 0- Absent

4: Grayanic acid
- 1- Present
- 0- Absent

5: Merochloropheaic acid
- 1- Present
- 0- Absent

6- Primary squamules
- 1- Permanent
- 0- Non-permanent

7- Primary squamules
- 1- Large
- 0- Small

8: Podetial squamules
- 1- Present
- 0- Absent

9: Podetial squamules
- 1- Largr
- 0- Small

10: Podetial squamules
- 1- Abundant
- 0- Rare

11: Podetia
- 1- Sorediate
- 0- Non-sorediate

12: Podetia
- 1- Non-corticate
- 2- Corticate

13: Podetial soredia
- 1- Granular
- 0- Farinose

14: Podetia
- 1- Blackened at the base
- 0- Not blackened at the base

15: Podetial width
- 1- Stout
- 0- Slender

16: Podetial branching
- 1- Branched (once or twice)
- 0- Not branched

17: Podetia
- 1- Smooth
- 0- Aerolate

18: Podetial
- 1- With large propagules
- 0- Without propagules

19: Cups
- 1- Present
- 0- Absent

20: Shape of podetial cups (Fig. 3)
- 1- Trumpet-shaped
- 0- Gublet-shaped

21: Podetial axils or cups
- 1- Perforated
- 0- Non-perforated

22: Cups
- 1- Proliferated
- 0- Not proliferated

23: Cups proliferation
- 1- Marginal
- 2- Central

Table 3: The binary (0, 1) data matrix built based on chemical (1-5) and morphological (6-23) characters of 17 Cladonia specimens used in this study.

TLC Number	Morphological and chemical data matrix 0 1 2 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3
SB12.	0 1 0 0 0 1 1 0 - - 0 0 - 0 1 0 0 1 1 1 0 0 -
SB13.	0 1 0 0 0 1 1 0 - - 0 0 - 0 1 0 0 1 1 1 0 0 -
SB16.	0 1 0 0 0 0 - 0 - - 0 1 - 1 1 0 0 0 1 0 1 1 0
SB17.	0 1 0 0 0 0 - 0 - - 0 1 - 1 1 0 0 0 1 0 1 1 0
SB19.	0 1 0 0 0 1 1 0 - - 0 1 - 1 0 0 0 0 1 0 1 1 ?
SB25.	0 1 0 0 0 1 0 0 - - 1 1 0 0 0 0 - 0 1 1 0 0 -
SB30.	0 1 0 0 0 0 - 1 0 0 1 1 - 0 0 1 1 0 1 0 - - -
SB33.	0 1 1 0 0 0 - 1 1 1 0 0 - 1 0 0 0 0 1 0 1 1 1
SB36.	0 1 1 0 0 1 1 0 - - 0 0 - 0 1 0 0 1 1 1 0 0 -
SB38.	0 1 1 0 0 1 1 0 - - 0 0 - 0 1 0 0 1 1 1 0 0 -
SB39.	0 1 0 0 0 1 1 0 - - 0 0 - 0 0 0 0 1 1 1 0 0 -
SB40.	0 1 0 0 0 1 0 0 - - 1 0 1 1 1 0 - 0 1 0 1 1 1
SB46.	0 1 0 0 0 1 1 1 1 0 0 1 - 0 1 0 1 0 1 0 1 1 1
SB49.	0 1 0 1 0 1 0 1 0 1 1 0 1 0 0 0 0 0 1 0 1 1 1
NS06.	0 1 0 0 0 1 0 1 0 0 1 0 1 0 1 0 - 0 1 1 0 0 -
NS05.	0 1 0 0 0 1 0 1 1 1 1 1 0 0 0 0 - 0 1 0 0 0 -
SB52.	0 1 0 0 1 1 0 0 - - 1 ? 0 0 1 0 - ? 0 - - - -

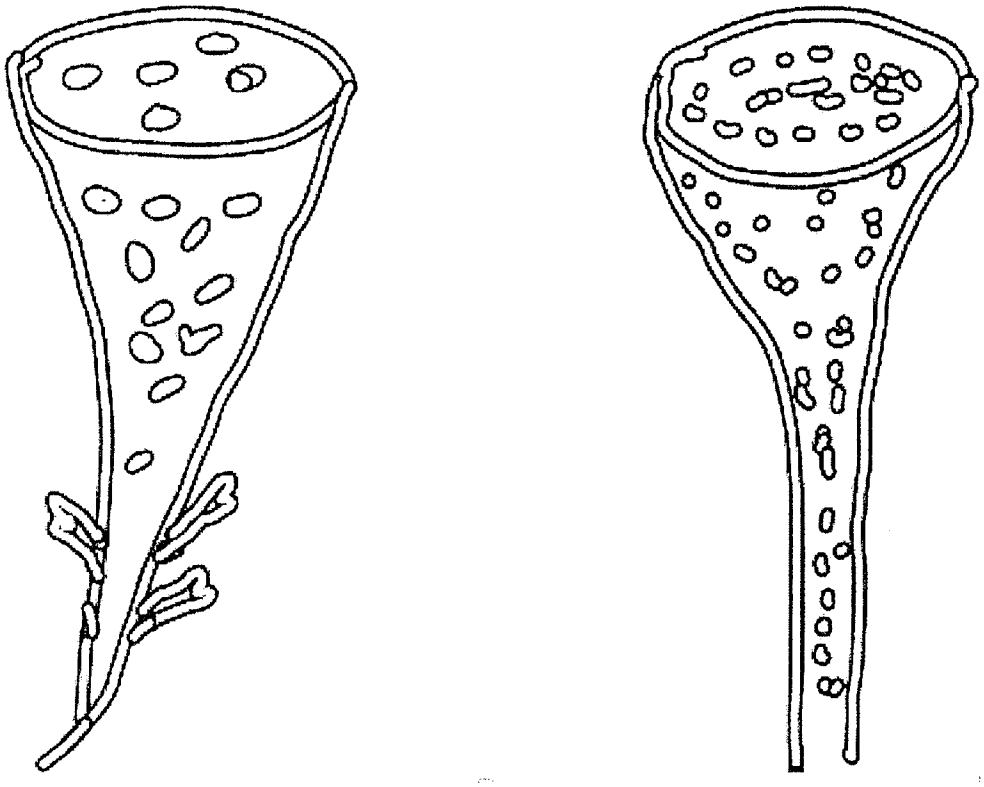

Fig. 3: Cup morphology in the genus *Cladonia.* Left, a trumpet-shaped cup, covered with coarse granular soredia; Right, a goblet-shaped cup, covered with farinose soredia.

aqueous phase was transferred to a clean eppendorf tube and the extraction was repeated. The DNA in the aqueous phase was precipitated with 0.2 volumes of 5M NaCl and 2.5 volume of 100% ethanol. After 20 minutes at 4°C the tubes were centrifuged for 10 minutes at 13,000 rpm. Pellets were washed with cold 80% ethanol, air-dried for 30 minutes and resuspended in 50μL sterile distilled water.

Genomic DNA was quantified by gel electrophoresis using a 1% agarose gel and stained with ethidium bromide. The gel was electrophoresed in1X TBE buffer (45mM Trizma base and Boric acid and 1mM EDTA [pH 8.0]) using 1 Kb Plus DNA Ladder (Invitrogen). The gel was run at 120 volt and visualized under UV transillumination (Spectoline CM-10). Density of bands was compared visually to estimate the amount of extracted DNA. All chemicals used in this study were provided by Fisher Sci. (Ottawa, ON) unless otherwise indicated.

DNA amplification

Isolated genomic DNA from lichens includes fungal nuclear and mitochondrial DNA as well as algal nuclear, mitochondrial and chloroplast DNA. To selectively amplify the internal transcribed spacer region 1 (ITS1), region 2 (ITS2) and the 5.8S from nuclear ribosomal DNA (rDNA), taxon-specific primers were designed in conserved regions (small subunit (SSU) and large subunit (LSU) rDNA) flanking the ITS regions that are specific to each of the fungal or algal nuclear ITS rDNA (Piercey-Normore and DePriest 2001), (Fig. 4). The primers, 1780F-5' (CTGCG GAAGG ATCAT TAATG AG) and ITS4-3′ (TCCTC CGCTT ATTGA TATGC) were used to amplify the ITS of the rDNA

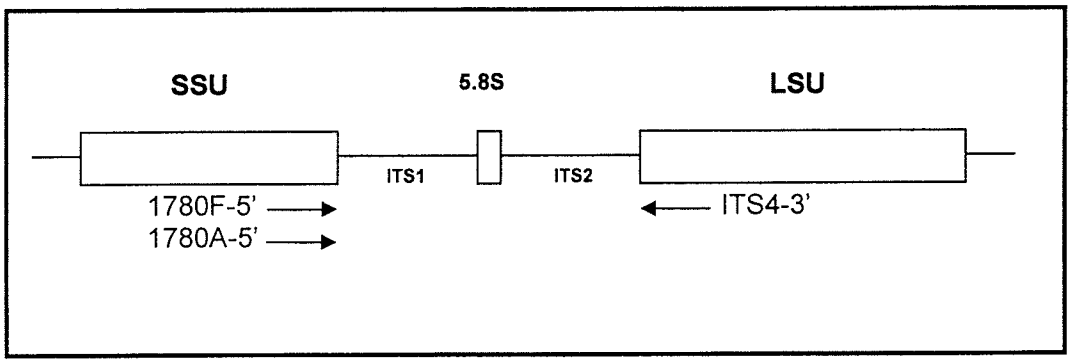

Fig 4. Illustration of a nuclear rDNA repeat unit showing annealing sites for amplification primers (1780F-5', 1780A-5' and ITS4-3') used in this study. These taxon-specific primers amplify algal and fungal ITS rDNA. The ambiguous sites for the taxon-specific primers are located on the small subunit of ribosomal DNA flanking the ITS1 region.

of the fungi. Primers, 1780A-5' (CTGCG GAAGG ATCAT TGATT C) and ITS4-3' were used to amplify the ITS of the rDNA of the algae.

Amplification reactions were done in a total reaction volume of 50 µl containing 10-50 ng DNA, PCR buffer (50 mM KCl, 100 mM Tris-HCl [PH 8.3], 1.5-2.0 mM $MgCl_2$ or $MgSO_4$, 2 µM of each deoxynucleotides (dNTPs), 0.5 µM of each primer and 2 units of Taq DNA polymerase (Invitrogen). For samples that were difficult to amplify 5% DMSO or 1X PCRx Enhancer (Invitrogen) were added to reactions.

DNA amplifications were performed on a Techne Genius thermal cycler (Fisher Sci., Ottawa, ON) or a T-Gradient Thermoblock thermocycler (Biometra, Goettingen, Germany). Both algal and fungal ITS rDNA regions were amplified with an initial denaturing temperature of 95°C for five minutes, followed by 33 cycles at a denaturing temperature of 94°C for 45 seconds, an annealing temperature of 56°C for 45 seconds and an extension temperature of 72°C for 1.5 minutes.

DNA Sequencing and alignment

Polymerase chain reaction (PCR) amplification products were gel purified by electrophoresis on 1% agarose in 1X TBE buffer. The band was excised from the gel, frozen overnight at –20°C and then crushed between Para film to collect the liquid as it melted. To remove loading dye and buffer, the DNA was precipitated with 0.2 volumes of 5M NaCl and 2.5 Volume of 100% ethanol. Purified DNA was quantified by the same method as the extracted genomic DNA.

DNA was sequenced using BigDye terminators on a 377 ABI DNA sequencer (University Core DNA and Protein Services, University of Calgary, Calgary, Alberta).

Additional ITS sequence of 32 *Cladonia*, *Cladia* and *Trebouxia* samples were downloaded for this study from GenBank (Table 4). Sequences were edited using Sequencher 4.2.2 (GenCodes Corp., MI, USA) and aligned visually using Se-Al v2.0a11 (Rambaut, 1996) and imported into PAUP 4.0 (Swofford 1998). There were no ambiguous sites in ITS sequences that would cause aligning problems.

Phylogenetic analyses

To explore the data, comparisons were made between subsets of the data. Accordingly nineteen datasets were constructed using aligned sequences of algal and fungal ITS entirely or partially, combined with chemical and morphological datasets.

Fungal datasets that included only sequences from this study:

1- ITS1, 5.8S and ITS2
2- ITS1
3- ITS2
4- ITS1, 5.8S and ITS2 combined with chemistry and morphology
5- ITS1, 5.8S and ITS2 of fungal sequences from symbiont partners only

Fungal datasets including sequences from this study and GenBank:

6- ITS1, 5.8S and ITS2
7- ITS1
8- ITS2
9- ITS1, 5.8S and ITS2 combined with chemistry and morphology (chemistry and morphology of GenBank sequences were determined from literature)
10- ITS1, 5.8S and ITS2 combined with chemistry and morphology with indels scored and incorporated into the dataset (insertions were coded as 1 and deletions as 0)

Table 4: List of 32 *Cladonia*, *Cladia* and *Trebouxia* species and their corresponding ITS sequence accession numbers in GenBank, downloaded for this study.

Species	**Acession No.**	**Species**	**Acession No./UTEX No.**
Cladia aggregata	AF453268	*C. merochlorophaea*	AF455227
C. ferdinandi	AF453269	*C. mitis*	AY170790
Cladonia asahinae	AF455229	*C. ocrochlora*	AF455192
C. cenotea	AF457896	*C. phyllophora*	AF455170
C. cenotea	AF457900	*C. pixydata*	AF455223
C. cariosa	AF455230	*C. pyxidata*	AF455208
C. cornuta	AF455196	*C. rangiferina*	AF458306
C. cornuta	AF455197	*C. rei*	AF455191
C. cristatella	AF453693	*C. subcervicornis*	AF517922
C. ecmocyna	AF455199	*C. subulata*	AF455180
C. fimbriata	AF455224	*T. erici*	AF345439/UTEX 910
C. gracilis	AF455194	*T. excentrica*	AF345433/UTEX 1714
C. gracilis	AF455198	*T. glomerata*	AF345382/UTEX 895
C. grayi	AF455226	*T. irregularis*	AF345411/UTEX 2236
C. grayi	AF455228	*T. magna*	AF345423/UTEX 67
C. maxima	AF455195	*T. pyriformis*	AF345406/UTEX 1712

Algal datasets including sequences from this study only:

11- ITS1, 5.8S and ITS2

12- ITS1

13- ITS2

Algal datasets including sequences from this study and GenBank:

14- ITS1, 5.8S and ITS2

15- ITS1

16- ITS2

Combined datasets for congruency tests

17- Fungal ITS from this study and Morphology

18- Fungal ITS1 and ITS2 from this study

19- Fungal and Algal ITS from this study

These datasets were analyzed by maximum parsimony, a commonly used phylogenetic method, as implemented in PAUP 4.0 (Swofford 1998). Maximum parsimony was performed using the tree bisection and reconnection (TBR) branch swapping option. Heuristic searches were conducted using 100 random addition replicates with a limit of 10 trees per search and 500 bootstrap replicates (Felsenstein 1985).

All algal trees were midpoint rooted since the ITS sequence of *Trebouxia* species associated with *Cladonia* section *Cladonia* cannot be aligned with ITS sequences obtained from other members of *Trebouxia*. Fungal trees produced based on ITS sequences from this study were also midpoint rooted since all ITS sequences obtained in this study belong to the *Cladonia* section *Cladonia* and could not be used as outgroup taxa. All fungal trees produced based on combinations of ITS sequences from this study

and GenBank sequences were rooted, using ITS sequences of *C. mitis* and *C. rangiferina* (members of *Cladonia* section *Cladina*) as the sister clade to section *Cladonia, C. cristatella* (member of *Cladonia* section *Cocciferae*), *C. cenotea* (member of section *Perviae*), and *Cladia ferdinandi* and *C. aggregate* (members of genus *Cladia*, family Cladoniaceae) as distant outgoup taxa.

The Kishino-Hasegawa (KH) test (Kishino and Hasegawa 1989) and Incongruence Length Difference (ILD) test (Farris *et al.* 1994) were implemented in PAUP 4.0. Three comparisons were tested to look for incongruencey in datasets

1- fungal ITS1 and ITS2
2- fungal morphology and ITS sequence
3- fungal and algal ITS sequence

The Incongruence Length Difference (ILD) test (Farris *et al.* 1994) assesses the heterogeneity of data sets by testing whether or not the two datasets produce the same topology and gives an indication of whether the data sets can be combined. The Kishino-Hasegawa (KH) test (Kishino and Hasegawa 1989) examines whether a topology derived from one dataset can possibly be derived from a second dataset. In other words, it examines congruence between a topology and a dataset and gives an indication of whether the data sets can be combined. The ILD test for DNA and morphological datasets resulted in a P- value of 0.01. The null hypothesis is that the DNA and the morphological datasets produce the same phylogenetic history and a P- value greater than 0.05 accepts the null hypothesis. A P-value of 0.01 implies that these two datasets are significantly different, thus they are not combinable. It also suggests they have different phylogenetic histories.

To compare the phylogenies resulted from phylogenetic and phenetic methods, datasets producing multiple topologies (fungal morphology, fungal ITS1 and Algal ITS1) were also analyzed by neighbor joining methods implemented in PAUP 4.0 (Swofford 1998) using uncorrected "P" distance model and ties were broken systematically.

4. Results

4.1. Fungal Phylogeny

There are three species complexes from section *Cladonia* represented in this study. The range of sequence similarities between members of each species complex is demonstrated in table 5. In some cases members of a species complex such as *C. chlorophaea* and *C. verticillata,* were more similar than members within a single species, such as *C. pyxidata* and *C. cornuta* (Table 5).

***Cladonia chlorophaea* species complex**

The *Cladonia chlorophaea* species complex is represented by *C. chlorophaea, C. merochlorophaea* and *C. grayi*. This group has members scattered throughout the tree (Fig. 5). Even members of the same chemospecies did not cluster together. *Cladonia grayi* and *C. chlorophaea* from the *C. chlorophaea* species complex clustered with *C. gracilis*, with 62% bootstrap support, and *C. fimbriata*, with 89% bootstrap support respectively. However, two members of *C. grayi* formed a sister clade with two members of *C. merochlorophaea* with 67% bootstrap support.

Two members of section *Cladina* clustered together, with 83% bootstrap support, as well as two members of the genus *Cladia*, with 100% bootstrap support, at the base of the trees (Fig. 5 & Append. 1). These results were supported by trees constructed from morphology and ITS sequence data from this study only (Appends. 2 & 3). *Cladonia merochlorophaea* clustered with *C. grayi;* and *C. chlorophaea* and *C. grayi* from *C. chlorophaea* species complex clustered with *C. coniocraea* , with 89% bootstrap support, and *C. gracilis* from *C. gracilis* species complex respectively.

Table 5: Range of pair wise similarities ("p" values) within members of species complexes and members of species within the Section *Cladonia* based on fungal ITS1, 5.8S and ITS2 nucleotide sequence. "n" is the number of sequences compared.

Taxon/Species complex	n	Fungal ITS sequence similarity
C. verticillata sp. complex	4	93.7 – 100%
C. chlorophaea sp. complex	7	93.9 – 99.5 %
C. gracilis sp. complex	9	91.7 – 99.9 %
C. cornuta	3	93.5 %
C. grayi	4	96.1 – 99.5 %
C. ochrochlora	2	97.4 %
C. pocillum	3	95.3 – 99.9 %
C. pyxidata	3	92.8 – 99.3 %

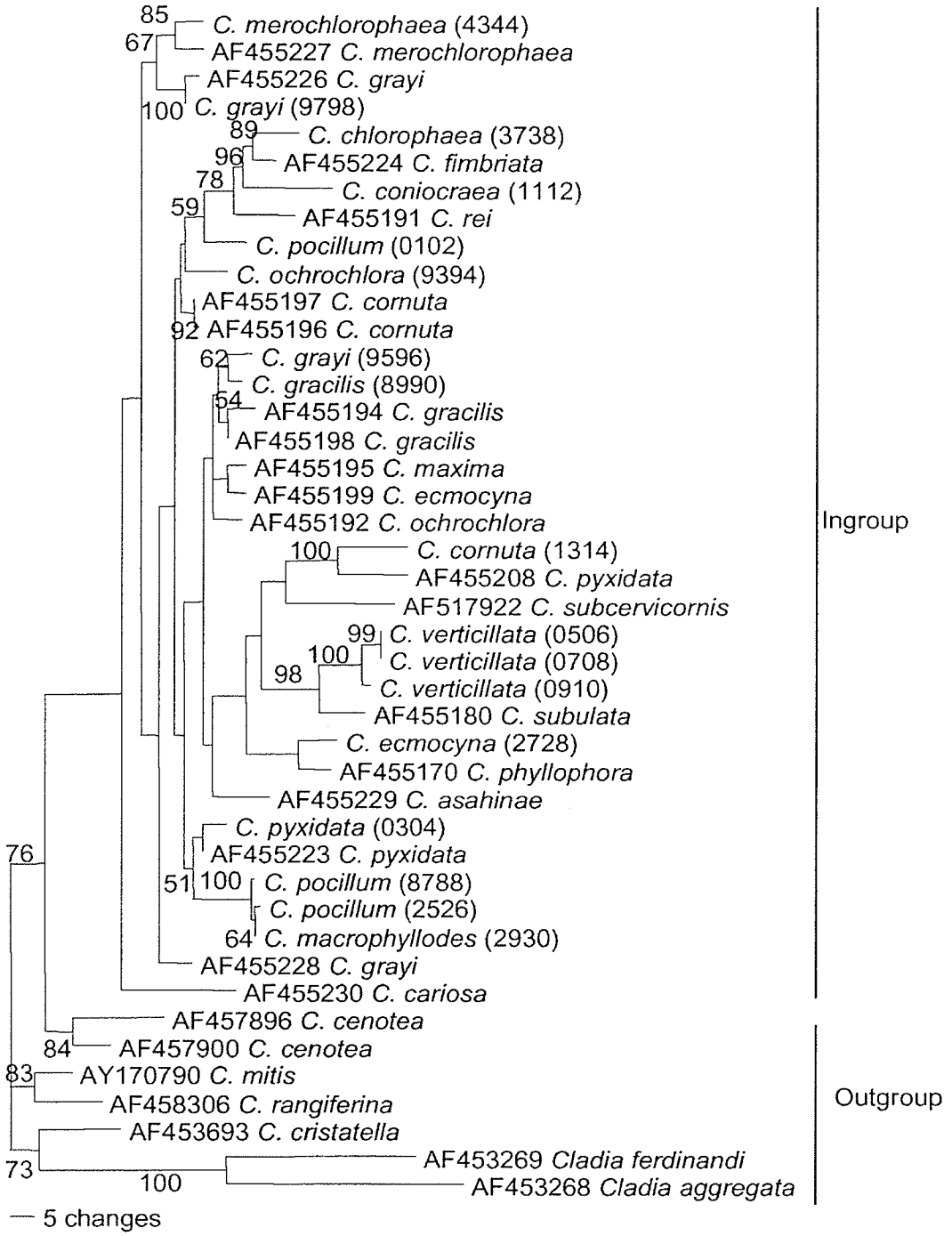

Fig. 5: One of 15 most parsimonious trees for the fungal morphology, ITS1, 5.8S and ITS2 combined nucleotide sequence data. Dataset includes 17 mycobionts of *Cladonia* from this study and 26 from GenBank. Indels in the sequence are scored and incorporated into the data. Numbers above branches are bootstrap support >50%. Numbers with species correspond to those in tables 1 & 4. CI: 0.6026, RI: 0.6630.

In the midpoint-rooted parsimony trees constructed from morphological data members of the *C. chlorophaea* species complex formed a polyphyletic group (Fig. 6 & Append. 4a). In the neighbor joining tree based on morphological data members of this group were also scattered (Append. 4b).

Cladonia pyxidata and *C. pocillum*

Cladonia pyxidata and *C. pocillum* are polyphyletic. In all trees *C. macrophyllodes,* a member of the *C. verticillata* species complex, clustered with four members of *C. pyxidata* and *C. pocillum,* with 51%-100% bootstrap support in different trees. Two members of these species always clustered outside this clade (Fig. 5 & Appends. 1-3). Even in trees based on morphological data (Fig. 6 & Append. 4) members of *C. pocillum* and *C. pyxidata* formed a clade along with *C. macrophyllodes* with 100% bootstrap support.

Cladonia gracilis species complex

The *Cladonia gracilis* species complex includes *C. cornuta, C. ecmocyna, C. gracilis* and *C. maxima*. Members of this group did not cluster together showing the polyphyletic nature of this group. Even members of *C. cornuta* did not cluster together. Two members of *C. cornuta* always grouped together, with 92% bootstrap support, and the third member clustered with *C. pyxidata* with 100% bootstrap support. Three members of *C. gracilis* species complex clustered with *C. grayi,* a member of the *C. chlorophaea* species complex with 62% bootstrap support. Other members of this group, *C. ecmocyna* and *C. maxima* did not show a consistent topological pattern. This inconsistent topology

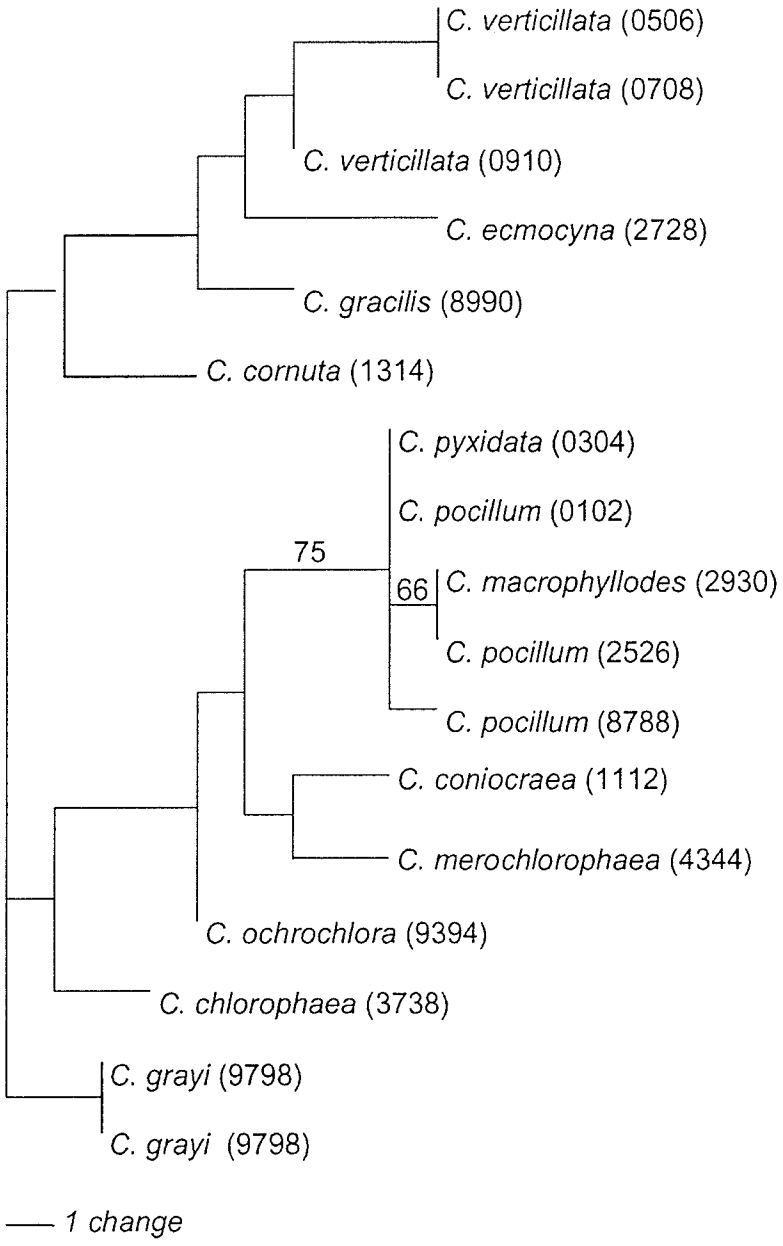

Fig. 6: One of 12 most parsimonious midpoint rooted trees for the fungal morphological data. Dataset includes 17 mycobionts of *Cladonia* from this study. Numbers with branches are bootstrap support >50%. Numbers with species correspond to those in tables 1 & 4. CI: 0.5676, RI=0.7538.

supports the polyphyletic nature of the *C. chlorophaea* species complex (Fig. 5 & Append. 1). In trees constructed from morphology and ITS sequence data combined (Appends. 2 & 3). A similar topological pattern was observed.

To compare the outcomes of a phylogenetic analysis (maximum parsimony) and a phenetic analysis for morphological dataset, a neighbor joining analysis was performed. In the neighbor joining tree based on morphological data members of the *C. chlorophaea* species complex did not cluster together (Append. 4b) forming a polyphyletic group. On the other hand in the parsimony trees constructed based on morphology data alone (Fig. 6 & Append. 4a), members of the *C. gracilis* species complex were basal to the clade containing *C. verticillata* forming a paraphyletic group.

Cladonia verticillata species complex

The *Cladonia verticillata* species complex was represented by *C. verticillata* and *C. macrophyllodes* in this study. In our results *C. macrophyllodes* clustered apart from members of *C. veticillata* and with *C. pyxidata* and *C. pocillum* in all trees with 51%-100% bootstrap support in different trees (Fig. 5 & Appends. 1-3) showing the polyphyletic nature of this group. In our results also the type species of the genus *Cladonia, C. subulata,* a member of the section *Cladonia*, is clustered with *C. verticillata* with 98% bootstrap support (Fig. 5 & Append. 1).

Comparing fungal ITS and morphology trees

In trees produced from ITS1, 5.8S, ITS2 and morphological combined data (Fig. 5 & Appends. 1-3) members of each of the *C. chlorophaea, C. gracilis* and *C. verticillata*

species complexes were polyphyletic. *Cladonia fimbriata* and *C. coniocraea* clustered with *C. chlorophaea* with 76% bootstrap support. *Cladonia grayi* from *chlorophaea* species complex and *C. gracilis* from *gracilis* species complex clustered together with 62% bootstrap support. *Cladonia macrophyllodes* a member of *C. verticillata* species complex, clustered with *C. pyxidata* and *C. pocillum* with 100% bootstrap support. *Cladonia subulata* the type species of the section *Cladonia* always grouped with *C. verticillata* with 98% bootstrap support.

Trees based on ITS1, ITS2, 5.8S sequences and morphological characters required that gaps be inserted within the sequence while aligning the sequences. These gaps were coded and incorporated into the dataset as additional characters. The two *Cladia* species fell within the ingroup of the genus *Cladonia* when gaps were not coded (Append. 5). When coded gaps were incorporated in the analysis *Cladia* species fell basal to the ingroup taxa (Fig. 5 & Append. 1).

Phylogenetic trees were topologically in agreement whether sequences from GenBank were included (Fig. 5 & Append. 1) or excluded (Appends. 2 & 3). Phylogenetic (maximum parsimony) and phenetic (neighbor joining) trees produced based on ITS sequence datasets both including and excluding GenBank sequences (Appends. 6, 7 & 8) show the same grouping pattern as trees based on the ITS sequence and morphology (Fig. 5 & Appends. 1-3).

Phylogenetic trees that were produced based on morphology only (Fig. 6 & Append. 4) showed a different topology from trees based on combined DNA and morphology data (Appends. 1-3). In the morphology trees (Fig. 6 & Append. 4) members of the *C. chlorophaea* species complex were polyphyletic. The *C. gracilis* species complex was

paraphyletic with its members clustered together and basal to the clade containing members of *C. verticillata* with 100% bootstrap support. The *C. verticillata* species complex was not monophyletic. One of its members, *C. macrophyllodes* clustered with *C. pocillum* and *C. pyxidata* with bootstrap support of 75% (Fig. 6).

The Kishino-Hasegawa (KH) test for combination of ITS sequence and morphology datasets and topologies resulted in a P-value ranging from 0.0004 to 0.0023. The ILD test for these datasets resulted in a P- value of 0.01 (Table 6). These values imply that these two datasets are significantly different and that they represent different phylogenetic histories.

Comparing fungal ITS1 and ITS2 trees

Topology of the 50% majority rule consensus of the most parsimonious trees produced from the fungal ITS1 and ITS2 separate sequence data sets were not similar to one another (Figs.7-8 & Appends. 9-10 and table 6). In the ITS1 tree four members of *C. chlorophaea* species complex clustered together, with 58% bootstrap support, as well as two members of *Cladonia* section *Cladina, C. mitis* and *C. rangiferina.* In this tree two *Cladia* species were also basal to the ingroup taxa. In the ITS2 trees, however, none of these patterns were observed (Fig. 8 & Append. 10). In general the number of informative characters in the fungal ITS1 sequence datasets was higher than that in the ITS2 sequence datasets (Table 7) reflecting better resolution in the ITS1 phylogenies (Fig. 7 & Append. 9). The ILD and KH tests for fungal ITS1 and ITS2 datasets resulted in P- values of 0.11, and a range of 0.0180 to 0.0706 (Table 6) respectively. These values imply that these two datasets are significantly different.

Table 6: Results of incongruence tests (ILD and KH tests) calculated for datasets in this study. P-values <0.05 resulted in rejection of null hypothesis.

Datasets	P-values	
	ILD test	KH test
Fungal ITS and Morphology	0.1	0.0004-0.0023
Fungal ITS1 and ITS2	0.11	0.180-0.706
Fungal and Algal ITS	0.1	<0.0001

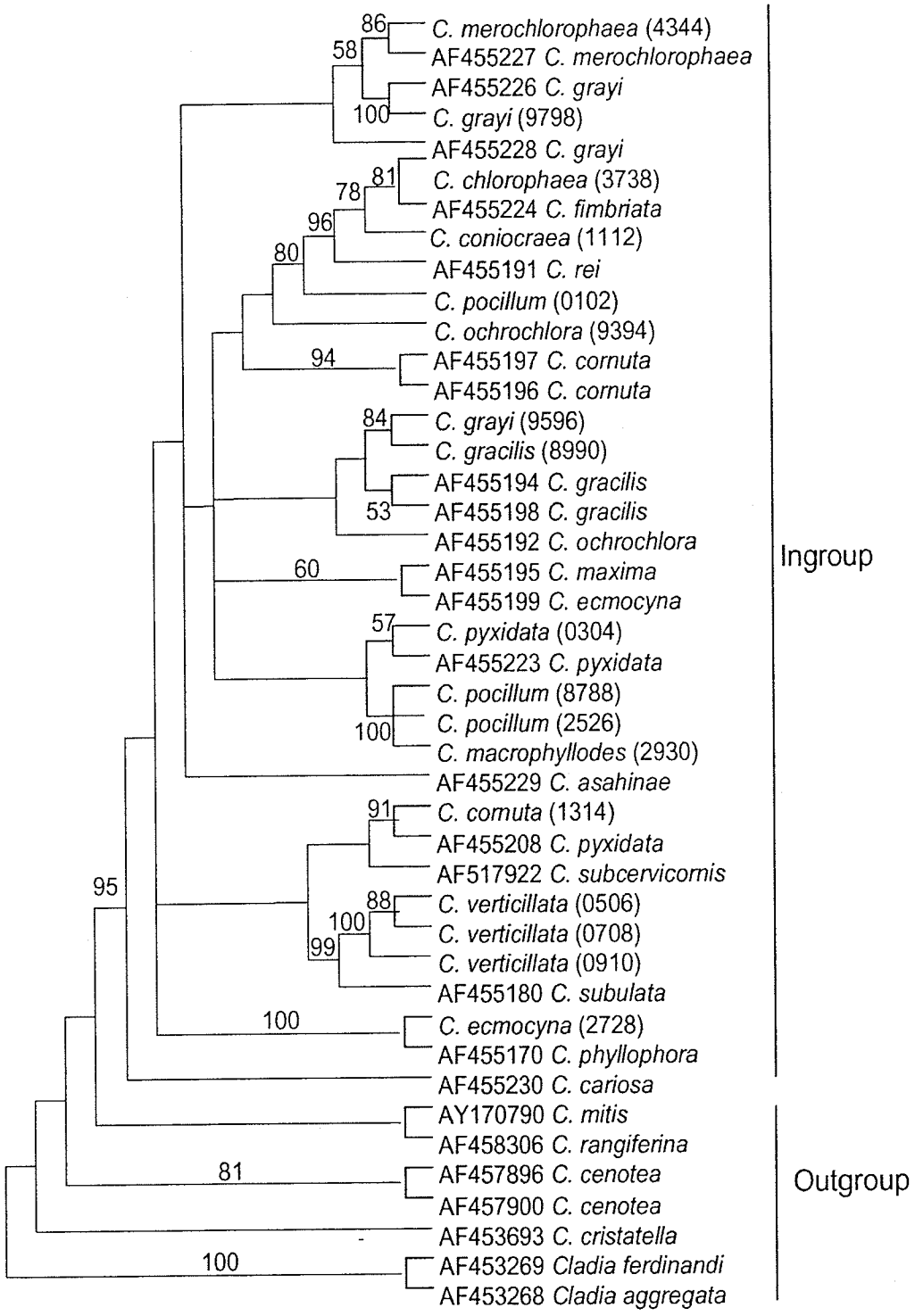

Fig. 7: 50% majority rule consensus of 38 most parsimonious trees for the fungal ITS1 nucleotide sequence data. Dataset includes 17 mycobionts of *Cladonia* from this study and 26 from GenBank. Numbers with branches are bootstrap support >50%. Numbers with species correspond to those in tables 1 & 4. CI: 0.6143, RI=0.6692.

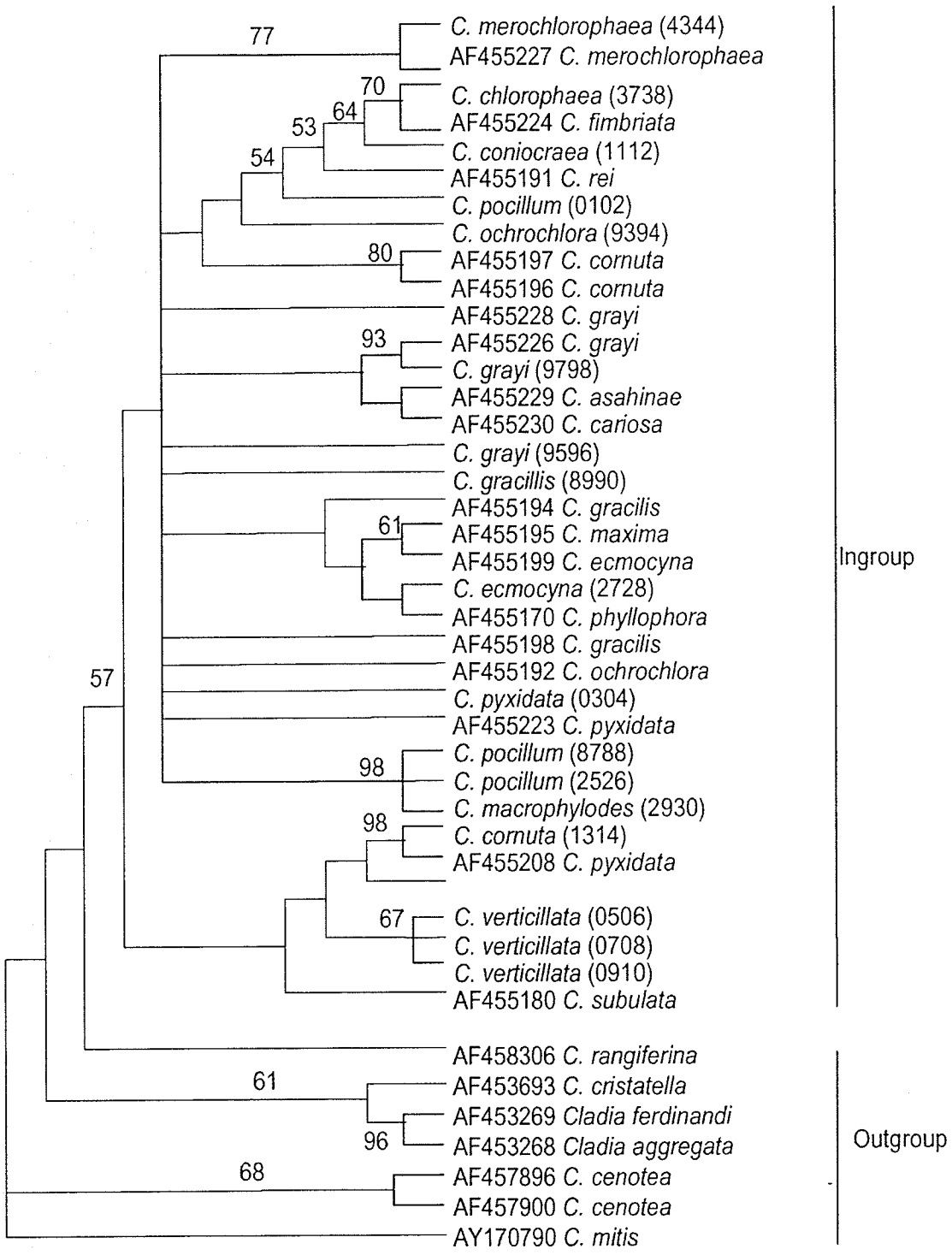

Fig. 8: 50% majority rule consensus tree of 29 most parsimonious trees for the fungal ITS2 nucleotide sequence data. Dataset includes 17 mycobionts of *Cladonia* from this study and 26 from GenBank. Numbers with branches are bootstrap support >50%. Numbers with species correspond to those in tables 1 & 4. CI=0.6889, HI=0.3111.

Table 7. A comparison of the variation in algal and fungal aligned ITS1 and ITS2 nucleotide sequences used in this study.

Dataset	Number of characters			
	Total	Constant	Uninformative	Informative
Mycobiont ITS1 (includes this study and GenBank species)	995	349	95	155
Mycobiont ITS2 (includes this study and GenBank species)	185	92	39	54
Mycobiont ITS1 (includes sequences obtained in this study only)	263	194	25	44
Mycobiont ITS2 (includes sequences obtained in this study only)	180	140	20	20
Natural photobiont ITS1 (includes this study and UTEX species)	194	178	11	5
Natural photobiont ITS2 (includes this study and UTEX species)	200	180	11	9
Natural photobiont ITS1 (includes sequences obtained in this study only)	194	185	4	5
Natural photobiont ITS2 (includes sequences obtained in this study only)	200	180	5	9

4.2. Algal Phylogeny

Photobionts of six species of *Cladonia* Section *Cladonia* (*C. verticillata, C. pyxidata, C. ochrochlora, C. merochlorophaea, C. ecmocyna* and *C. grayi)* clustered with *T. glomerata* or *T. pyriformis* with 88% bootstrap support forming a clade A (Fig. 9, Table 8) in the combined analysis. Photobionts of five species of *Cladonia* (*C. cornuta, C. fimbriata C. coniocrea, C. pocillum* and *C. macrophyllodes*) clustered with *T. magna* and *T. excentrica* with 99% bootstrap support, forming a clade B (Fig. 9). In clade B, photobionts of *C. cornuta* and *C. fimbriata* clustered with *T. excentrica* although with low bootstrap support. Sister to this clade is a clade containing photobionts of *C. coniocraea, C. pocillum* and *C. macrophyllodes* with 64% bootstrap support. *Trebouxia magna* clustered basal to these sister clades but with low bootstrap support.

The same topological pattern was observed in clade A of the ITS1 nucleotide sequence tree (Fig. 10). The only difference between the tree constructed from the entire ITS sequence data (Fig. 9) and the tree based on ITS1 sequence data (Fig. 10) is that in the former tree *T. erici* is basal to Clade A and *T. excentrica* is in clade B while in the latter tree they switch their positions.

In clade B of the ITS1 nucleotide sequence tree (Fig. 10), *T. magna* clustered with photobionts of *C. coniocraea, C. pocillum, C. macrophyllodes, C. cornuta* and *C. fimbriata* and *T. erici,* with 52% bootstrap support, is located basal to this clade with 61% bootstrap support.

The ITS2 dataset was less resolved and resulted in 22 most parsimonious trees (Fig. 11). In the 50% majority rule consensus tree of all 22 most parsimonious trees part of the *T. magna* clade was present, however, the *T. excentrica* clade was unresolved (Fig. 11).

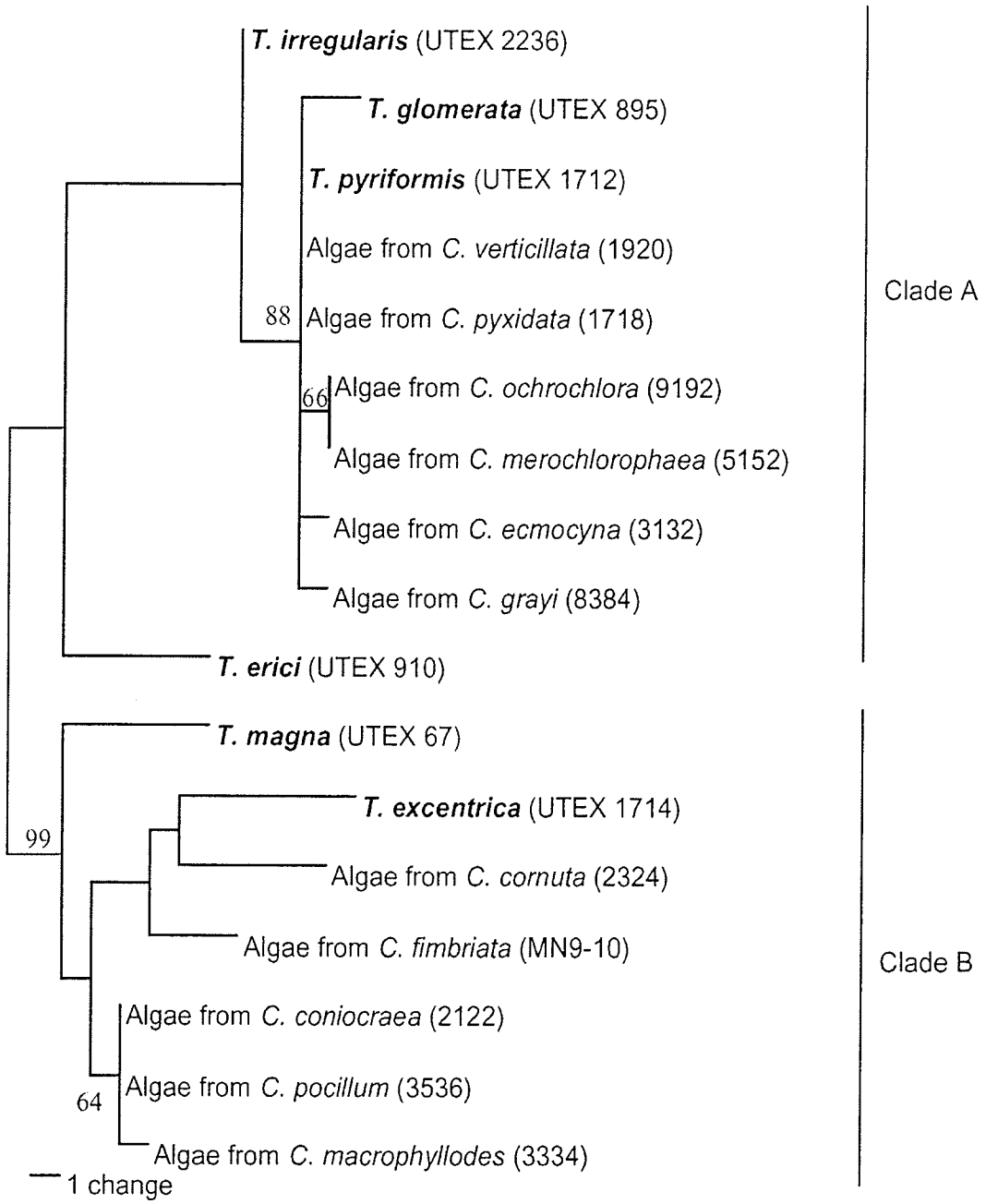

Fig. 9: One of two most parsimonious midpoint rooted trees for the algal ITS1, 5.8S and ITS2 combined nucleotide sequence data. Dataset includes 11 photobionts of *Cladonia* from this study and six known *Trebouxia* species from GenBank. Numbers with branches are bootstrap support >50%. Numbers with species correspond to those in tables 1 & 4. CI: 0.9111, RI: 0.9255.

Table 8: Comparison of pairwise distances, using the uncorrected "P" distance method, and number of sequence substitutions between natural photobionts and two known *Trebouxia* species in clade A of fig. 9.

Natural photobiont	**Distance** *T. pyriformis*	**Substitutions** *T. pyriformis*	**Distance** *T. glomerata*	**Substitutions** *T. glomerata*
From *C. verticillata*	0.0	0	0.004	2
From *C. pyxidata*	0.0	0	0.004	2
From *C. ochrochlora*	0.002	1	0.006	3
From *C. merochlorophaea*	0.002	1	0.006	3
From *C. ecmocyna*	0.002	1	0.006	3
From *C. grayi*	0.002	1	0.006	3

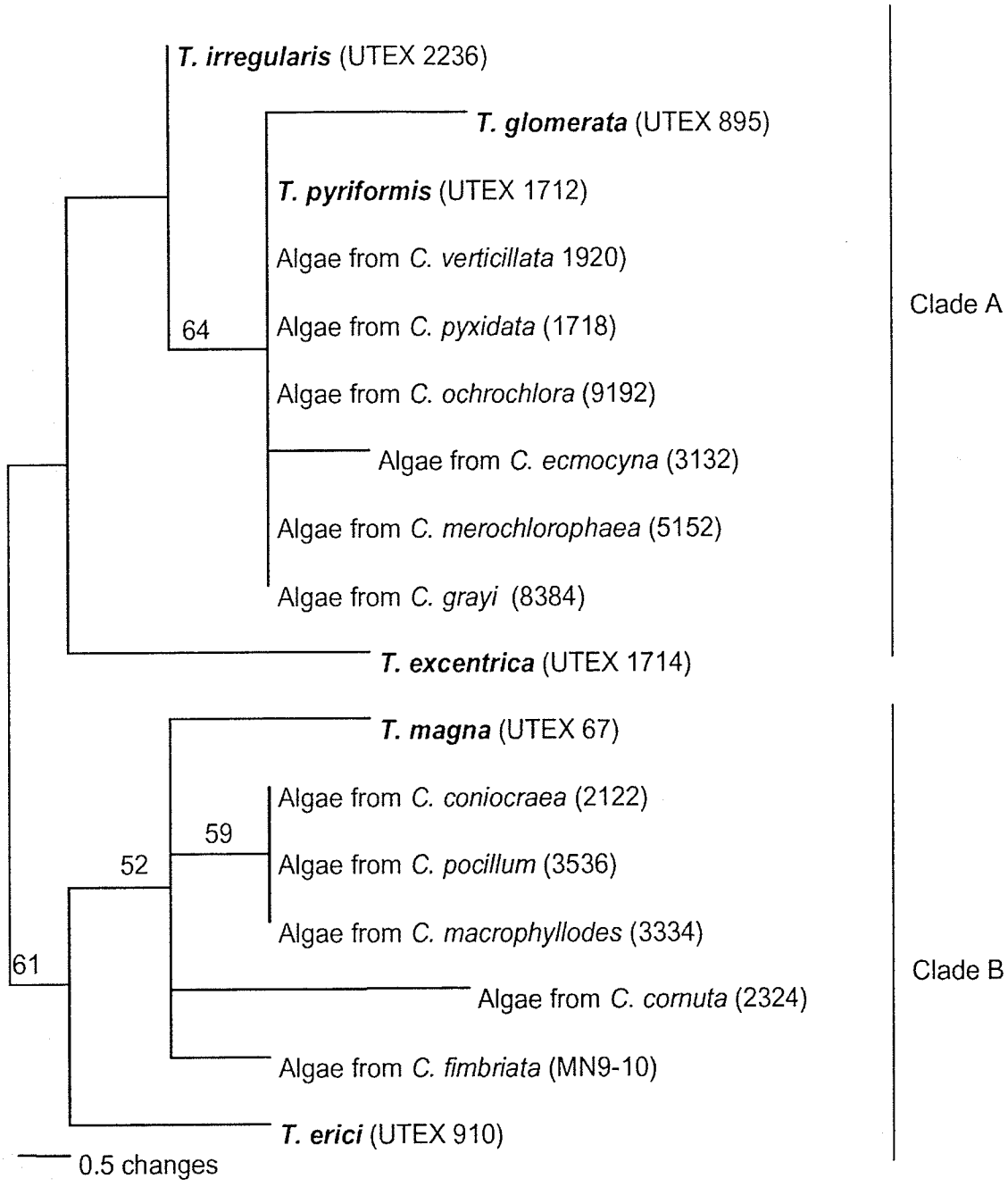

Fig. 10: The single most parsimonious midpoint rooted tree for the algal ITS1 nucleotide sequence data. Dataset includes 11 photobionts of *Cladonia* from this study and six known *Trebouxia* species from GenBank. Numbers with branches are bootstrap support >50%. Numbers with species correspond to those in tables 1 & 4. CI: 1.0, RI: 1.0.

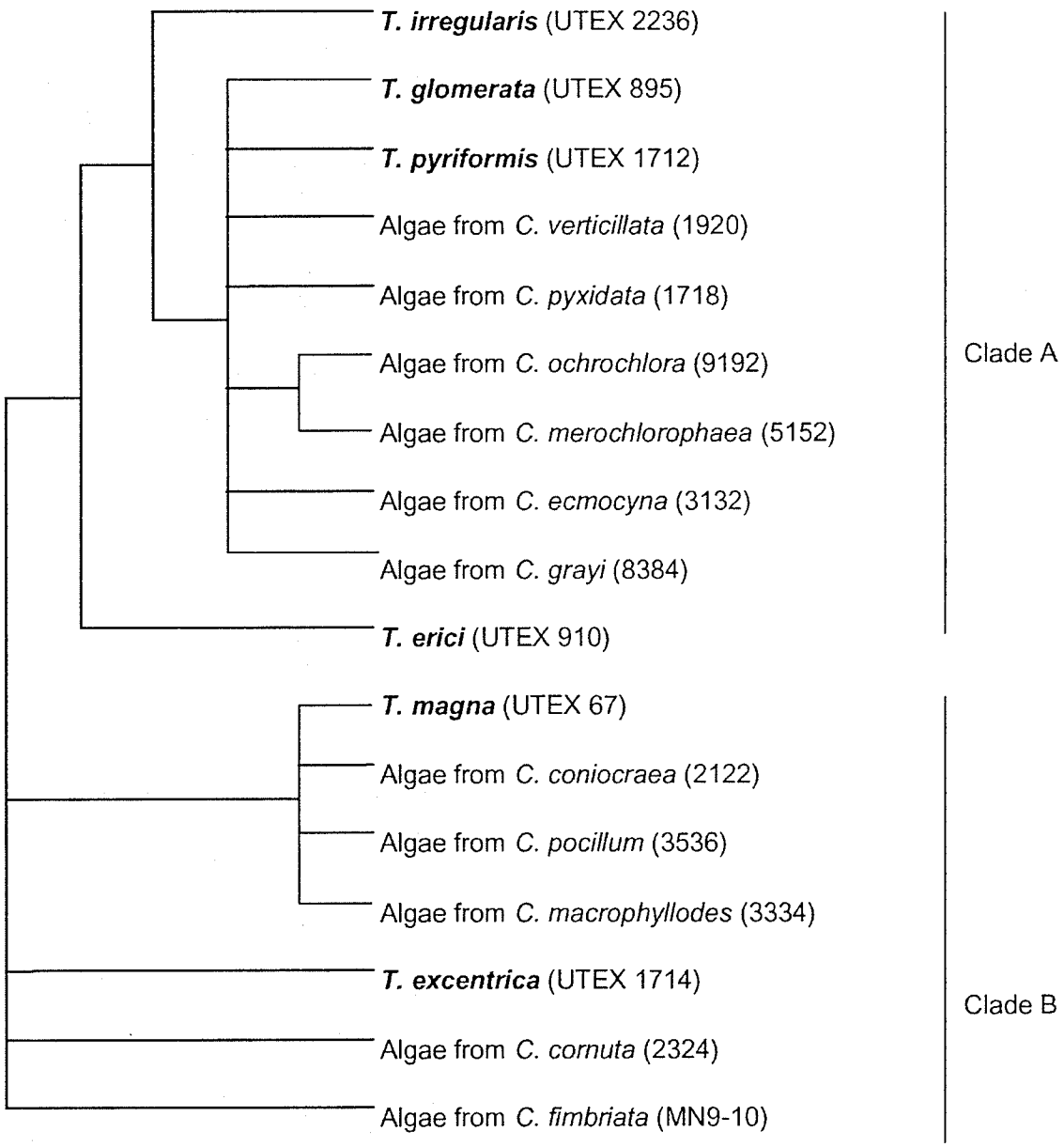

Fig. 11: 50% majority rule consensus of 22 most parsimonious midpoint rooted trees for the algal ITS2 nucleotide sequence data. Dataset includes 11 photobionts of *Cladonia* from this study and six *Trebouxia* species from GenBank. Numbers with species correspond to those in tables 1 & 4. CI: 0.8750, RI: 0.9333.

In the neighbor joining tree based on ITS2 sequence data *T. erici, T. excentrica* and *T. magna* are all located within Clade B (Append. 11). Bootstrap support analysis was performed for all neighbor joining trees and it resulted in no support >50%.

Comparison of algal ITS1 and ITS2 sequence phylogenies

The ITS1 and ITS2 phylogenies produced similar topologies except for the placement of *T. erici* and *T. excentrica* (Figs. 10 & 11). In the ITS1 tree, *T. excentrica* is basal to the clade containing *T. glomerata* and *T. pyriformis* and related natural photobionts, as opposed to the unresolved location of this species in the 50% majority rule consensus of ITS2 trees (Fig. 11). The location of *T. erici* basal to the clade containing *T. magna* and related natural photobionts in the ITS1 tree is also supported (bootstrap value of 61%) compared to the basal location of this species in clade A of the ITS2 tree with no support. In general, ITS1 sequence data constructed clades with higher bootstrap supports. However, the number of informative characters in the algal ITS2 dataset was higher than the ITS1 dataset (9 and 5 informative characters respectively, table 7).

When sequence data of 11 natural photobionts was analyzed, two most parsimonious trees were constructed based on the entire ITS sequence data (Append. 12). The only difference between these trees was the position of the photobiont of *C. cornuta* relative to the photobiont of *C. fimbriata.* In one of the trees both taxa clustered together (Append. 12a) while in the other tree the former taxa was basal to the latter (Append. 12b).

ITS1 sequence dataset analysis showed the basal position of *C. cornuta* relative to the photobiont of *C. fimbriata* (Append. 13a) and ITS2 sequence dataset analysis resulted in a clade containing both taxa as sister taxa (Append. 13b).

4.3. Overall Evolution

For eight of the natural lichen associations in Section *Cladonia* that we compared, the phylogenies of the algal and fungal partners (Fig. 12 and table 6) were not congruent and there were no evidence of overall parallel cospeciation. The ILD test for these datasets resulted in a P- value of 0.01. The KH test for Algal ITS and fungal ITS sequence datasets resulted in a P-value <0.0001. The null hypothesis is that the algal and fungal datasets derived from the ITS sequence data were similar, implying there was parallel cladogenesis in the algal and fungal phylogenies. The low P-values implied that these two datasets were significantly different and there was no evidence for overall parallel cladogenesis in species sampled in this section.

Isolated events of coevolution

Cladonia pocillum and *C. macrophyllodes* formed a clade with 100% bootstrap support. Photobionts of these two lichens form a clade along with the photobiont of *C. coniocraea* with 98% bootstrap support. However, *C. pyxidata* which is a close ally to the clade containing *C. pocillum* and *C. macrophyllodes* becomes associated an algal genotype different from those in the clade with 98% bootstrap support. It also suggests that *C. coniocraea*, not part of the fungal clade, has become associated with an algal genotype in the clade with 98% bootstrap support.

Error!

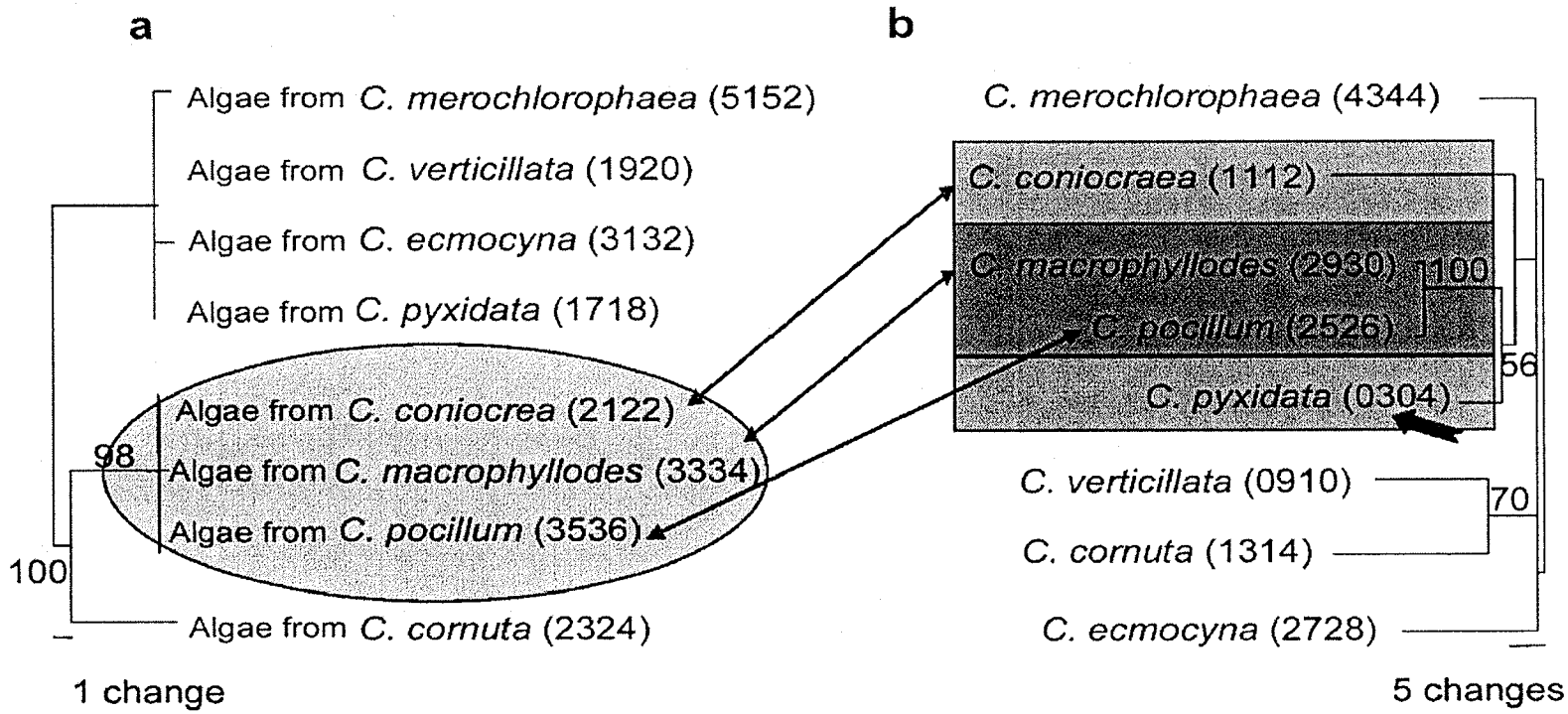

Fig. 12: Comparison of the photobiont and mycobiont phylogenies of natural lichen associations based on ITS1, 5.8S and ITS2 combined sequence data. Datasets include eight *Cladonia* samples. Both photobionts and mycobionts are isolated from the same specimen. Numbers with branches are bootstrap support >50%. Numbers with species correspond to those in tables 1 & 4. a: The single most parsimonious midpoint rooted tree for the algal ITS sequences. CI: 1.0, RI: 1.0; and, b: The single most parsimonious midpoint rooted tree for the fungal ITS sequences. CI: 0.8226, HI: 0.8832. Fine arrows connect species with suspected parallel speciation and the bold arrow indicates and algal switch.

5. DISCUSSION

5.1. Phylogenetic History of the Fungal Partner

Cladonia chlorophaea species complex

Ahti (1966) recognized 10 species in the *C. chlorophaea* species complex, namely *C. chlorophaea, C. grayi, C. merochlorophaea, C. cryptochlorophaea, C. cyathomorpha, C. conista, C. fimbriata, C. magyarica, C. pocillum, C. pyxidata* and some unnamed chemotypes. Sandstede (1931 in Ahti 1966) documented only two species in this group, *C. chlorophaea* and *C. grayi,* and Asahina (1940, 1943 in Ahti 1966) recognized four species in this group, *C. chlorophaea, C. grayi, C. merochlorophaea* and *C. cryptochlorophaea* exclusively based on their chemistry. Members of this species complex all contain fumarprotocetraric acid as either a constant or accessory substance, *C. chlorophaea* and can be distinguished by exclusive presence of fumarprotocetraric acid. Presence of other chemical compounds such as merochlorophaeic acid and sometimes novochlorophaeic acid are in *C. merochlorophaea*, cryptochlorophaeic acid is in *C. cryptochlorophaea,* and grayanic acid is in *C. graryi.* In the past chemotypes within *C. chlorophaea* complex have been recognized either as members of the same species (Krog 1967) or as separate chemospecies supported by minor morphological differences (Ahti 1966).

Cladonia chlorophaea was first described as an intermediate species between *C. pyxidata* and *C. fimbriata* (Sommerfelt 1826, Florke 1828 in Ahti 1966) due to sorediate characters. *Cladonia pyxidata* is a non-sorediate species with areoles on the podetia while *C. chlorophaea* has coarsely granular soredia and *C. fimbiriata* is covered with farinose soredia. In this study, this group has been represented by three species, *C. chlorophaea,*

C. grayi and *C. merochlorophaea,* with members scattered over the tree (Fig. 5 & Appends. 1-3). *Cladonia pocillum*, and *C. pyxidata* were not included in this species complex. Despite their morphological similarities, this group is a polyphyletic group since its members are not clustered together in a clade (Fig. 5 & Appends. 1-3). Even three samples of *C. grayi* did not cluster together. *Cladonia grayi* and the *C. chlorophaea* from *C. chlorophaea* species complex clustered with *C. gracilis* and *C. fimbriata* respectively. However, two members of *C. grayi* formed a sister clade with two members of *C. merochlorophaea* group. Two members of section *Cladina* clustered together, as well as two members of the genus *Cladia* at the base of the tree (Fig. 5 & Append. 1). The distance between members of one species (Table 5) and the separation of these taxa in the trees could be due to hybridization and gene flow or even the presence of cryptic species within species. Culberson *et al.* (1988) studied gene flow in the *C. chloropheaea* complex and showed that the grayanic acid and merochlorophaeic acid chemotypes interbreed. Clustering of these two chemotypes may be explained by potential gene flow between them. *Cladonia chlorophaea* clustered with *C. fimbriata* and *C. coniocraea* (Fig. 5 & Appends. 1-3). *Cladonia coniocraea* differs from *C. chlorophaea* and *C. fimbriata* morphologically and is part of *C. gracilis* species complex. Our results also agree with the results of a previous study by Stenroos *et al.* (2002) in presenting the polyphyletic nature of this group.

Morphologically, the *C. chloropheaea* complex is recognized by usually persistent primary squamules; unbranched or rarely branched podetia; podetia corticate at the base and sorediate in upper portions and inside the cups; goblet-shaped cups (Fig. 3), and sometimes with few marginal proliferations (Ahti 1966). *Cladonia chlorophaea* is

distinguished by its grayish green color and coarsely sorediate podetia and cups. *Cladonia grayi* is greenish brown and coarsely sorediate as well. *Cladonia cryptochlorophaea* is known to be light green and covered with coarse soredia. *Cladonia merochlorophaea* has two morphological types, one with abundant soredia and containing fumarprotocetraric acid and merochlorophaeic acid. This type is known only in North America. The other type is esorediate and contains merochlorophaeic acid, sometimes accompanied with novochlorophaeic acid as well as fumarprotocetraric acid. This type is known in boreal regions of the northern hemisphere. Both morphological types of *C. merochlorophaea* are very similar and despite these subtle morphological differences the secondary chemistry distinguishes all chemospecies. This could be due to high gene flow and hybridization shown in this group (Culberson *et al.* 1988).

In parsimony trees constructed from morphological data only, members of the *C. chlorophaea* complex formed a polyphyletic group (Fig. 6 & Append. 4a) with species outside the *C. chlorophaea* species complex. This is significant because differences are very subtle and members of species complex would be expected to cluster together. However this topology could be a result of character are scoring and is subject to change if characters scored differently. In the neighbor joining tree based on morphological data members of the *C. chlorophaea* species complex formed a paraphyletic group with members of *C. verticillata* (Append. 9b). The difference between phylogenetic and phenetic methods may account for this variation.

Cladonia pyxidata* and *C. pocillum

Cladonia pyxidata and *C. pocillum* are polyphyletic. Of six samples of *C. pyxidata* and *C. pocillum* in this study, four of them always clustered with *C. macrophyllodes,* a member of the *C. verticillata* complex, and two clustered outside this clade Fig. 5 & Appends. 1-3). Even in trees based on morphological data (Fig. 6 & Append. 4) members of *C. pocillum* and *C. pyxidata* along with *C. macrophyllodes* clustered together.

It is possible that members of *C. pocillum* and *C. pyxidata* are actually a group of morphologically similar but genetically divergent phylogenetic species. Aptroot *et al.* (2001) investigated *C. pyxidata* and *C. pocillum* and reported that the type species of *C. pyxidata* resembles *C. pocillum.* They were uncertain whether these two species would always be distinguishable based on morphology. In addition they reported that they did not come across co-occurrence of *C. pyxidata* and *C. pocillum* in their sampling locations. This may suggest that these two species are actually variations of one species. These two species are very similar morphologically, containing persistent primary squamules, and cups with marginal proliferations on esorediate podetia. Both taxa are gray to olive green or with brown shades. Fumarprotocetraric is the only secondary compound in both species; however traces of atranorin have been detected in some samples of *C. pocillum.* Apothecia are uncommon in these taxa (Ahti 1966, 2000a). *Cladonia pyxidata* and *C. pocillum* are expected to cluster together due to their morphological similarities. The polyphyly of these species in trees based on ITS sequence data (Fig. 5 & Appends. 1-3) could be explained by the existence of cryptic phylogenetic species within the two species, and in trees based on morphological data (Fig. 6 & Append. 4) by the scoring scheme.

***Cladonia gracilis* species complex**

Ahti (1980) placed seven species of *Cladonia* in *C. gracilis* species complex. *Cladonia macroceras, C. maxima* and *C. squamosissima* are distributed in the Northern hemisphere, and *C. subchordalis* in the Southern hemisphere. *Cladonia cornuta* and *C. ecmocyna* each with one subspecies as well as *C. gracilis* with six subspecies are distributed in both hemispheres.

The *C. gracilis* species complex is recognized by primary squamules that disappear as the podetia become taller. Podetia are often clubs without a cup but some subspecies contain cups. The podetial cortex is continuous to areolate and in some species sorediate patches can be observed in higher portions of the podetia. Chemically the only secondary compound present in this group was fumarprotocetraric acid and sometimes traces of atranorin in some species like *C. maxima, C. ecmocyna* and *C. gracilis* var. *gracilis* (Ahti 1980). This group is represented in our study by *C. gracilis, C. cornuta, C. asahinae, C. maxima* and *C. ecmocyna*.

Cladonia ochrochlora often clustered with members of the *C. gracilis* species complex (Fig. 5 & Append. 1). Morphologically *C. cornuta* and *C. ochrochlora* are very similar in having cupless podetia covered with farinose soredia in upper portions. However, Stenroos *et al.* (2002) believed that this group is a monophyletic group despite inclusion of *C. ochrochlora* in the clade containing the *C. gracilis* complex. In addition, they found three subspecies of *C. gracilis* to be polyphyletic. This indicates that the current classification of this group needs to be reconsidered.

Cladonia verticillata species complex

The *Cladonia verticillata* species complex (Ahti 2000a), is defined by centrally proliferating cups and was represented by *C. verticillata* and *C. macrophyllodes* in this study. In our results *C. macrophyllodes* was separated from members of *C. veticillata.* It clustered with *C. pyxidata* and *C. pocillum* in all trees (Fig. 5 & Appends. 1-3) showing the polyphyletic nature of this group. This again agrees with the results of a study based on ITS of the nuclear rDNA and partial sequences from protein-coding beta-tubulin, by Stenroos *et al.* (2002). In our analysis, the three samples of *C. verticillata* showed no vatiation in ITS1, ITS2 and 5.8S sequences. Stenroos *et al.* (2002) also found a low sequence variation in this group as well as a close phylogenetic relationship between members of this group and *C. subulata.* However *C. verticillata* and *C. subulata* are quite different morphologically. *Cladonia subulata* is cupless or with irregular cups. Podetia has no cortex and are covered with farinose soredia. *Cladonia verticillata* on the other hand is esorediate with continuous or areolate cortex. Podetia are always cup-bearing with central proliferations (Ahti 2000a).

Cladonia verticillata species complex is not monophyletic group and its members cluster with members of other complexes. However *C. verticillata* is a monophyletic species.

Comparing fungal ITS and morphological trees

In trees produced from ITS1, 5.8S, ITS2 sequence and morphological characters (Fig. 5 & Appends. 1-3) members of *C. chlorophaea, C. gracilis* and *C. verticillata* species complex were polyphyletic. *Cladonia fimbriata* and *C. coniocraea* clustered with *C.*

chlorophaea. Cladonia grayi from the *C. chlorophaea* species complex and *C. gracilis* from the *C. gracilis* species complex clustered together. *Cladonia macrophyllodes* a member of *C. verticillata* species complex, clustered with *C. pyxidata* and *C. pocillum. Cladonia subulata* the type species of the section *Cladonia* always grouped with *C. verticillata.*

Cladia is a good candidate as the outgroup taxon in phylogenetic studies of the genus *Cladonia* (Stenroos *et al.* 2002). *Cladia* is a distinct genus from genus *Cladonia* and belongs to the family Cladoniaceae. However, trees based on ITS1, ITS2, 5.8S sequences and morphological characters required that gaps be inserted within the sequence while aligning the sequences. These gaps were coded and incorporated into the dataset as additional characters. The two *Cladia* species fell within the ingroup of the genus *Cladonia* when gaps were not coded (Append. 5). When coded gaps were incorporated in the analysis *Cladia* species fell basal to the ingroup taxa (Fig. 5 & Append. 1).

Trees produced based on morphological characters only (Fig. 6 & Append. 4) showed different topologies from trees based on DNA and morphological data (Fig. 5 & Appends. 1-3). In the morphology trees (Fig. 6 & Append. 4) the *C. gracilis* complex was paraphyletic with its members clustered together basal to the clade containing members of *C. verticillata*. The *C. verticillata* species complex was not monophyletic with *C. macrophyllodes* clustering with *C. pocillum* and *C. pyxidata.* These three species are very similar morphologically containing persistent primary squamules, and cups with proliferations on esorediate podetia. The *C. chlorophaea* species complex seems to be polyphyletic in the morphological trees (Fig. 6 & Append. 4). Members of *C. chlorophaea* were located basal to the clade containing *C. pocillum* and *C. pyxidata.*

The KH test for DNA and morphology datasets resulted in a P-value ranging from 0.0004 to 0.0023 implying that molecular and morphological datasets are significantly different, therefore they are not combinable. This difference between these two datasets could be due to a low resolution in one tree (Append. 7) and high resolution in the other tree (Fig. 6) (Clark *et al.* 2000).

Sometimes independent datasets can be combined and analyzed simultaneously to increase the number of characters and to incrase the chance of finding "the true phylogeny". This requires an assessment of the overall congruence of characters from all data sets. On the other hand, if there is heterogeneity among datasets that affects the phylogeny estimation, for example differences in substitution rates, combining data sets could result in a misleading phylogenetic assessment (de Queriroz *et al.* 1995). In this study the ITS1 sequence offered a significantly higher number of informative characters than the ITS2 sequence dataset (Table 6). This implies a higher rate of evolution in the ITS1 sequence. However based on the ILD and KH tests these two datasets are not significantly different and can therefore be combined.

Comparing fungal ITS1 and ITS2 trees

Topology of the 50% majority rule consensus of the most parsimonious trees produced from the fungal ITS1 and ITS2 separate sequence data sets were not similar (Figs. 7-8 & Appends. 9-10). In the ITS1 tree, five members of the *C. chlorophaea* complex clustered together, as well as two members of *Cladonia* section *Cladina, C. mitis* and *C. rangiferina.* In this tree, two *Cladia* species were also located basal to the ingroup taxa. In the ITS2 tree, however, none of these groupings were observed. In

general, the number of informative characters in the fungal ITS1 sequence datasets was higher than in ITS2 sequence datasets (Table 6) producing better resolution in the ITS1 phylogenies (Fig. 7 & Append. 9). Dodd *et al.* (2000) also reported a higher number of informative characters in the ITS1 than the ITS2 nucleotide sequences among 50 *Trichoderma* isolates representing seven species.

The ILD test for the fungal ITS1 and ITS2 datasets resulted in a P- value of 0.11. The null hypothesis is that ITS1 and ITS2 datasets are the same and a P- value greater than 0.05 accepts the null hypothesis. A P-value of 0.11 implies that the ITS1 and ITS2 datasets are not significantly different. They represent the same phylogenetic history and the datasets can be combined. The KH test for ITS1 and ITS2 datasets including the GenBank sequences resulted in a P-value ranging from 0.0180 to 0.0706. The Kishino-Hasegawa test for ITS1 and ITS2 sequences excluding the GenBank species resulted in P-values ranging from 0.0321 to 0.0706. These P-values also show that some of the trees produced based on one of the datasets could be reproduced by sequences in the other dataset implying that results produced from the two datasets are contradictory and some of the trees are combinable. However, in order to be able to use all the evidence available (total evidence) these trees were combined.

5.2. Phylogenetic history of the algal partner

Six species of *Trebouxia*, namely, *T. irregularis, T. glomerata, T. pyriformis, T. erici, T. magna* and *T. excentrica* have been shown to form lichen associations with species in the ascomycete genus *Cladonia* (Tschermak-Woess, 1988). When taxa clustered together based on nucleotide sequence similarities, they are assumed to belong to the same or very

closely related species. Photobionts of six species of *Cladonia* section *Cladonia* (*C. verticillata, C. pyxidata, C. ochrochlora, C. merochlorophaea, C. ecmocyna* and *C. grayi)* clustered with *T. glomerata* or *T. pyriformis* with 88% bootstrap support (clade A) (Fig. 9) implying that the natural photobionts of these *Cladonia* mycobionts are closely related to either of these *Trebouxia* species.

Photobionts of five species of *Cladonia* (*C. cornuta, C. fimbriata C. coniocraea, C. pocillum* and *C. macrophyllodes*) clustered with *T. magna* or *T. excentrica* with 99% bootstrap support (clade B) (Fig. 9). In this clade, photobionts of *C. cornuta* and *C. fimbriata* clustered with *T. excentrica.* Sister to this clade is a clade containing photobionts of *C. coniocrea, C. pocillum* and *C. macrophyllodes. Trebouxia magna* clustered basal to these sister clades. Based on ITS sequence similarities we assume that *T. excentrica* is associated with *C. cornuta* and *C. fimbriata* and *T. magna* is associated with *C. coniocrea, C. pocillum* and *C. macrophyllodes.*

In all phylogenetic reconstructions clade A (*T. pyriformis/T. glomerata* clade) was less resolved than clade B (*T. magna/T. excentrica* clade). This lack of resolution can be explained by the highly similar ITS sequences of the taxa in this clade (Table 8).

Comparison of algal ITS1 and ITS2 sequence phylogeny

The ITS1 and ITS2 topologies placed *T. erici* and *T. excentrica* in different clades (Figs. 10 & 11). In the ITS1 tree, *T. excentrica* is closely related to the clade consisting of *T. glomerata* and *T. pyriformis* and related natural photobionts, as opposed to the basal and unresolved location of this species in the ITS2 tree. The location of *T. erici* basal to the clade containing *T. magna* and related natural photobionts in the ITS1 tree is also

supported (bootstrap value of 61%) compared to the unresolved basal location of this species in the ITS2 tree. In general, the ITS1 sequence dataset produced more phylogenetic information despite a lower number of informative characters (Table 8) in the algal ITS1 dataset (Fig. 10 & Appends. 11 & 13a) compared to the ITS2 dataset (Fig. 11 & Append. 13b). A higher number of informative characters may produce homoplasy and account for lower resolution in the ITS2 trees.

Hausner and Wang (2005) suggested that the secondary structures of ITS regions are more conserved than the actual nucleotide sequence. Coevolutionary processes within and between the ITS1 and ITS2 regions maintain the secondary structures and potential interactions between the two ITS regions. This could serve as one reason for the incongruency observed in the output of the ITS1 and ITS2 analyses (Figs. 10 & 11).

When sequence data of 11 natural photobionts, isolated for this study, were analyzed, two most parsimonious trees were constructed based on the entire ITS sequence data, both of them are shown in Append. 12. The only difference between these trees is the position of the photobiont of *C. cornuta* relative to the photobiont of *C. fimbriata.* In one of the trees both taxa clustered together (Append. 12a) while in the other tree the former taxon is basal to the latter (Append. 12b).

When ITS1 and ITS2 datasets were analyzed separately, again two most parsimonious trees were constructed for each dataset. In both cases the tree with higher resolution was demonstrated (Append. 13). The ITS1 tree (Append. 13a) was identical to one of the most parsimonious trees constructed based on the entire ITS sequence (Append. 12a) while topology of the ITS2 tree (Append. 13b) was identical to the other most parsimonious tree based on the entire ITS sequence (Append. 12b).

***Trebouxia glomerata/T. pyriformis* clade**

The algal ITS rDNA of six species of lichen forming fungi fall into the T. *glomerata/T. pyriformis* clade. This includes *C. ecmocyna, C. grayi, C. merochlorophaea, C. ochrochlora, C. pyxidata* and *C. verticillata.* Therefore, based on ITS rDNA nucleotide sequence data the algal partners of these species may be considered to be either *T. glomerata* or *T. pyriformis* (Fig. 9). This agrees with results of a study on the entire genus *Cladonia* based on ITS sequence data in which *T. glomerata* and *T. pyrifomis* clustered together along with photobionts of *C. grayi* and *C. verticillata,* and *T. irregularis* has a basal position to this clade (Piercey-Normore and DePriest 2001). Further investigation of these species, using sequence substitution comparisons and a distance matrix showed a greater similarity between the ITS sequence of natural photobiont in clade A and the ITS sequence of *T. pyriformis* than between taxa in clade A and the ITS sequence of *T. glomerata* (Table 8). Based on ITS sequence comparisons and the distance matrix, the natural photobionts of *C. verticillata* and *C. pyxidata* are identical to *T. pyriformis*. This also agrees with Meisch (1981) who isolated *T. pyriformis* from *C. verticillata.*

Comparison of the natural photobionts of *C. ochrochlora, C. merochlorophaea, C. ecmocyna* and *C. grayi* with *T. pyriformis* and *T. glomerata* showed one substitution between the natural photobionts and *T. pyriformis,* as opposed to three substitutions between the photobionts and *T. glomerata* (Table 8). This suggested that the species, *C. ochrochlora, C. merochlorophaea, C. ecmocyna* and *C. grayi* are associated with the algal genus *T. pyriformis.* However, according to Piercey-Normore and DePriest (2001)

the photobiont of *C. pyxidata* is more closely related to *T. excentrica* and the photobiont of *C. ochrochlora* with *T. magna. Trebouxia glomerata* has been isolated from several *Cladonia* species such as *C. cornuta* and *C. gracilis* var. *chodalis* (Waren 1918-1919, Ahmadjian 1960). In our results, however, the algal ITS sequence of *C. cornuta* did not cluster with *T. glomerata.* The photobiont of *C. cornuta* is more closely related to *C. excentrica* (Fig. 9). Wang-Yang (1970) reported the association of *C. cornuta* with *T. impressa*. These results suggest that a single fungal species can associate with more than one algal species. It also suggests that a single algal species can associate with more than one fungal species, a situation known as "low selectivity" (Beck *et al.* 2002). The association of more than one photobiont species with one mycobiont species is not uncommon in lichens. *Cladonia chlorophaea* is reported to be associated with both *T. pyriformis* and *T. excentrica* (Meisch 1981, Piercey-Normore and DePriest 2001). *Cladia aggregata* has been found to have two algal partners, *T. erici* and *T. glomerata* (Takeshita *et al.* 1991). *Cladonia ramulosa* and *C. squamosa* also have been reported to have two partners *T. erici* and *T. pyriformis* also *C. coccifera* and *C. deformis* seem to be associated with two algal species, *T. glomerata* and *T. pyriformis* (Nakano and Iguchi 1994). Therefore, it seems that the lichenization of some *Cladonia* species is less selective and not restricted to a unique algal partner. This low selectivity toward photobionts in vegetatively reproducing lichens is not surprising where there is no relichenization and algal switch (horizontal transfer of algae) involved. The photobionts of different lichen thalli may be clonal from the parental thalli but different populations contain different algal partners. Therefore the same fungal species in different lichen populations may be associated with different species of algae. In sexually reproducing

lichens this variation can be due to the unavailability of the more compatible algae in the environment, so the mycobionts must recruit a less compatible but available alga.

Trebouxia irregularis and *T. erici*

Trebouxia irregularis and *T. erici* are not represented by any species in section *Cladonia* in this study, although *T. irregularis* is part of the monophyletic clade of *T. glomerata/T. pyriformis* in all trees and *T. erici* is clustered together with members of clade A and clade B in different trees (Figs. 9-11 & Append. 11). Piercey-Normore and DePriest (2001) also showed that these two taxa are not closely related to the natural photobionts isolated from lichens in *Cladonia* section *Cladonia.*

Trebouxia erici has previously been isolated from several species of *Cladonia* such as *C. chlorophaea, C. coniocraea, C. gracilis* ssp. *turbinata, C. grayi, C. subulata, C. cristatella* as well as *Cladia aggregata* (Archibald 1975, Meisch 1981, Takeshita *et al.* 1991, and Nakano and Iguchi 1994). However, in our study the ITS sequence of the photobiont of *C. coniocraea* is more closely related to *T. magna* than *T. erici* (Fig. 9).

Trebouxia irregularis and *T. erici* have divergent ITS sequences (Figs. 9-11 & Append. 11). They also differ morphologicaly (Table 9).

Trebouxia magna and *T. excentrica* clade

The algal ITS sequence of *Cladonia pocillum, C. macrophyllodes* and *C. coniocraea* is more similar to the ITS sequence of *T. magna* than to *T. excentrica. Cladonia fimbriata* and *C. cornuta* seem to be associated with *T. excentrica* (Fig. 9). However, according to Piercey-Normore and DePriest (2001) the photobiont of *C. fimbriata* was *T. magna.*

Although clade B is more divergent than clade A, it is supported by 99% bootstrap . 18). More homoplasy may account for more synapomorphies resulting in strong support for a clade.

Pyrenoid structure and its application to identification of *Trebouxia* species

Chloroplast thylakoid lamellae entering the pyrenoid matrix may become structurally modified. Pyrenoids are distinct areas within the chloroplast that are filled by a proteinaceous matrix. Based on forms and arrangement of thylakoid lamellae within the pyrenoid, Friedl (1989) distinguished eight pyrenoid types (see Fig. 1 in Friedl 89), of which three are seen in *Cladonia* photobionts (*irregularis-, erici-* and *magna*-type pyrenoids). The remaining five pyrenoid types (*gigantean-, impressa-, arboricola-, gelatinosa-* and *corticola*-type pyrenoids) have not been observed in algal cells associated with *Cladonia* spp. (Tschermak-Woes 1988, Friedl 1989). The advantage of using pyrenoid characters as diagnostic features is that these structures are not influenced by different culture media and are even stable within the lichen thallus (Friedl 1989).

Irregularis-type pyrenoids can be observed in *T. excentrica, T. glomerata, T. irregularis* and *T. pyriformis*. This type of pyrenoid has an irregular form with non-modified thylakoids as well as a small number of thin and curved thylakoid tubules invaginating the pyrenoid matrix. Numerous pyrenoglobuli (apparently empty vesicles located between thylakoids) are also associated with thylakoid tubules. However there are no pyrenoglobuli attached to the thylakoids located at the periphery of the pyrenoid matrix. Species with *irregularis*-type pyrenoids are so uniform in morphology (shape, chloroplast characters and autospore and zoospore features) that no distinctive characters

exist, therefore these taxa could be included in one species (Friedl 1989). However, based on ITS sequences, species with *irregular*-type pyrenoids seem to be polyphyletic (Fig. 9).

The *erici*-type pyrenoid is specific to *T. erici* and *magna*-type pyrenoids can be observed only in *T. magna*. Since thylakoid lamellae entering the pyrenoid matrix in these species are not structurally modified, neither *T. erici* nor *T. magna* exhibit a true pyrenoid. Only their indistinct areas within the chloroplast are thought to function as pyrenoids. The absence of true pyrenoids in these two species is in contrast with other species within this genus (Friedl 1989).

These structural features link *T. magna* and *T. erici* together in the ITS1 tree as well as the ITS2 neighbor joining tree (Figs. 10 & 11), but not in the parsimony ITS2 tree (Fig. 11) or the entire ITS region tree (Fig. 9). The separation of the two taxa in the latter two trees is supported by other features. In *T. magna* pyrenoglobuli are concentrated in the centre and starch grains are deposited at the periphery of pyrenoglobuli. Pyrenoglobuli in *T. erici* are higher in number but smaller in size.

5.3. Coevolution

Overall evolution

For the eight of the natural lichen associations in section *Cladonia* that we compared, the phylogenies of the algal and fungal partners (Fig. 12) were not congruent and there was no evidence of overall parallel cospeciation. The ILD and KH tests for Algal ITS and fungal ITS sequence datasets resulted in rejection of the null hypothesis. The two datasets were significantly different and there was no evidence for overall parallel cladogenesis in this section of the genus *Cladonia*. This incongruency could be a

result of insufficient sample size and broad geographic region. We may increase the chance of finding a coevolutionary hot spot by choosing a smaller geographic region and increasing the number of samples within that region because isolated events were detected in some sections of *Cladonia* (Piercey-Normore and DePriest 2001).

When reciprocal evolution leads to cospeciation equal numbers of fungal and algal species are expected to evolve, a situation not seen in our results. For example, based on identical ITS sequences of algae associated with *C. vertcillata* and *C. pyxidata,* these species seems to be associated with one strain of *T. pyriformis* (Table 8). Cospeciation would be expected with highly specific associations between algal and fungal partners at the population level. This might suggest that some or all of the eight species in this study were not specific in the symbiotic association at the population level. Population studies would be required to examine this theory.

If species of either symbiont contains cryptic phylogenetic species, then detection of parallel cladogenesis would be obscured. One example is the genus *Letharia* and its photobiont *Trebouxia jamesii*. Based on actin gene sequence, Kroken and Taylor (2000) suggest that members of the morphospecies *T. jamesi* consisted of several phylogenetic species. A clade containing six of the closely related phylogenetic species of *T. jamesi* is associated with five of the six phylogenetic species of *Letharia*.

In cases where photobionts of each species have been assigned to a species based on morphology, each species can include several cryptic phylogenetic species. In such cases applying phylogenetic species concept alternative could provide explanation cospeciation studies than biological or morphological species concepts. Hybridization also can obscure coevolution by altering phylogenetic history of one biont. This phenomenon has been

shown in members of the *C. chlorophaea* complex (Culberson *et al.* 1988) and *Ramalina* (Culberson *et al.* 1993). In other words, lack of clarity in species definition could be problematic in examination of cospeciation. Taxonomic issues on *C. pyxidata* and *C. pocillum,* such as existence of ecological species within *C. pyxidata,* can cover up patterns of parallel cladogenesis in section *Cladonia*. Rejection of parallel cladogenesis suggests that either taxonomic issue may exist within species of the genus or horizontal transfer of algae such as algal switching must be ongoing even within section *Cladonia* (Fig. 12).

Horizontal transfer can be carried on by existence of sexual reproduction in Cladoniaceae. Sexual reproduction increases the chance of horizontal transfer and photobiont exchange due to the obligate relichenization process in sexually reproductive lichens. However the exchange of photobionts may also be possible in strile lichens by incorporation of symbiotic propagules from different lichens with a different photobiont. The accessory photobiont may become the dominant photobiont later on developmetal stages (Beck *et al.* 1998).

Isolated events of coevolution

The rejection of parallel cladogenesis in the entire data does not rule out the possibility of detecting cospeciation in a subset of the data. Parallel cladogenesis was evident in *C. macrophyllodes* and *C. pocillum* (Fig. 12). In the fungal phylogeny *C. macrophyllodes,* and *C. pocillum* form one clade with bootstrap support of 100%, *C. pyxidata* is basal to this clade with 56% bootstrap support. While in the algal phylogeny photobionts of *C. coniocraea, C. macrophyllodes,* and *C. pocillum* formed a clade with

98% bootstrap support. Placement of the algae from *C. pyxidata* in another clade shows that the mycobiont of *C. pyxidata* may have undergone an algal switch and selected a different alga. Although *C. pyxidata* produces aeroles, it also produces apothecia, so it is assumed that it reproduces sexually. The fungal spore will grow into a prethallus and must recruit new algal partners in order to transform into a lichen thallus. During this relichenization process, algal switching may occur and the mycobiont of *C. pyxidata* may recruit a new photobiont. This new photobiont of *C. pyxidata* would be different from the original photobiont. This new photobiont may be more compatible under some environmental conditions than in other conditions.

Members of *C. chlorophaea* complex are all sorediate but sometimes produce apothecia, thus it is assumed that they reproduce asexually more often. Therefore vertical transfer of algae should be common, increasing the chance of parallel cladogenesis in this group. However, Piercey-Normore and DePriest (2001), did not find parallel evolution in four closely related taxa in the *C. chlorophaea* complex (*Cladonia* section *Cladonia*).

Selectivity and specificity in the genus *Cladonia*

Selectivity of the mycobiont in *Cladonia* lichens leads to a higher number of fungal species associated with a lower number of algal species in *Cladonia*. In this case lichen fungi may be selecting for optimal symbiotic algal partners rather than undergoing strict cospeciation that results in the equal number of algal species associated with fungal species. Resource tracking, the process whereby one symbiont tracks a particular feature of the other symbiont (such as physiological or morphological features) rather than tracking the taxonomic features may be common in these species. If fungi can select free-

living algae, or algae from other lichen associations, then this horizontal transfer can explain the symbiotic association of algae and fungi showing incongruent phylogenetic histories. In this case, the underlying phylogeny of the mycobionts and photobionts would not need to be congruent.

Comparing results of this study with the literature (Archibald 1975, Hildreth and Ahmadjian 1981, Nakano and Iguchi 1994, Piercey-Normore and DePriest 2002, and Tschermak-Woes 1988) shows that *C. pyxidata* can be associated with *T. pyriformis* and *T. exentrica; C. grayi* with *T. pyriformis* and *T. erici; C. cornuta* with *T. glomerata, T. excentrica* and *T. impressa; C. gracilis* with *T. glomerata* and *T. erici; C. chlorophaea* with *T. pyriformis, T. excentrica* and *T. erici, C. coniocreae* with *T. erici* and *T. magna;* and *C. fimbriata* with *T. magna* and *T. excentrica.* This shows low selectivity of each symbiont toward the other biont in section *Cladonia,* genus *Cladonia.*

Yahr *et al.* (2004) showed that of eight species of *Cladonia* that they studied, six are associated with members of only one genotype of photobiont, whereas the other two species are associated with two genotypes of photobionts. They suggest that *Cladonia* is highly selective for the *Asterochloris* group at the genus level, whereas individual species or genotypes within the genus may show a high to low degree of selectivity for individual clades or genotypes of the *Asterochloris* group.

6. Conclusions and Future Research

Four *Trebouxia* species (*T. glomerata, T. pyriformis, T. magna* and *T. erici*) are associated with lichens of genus *Cladonia* in Manitoba.

All species complexes, most species and even some subspecies within *Cladonia* section *Cladonia* are polyphyletic; therefore, species concepts in regard to lichens of the genus *Cladonia* should be revisited. Also, further work with an increased number of specimens per species and species complexes is needed to resolve the taxonomy of this genus. The polyphyletic nature of mycobionts in lichens of the genus *Cladonia* may be obscuring existence of parallel cladogenesis between the mycobionts and the photobionts of *Cladonia* section *Cladonia*. In this respect, taxonomy of the species involved should be resolved prior to further coevolutionary investigations.

The taxonomic aspect of coevolution, cospeciation or parallel cladogenesis, was investigated in this study. Cospeciation was not detected at the species level between the algal and fungal partners of lichen associations, in the genus *Cladonia,* section *Cladonia.* However, isolated events of coevolution were observed in *Cladonia* section *Cladonia* (in *C. macrophyllodes* and *C. pocillum*) as well as an algal switch by *C. pyxidata.*

The ecological and/or environmental aspects of coevolution also need to be considered in future works. Habitat and climate conditions may influence algal survival. Environmental conditions as well as algal genotypes associated with certain fungi may influence morphology. Mycobionts in section *Cladonia,* and lichens in general seem to be selective toward photobionts depending on environmental factors as well as availability of potential photobionts. Selection of photobionts seems to be more related to habitat conditions rather than to the phylogenetic history of photobionts and mycobionts.

Algal switching seems to be common in lichens and it occurs under several conditions. When more compatible algae become available, the alga may be taken by the mycobiont as the accessory photobiont, and later become the primary photobiont. In this case, algae that are less suitable to fungi in certain environmental conditions might become more favorable to fungi under the new environmental conditions.

Future research requires genetic screening of populations and incorporating genotypes with environmental data to illustrate effects of habitats and environmental factors in photobiont selection of mycobionts in lichens.

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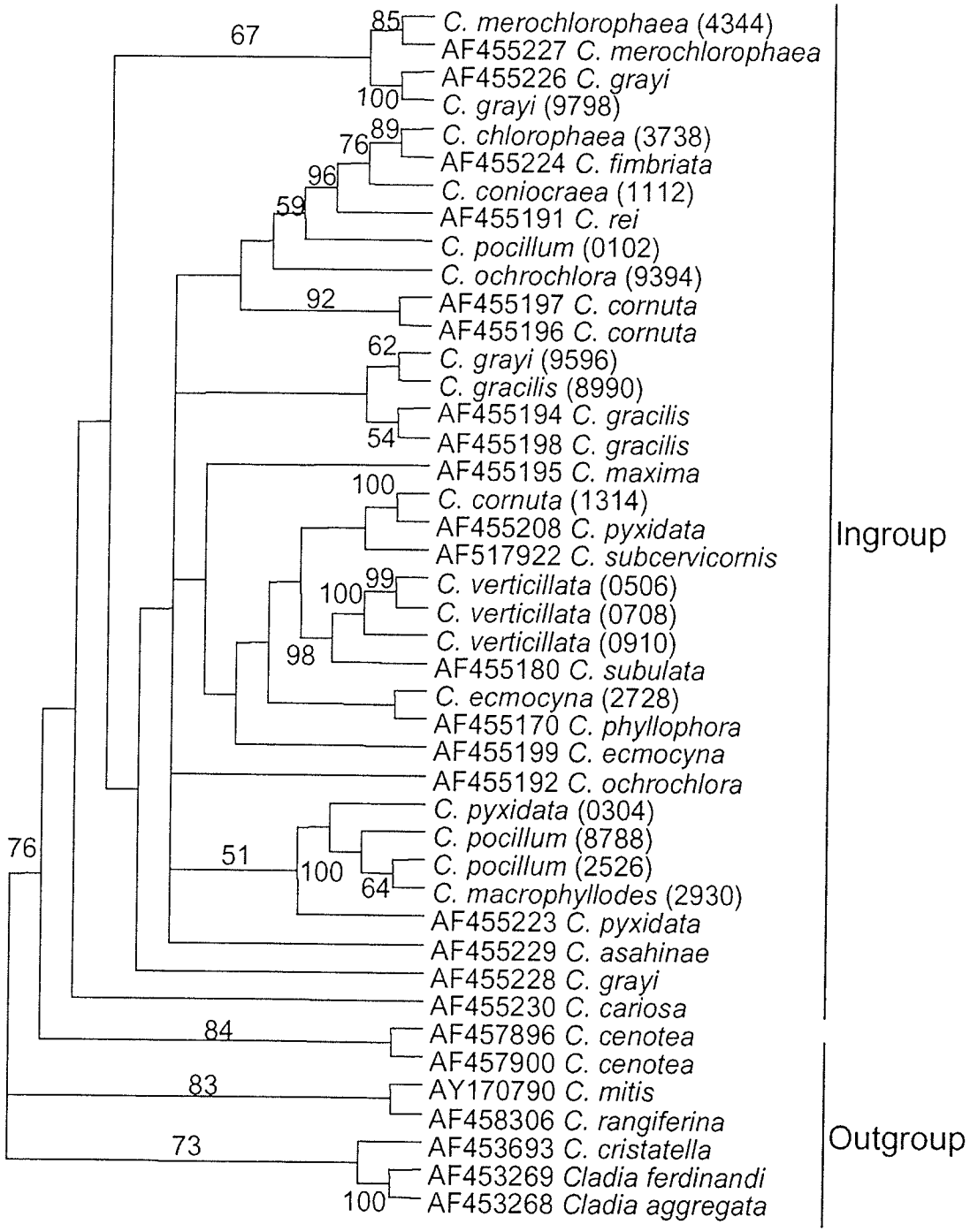
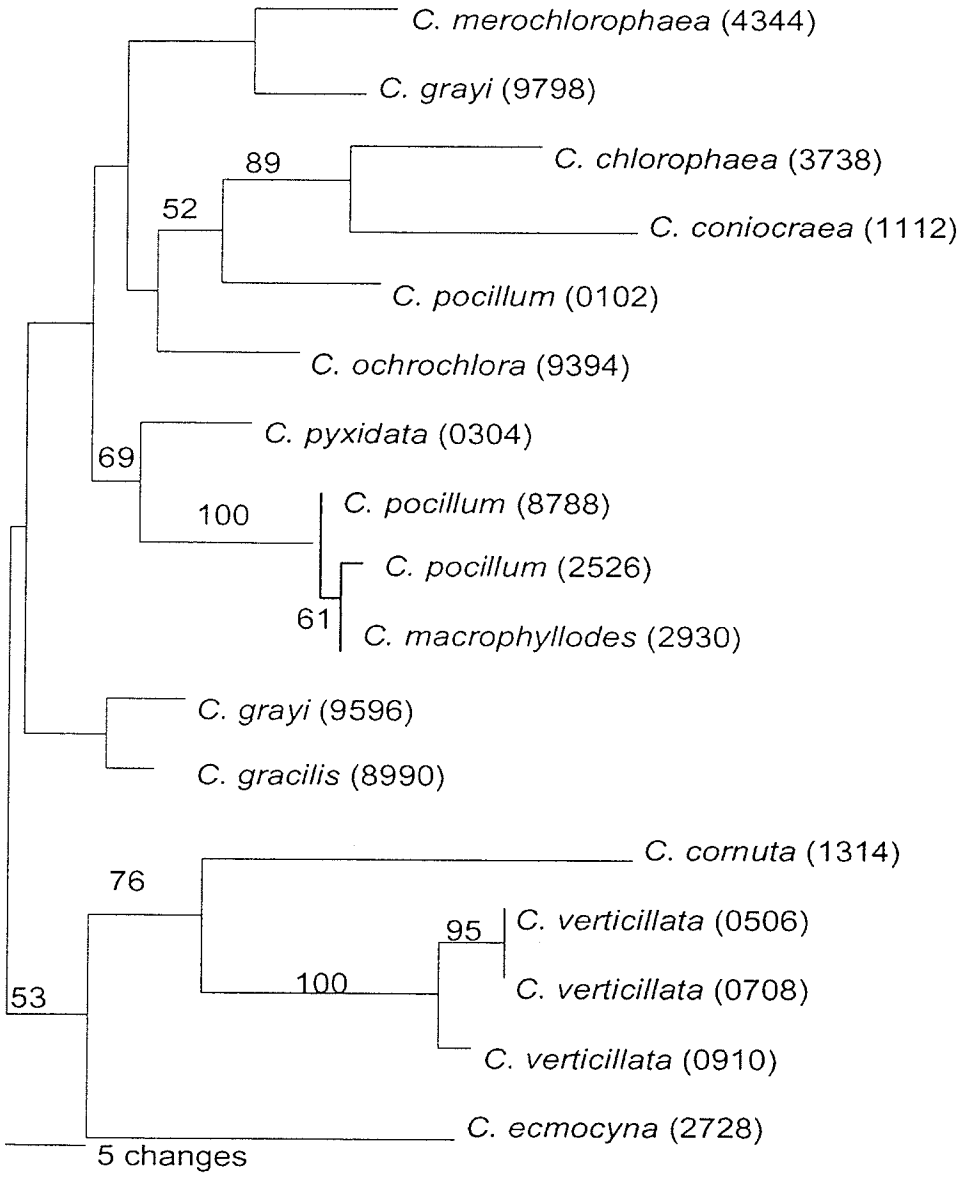
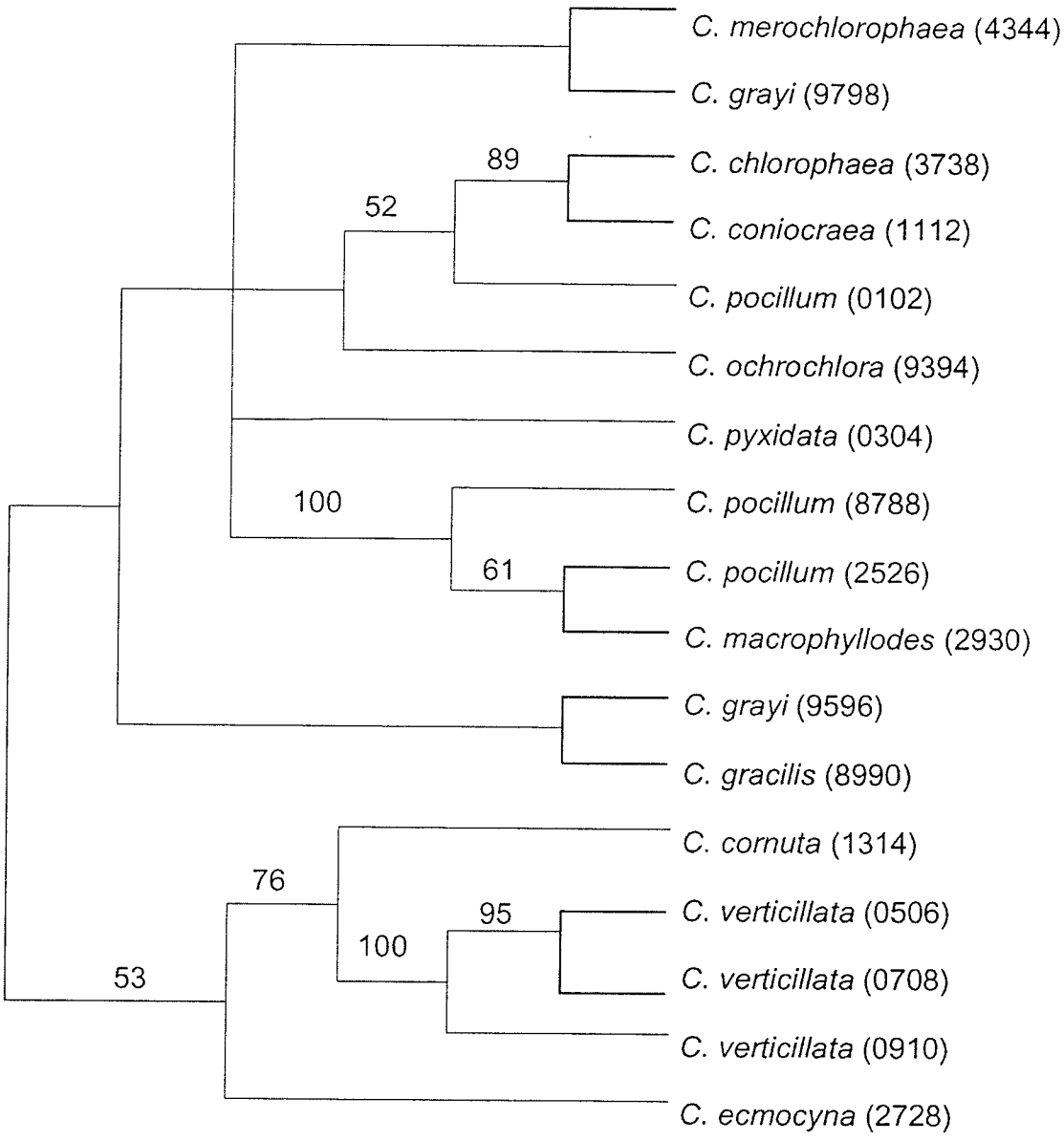
APPENDICES 1-13



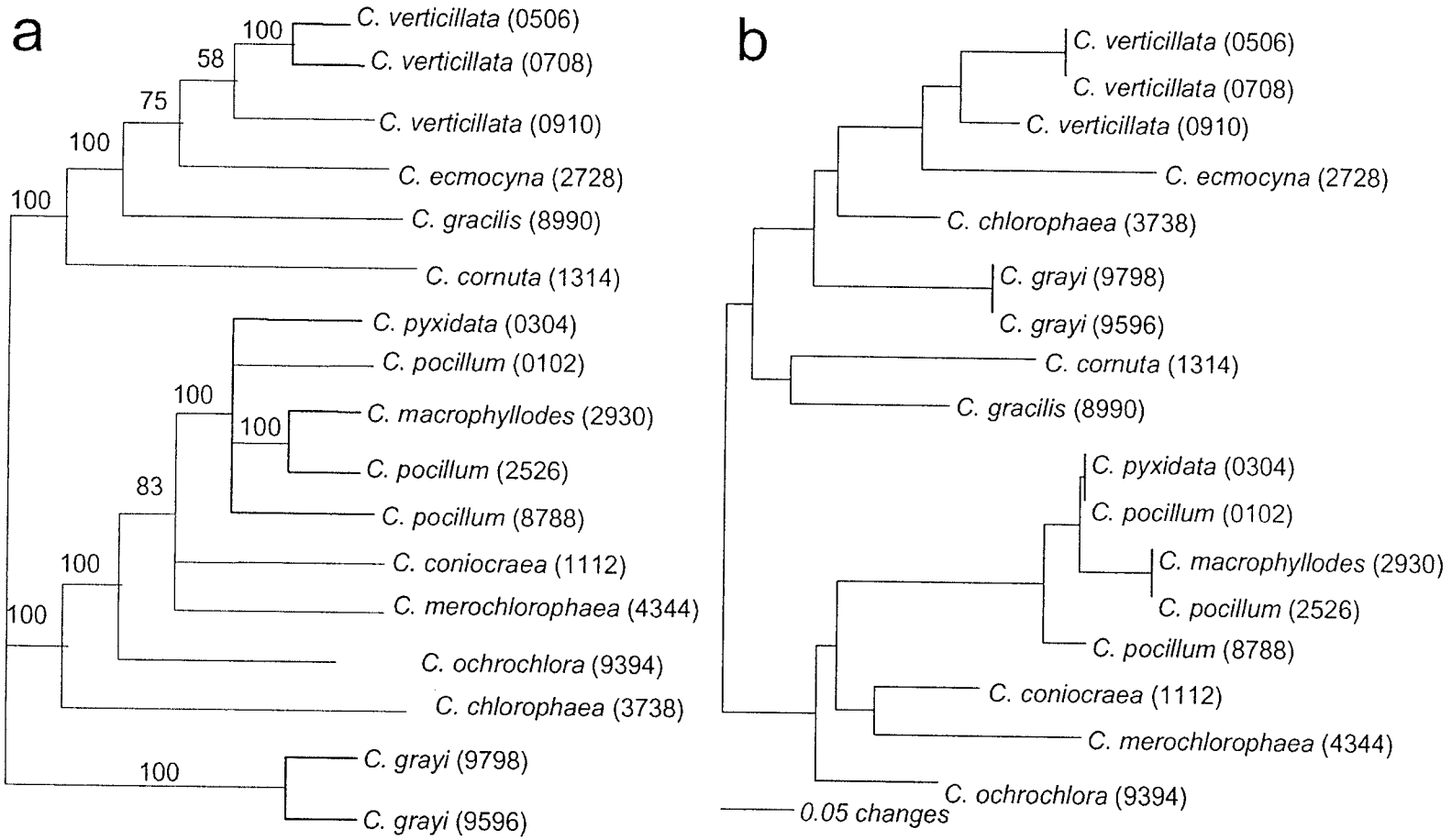
Append. 1: 50% majority rule consensus of 15 most parsimonious trees for the fungal morphology, ITS1, 5.8S and ITS2 nucleotide sequence data. Dataset includes 17 mycobionts of *Cladonia* from this study and 26 from GenBank. Indels in the sequence are scored and incorporated into the data. Numbers with branches are bootstrap support >50%. Numbers with species correspond to those in tables 1 & 4. CI: 0.6026, RI: 0.6630.



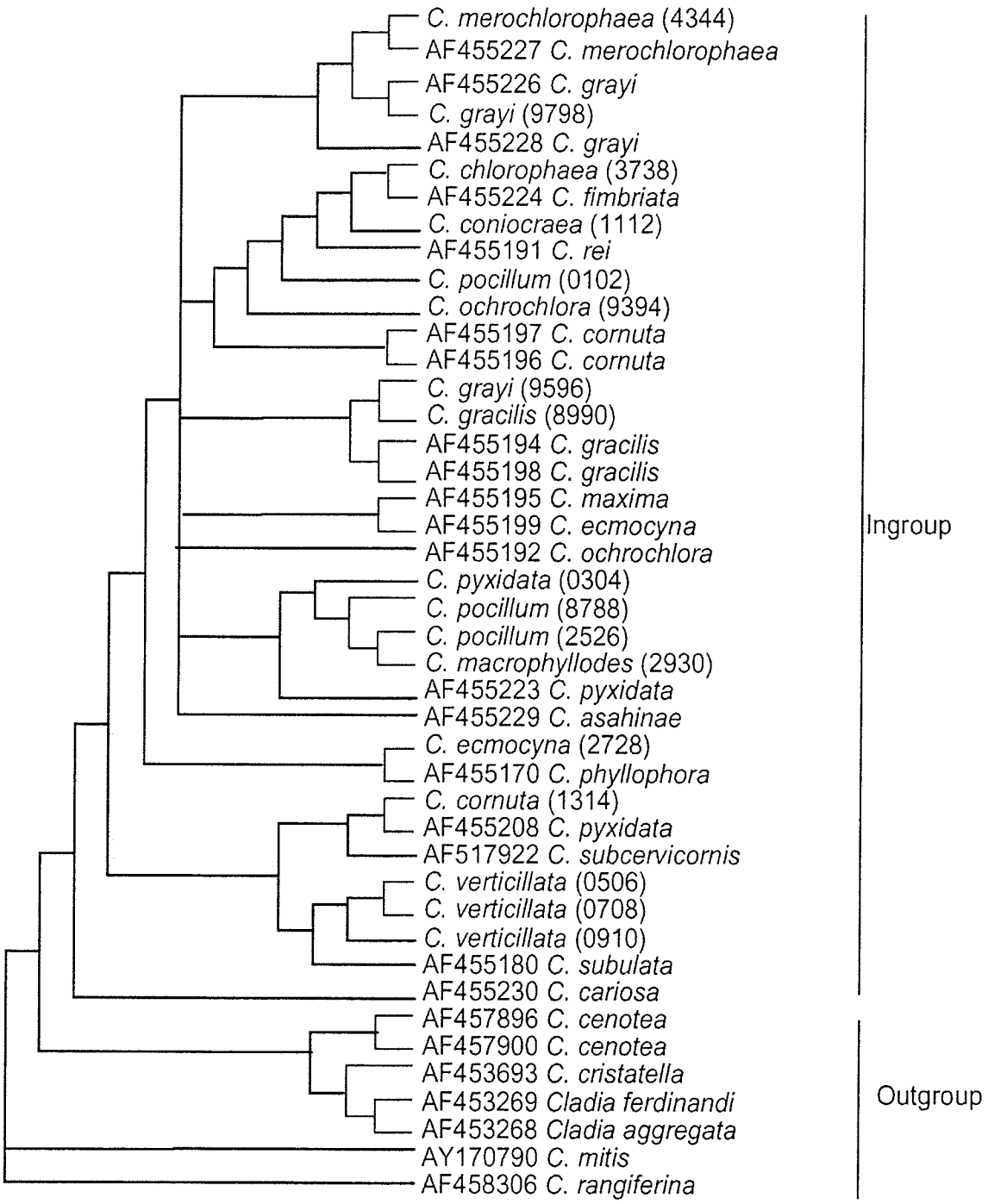
Append. 2: One of three most parsimonious midpoint rooted trees for the fungal morphology, ITS1, 5.8S and ITS2 sequence data. Dataset includes 17 mycobionts of *Cladonia* from this study. Numbers with branches are bootstrap support >50%. Numbers with species correspond to those in tables 1 & 4. CI: 0.6026, RI: 0.6630.



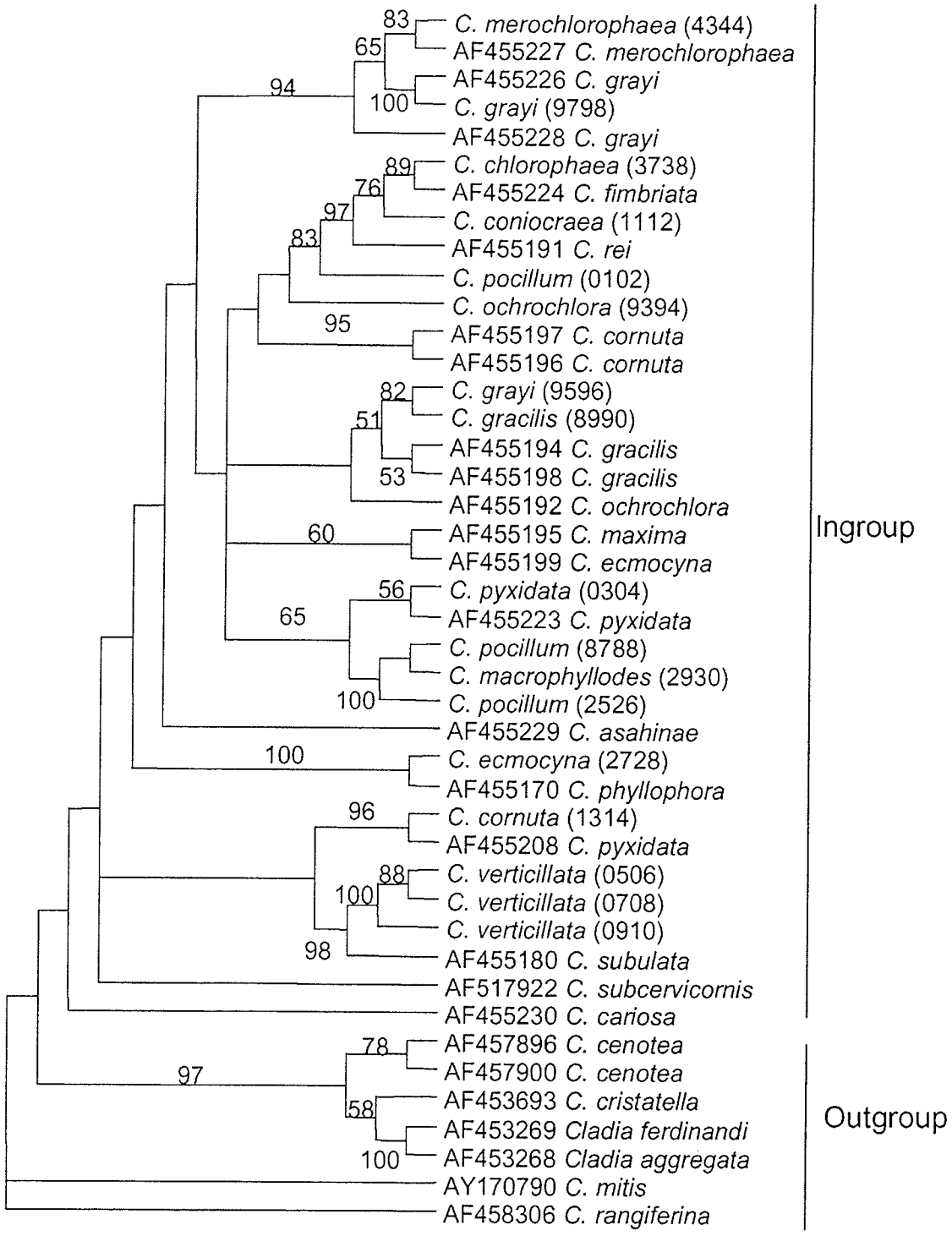
Append. 3: 50% majority rule consensus of three most parsimonious midpoint rooted trees for the fungal morphology, ITS1, 5.8S and ITS2 sequence data. Dataset includes 17 mycobionts of *Cladonia* from this study. Numbers with branches are bootstrap support >50%. Numbers with species correspond to those in tables 1 & 4. CI: 0.6938, RI: 0.6960.



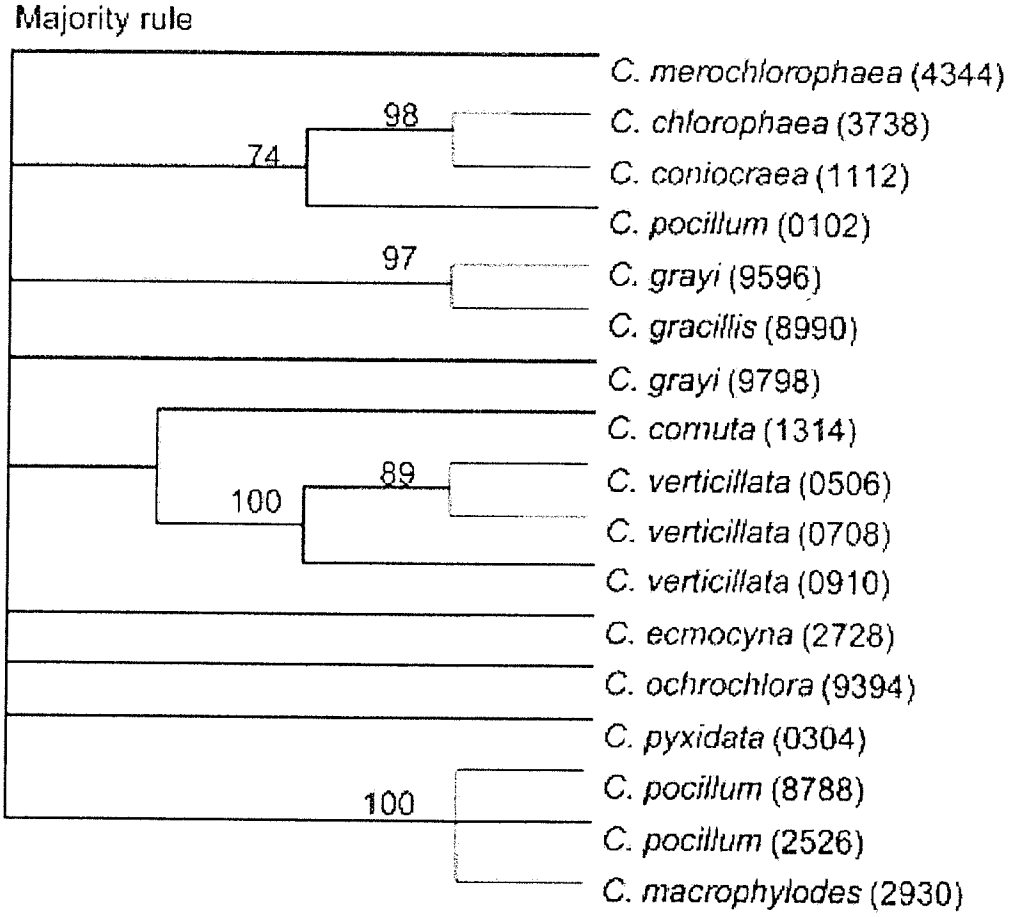
Append. 4: a: 50% majority rule consensus of 12 most parsimonious midpoint rooted trees for the fungal morphological data. Dataset includes 17 mycobionts of *Cladonia* from this study. Numbers with branches are bootstrap support >50%. Numbers with species correspond to those in tables 1 & 4. CI: 0.5676, RI: 0.7538; and, b: Neighbor joining midpoint rooted tree for the fungal morphological data. Dataset includes 17 mycobionts of *Cladonia* from this study. Numbers with species correspond to those in tables 1 & 4.



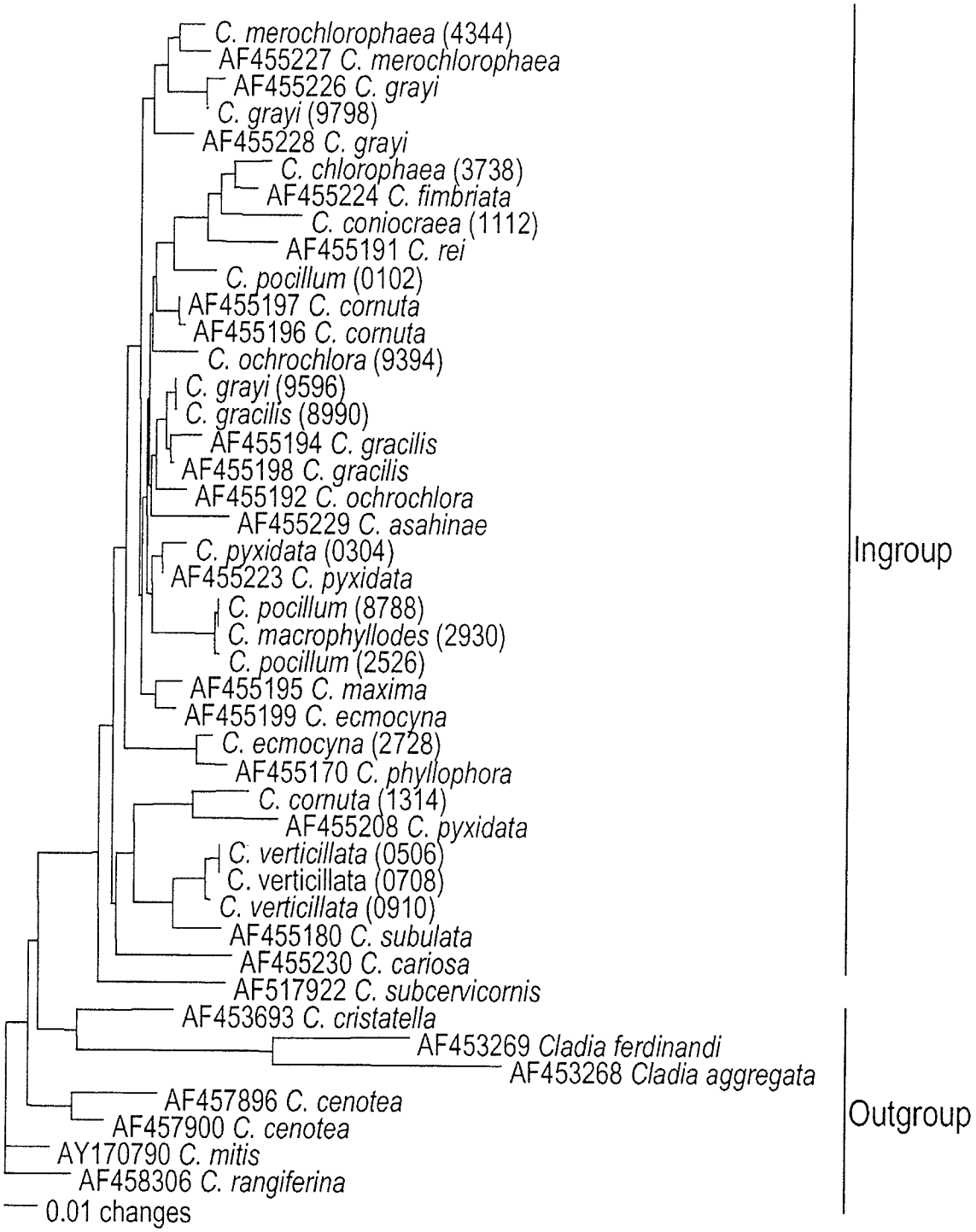
Append. 5: 50% majority rule consensus tree of 27 most parsimonious trees for the fungal morphology, ITS1, 5.8S and ITS2 nucleotide sequence data. Dataset includes 17 mycobionts of *Cladonia* from this study and 26 from GenBank. Numbers with species correspond to those in tables 1 & 4. CI: 0.5980, RI: 0.6568.



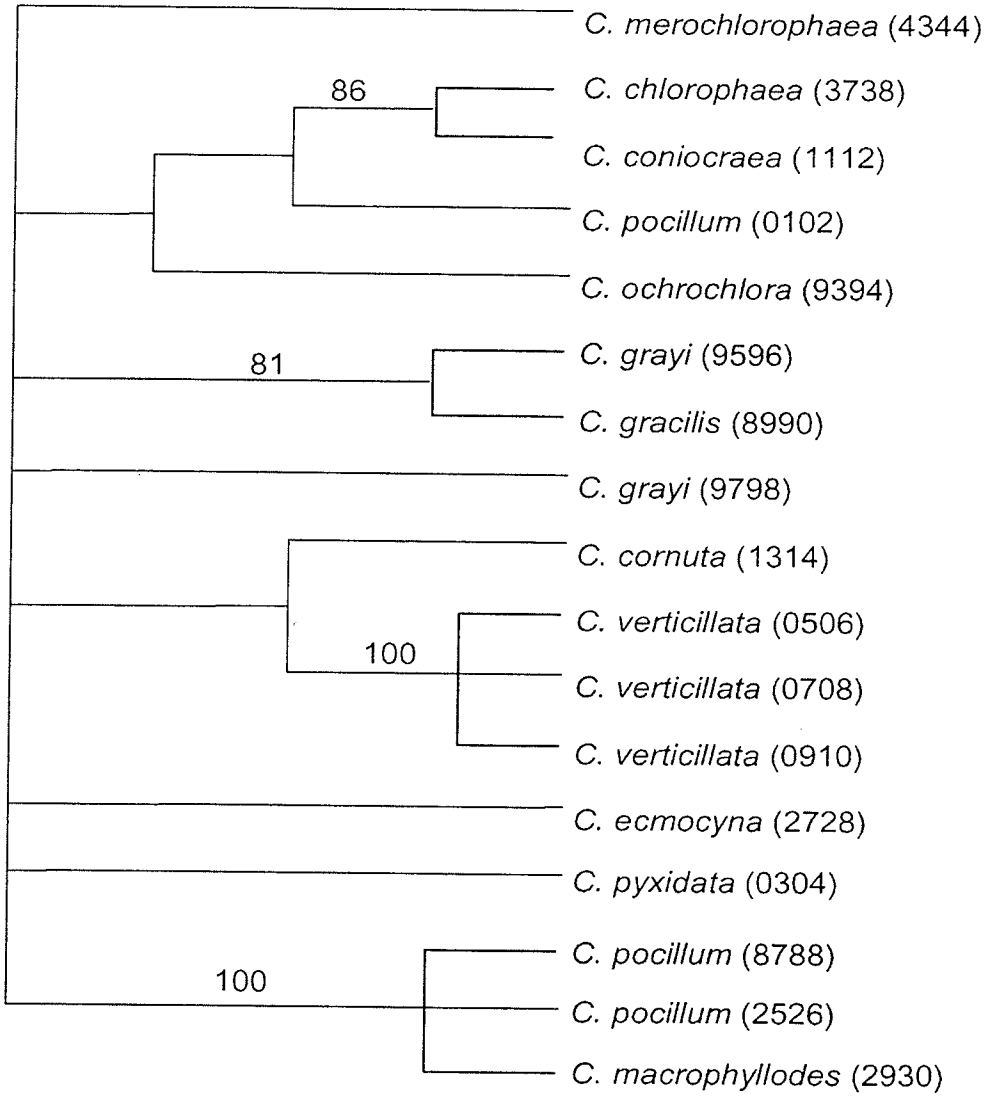
Append. 6: 50% majority rule consensus tree of 34 most parsimonious trees for the fungal ITS1, 5.8S and ITS2 combined nucleotide sequence data. Dataset includes 17 mycobionts of *Cladonia* from this study and 26 from GenBank. Numbers with branches are bootstrap support >50%. Numbers with species correspond to those in tables 1 & 4. CI: 0.6118, RI: 0.6672.



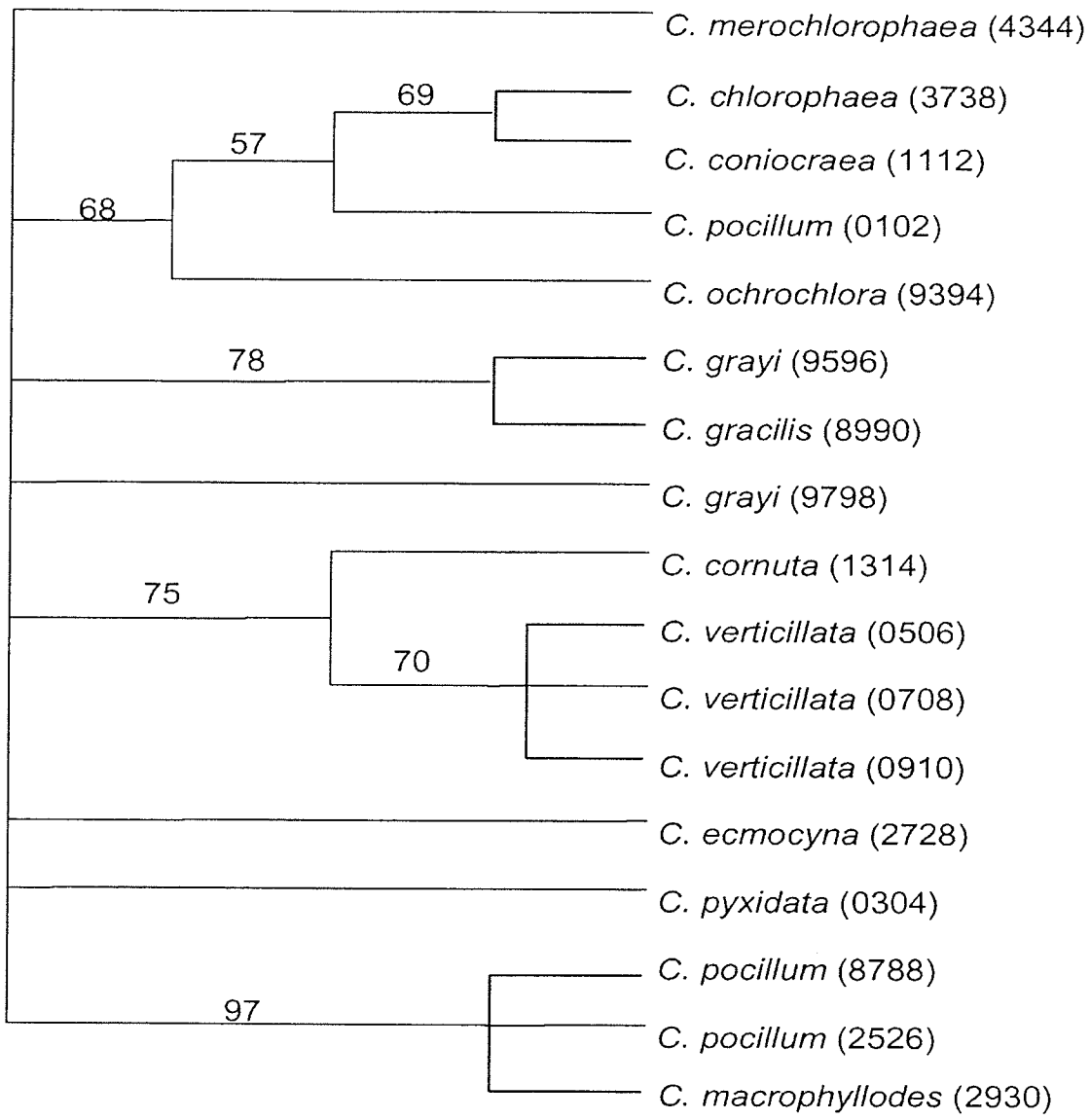
Append. 7: 50% majority rule consensus of four most parsimonious midpoint rooted trees for the ITS1, 5.8S and ITS2 combined sequence data. Dataset includes 17 mycobionts of *Cladonia* from this study. Numbers with branches are bootstrap support >50%. Numbers with species correspond to those in tables 1 & 4. CI: 0.7561, RI: 0.7619.



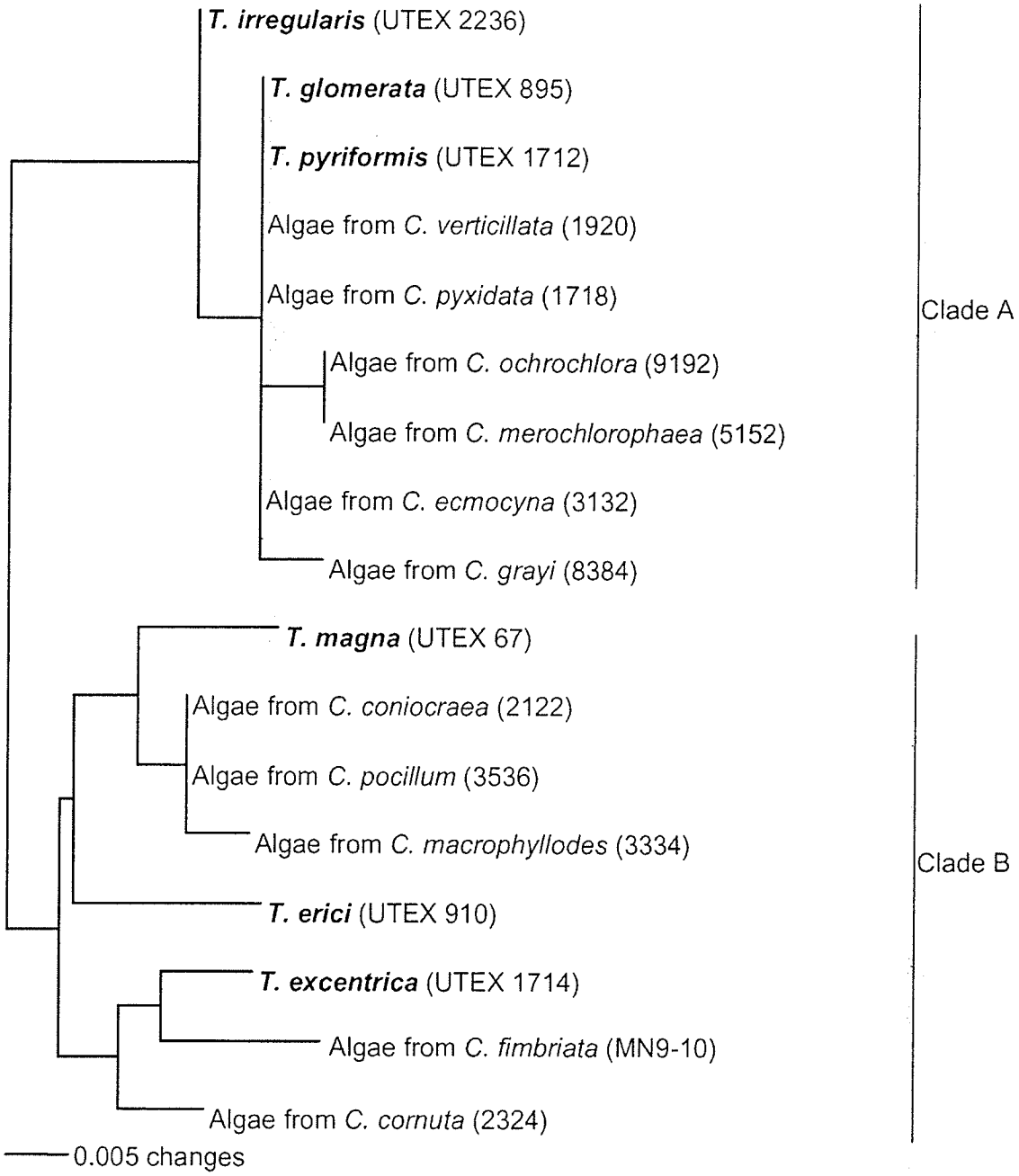
Append. 8: Neighbor joining tree for the fungal ITS1, 5.8S and ITS2 combined nucleotide sequence data. Dataset includes 17 mycobionts of *Cladonia* from this study and 26 from GenBank. Numbers with species correspond to those in tables 1 & 4.



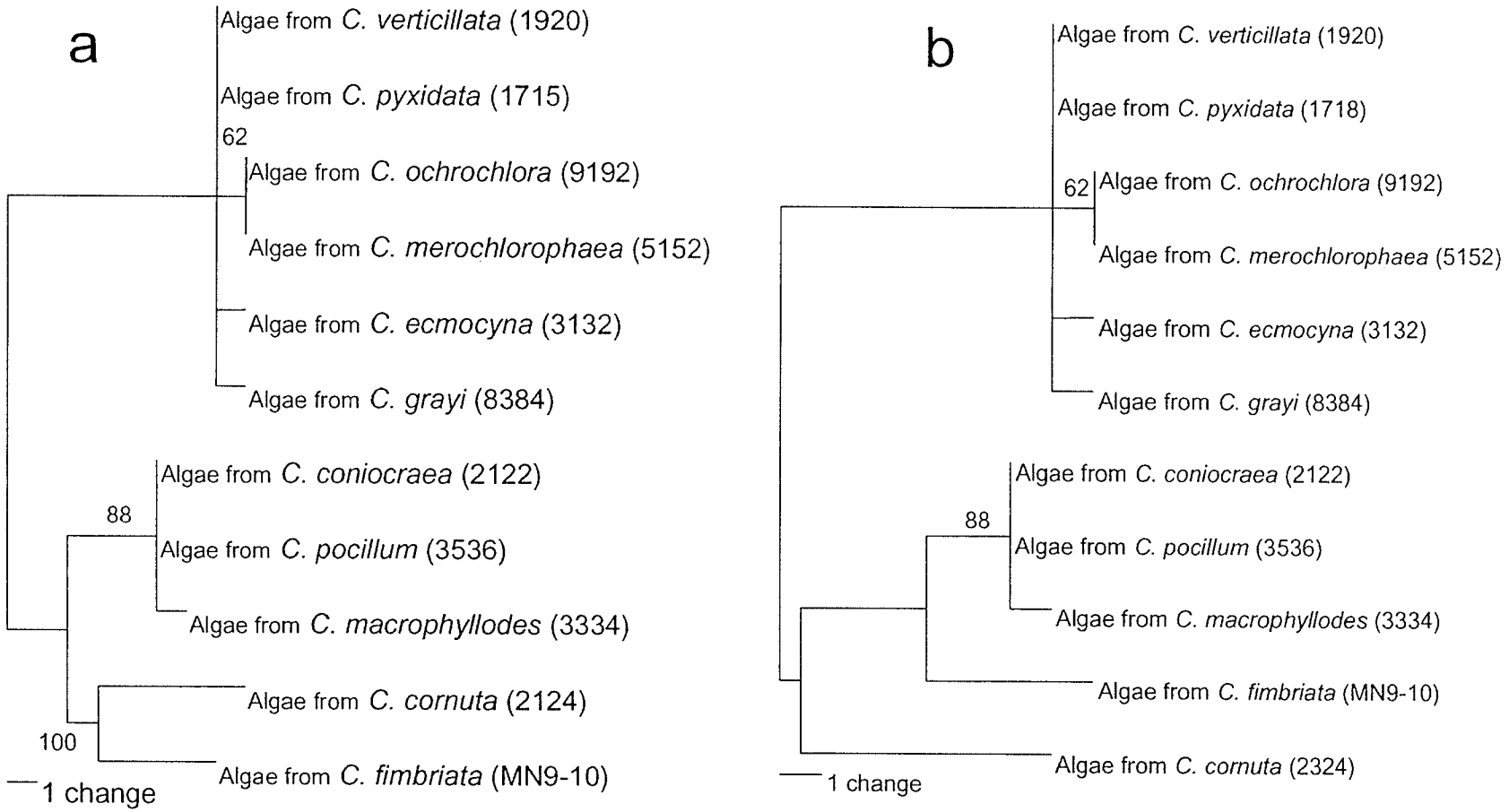
Append. 9: 50% majority rule consensus of 46 most parsimonious midpoint rooted trees for the fungal ITS1 nucleotide sequence data. Dataset includes 17 mycobionts of *Cladonia* from this study. Numbers with branches are bootstrap support >50%. Numbers with species correspond to those in tables 1 & 4. CI: 0.7619, RI: 0.7472.



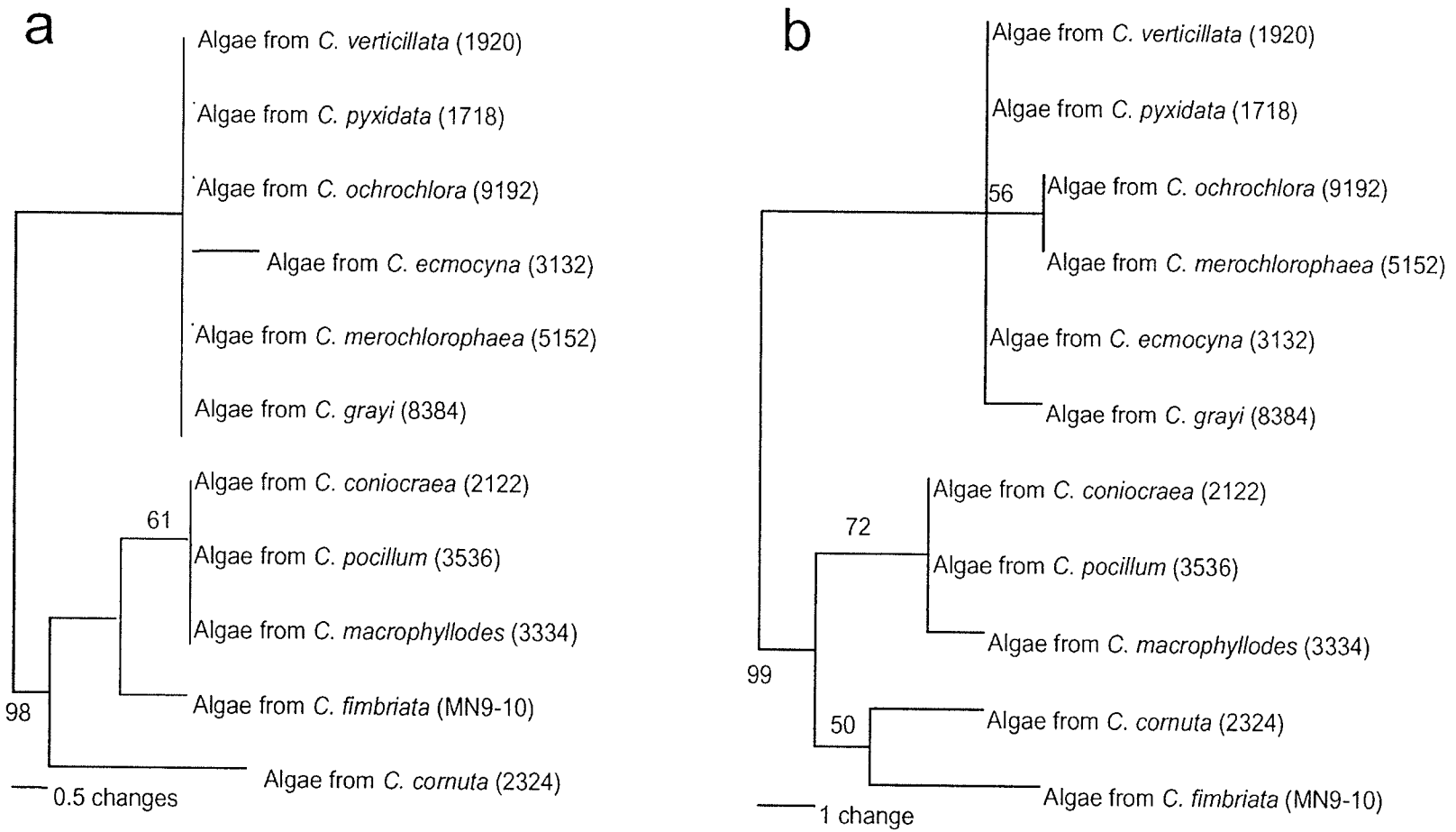
Append. 10: 50% majority rule consensus of 31 most parsimonious midpoint rooted trees for the fungal ITS2 nucleotide sequence data. Dataset includes 17 mycobionts of *Cladonia* from this study. Numbers with branches are bootstrap support >50%. Numbers with species correspond to those in tables 1 & 4. CI: 0.7636, RI: 0.7593.



Append. 11: Neighbor joining midpoint rooted tree for the algal ITS2 nucleotide sequence data. Dataset includes 11 photobionts of *Cladonia* from this study and six known *Trebouxia* species from GenBank. Numbers with species correspond to those in tables 1 & 4.



Append. 12: The two most parsimonious midpoint rooted trees (a and b) for the algal ITS1, 5.8S and ITS2 combined nucleotide sequence data. Datasets include 11 photobionts of *Cladonia* from this study. Numbers with branches are bootstrap support >50%. Numbers with species correspond to those in tables 1 & 4. CI: 0.9615, RI: 0.9767.



Append. 13: a: One of two most parsimonious midpoint rooted trees for the algal ITS1 nucleotide sequence data. CI: 1.0, RI: 1.0; and, b: One of two most parsimonious midpoint rooted trees for the algal ITS2 nucleotide sequence data. CI: 0.9375, RI: 0.9615.
Datasets include 11 photobionts of *Cladonia* from this study. Numbers with branches are bootstrap support >50%. Numbers with species correspond to those in tables 1 & 4.