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Phylogenetic, taxonomic and biogeographical studies in the Pithophoraceae (Cladophorales, Chlorophyta)

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General introduction

Algal evolution has resulted in a diverse array of morphologies, reproductive strategies and life histories, physiological reactions, biosynthetic products, and biotic interactions. These features serve as the basis for traditional systematics. However, some groups have proven to be notoriously difficult to treat taxonomically due to morphological simplicity, phenotypic plasticity, and convergent evolution of characters. One prime example for such taxonomic difficulties is the green algal order Cladophorales (Ulvophyceae, Chlorophyta).

The green algal order Cladophorales and its phylogenetic position

The order **Cladophorales** is characterized by a siphonocladous organization of uniseriate filaments or more complex thalli consisting of multinucleate cells; decoupled nuclear and cytoplasmic divisions; closed mitosis; no cytoplasmic streaming resulting in nuclei and chloroplasts with fixed positions; single or multiple chloroplasts, often forming a reticulum; alternation of isomorphic generations in sexually reproducing species; cell walls composed of cellulose I with parallel microfibrils; a flattened flagellar apparatus; a cruciate zooid type; and absence of terminal caps (O'Kelly & Floyd 1984, van den Hoek 1984, van den Hoek *et al.* 1995).

The group currently known as Cladophorales had been separated from the Ulvophyceae and classified as a class of their own, Cladophorophyceae nom. nud. (van den Hoek *et al.* 1995). The elevation of the ulvophycean orders (Ulvales, Cladophorales, Bryopsidales, Dasycladales and Trentepohliales) to the class level seemed sensible at the time based on significant cytomorphological and cytochemical differences (van den Hoek 1984, van den Hoek *et al.* 1995), but there is mounting molecular evidence that the Ulvophyceae *sensu* Mattox and Stewart (1984) represents a natural group (López-Bautista & Chapman 2003, Watanabe & Nakayama 2007, Cocquyt *et al.* 2010) that includes the Cladophorales. Together with filamentous marine green algae and larger green seaweeds, this class also contains the filamentous aeroterrestrial Trentepohliales and several obscure organisms such as *Blastophysa*, *Ignatius*, *Oltmannsiellopsis* or *Pseudocharacium* (Mattox & Stewart 1984, Watanabe & Nakayama 2007, Cocquyt *et al.* 2010). Cocquyt *et al.* (2010) inferred monophyly of the Ulvophyceae for the first time with high support. The monophyly of the Ulvophyceae had been questioned because it lacks unique ultrastructural synapomorphies (Mattox & Stewart 1984, O'Kelly & Floyd 1984, Zuccarello *et al.* 2009) and because earlier molecular phylogenetic studies did not fully resolve the relationships among the orders and the positions of some enigmatic genera (Chappell *et al.* 1991,

Watanabe & Nakayama 2007).

Relationships within the Cladophorales

Controversy with regards to the recognition at the ordinal level based on repeated re-interpretation of pre-molecular characters has led to the recognition of either two or just one order. The following scenarios have been proposed: only one order Siphonocladales (Børgesen 1913, Feldmann 1938, Jónsson 1965, O'Kelly & Floyd 1984), only one order Cladophorales (Chapman & Chapman 1973, van den Hoek 1984, van den Hoek & Womersley 1984), or two separate orders Siphonocladales s.s. and Cladophorales s.s. (Børgesen 1948, Egerod 1952, Womersley 1984, Bold & Wynne 1985). Currently the recognition of two separate lineages corresponding to the Siphonocladales s.s. and Cladophorales s.s. has been established (Leliaert *et al.* 2003). The term Cladophorales s.s. is equivalent to Cladophoraceae (sensu Wille). However, the Cladophoraceae have a sister relationship with the Siphonocladales s.s., a lineage that currently has the status of an order, and that contains several small families. To avoid confusion, these two lineages are referred to as the **Cladophora-clade** and the **Siphonocladus-clade**, respectively. Members of the *Siphonocladus*-clade are distributed mainly in the tropics and include forms with highly specialized thallus architecture such as pseudoparenchymatic clusters, blades and three-dimensional networks in addition to *Cladophora*-like filamentous taxa. Members of the *Cladophora*-clade extend their distribution from the tropics into cold temperate and (ant)arctic waters and consist of branched or unbranched filaments, represented by the genera *Chaetomorpha* Kützinger, *Cladophora* Kützinger and *Rhizoclonium* Kützinger. Persistent problems in the taxonomy of the Cladophorales at the level of families and genera have originated from the morphological simplicity with the associated lack of diagnostic characters, the large extent of phenotypic plasticity in relation to environmental conditions, and are also largely connected to the interpretation of the species-rich and variable genus *Cladophora*.

Cladophora is one of the largest and taxonomically and nomenclaturally most frustrating genera of green macroalgae. Pioneering work in the genus and its allies was done by van den Hoek (1963) who reduced the vast number of *Cladophora* species described to 34 species for Europe and classified them into 11 sections of the genus (12 sections in later treatments). While some species have relatively narrow morphological amplitudes and are easily identified, the majority of species is morphologically plastic and difficult or in some cases even impossible to identify at the species level. A photograph of branched apical filaments of two large-celled marine *Cladophora* species is shown in Fig. 1. Approximately 100 species are currently recognised worldwide, but this probably does not reflect the actual number of species. Based on the development of morphological series, van den Hoek postulated a system in which *Cladophora* represents an ancestral morphotype from which more complex forms have evolved (van den Hoek 1963, 1982, 1984). It was hypothesized that 'satellite genera' such as *Anadyomene* V.J. Lamouroux, *Boodlea* (Dickie) G. Murray & De Toni, *Chaetomorpha*, *Chaemaedoris* Montagne, *Cladophoropsis* Børgesen, *Ernodemsis* Børgesen, *Microdictyon* Decaisne, *Rhizoclonium*, *Struvea* Sonder, *Valonia* C. Agardh, *Willeella* Børgesen and *Wittrockiella* Wille have evolved from eight of these sections of *Cladophora* (van den Hoek 1963, 1982, 1984; Fig. 2). The genus *Cladophora* is clearly polyphyletic (Bakker *et al.* 1994, Hanyuda *et al.* 2002, Leliaert *et al.*

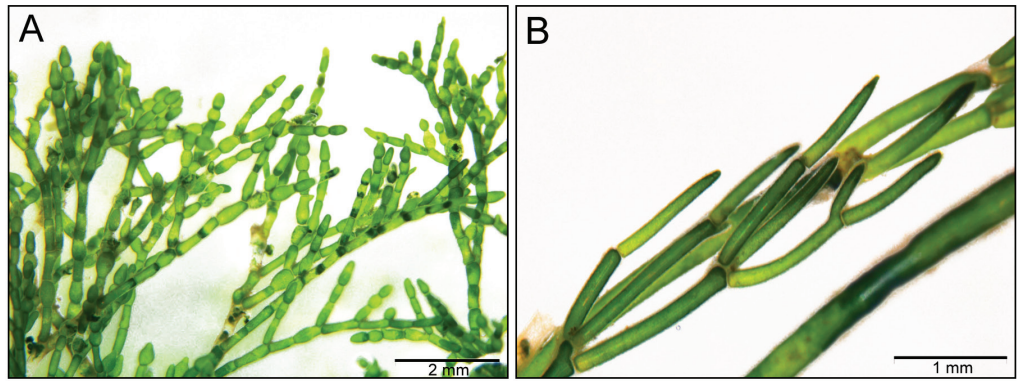


Fig. 1. Densely branched apical filaments of two large-celled marine *Cladophora* species. Both species grow mainly by acropetal cell divisions, and are among the easily identified *Cladophora* species. **A.** *Cladophora lehmanniana* (Lindenberg) Kützing. **B.** *Cladophora pellucida* Dickie.

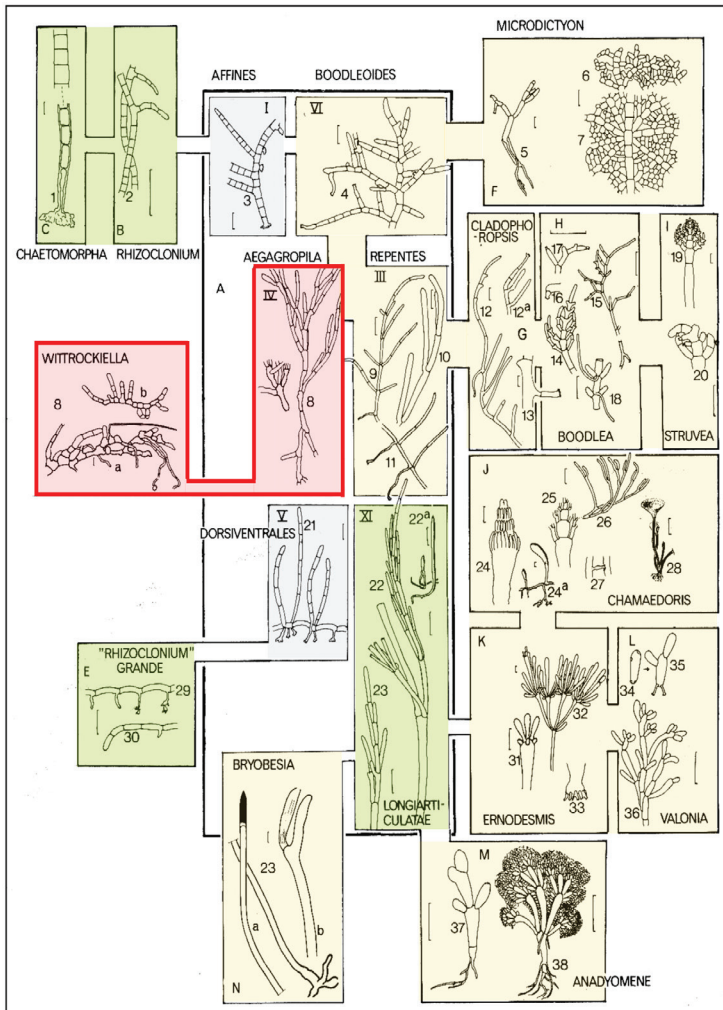


Fig. 2. Morphological relationships between sections of the genus *Cladophora* and other genera of the Cladophorales (from van den Hoek 1984). Sections of *Cladophora* are shown within the central box, satellite genera are arranged outside of the *Cladophora*-box. Different colours indicate taxonomic affinities: yellow indicates systematic placement in the *Siphonocladus*-clade (but see Leliaert et al. 2007a & b, 2008, 2009b for molecular relationships within this lineage), green indicates the *Cladophora*-clade, red indicates the *Aegagropila*-clade, and grey represents unknown taxonomic affinity. At the species level, however, many taxa have been found to belong to a different main clade. For example, some species (e.g., *C. prolifera*) placed in the *Cladophora* section *Longi-Articulatae* are members of the *Siphonocladus*-clade (Bakker et al. 1994, Leliaert et al. 2007). Diagram reproduced with permission from the author and the KNAW.

2003, 2007a) and molecular sequence data have shown that so far none of the *Cladophora* sections represents a natural group. However, in molecular phylogenies, all genera with specialized thallus architecture and mode of cell division group in a single lineage, the *Siphonocladus*-clade, which largely corresponds to the traditional circumscription of the Siphonocladales, with the exception of some anomalous *Cladophora* species (Leliaert *et al.* 2003, 2007a). The notion that the genus *Wittrockiella* is closely related to *Aegagropila* (van den Hoek *et al.* 1984; see Fig. 2) has been confirmed by molecular data (Hanyuda *et al.* 2002).

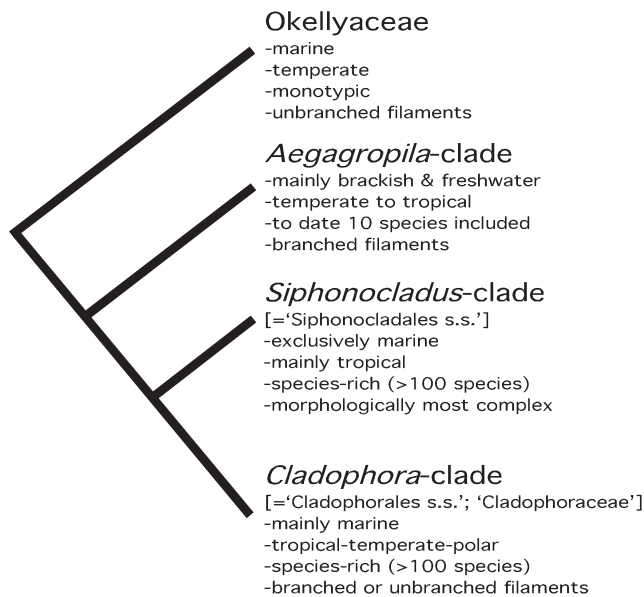


Fig. 3. Schematic phylogenetic tree of the Cladophorales with gross information for the four main lineages on habitat, species numbers and morphology. Based on the studies of Hanyuda *et al.* 2002, Leliaert *et al.* 2003, Yoshii *et al.* 2004, and Leliaert *et al.* 2009a).

In molecular phylogenies of the Cladophorales, four lineages have been recovered (Fig. 3): the *Siphonocladus*-clade and the *Cladophora*-clade are species-rich, predominantly marine lineages that have a sister relationship (Leliaert *et al.* 2003), and the **Aegagropila-clade** which is sister to those (Hanyuda *et al.* 2002, Yoshii *et al.* 2004). A fourth lineage, **Okellyaceae**, was recently discovered and is sister to the three main clades and includes *Okellya curvata* Leliaert, an unbranched, marine microfilamentous species occurring in temperate subtidal habitats (Leliaert *et al.* 2009a). Species of *Cladophora*

are (still) found in the three main clades of the Cladophorales. The lectotype species of *Cladophora* and the Cladophoraceae is *Cladophora oligoclona* (Kützinger) Kützinger (Setchell & Gardner 1920, van den Hoek 1963), which van den Hoek (1963) placed in synonymy with *Cladophora rivularis* (L.) Hoek. Molecular data place *C. rivularis* in a freshwater clade together with *C. glomerata* (L.) Kützinger and *C. fracta* (O.F. Müller ex Vahl) Kützinger (possibly these three morphospecies actually represent only one polymorphic species) within the *Cladophora*-clade (Leliaert & Boedeker 2007). Thus, *Cladophora* species that are placed in the *Siphonocladus*- or the *Aegagropila*-clade need to be transferred to other or new genera. While significant progress has been made in untangling the relationships within the *Siphonocladus*-clade by molecular phylogenetic studies and morphological revisions (e.g., Leliaert & Coppejans 2006, 2007, Leliaert *et al.* 2007a & b, 2008, 2009b), both the *Cladophora*- and the *Aegagropila*-clade require a lot of systematic work. A molecular phylogenetic and taxonomic treatment of the *Aegagropila*-clade is provided in **Chapter 7** of this thesis and the family name **Pithophoraceae** is proposed for this lineage.

The *Aegagropila*-clade (= *Pithophoraceae*, see Chapter 7)

The *Aegagropila*-clade includes taxa from six monotypic or species-poor genera and a small number of freshwater *Cladophora* species, and its members are primarily distributed in brackish and freshwater environments (Hanyuda *et al.* 2002, Yoshii *et al.* 2004, Rindi *et al.* 2006). Its members occur in narrow niches such as on the carapaces of freshwater turtles (some members of *Basycladia* Hoffmann & Tilden, Fig. 4), on freshwater snails and bivalves (monotypic *Arnoldiella* Miller and several *Basycladia* species), on and endophytically in saltmarsh plants and mangrove pneumatophores (some members of *Wittrockiella* Wille), or on marine intertidal snails (*Cladophora conchopheria* Sakai). Based on morphological similarities, it has been proposed that *W. paradoxa* might be



Fig. 4. *Basycladia* sp. growing on the carapace of a snapping turtle (*Chelydra serpentina*) in southeast Michigan (photograph by Jay Fitzgerald, with permission).

Previous molecular phylogenetic studies by Hanyuda *et al.* (2002) and Yoshii *et al.* (2004) showed that the marine species *Cladophora conchopheria* and *C. horii* form a sister-clade to the rest of the *Aegagropila*-clade, that *Wittrockiella lyallii* and *W. amphibia* (as *W. paradoxa* Wille) group together, that *C. kosteriae* is allied to a species of *Basycladia*, and that *Aegagropila* and *Pithophora* have a close relationship (Fig. 6). The relation of *Arnoldiella conchophila* and *C. okamurae* (as *Chaetomorpha okamurae*) to the other taxa was not resolved (Hanyuda *et al.* 2002). The aerophytic unicellular alga *Spongiochrysis hawaiiensis* was recovered on a basal polytomy with *C. conchopheria* (Rindi *et al.* 2006). Despite this advancement in our understanding of the evolutionary relationships within the Cladophorales, the *Aegagropila*-clade is in need

to the warm-temperate to tropical species *Cladophorella calcicola* Fritsch (van den Hoek *et al.* 1984). Recently, an aerophytic unicellular organism occurring on tree bark has been described and included in this lineage based on DNA sequence data (monotypic *Spongiochrysis hawaiiensis* Rindi *et al.* 2006). *Aegagropila linnaei* is the best known representative of the lineage and gained considerable scientific and cultural significance due to the peculiar lake balls (also known as ‘Marimo’, e.g., Fig. 5) formed under specific conditions (van den Hoek 1963, Kurogi 1980, Niiyama 1989).



Fig. 5. Japanese postal stamp showing the ball-form of *Aegagropila linnaei*, a species that has gained considerable cultural and economic value in Japan.

of increased taxon sampling and a taxonomic revision (see Fig. 6).

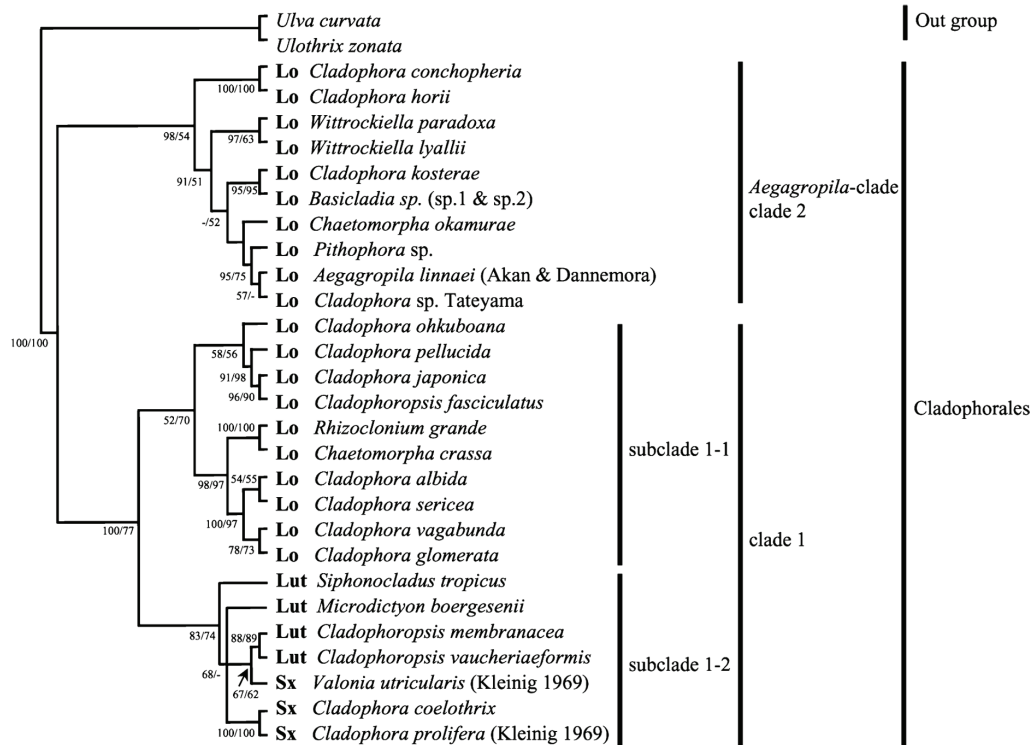


Fig. 6. Phylogenetic relationships within the Cladophorales, providing the most information on members and relationships to date on the *Aegagropila*-clade (Yoshii *et al.* 2004). Shown is a tree from a maximum likelihood analysis using SSU rDNA sequences, numbers at nodes represent bootstrap values (maximum parsimony/maximum likelihood). Subclade 1-1 corresponds to the *Cladophora*-lineage, subclade 1-2 to the *Siphonocladus*-lineage. Reproduced with permission from John Wiley & Sons.

Phenotypic plasticity in the Cladophorales

The taxonomic uncertainties within the Cladophorales result to a large extent from extensive phenotypic plasticity (especially from the variability of *Cladophora*) and from convergent evolution (the existence of *Cladophora* look-alikes in all three main clades, e.g., *Cladophoropsis* in the *Siphonocladus*-clade and *Aegagropila* in the *Aegagropila*-clade). Extensive morphological variability resulting from phenotypic plasticity, developmental variability and polymorphism exists in various taxa of the *Siphonocladus*-clade and obscures traditional morphological concepts of species delineation (Leliaert & Coppejans 2006, 2007, Leliaert *et al.* 2008, 2009b). The variability of *Cladophora* species depending on environmental conditions has been well documented (e.g., van den Hoek 1963, Parodi & Cáceres 1991). Despite extremely simple thallus architecture, more than 900 species of *Cladophora* had been described based on subtle morphological differences between specimens. The main diagnostic features are cell dimensions, branching pattern, rhizoid formation and overall thallus organisation. However, these morphological characters are greatly plastic in a large number of *Cladophora* species (see Fig. 7), and have also evolved

independently in different taxa.



Fig. 7. Morphological variability of a freshwater *Cladophora* (*Cladophora "rivularis"*) in culture. After being transferred from the field to culture conditions, the main axis continued to grow with newly produced cells being about half the diameter of the original size.

1930). Identification at the species level is virtually impossible due to overlap of character states (see Möbius 1895, van Oye 1922). Various synonymy scenarios were proposed over the years and at the same time new species and varieties were described (e.g., Collins 1909, Sula 1930, Silva 1954, van den Hoek 1959). It was shown that the most important diagnostic characters, namely the shape, size and arrangement of the cyst-like akinetes are not stable but rather controlled by a wide range of environmental conditions. Akinete formation and germination is inducible by various external factors (Ernst 1908, Agrawal 1986, Stevens & Neilson 1987), the size of akinetes is age-dependent (Brand 1904), akinete and branch formation are the same, reversible process (Mothes 1930), and helicoid formation is inducible as a wounding response (Mothes 1930). Based on filament diameters, another diagnostic character, van Oye (1922) could identify a single *Pithophora* specimen from Java as ten different described species.

From a modern point of view, it is obvious that only methods of molecular phylogenetic inference have the power to unravel systematic problems within the Cladophorales, e.g., to identify conspecifics (or closely related species) and also to identify cryptic species within morphospecies in these highly plastic and morphologically simple filamentous green algae.

Aims and outline of the thesis

This study extends the previous phylogenies of the *Aegagropila*-clade by increased taxon sampling and by combining SSU (small subunit = 18S) and LSU (large subunit = 28S) rDNA sequence data, which has been shown to lead to better resolved phylogenies in the Cladophorales (Leliaert *et al.* 2007). The confused taxonomy of the *Aegagropila*-lineage was clarified using methods of molecular phylogenetic inference. In selected taxa, topics such as phylogeography and dispersal abilities, historical biogeography and glacial refugia, and ecology and declining populations were studied in detail.

In **Chapter 2**, it is investigated whether *Aegagropila linnaei* still occurs in the Netherlands. The ball-form of this species had been reported from eight locations before the 1970s, but all habitats have drastically changed during the last 50 years and were severely affected by eutrophication. All historical locations were searched by snorkelling, SCUBA diving, dredging and shore surveys.

Chapter 3 provides a detailed account of the worldwide distribution of *Aegagropila linnaei*, reconstructed from more than 1200 herbarium specimens from 28 herbaria, an extensive literature survey and recent field observations. An assessment of the global decline of this species is given, based on a survey of extinct and extant populations in connection with changes in trophic conditions of the lakes the species is known from.

The dispersal potential of *Aegagropila linnaei* was examined in desiccation experiments and compared to other widespread freshwater Cladophorales in **Chapter 4**. Furthermore, ITS rDNA sequences were generated from populations throughout the distribution range and the recovered ribotypes analysed in a haplotype network. The global distribution of the species is interpreted with regards to the inferred dispersal capacity, the distribution of the ribotypes and past glacial events.

In **Chapter 5**, *Cladophora amphibia* and *Wittrockiella paradoxa* are synonymised, based on morphological, ecological and molecular evidence. In addition, the heterotrichous mode of growth is characterised.

In **Chapter 6**, it is shown that the South American species *Cladophoropsis brachyartra* is morphologically and ecologically equivalent to the presumed New Zealand endemic *W. lyallii*. The resulting disjunct distribution of the species is discussed in the light of DNA sequence divergence, sea surface temperatures, habitat availability in the southern ocean and winter sea ice extent during the last glacial maximum.

Chapter 7 provides the first multi-gene phylogeny of the *Aegagropila*-lineage, and taxon sampling was increased compared to earlier works. LSU and SSU sequences were analysed with state of the art methods of phylogenetic inference. The morphology was re-examined in search of novel diagnostic characters, and thallus evolution within the group was investigated with regards to the taxa's ecological niches. The pyrenoid ultrastructure was studied by transmission electron microscopy in members of all genera within the lineage. The family Pithophoraceae is re-instated for this group of organisms, and descriptions of two new genera and several new combinations are given.

A list of all nomenclatorial changes proposed in this thesis is given in **Appendix S8**.