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

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Contents

Chapter 1

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* Kützing, *Cladophora* Kützing and *Rhizoclonium* Kützing. 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.*

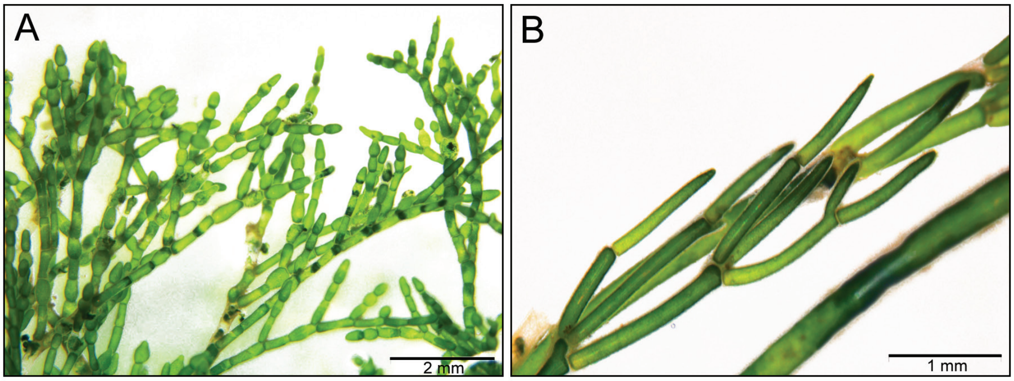

Fig. 1. Densely branched apical filaments of two large-celled marine *Cladophora* species. Both species grow mainly by acropetal cell divisions, and are among the easily identified *Cladophora* species. **A.** *Cladophora lehmanniana* (Lindenberg) Kützing. **B.** *Cladophora pellucida* Dickie.

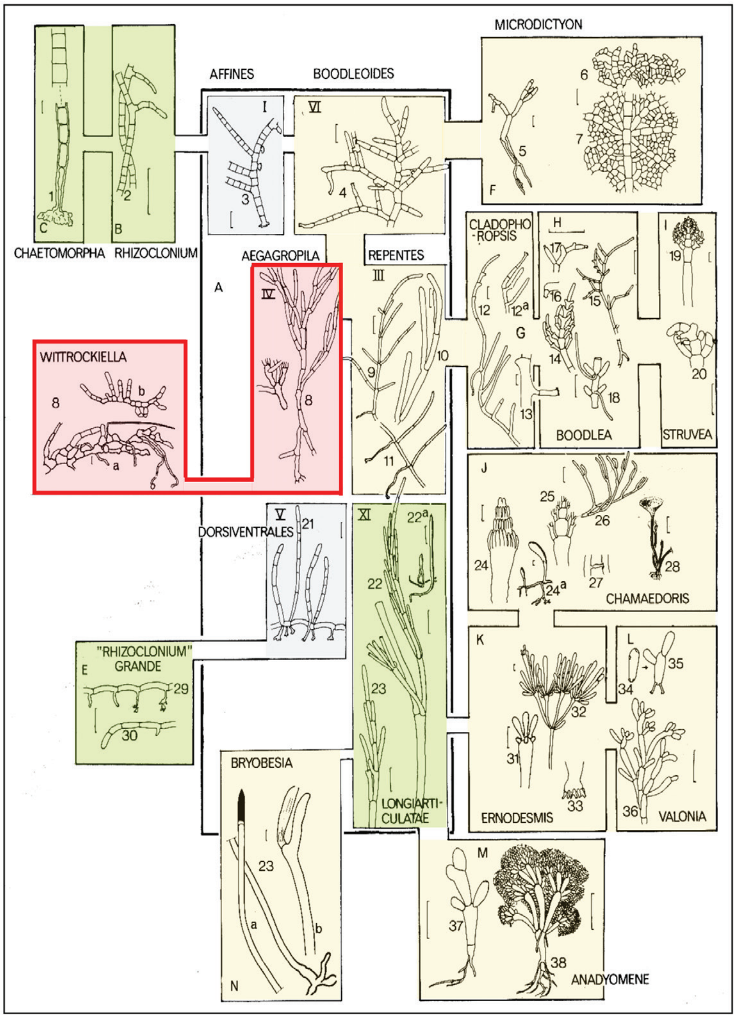

Fig. 2. Morphological relationships between sections of the genus *Cladophora* and other genera of the Cladophorales (from van den Hoek 1984). Sections of *Cladophora* are shown within the central box, satellite genera are arranged around the outside of the *Cladophora*-box. Different colours indicate 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).

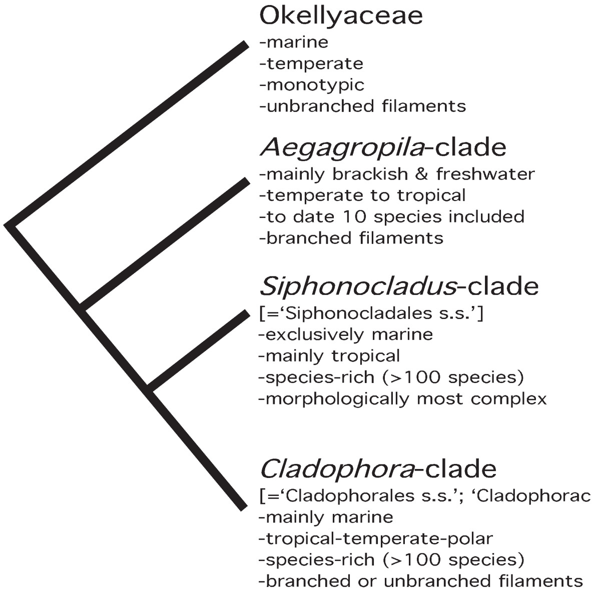

Fig. 3. Schematic phylogenetic tree of the Cladophorales with gross information for the four main lineages on habitat, species numbers and morphology. Based on the studies of Hanyuda *et al.* 2002, Leliaert *et al.* 2003, Yoshii *et al.* 2004, and Leliaert *et al.* 2009a).

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* (Kützing) Kützing (Setchell & Gardener 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.) Kützing and *C. fracta* (O.F. Müller ex Vahl) Kützing (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 *Aegegropila*-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 ***Basicladia*** Hoffmann & Tilden, Fig. 4), on freshwater snails and bivalves (monotypic ***Arnoldiella*** Miller and several *Basicladia* 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 closely related 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. 4. *Basicladia* 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. kosterae* is allied to a species of *Basicladia*, 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

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).

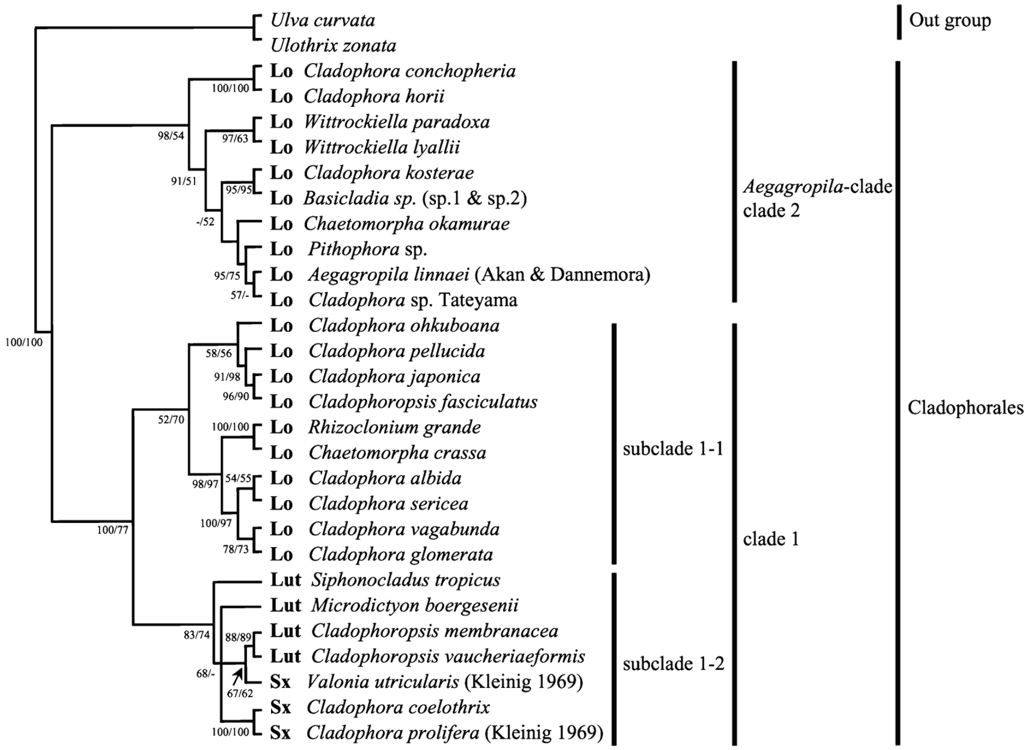

Fig. 6. Phylogenetic relationships within the Cladophorales, providing the most information on members and relationships to date on the *Aegagropila*-clade (Yoshii *et al.* 2004). Shown is a tree from a maximum likelihood analysis using SSU rDNA sequences, numbers at nodes represent bootstrap values (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 *Siphonocadus*-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.

More than 30 taxa of *Aegagropila* have been described, which are currently all regarded as synonyms of a single species, *A. linnaei*. In another member of the *Aegagropila*-lineage, the (sub)tropical, unattached genus *Pithophora*, phenotypic plasticity and taxonomic uncertainty are also very pronounced. Most other taxa of the *Aegagropila*-lineage are not known sufficiently enough to assess the degree of phenotypic plasticity. More than 35 taxa of *Pithophora* have been described, including a large number of varieties. Phenotypic plasticity in the few morphological characters led to an inflation in the number of described species (Ernst 1908, Mothes 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**.

The current situation of the freshwater green alga *Aegagropila linnaei* Kützing (Cladophorales, Chlorophyta): no more lake balls in the Netherlands

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Published as

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and

Boedeker, C. 2010. The attached form of the endangered freshwater alga *Aegagropila linnaei* Kützing (Chlorophyta) is found in the Zuideindigerwiede, The Netherlands. *Aquatic Botany* 92:75-77.

Abstract

Aegagropila linnaei, a freshwater green macroalga, had been abundant in several locations in the Netherlands before the 1960s. Both the ‘lake ball’ form of this alga and dense unattached mats floating over the sediment have been described from these locations. After 1967, this species has not been recorded anymore from the Netherlands. In 2007 and 2009, the historical collection sites were surveyed for extant populations of *A. linnaei*. All habitats have changed drastically during the last 50 years and were affected severely by eutrophication. Populations of *A. linnaei* seem to have become extinct in all but two locations (Boven Wijde and Zuideindigerwiede, province Overijssel). In the Boven Wijde, we found only very small amounts of attached filaments, while the attached form was widespread on solid substrates in the Zuideindigerwiede. The attached form had not been reported previously from the Netherlands. Environmental conditions do not seem suitable anymore to maintain extensive unattached growth forms including the enigmatic lake balls and the species must be regarded as threatened in the Netherlands. We therefore propose to include *A. linnaei* in a national red list. The decline of populations outside the Netherlands is reviewed and discussed. In addition to morphological identification of the attached filaments, partial sequences of the nuclear large subunit rDNA were generated and compared with different growth forms and habitats from several other locations outside the Netherlands. The sequences confirm the identity of the Dutch material and indicate very little divergence both between populations in different locations and between different growth forms.

Introduction

The green macroalga *Aegagropila linnaei* Kützing (Cladophorales, Ulvophyceae) occurs in freshwater and some brackish environments and has essentially a palaearctic distribution (van den Hoek 1963, Pankow 1965). The simple morphology consists of stiff, branched uniseriate filaments and is very similar to members of the related genus *Cladophora* Kützing. *Aegagropila linnaei* is morphologically distinct from *Cladophora* spp. by the lateral or subterminal, often opposite, sometimes serial insertion of branches; irregular cell shape and unrestricted insertion positions of branches especially in the basal parts; and development of rhizoids from the base of cells which can produce terminal haptera (van den Hoek 1963, Leliaert & Boedeker 2007, Soejima *et al*. 2008). The species is presumed to reproduce mainly or entirely vegetatively by means of fragmentation (Brand 1902, Acton 1916, van den Hoek 1963). Based on morphological similarities, the species was placed in the genus *Cladophora* (as *C. aegagropila* (L.) Rabenhorst, van den Hoek (1963)), but has recently been recognized as its own monotypic genus based on molecular evidence. It is placed in a separate clade that is sister to the orders Siphonocladales and Cladophorales, together with several other species-poor, predominantly freshwater genera such as *Arnoldiella*, *Basicladia*, *Pithophora* and *Wittrockiella* (Hanyuda *et al*. 2002, Leliaert *et al*. 2003).

Aegagropila linnaei occurs in several different growth forms, depending on environmental conditions (Waern 1952, Sakai & Enomoto 1960, Niiyama 1989). The species is best known for the formation of spherical balls several centimeters in diameter, the so- called lake balls, Marimo (in Japan), or 'moss balls' (in aquarium shops). These balls consist of many interwoven filaments. In Japan, the lake balls are very popular and have been designated a 'special natural monument' (Kurogi 1980). The ball forms have also become very popular in the aquarium trade.

The species can grow as unattached mats, floating above the substrate in shallow water, or as attached epilithic or epizoic filaments. Different theories about the formation of the ball form have been proposed, but it is generally assumed that the formation is a mixture of mechanic processes through water motion and features intrinsic to the species that help entanglement, such as the stiff texture and the formation of rhizoids, and the growth patterns following the abrasion of apical cells while rolling on the sediment (Wesenberg-Lund 1903, Acton 1916, van den Hoek 1963, Kurogi 1980, Niiyama 1989, Einarsson *et al*. 2004). It has been shown in culture experiments that stable balls can be produced when exposing loose filaments to a rolling motion (Nakazawa 1973).

A recent study from Japan also contributing to the conservation of *A. linnaei* and the management of its habitats indicates the possibility of genetic differentiation between different growth forms, and it emphasizes the necessity to extend conservation efforts to epilithic populations as well (Soejima *et al*. 2008).

Floating balls or unattached mats of *A. linnaei* have been found in eight locations in the Netherlands: in Lake Naardermeer in North Holland (Koster 1959), in the lake system Loosdrechtse Plassen in North Holland (Koster 1959), in Lake Zwarte Broek in Friesland (Kops *et al*. 1911, Koster 1959), and in several parts of the lake system 'De Wieden' in the province Overijssel (Koster 1959, Segal & Groenhart 1967). Since then, *Aegagropila* balls or mats have not been reported again, so the last record of this species in the Netherlands is more than 40 years old. Attached forms had never been reported from the Netherlands.

Taking into account the long period during which *A. linnaei* had not been recorded and the changes in the environment in the meantime, it is assumed that this species had disappeared from its original habitats. In all locations in The Netherlands where *A. linnaei* used to occur the water quality deteriorated from the 1960s onwards, mainly due to eutrophication effects. These locations were subsequently characterised by a reduction in water transparency, by the decline and absence of charophyte vegetation, and by cyanobacterial blooms (e.g., Hofstra & van Liere 1992, Maasdam & Claassen 1998, Riegman 2004, Boosten 2006, M. Thannhauser – pers. comm.). We visited the historical collection sites to check whether this species and its characteristic ball-shaped growth form still might exist in the Netherlands. We further discuss the situation and threat to this species elsewhere. Partial LSU rDNA sequences were generated to verify morphological identifications of putative material of *A. linnaei* and to investigate possible genetic divergence between different growth forms, as well as between populations from different habitats and locations.

Materials and Methods

Herbarium and literature survey of Dutch locations

During a survey on the global distribution of *Aegagropila linnaei* based on herbarium specimens, it became obvious that there are very few recent collections of this species in general, with the majority of the herbarium specimens being more than 100 years old. For findings from the Netherlands that are neither mentioned in available literature nor deposited in the collections of herbaria, we contacted several Dutch organizations that carry out monitoring of aquatic vegetation and/or water quality in different areas of the Netherlands: These include Stichting Natuurmonumenten, Stichting Floron, Landelijk Informatiecentrum Kranswieren (LIK), Waternet, Waterschap Reest and Wieden, and Wetterskip Fryslân. Also several boat rental businesses and dive clubs in the Netherlands were contacted for possible amateur sightings of 'lake balls'. The locations in the resulting list (Table 1) were the candidates for field work to search for extant populations in the Netherlands.

Field work

We visited eight locations during eight field trips, covering all but one location (Beulaker Wijde) where *A. linnaei* had been found previously (Fig. 1), plus one additional location that was pointed out by amateur divers as a location for 'lake balls' ('De Groene Heuvels'). Information about the locations and employed field methods are summarized in Table 2. Field methods depended on local conditions, *i.e.,* size of the lake, shore accessibility, underwater visibility, and availability of a boat. Wherever information on the precise location of previous collections was available, field work was focused in those areas of the lakes (*i.e.,* the Mennegat in the Naardermeer and the A. Lambertskade in the Loosdrechtse Plassen). If the underwater visibility was too poor for snorkeling or SCUBA diving, the sampling consisted of dredging with a standard garden rake using a boat or wading in shallow water, and shore observations. Special attention was paid to submerged solid substrates, *i.e.,* bivalve shells and stones. Shore observations were concentrated to areas with reed stands and leeward bays or shores. Material resembling *A. linnaei* was brought back to the laboratory and examined under a microscope. Verified material of *A.*

linnaei was vouchered and stored in L (the National Herbarium of the Netherlands, Leiden branch; see Table 3 for details).

Fig. 1. Map of the Netherlands indicating the historical collection sites of *Aegagropila linnaei* by numbers. 1 Naardermeer, 2 Loosdrechtse Plassen, 3 De Wieden area, 4 Zwarte Broek

DNA sequence analysis

Partial large subunit (LSU) rDNA sequences from six specimens of *Aegagropila linnaei* were analyzed to check the morphological identifications, covering different growth forms, habitats, and locations (including one Dutch site). Sample and collection information, voucher specimens, and GenBank accession numbers are given in Table 3. DNA was extracted from fresh material or specimens that had been desiccated in silica gel after collection (Chase & Hills 1991). Fresh material was processed as herbarium vouchers now deposited in L, plus additional liquid preservation in formaldehyde solution or ethanol. Total genomic DNA was isolated using the Chelex method (Goff & Moon 1993).

PCR amplifications were performed in a Biomed thermocycler with an initial denaturation step of 94°C for 5 min followed by 31 cycles of 30s at 94°C, 30s at 57°C, and 30s at 72°C, with a final extension step of 5 min at 72°C. The reaction volume was 25µL and consisted of 0.1-0.4 µg genomic DNA, 1.25 nmol of each dNTP, 6 pmol of each primer, 2.5 µL of 10 x reaction buffer containing 1.5 mM $MgCl_2$ (Qiagen), 1 µL BSA (2.5%), 17.7 µL H_2O and one unit of *Taq* polymerase (Qiagen). The first ~ 590 nucleotides of the LSU rDNA were amplified using the universal primers C’1 forward (5’-ACCCGCTGAATTTAAGCATAT-3’) and D2 reverse (5’-TCCGTGTTTCAAGACGG-3’; Hassouna *et al.* 1984, Leliaert *et al.* 2003). Amplifications were checked for correct size by electrophoresis on 1% agarose gels and subsequent staining with ethidium bromide. PCR products were purified with Montage PCR filter units (Millipore) or with ExoSAP-IT (USB Corporation) following the manufacturers’ protocols. Cleaned PCR products were sent to Macrogen, South Korea, for sequencing. The final consensus sequences were constructed with Sequencher 4.0.5 software (GeneCodes), subsequently aligned by eye in Se-Al v2.0a11 (Rambaut 2007) and submitted to GenBank (see Table 3).

Table 1. Locations in the Netherlands where *Aegagropila linnaei* has been reported from, with information on reported growth forms and abundance of *A. linnaei*, the date of the last record, and sources. The location numbers correspond to Fig. 1.

Location (province)	**Lat.**	**Long.**	**Depth**	**Reported growth forms of *A. linnaei***	**Notes on abundance**	**Last record**	**Herbarium collections**[a]	**Additional references**
1 Naardermeer (North-Holland)	N52°17′60″	E05°07′12″	1 m	unattached mats, balls	very abundant	1962	L, LD	Koster 1959
2 Wijde Blik (North-Holland)	N52°13′12″	E05°02′60″	1-2 m	ball		1955	L	
3 De Wieden (Overijssel)								
- Beulaker Wijde	N52°42′03″	E06°03′36″	1 m	ball		1938		Koster 1959
- Boven Wijde	N52°43′48″	E06°06′36″	1 m	tufts (herbarium), ball (literature)		1944	L	
- Duinigermeer	N52°43′12″	E06°00′00″	1 m	ball		1967		Segal & Groenhart 1967
- Zuideindigerwiede	N52°42′36″	E06°04′48″	1 m	balls		1951	L	Segal & Groenhart 1967
- Molengat	N52°44′24″	E6°05′24″	1-(3) m	unattached mats	numerous; dense masses	1960	L, LD	Segal & Groenhart 1967, van den Hoek 1963
4 Zwarte Broek and nearby ditches (Friesland)	N53°15′00″	E05°56′24″	1 m	unattached tufts, balls	not common, but numerous	1906	BI, BR, BRNU, L, NY, PC	Kops *et al.* 1911

[a] herbarium acronyms follow Holmgren *et al.* (1990)

Table 2. Locations in the Netherlands that were visited in search of extant populations of *Aegagropila linnaei*, with information on fieldwork details, visibility, sediment, and observed vegetation. The location numbers correspond to Fig. 1.

Location (province)	Field dates	Field methods	Visibility	Sediment	Observations
1 Naardermeer (North-Holland)	July 2007	snorkeling	1 m	sand	Dense vegetation of Characeae, numerous ball-shaped colonies of *Nostoc pruniforme*.
2 Wijde Blik (North-Holland)	May 2007	snorkeling, shore survey	0.5–1 m	sand, mud in sheltered areas	No floating algae; barren sediment. Only macroalgae observed: *Cladophora glomerata*, *Gongrosira* sp. (on *Dreissena polymorpha*), *Vaucheria* sp.
3 De Wieden (Overijssel)					
- Boven Wijde	June 2007	snorkeling, shore survey	0.5 m	mainly mud, sand on eastern shore	Floating on surface: *Cladophora glomerata*, *Oedogonium* sp., *Spirogyra sp*. On reed: *Chaetophora elegans*, *Rhizoclonium* sp. On *Anodonta anatina*: *Gongrosira* sp., one single tuft of ***Aegagropila linnaei***.
- Molengat	June 2007	raking, shore survey (boat)	< 0.5 m	mainly mud, sand on eastern shore	No floating algae. On sand: *Vaucheria* sp. Attached on wood and waterplants: *Cladophora glomerata*, *Cladophora fracta*, *Oedogonium* sp. *Anodonta anatina* and *Dreissena polymorpha* without epiphytic growth.
	Nov 2007	SCUBA diving	< 0.5 m		
- Duinigermeer	July 2009	snorkeling, shore survey	1 m	(sand and mud)	No macroalgae, barren sediment. Reed stalks and other macrophytes covered with *Oscillatoria* sp.
- Zuideindigerwiede	July 2009	snorkeling	0.5 m	sand and mud	Dense vegetation of Characeae, ***Aegagropila linnaei*** present on numerous *Anodonta*/*Unio* shells and rocks.
4 Zwarte Broek (Friesland)	Aug 2007	raking, shore survey	< 0.1 m	thick layer of mud	Floating on surface: *Cladophora glomerata*, *Hydrodictyon reticulatum*, *Lemna minor*, *Spirogyra* sp. *Anodonta* sp. without epiphytic growth.

Table 3. List of specimens for which partial LSU rDNA sequences have been determined.

Location	Lat.	Long.	Growth form	Collectors	Coll. date	No.	Voucher	GenBank accession numbers
Lake Myvatn, Iceland	N65º36′36″	E17º02′36″	ball	Á. Einarsson	2002	B54	L0793580	EU655697
Ukraine (aquarium shop)			ball		(2005)	C01	L0793577	EU655698
Holmön, Baltic Sea, Sweden	N63º46′48″	E20º53′24″	attached filaments (on rock)	L. Bergström & J. Ask	2004	C41	L0793571	EU655702
Ramsholmen, Baltic Sea, Finland	N60º02′50″	E23º28′48″	unattached, matted	R. Munsterhjelm	2007	L68	L0793579	EU655699
Sällvik, Baltic Sea, Finland	N60º01′48″	E23º30′00″	unattached, matted	R. Munsterhjelm	2007	L69	L0793578	EU655701
Boven Wijde, the Netherlands	N52º43′48″	E06º06′36″	attached filaments (on *Anodonta*)	C. Boedeker & A. Immers	2007	AI15	L0793572	EU655700

Results

Herbarium and literature survey of Dutch locations

In total, 27 herbarium specimens of *A. linnaei* from Dutch locations could be traced. The collection in L (see Holmgren *et al.* (1990) for herbarium abbreviations) contains the majority of the material (11 specimens) and covers six locations in the Netherlands: Boven Wijde, Molengat, Zuideindigerwiede (all in an area called 'De Wieden', province Overijssel), Mennegat (Naardermeer, province North-Holland), Wijde Blik (Loosdrechtse Plassen, province North-Holland) and Zwarte Broek (near Roodkerk, province Friesland) (Fig. 1). Additional specimens from the same locations of *A. linnaei* were housed in nine other herbaria (BM, BR, BRNU, LD, M, NY, PC, UBC, W). All herbarium specimens consist of unattached material, either ball-shaped or free-floating entangled masses. The collections span a 110 year period (1852-1962). Lake balls of this kind have also been reported in the literature from the Beulaker Wijde (Koster 1959) and Duinigermeer (Segal & Groenhart 1967), both in the 'De Wieden' area, province Overijssel. All locations are listed with additional information in Table 1. None of the contacted monitoring organizations had ever recorded *A. linnaei*. While most reports of 'lake balls' by boat rental businesses could be instantly regarded as being cyanobacterial by how they were described, there was initially convincing information given by amateur divers about *Aegagropila*-like balls in recent years from the lake 'De Groene Heuvels'.

Field work

The results of the field trips are summarized in Table 2. All historical locations of *A. linnaei* gave a similar impression with signs of eutrophication as indicated by poor underwater light climate and organic muddy sediments, except the Naardermeer and to some extent the Duinigermeer (see below). Unattached mats or balls of *A. linnaei* were not encountered anywhere. The attached form of *A. linnaei* was found in two locations, the Boven Wijde and the Zuideindigerwiede, both located in the province Overijssel. In the Boven Wijde, a single attached tuft of *A. linnaei* was found on a shell of the freshwater shellfish *Anodonta anatina* (Fig. 2). The filaments displayed all the typical morphological characters of the species (van den Hoek 1963, Leliaert & Boedeker 2007): subterminal insertion of branches (Figs. 2a-d), rhizoids in distal parts of the thallus (Fig. 2a), often opposite branches (Fig. 2b), irregular cell shape and unrestricted insertion of branches in basal parts (Fig. 2c), and serial insertion of branches (Fig. 2d). In the Zuideindigerwiede the bottom was covered with a dense charophyte vegetation, and attached tufts of *A. linnaei* were present on numerous dead *A. anatina* shells (Fig. 3). The shells seemed to randomly distributed throughout the lake. The few living bivalves encountered showed no algal growth on their shells. On one large boulder *A. linnaei* had a covering degree of more than 50%.

The Naardermeer differed from the other sampling locations in having clearer water and a dense charophyte vegetation. Hundreds of unattached ball-shaped colonies of the cyanobacterium *Nostoc pruniforme* C. Agardh ex Bornet & Flahault were floating just above the bottom, resembling *A. linnaei* underwater (Figs. 4a-b). The Duinigermeer also differed from the other locations in the 'De Wieden' area by better underwater light climate. No macroalgae were found in the Duinigermeer, however, only a very small part was searched. Even though the visibility was >50 cm, the sediment was barren except for submerged higher plants and only a layer of filamentous cyanobacteria (*Oscillatoria* sp.) was discovered on submerged reed stalks. The few dead *Anodonta anatina* shells found showed no algal cover.

The recreational lake 'De Groene Heuvels', even though not a location from which the occurrence of *A. linnaei* had been reported earlier, was visited to verify reports of 'lake balls' by divers. Ball-shaped colonies of the ciliate *Ophridium versatile* (Müller) Ehrenberg and the cyanobacteria *Rivularia* sp., *Gloeotrichia pisum* (C. Agardh) Thuret and *Tolypothrix polymorpha* Lemmermann were observed. The latter formed free-floating, dark-green tufts up to 4 cm in diameter (Figs. 4c-d), resembling *A. linnaei*. *Aegagropila linnaei* was not found and there is no evidence that the species ever occurred there.

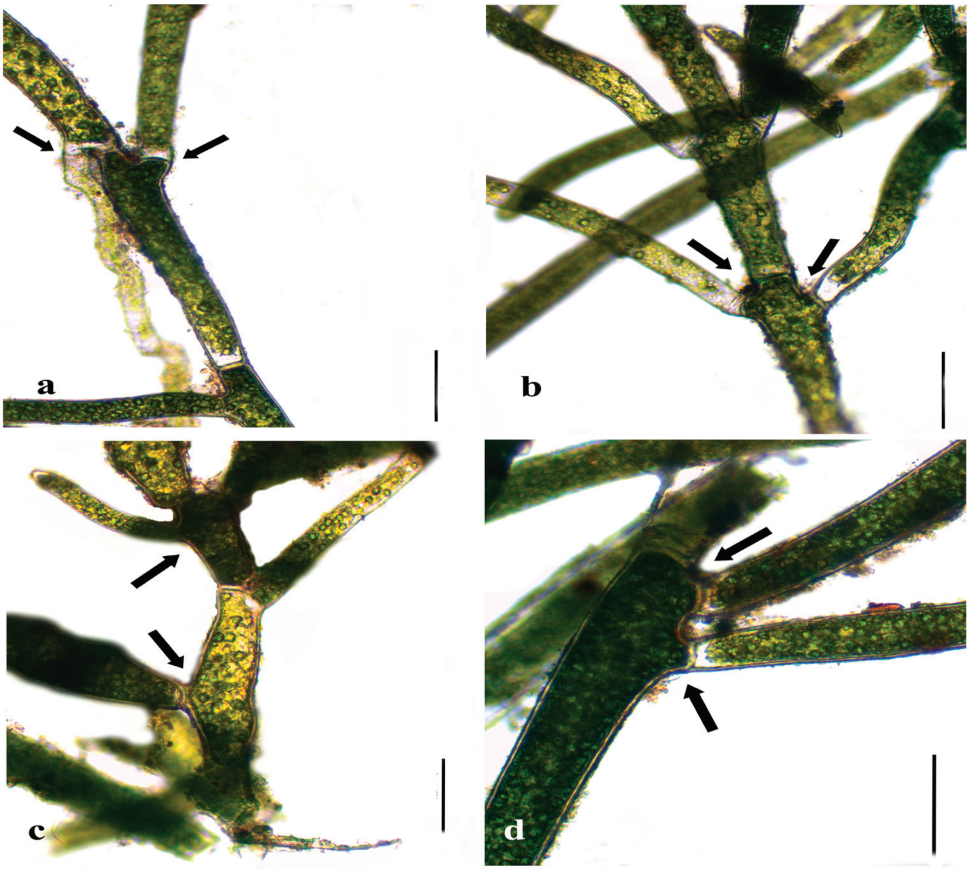

Fig. 2. Morphological characters of *Aegagropila linnaei* from the Boven Wijde, the Netherlands. **a** Subterminal insertion of branches and a rhizoid sprouting from the distal part of the thallus. **b** Subterminal insertion of branches, opposite branching, irregular cell shape. **c** Irregularly shaped cells in the basal region, branching from the middle of a cell, thick cell walls. **d** Serial insertion of branches. Scalebars = 100 µm

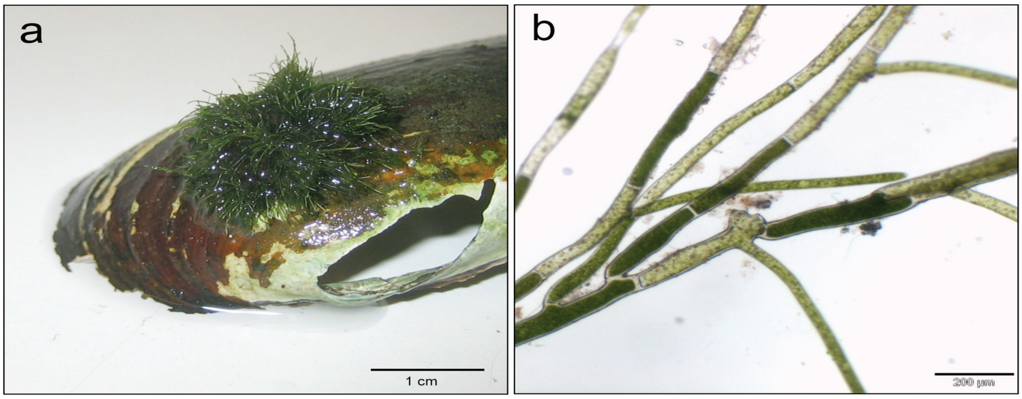

Fig. 3. *Aegagropila linnaei* from the Zuideindigerwiede. **a** tuft attached to dead shell of *Anodonta anatina*. **b** microscopic details of filaments showing typical subterminal as well as serial insertion of branches, and irregular cell shape.

DNA sequences

All six samples of *A. linnaei* (see Table 3), representing different growth forms, habitats, and locations showed identical nucleotide sequences in the LSU, except for one point mutation at position 178 (reference sequence: *Chlorella ellipsoidea* Gerneck, GenBank no.: D17810) in two samples. One of the two sequences of *A. linnaei* from the brackish Pojo Bay, Finland (L69, unattached populations) and the sequence from the Netherlands (AI15, attached) displayed an A instead of a G at that position. Close examination of the electropherograms at this variable position showed underlying peaks matching the corresponding base of the other sequence in three out of the six sequences, suggesting that the observed differences from direct cycle sequencing might be attributable to intragenomic variation.

Discussion

There are no Dutch records of the enigmatic green alga *Aegagropila linnaei* dated later than 1962 preserved in any official herbarium. The last report of the species in the literature is dated five years later (Segal & Groenhart 1967). After 1967, there are no reports until our present study. During a survey of the collections of *A. linnaei* from 29 herbaria, only 9 out of more than 1000 specimens had been collected in Europe in the last 30 years (data not shown). Even though this reflects a change in the tradition of collecting and depositing freshwater algal specimens during the last century to some degree, the obvious anthropogenic changes in the natural habitats most probably have led to a decline in populations in the Netherlands and elsewhere.

Field work

Searching for unattached algae floating over the bottom, such as *A. linnaei*, in peaty or eutrophicated lakes with poor visibility is very difficult. Although the lake balls have been frequently reported to occur in large numbers, the populations often seem to be restricted to a specific part of the lakes (Koster 1959, Pankow 1965, Kurogi 1980, Pankow & Bolbrinker 1984, Einarsson *et al.* 2004). In those areas, light supply, depth, sediment type, slope, and water movements obviously allow the unattached forms to thrive. Even when targeting typical habitats such as shallow sandy bottoms, reed belts, downwind bays or specifically mentioned historic collection sites, and deploying a range of methods, populations could be entirely missed. This is even more likely if the unattached forms (mats and balls) have disappeared and if the remaining population of attached individuals is small and patchy. Finding the alga on one single unionid mussel shell in the Boven Wijde may suggest a small and scattered population in that location. The population in the Zuideindigerwiede appears to be much larger. *Aegagropila linnaei* has also been found attached on unionids in Japan (Niiyama 1989, Wakana *et al.* 2001b, 2005). Growing on shellfish in shallow water might be one way how populations can survive when not enough light penetrates into deeper waters anymore due to eutrophication effects.

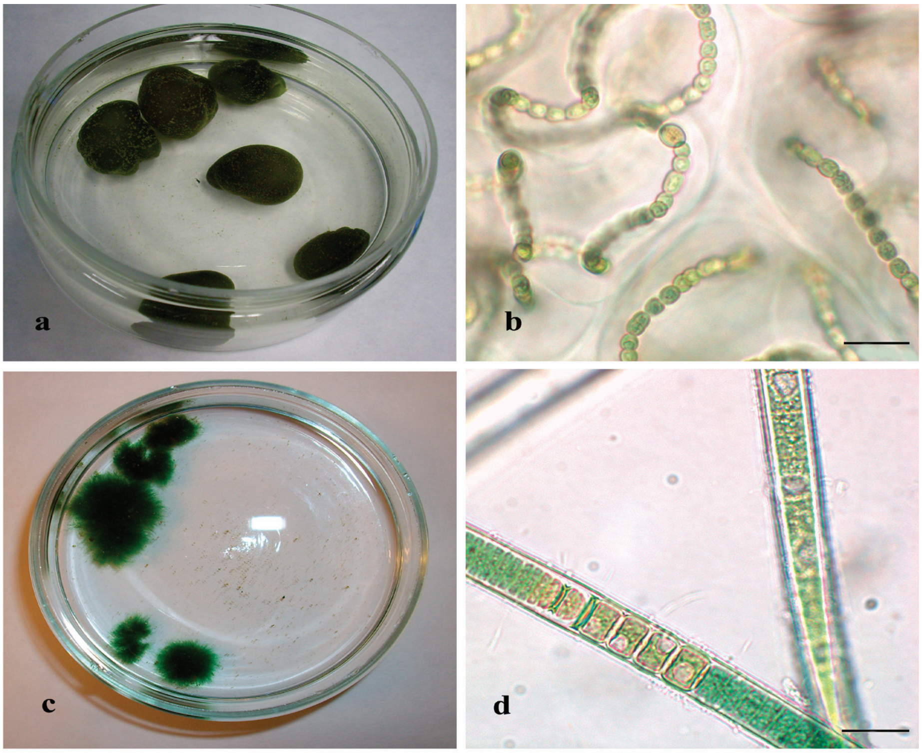

Fig. 4. Cyanobacterial 'lake balls', resembling *Aegagropila linnaei* underwater. **a, b** *Nostoc pruniforme* from the Naardermeer. **c, d** *Tolypothrix polymorpha* from 'De Groene Heuvels', Wijchen. Diameter of the Petri dishes = 10 cm, scalebars = 20 µm.

Growth forms and habitats

The range of different growth forms, the morphological plasticity of the species, and the range of habitats have led to the description of a large number of species and forms, but these were later synonymized by van den Hoek (1963). Lakes are generally regarded as the typical habitat of *A. linnaei*, and the species can be locally abundant or even dominant (van den Hoek 1963). Attached populations also occur in several rivers. The epilithic form has been found in the Seine and the Moine in France (van den Hoek 1963), in the river Sasso in Switzerland (van den Hoek 1963), the Fényes spring in Hungary (Palik 1963), in the Oslava river in the Czech Republic (as *Cladophora moravica* (Dvořák) Gardavský, Gardavsky (1986)), and in the rivers Tees, Tyne, Tweed, and Wear in the UK (Holmes & Whitton 1975, Whitton *et al*. 1998). Another very different habitat of *A. linnaei* is the brackish northern part of the Baltic Sea, where it grows mostly attached on rocks. *Aegagropila linnaei* is widespread and locally abundant in the Gulf of Bothnia (Waern 1952, van den Hoek 1963, Bergström & Bergström 1999), where the salinity is below 6 psu.

Two ball-shaped samples of *Aegagropila linnaei* from two lakes as distant as Japan and Sweden were previously shown to have identical small subunit (SSU) rDNA gene sequences (Hanyuda *et al*. 2002). Here, we present six partial sequences of the more variable large subunit (LSU) rRNA gene. These sequences represent the three different growth forms (ball-shaped, unattached mats, epilithic/epizoic), a range of habitats (brackish and freshwater) and distant locations (Table 3). All sequences are identical except for one shared point mutation in the sample from the Netherlands and in one of the samples from Finland, confirming that *A. linnaei* has a wide distribution and spans a range of habitats. Therefore, it seems justified to refer to all growth forms as one species. An isozyme study of *A. linnaei* populations in Japan revealed genetic differentiation and limited gene flow between attached and unattached populations, emphasizing the need for conservation efforts for all growth forms (Soejima *et al*. 2008).

Eutrophication in the Netherlands

All lakes in the Netherlands where *A. linnaei* had been collected in the past were severly eutrophicated, starting around the 1960s and 1970s. Characteristic for these eutrophicated systems were the reduction in water transparency and the absence of charophyte vegetation (Leentvaar & Mörzer Bruijns 1962, Maasdam & Claassen 1998, Riegman 2004, Boosten 2006, Kiwa Water Research 2007, Waternet - pers. comm., Wetterskip Fryslân - pers. comm.). Restoration measures undertaken in the 1990s led to improved water quality and a return of charophyte vegetation only in the Duinigermeer (van Berkum *et al*. 1995), the Zuideindigerwiede (E. Nat - pers. comm.), and the Naardermeer (Boosten 2006), while restoration attempts have not improved the situation in the Loosdrechtse Plassen so far (Hofstra & van Liere 1992, Van Liere & Gulati, 1992).

From the herbarium survey of locations worldwide and the potential natural state of those lakes, as well as from many literature reports, it is apparent that *A. linnaei* typically occurs in oligo- to mesotrophic lakes, but can also occur in dystrophic and slightly eutrophic habitats. Only the attached form has been encountered sporadically in eutrophic or disturbed habitats, such as the rivers Seine and the Moine (van den Hoek 1963) or the Boven Wijde (see above). Due to self-shading, the unattached forms need good light conditions to grow (Yoshida *et al*. 1994), and they might therefore be more sensitive to eutrophication due to the invariable deterioration in underwater light climate. A reduction in light availability has been shown to cause the decline in charophytes in eutrophicated lakes in Sweden (Blindow 1992). Another negative effect on submerged vegetation in eutrophicated waters can be damage by bottom feeding fish such as bream (ten Winkel & Meulemans 1984). In the case of *A. linnaei*, the change from sandy to muddy sediments in eutrophicated waters would have a negative impact, especially on the formation of balls. In addition to eutrophication, changes in hydrology and turbidity could also have a strong negative effect on unattached growth forms.

The only locations where *A. linnaei* appears to still occur in the Netherlands are located in the 'De Wieden' area in north-west Overijssel. The shallow lakes in this area originate from peat production in the 16th-18th century. Nowadays, 'De Wieden' is a very popular recreational area, especially for boat tours. Generally, the lakes in the 'De Wieden' area have improved from strongly eutrophicated systems in the 1970s to a moderate water quality in recent years (Riegman 2004). The Zuideindigerwiede has a surface area of approximately 50 ha, plus surrounding reed stands, and a mean depth of 1m (Segal & Groenhart 1967), and is connected by canals to larger lake Boven Wijde. The Zuideindigerwiede was known for remarkably clear water and a diverse charophyte flora prior to the 1960s (Segal & Groenhart 1967, van Raam 1998). The water began to become more turbid in the mid-1960s and charophyte stands started to decline, until basically no submerged vegetation was left in the lake system 'De Wieden' as a consequence of water pollution and eutrophication, the increasingly popular (boat) tourism, and terrestrialization of some lakes (van Raam 1998). The water quality in the Zuideindigerwiede has improved again in recent years, which is also illustrated by the finding of the rare and endangered charophyte *Nitella hyalina* (De Candolle) Agardh in 2002 (Nat 2002). Other noteworthy charophytes that returned to the Zuideindigerwiede are *Chara aspera* Detharding ex Willdenow, *Nitella mucronata* (A. Braun) Miquel and *Nitellopsis obtusa* (Desvaux) J. Groves. *N. hyalina* had last been observed in the Zuideindigerwiede in 1969 (Raam & Maier 1995). The species had also disappeared from all other of the very few locations it was known from (Naardermeer, Loosdrechtse Plassen/Vechtstreek, small lakes in Friesland), but has returned to all original areas in recent years (Raam & Maier 1995, Nat 2002, Waternet 2005). Interestingly, these are the same locations where *A. linnaei* used to occur in the Netherlands.

It is most likely that the populations of *A. linnaei* died out during the 1960s and 1970s in the Naardermeer, the Loosdrechtse Plassen, the Duinigermeer, the Molengat, and the Zwarte Broek, because of the effects of eutrophication. The finding of the species in the eutrophicated Boven Wijde and Zuideindigerwiede might represent the remainders of a once larger population. An alternative, less likely, explanation is that *A. linnaei* could have become extinct everywhere in the 1970s and could have recently recolonized the Boven Wijde and the Zuideindigerwiede. There is no indication that habitats with an improved water quality such the Naardermeer have been recolonized, and the recolonization potential of the species might be very low. The restored Naardermeer seems like a suitable habitat for *A. linnaei*, as judged by the trophic level (mesotrophic), the charophyte vegetation, and especially the presence of *Nostoc pruniforme* (see Mollenhauer *et al.* 1999). Dispersal by water birds should theoretically make recolonization of suitable habitats possible (e. g., Schlichting 1960). On the other hand, the assumed small size of source populations, the rarity or complete absence of sexual reproduction (Soejima *et al*. 2008), and the very slow growth rates (van den Hoek 1963, pers. obs.) reduce the likelihood of re-establishing populations. Fragmentation can be regarded as an efficient way of dispersal in some groups of algae. However, fragmentation as the only means of reproduction in a slow-growing organism such as *A. linnaei* i.e, without any form of additional spore release, results in a low dispersal potential.

Decline and threat of Aegagropila linnaei

Aegagropila linnaei is seemingly extinct in most of the original locations in the Netherlands, where this species used to be quite abundant (see Table 2), as already reported for the Zuideindigerwiede (Segal & Groenhart 1967). Extinct populations have also been reported in the literature from the German Baltic Sea coast (Schories *et al.* 1996) and from Lake Galenbecker in northeastern Germany (Pankow 1985). In some instances, the ball form became extinct while the epilithic filamentous form still persisted, *i.e.,* in Lake Zeller in Austria (Nakazawa 1974, Kann & Sauer 1982) and in Lake Akan in Japan (Wakana 1993, Wakana *et al.* 1996). Also this seems to be true for our study. A decline in population numbers and/or size is known from Lake Myvatn in Iceland (Einarsson *et al.* 2004), from Takkobu Marsh and other small swamp lakes in Japan (Wakana *et al.* 2001b, Wakana *et al.* 2005), and from Denmark, where the only population left occurs in Sorø Sø, Sjæland (R. Nielsen - pers. comm.). The observed decline in populations or the natural habitats being under threat led to the inclusion of the species in several national red lists or other conservation instruments. *Aegagropila linnaei* has a status as an endangered or protected species in Japan (Environment Agency of Japan 2000), Iceland (Á. Einarsson - pers. comm.), United Kingdom (Brodie *et al.* 2008), Germany (Ludwig & Schnittler 1996, Schories *et al.* 1996), Sweden (Gärdenfors 2005), Estonia (Lilleleht 1998), and Russia (Noskov 2000).

Eutrophication of aquatic systems is a common process worldwide and leads to the loss of unique habitats and a reduction in biodiversity (*i.e.,* Bayly & Williams 1973). Restoration of affected water bodies once polluted is difficult, costly, and takes a long time. Even after restoration, ecosystems do not necessarily return to their original diversity and community structure (Entwisle 1997). If a habitat is successfully recolonized by *A. linnaei*, it might take decades before populations abundant enough will have built up to develop into the typical unattached mats or the lake balls. On the other hand, there is some indication for genetic differentiation between unattached and attached populations, as well as between different attached populations (Soejima *et al.* 2008). Therefore, loss of genetic diversity within *A. linnaei* might have already occurred in the Netherlands. The ball shapes that *A. linnaei* can produce have led to its popularity among scientists and naturalists, as well as in the aquarium trade, and Japanese society. This popularity could be the key to its conservation, and *A. linnaei* could possibly function as a flagship species of endangered freshwater algae other than desmids or charophytes. The situation in the Netherlands would certainly justify the inclusion of this species in a red list of the Netherlands or similar conservation instruments. At the moment, red lists only exist in the Netherlands for animals, fungi, vascular plants, and lichens. Red lists are possibly a first step in conservation measures, but it is impossible to protect individual algal species, thus only conservation of the natural habitats and catchment management could be successful. The future of *A. linnaei* in the Netherlands, and in some other regions, is uncertain.

Conclusions

Aegagropila linnaei had not been found in the Netherlands for more than 40 years, but is not extinct since the species was encountered again during a recent field survey, for the first time in the attached form. In all but two of the original locations, the species could not be found anymore, and the Zuideindigerwiede seems to be the only location in the Netherlands where *A. linnaei* still occurs in fair abundances. Taking into account the history of eutrophication in these lakes, it must be assumed that the species became extinct in those locations during the 1960s and 1970s. Unattached growth forms such as the enigmatic lake balls do not occur in the Netherlands anymore. Some of the original habitats have been restored and are candidates for possible recolonization, but the species might have a poor recolonizing potential. We propose to include this species in a national red list. Protective measures should be considered where *A. linnaei* still occurs. Some conservational status should be considered for the Zuideindigerwiede, since also the red list category 1 species *N. hyalina* and several other rare charophytes have recently been discovered there. If conditions remain suitable for *A. linnaei* or improve further, unattached growth forms may eventually develop again.

In addition, it was shown that specimens with different growth forms from different locations and habitats differ by one basepair at most in the partial LSU rDNA sequences and either represent a single species or a complex of closely related cryptic species.

Acknowledgements

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Chapter

3

Global decline of and threats to *Aegagropila linnaei*, with special reference to the lake ball habit

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Abstract

The freshwater macroalga *Aegagropila linnaei* can occur attached or unattached, but is best known for its loose-lying spherical growth form ('lake balls', '*Cladophora* balls', or 'Marimo'). The worldwide distribution of this species was reconstructed based on more than 1200 specimens from 28 herbaria, an extensive literature survey, and field observations. *Aegagropila linnaei* was found in 283 locations, with the majority located in central and northern Europe. Few records of *A. linnaei* exist from North America, but it is relatively widespread in Japan. The preferred habitat is inferred to be oligo-mesotrophic lakes with moderate to high calcium levels. In more than 50% of the known locations, *A. linnaei* has not been found in the last 30 years, and this decline is assumed to be correlated with changes in trophic conditions in those lakes. The unattached growth forms show a stronger decline than attached populations as a result of the habitat types in which they can occur.

Introduction

The freshwater green alga *Aegagropila linnaei* (Cladophorales, Ulvophyceae) is best known for its unattached spherical growth form, also referred to as “Marimo”, “lake balls”, or “*Cladophora* balls” (Fig. 1a). These lake balls can develop under certain hydrographic and topographic conditions either directly from the attached growth form (Fig. 1b), from floating unattached mats, or from fragmented older balls (Brand 1902, Kurogi 1980). Individual filaments of *A. linnaei* have a simple morphology of uniseriate branched filaments similar to *Cladophora* species (Fig. 1c). The filaments can be attached by a holdfast or secondary rhizoids to the substratum, forming low turfs on submerged rocks or bivalves, or can be intermingled and free floating as mats or balls (van den Hoek 1963, Leliaert & Boedeker 2007). The lake balls are more or less radially organized, and increase in size by the vegetative growth of the interwoven filaments. The stiff texture of the filaments and the production of secondary rhizoids are features that facilitate entanglement and subsequent ball formation. Water motion is the main shape-giving factor, and balls occur typically in shallow lakes with sandy bottoms. Rolling movements on the sediment and abrasion of apical cells induce radial growth and profuse lateral branching (Acton 1916). Different growth forms can co-occur under different conditions in the same lake (Luther 1951, Kurogi 1980).

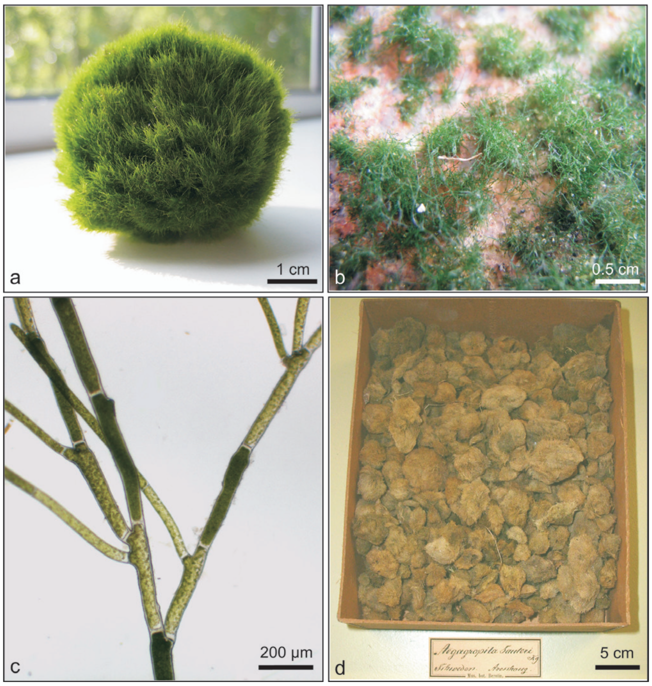

Figure 1. (a) Lake ball, ordered from an online aquarium shop. (b) Attached growth form of *A. linnaei* from Lake Erken, Sweden (photo: Roland Bengtsson). (c) Microscopical characters of *A. linnaei* filaments with typical subterminal and serial insertion of branches. (d) Herbarium box (in Botanical Garden and Museum Berlin-Dahlem, Germany) with old specimens (as *Aegagropila sauteri*) from a lake in Sweden.

The spherical growth form has attained considerable popularity. *Aegagropila linnaei* has been given the status of a “special natural monument” in Japan (Kurogi 1980), and the lake balls are shown on postage stamps from Japan and Iceland. In Japan, a whole range of “Marimo merchandise” exists, ranging from souvenirs and lucky charms with live specimens to toys and cartoons. *Aegagropila linnaei* balls have also become extremely popular in the aquarium trade in recent years.

In contrast to most other freshwater algae, *A. linnaei* has a restricted distribution, and is found mainly in previously glaciated areas of Europe. In addition, *A. linnaei* has been identified in a range of locations in Japan, whereas only few records are known from North America. In most countries it is regarded as a rare species. Freshwater algae that are not widespread are often restricted in their distribution by specific ecological demand, such as desmids (Coesel *et al.* 1978) or macroscopic *Nostoc* species (Mollenhauer *et al.* 1999). On the other hand, a restricted distribution can also be the result of a limited dispersal capacity. Migrating birds are one of the most important dispersal vectors for freshwater algae (Schlichting 1960, Green *et al.* 2002), transporting desiccation-resistant stages such as akinetes. However, akinetes are basically unknown in *A. linnaei* (Brand 1902, Acton 1916, van den Hoek 1963). It can be speculated that limited dispersal abilities and competition effects have shaped the current distribution of *A. linnaei* more than has the availability of habitats.

Even though the species was initially assumed to occur mainly in eutrophic waters (van den Hoek 1963), eutrophication is mentioned as a threat in several more recent studies reporting declining populations of *A. linnaei*. Partly because of the popularity of the intriguing ball form, this is one of the few freshwater algal species that has been listed threatened on several red lists of endangered species. *Aegagropila linnaei* is included in the national red lists of Belarus (MINPRIRODA 2006), Estonia (Lilleleht 1998), Germany (Ludwig & Schnittler 1996), Japan (EAJ 2000), Russia (Noskov 2000), and Sweden (Gärdenfors 2005). In addition, *A. linnaei* is explicitly mentioned in the Ramsar specifications of Lake Akan, Japan (Moriwake 2005), and Lake Myvatn, Iceland (Gíslason 1992). In the United Kingdom, *A. linnaei* is included the list of rare algae and in the assessments of Important Plant Areas by Plantlife (Brodie *et al.* 2008).

For several locations, this species is already assumed or known to be extinct. In four out of the five locations in The Netherlands where *Aegagropila linnaei* was found 60 years ago, it is now absent, and the unattached forms seem to have disappeared altogether as a result of the effects of eutrophication (Boedeker & Immers 2009). Balls of *A. linnaei* have been extinct in Lake Zeller in Austria since around 1910, most likely due to effects of human activity (Nakazawa 1974), and only the attached filamentous form has been reported since then (Kann & Sauer 1982). The attached growth form is still present in several larger rivers of northern England and Scotland, but the ball-shaped growth form is found only in a few unchanged locations (John 2002). There is only one location in Denmark (Sorø Sø, Sjæland) where *A. linnaei* occurs, although the species had been found previously in more locations (van den Hoek 1963). In Lake Galenbecker, in northeastern Germany, carp cultivation and intensified agriculture in the 1960s led to eutrophication and extinction of the species (Pankow 1985). Also, in Japan, where the species is still relatively widespread, human activities have led to the destruction of many of *A. linnaei*'s natural habitats. Populations of *A. linnaei* have disappeared from two bays in Lake Akan, most likely as a result of increased mud deposition (Wakana *et al.* 2006). Population declines (both in population density and distribution area) have been monitored in the Takkobu Marsh, Japan, between 1996 and 2004, and are probably a result of the effects of eutrophication, such as higher nutrient load, accumulating layers of mud and silt on the marsh bottom, shading by phytoplankton blooms, and an increase in the depth limit of freshwater bivalves (Wakana *et al.* 2005). Declining populations have also been observed in several other small swamp lakes for the same reasons (Wakana *et al.* 2001b). The *Red Data Book of Estonia* lists eutrophication, changes in water hydrology, and dredging as factors that threaten the survival of *A. linnaei* (Lilleleht 1998).

In this study, detailed information on the distribution of *A. linnaei* based on herbarium specimens (Fig. 1d), literature data, and field observations is used to assess whether there is a global trend of declining populations. Habitat preferences with regard to trophic level are inferred, and we discuss possible deleterious effects of eutrophication. Furthermore, we evaluated whether unattached populations, including the ball form, are more threatened than the attached growth form.

Materials and Methods

Location survey

We surveyed herbarium collections to produce detailed distribution maps of *A. linnaei*. Fifty-nine herbaria responded to our inquiries, of which 28 had collections of *A. linnaei* (for a list of *A. linnaei* synonyms, see Appendix S1 in the Supplementary Materials; see Appendix S2 for a complete list of herbaria). We borrowed from or visited collections, and identified the samples using a light microscope (Olympus BH2) after rehydrating fragments in water. In total, we checked approximately 1200 herbarium specimens of *A. linnaei*, about half of which had location details, and a third of which (about 400 specimens) had a collection date (Table 1). Almost all specimens with missing information seem to be about a century old, judging from the general appearance of the vouchers and the handwriting. In addition to the location and collection date, we recorded the growth form wherever possible. The resulting data set was amended by an extensive literature survey and direct inquiries with local and national water-monitoring organizations or with limnological departments in countries where *A. linnaei* was known or suspected to occur. These inquiries were particularly useful in obtaining information on the existence of extant populations and on past and present trophic levels of the habitats. We reduced this data set to a digest listing all locations (see Appendix S3), and georeferenced the locations. Excluded specimens, locations, and synonyms are listed in Appendices S1 and S3. We created maps using Manifold GIS (geographic information system) software, and displayed data using the World Geodetic 1984 projection. We treated multiple records from the same lake, that is, in different years or in different areas of the same lake, as one location. Almost one-fifth of the locations are from the Baltic Sea, so these locations were grouped together as Baltic proper, western Gulf of Bothnia, eastern Gulf of Bothnia, Gulf of Finland, and Bay of Bothnia, each counting as one location to deflate total location numbers, since individual lakes represent locations in freshwater environments. Records younger than 30 years have been classified as “extant populations” and are listed separately in Appendix S4.

Origin of Aegagropila linnaei *balls in the aquarium trade*

We contacted more than 20 aquatic plant traders in Belgium, Denmark, Germany, Japan, the Netherlands, Singapore, Ukraine, and the United States to find out about the origin of *A. linnaei* balls for sale in commercial aquarium shops, and to learn about the possible supply from wild stocks.

Table 1. Number of herbarium specimens, locations, and extant populations of *Aegagropila linnaei*.

Herbarium specimens	**Number**
Herbarium specimens	1200
Herbarium specimens with recorded locations	661
Herbarium specimens with recorded dates	404
"Ball-shaped" herbarium specimens	310
Locations	
Locations (including literature and field observations)	283
Locations corrected for Baltic Sea[a]	233
Locations with known growth forms[a]	185
Locations with attached growth form[a]	61
Locations with unattached growth form (ball-shaped)[a]	124 (90)
Extant locations (< 30 years)	
Extant locations[a] (percent decline)	101 (57%)
Extant locations with attached growth form[a] (percent decline)	33 (46%)
Extant locations[a] with unattached/ball-shaped form (percent decline)	51/36 (60%)
Top six countries: no. locations (no. extant locations)	
Sweden[a]	51 (23)
Germany	33 (8)
Great Britain and Ireland	32 (16)
Japan	21 (20)
Russia[a]	19 (2)
Iceland	10 (9)

[a]Baltic Sea is divided into five areas: Baltic proper, Gulf of Bothnia West, Gulf of Bothnia East, Gulf of Finland, Bay of Bothnia, each counting as one location.

Results

Location survey

Table 1 shows an overview of the herbarium and literature survey. In total, *A. linnaei* had been recorded from 283 locations (Appendix S3), or rather 233 locations when the list is corrected for the continuous coastline of the Baltic Sea. We sorted the dated herbarium specimens of *A. linnaei* in intervals of 25 years to illustrate the distribution of collections over time (Figs. 2a, b, c). The first two time intervals in each diagram are underrepresented, since about 600 herbarium specimens did not have a date but were clearly very old, as indicated by the dashed lines. Both the number of specimens (Fig. 2a) and their locations (Figs. 2b, c) show a decrease since the beginning of the last century. This decrease is most pronounced from 1900 onward, and again from 1975 onward. The number of locations represented by herbarium specimens dropped from 32 (1875–1899) to 7 (1975–2007), representing a decline in locations of 78%. The number of locations for ball-shaped specimens dropped from 27 to 5 in the same time interval (Figs. 2b, c). Since herbarium collections reflect not only the occurrence of species at a particular time and place but also the degree of collecting activity, we complemented the herbarium data set with a comprehensive literature survey and field observations, which yielded an additional 152 locations (see Appendix S3 combined data shown in Fig. 2d).

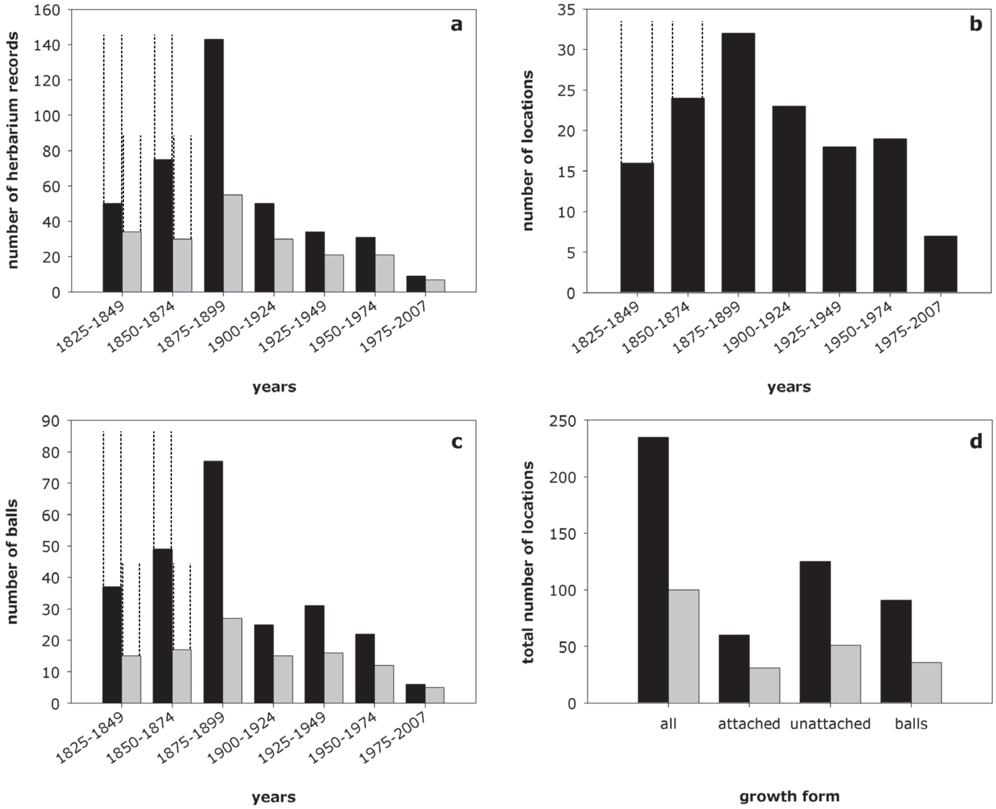

Figure 2. (a) Number of herbarium specimens of *A. linnaei* over time. Black bars indicate the total number of herbarium specimens, gray bars indicate the number of herbarium specimens corrected for duplicates. (b) Number of locations of *A. linnaei* over time based on herbarium specimens. (c) Number of herbarium specimens (black bars) and locations based on herbarium specimens (gray bars) of the ball-shaped growth form of *A. linnaei*. (d) Total number of known locations of *A. linnaei* (black bars) and known extant populations (< 30 years; gray bars), based on herbarium specimens, literature, and field data. Dashed lines indicate the large number of very old herbarium specimens that had no date.

We classified records from less than 30 years ago as "extant populations," and these are listed with the most recent collection date in Appendix S4. *Aegagropila linnaei* has been found in 107 locations since 1976, or 101 locations when correcting this number for multiple locations within the same area of the Baltic Sea (Table 1; Appendix S4). Of the 235 total locations where *A. linnaei* had been found originally, it has not been recorded in the last 30 years in about 57% of those locations (Fig. 2d). All extant populations are indicated in Fig. 3. Outside Europe, extant populations of *A. linnaei* can be found in a considerable number of locations in both Iceland and Japan (Fig. 3a). Within Europe, the majority of extant populations exist in Sweden, Great Britain, Ireland, and northeastern Germany (Fig. 3b) - thus in areas that also had the highest density of original habitats. In the Alps, one of the clusters of original habitats, *A. linnaei* has been reported from only a single location in the last 30 years (Lake Zeller). For the majority of European locations, no recent findings of *A. linnaei* have been made.

Differences in *A. linnaei* population declines have been found for the different growth forms (Fig. 2d). The presumed loss of locations is less pronounced for the attached growth form (48% decline) than for the unattached forms (59% decline; ball form only: 60% decline).

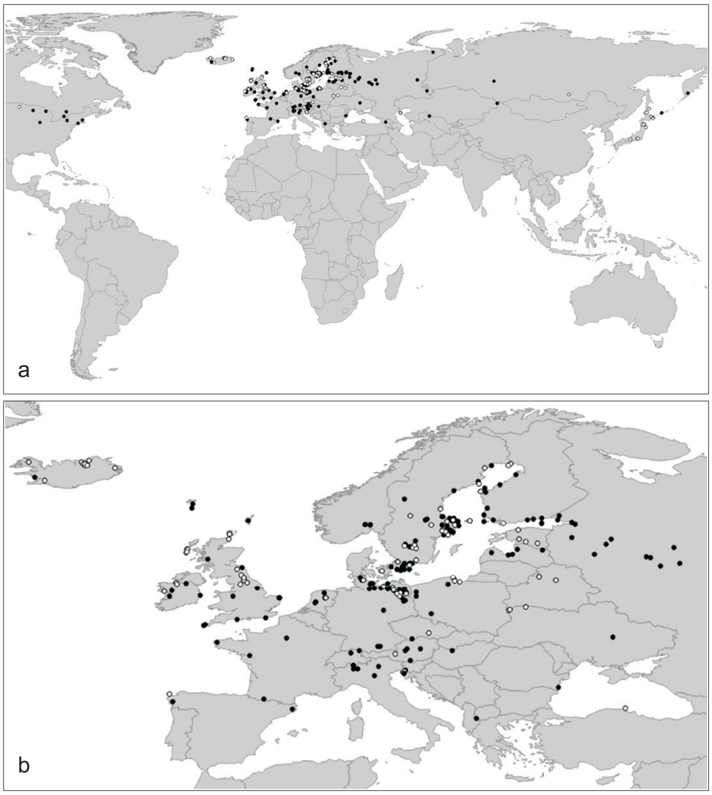

Figure 3. (a) All known locations of *A. linnaei* (black dots) and extant populations (< 30 years; white dots). (b) Shows a more detailed map of Europe.

A direct comparison of original and extant European locations in relation to the growth forms of *A. linnaei* is given in Fig. 4. Figs. 4a and 4b show all known attached populations (historical and current) and the extant attached populations, respectively. For about 48% of the locations (corrected) with the attached growth form, *A. linnaei* has not been reported in the last 30 years. In Figs. 4c and 4d, both the original and the extant unattached populations are compared, further broken down into floating and ball-forming populations. Unattached growth forms have been found more commonly in Europe than the attached growth form (Figs. 4a, 4c). However, when comparing the number of extant ball-forming populations with the number of historical habitats for this growth form, for about 60% of those locations *A. linnaei* has not been reported in the last 30 years (59% for all unattached growth forms combined).

Origin of A. linnaei *balls in the aquarium trade*

Of more than 20 contacted aquarium shops and aquatic plant trading companies, 13 were willing to share at least some information concerning the origin of the “lake balls” in their product line. Generally, companies were very reluctant to reveal their suppliers, growers, or wholesalers. However, in all instances where information was obtained, Ukraine was given as the country of origin, sometimes specified as Lake Svityaz, or the Shatsk lakes, in northwestern Ukraine. Local Ukrainian sellers of *A. linnaei* balls in Odessa and Kyiv said that they receive their material periodically from Lake Svityaz, but claimed that they grow the majority of balls themselves. Most companies grow balls vegetatively from fragments to market size, but receive stocks from Ukraine. Balls from Lake Svityaz are shipped even to aquatic plant-breeding facilities in Southeast Asia before they are returned to the European market. Japanese aquarium shops sell only balls of European origin. No other natural source of *A. linnaei* balls other than the Shatsk lakes (including Lake Svityaz) was ever mentioned by people in the aquarium plant-trade business.

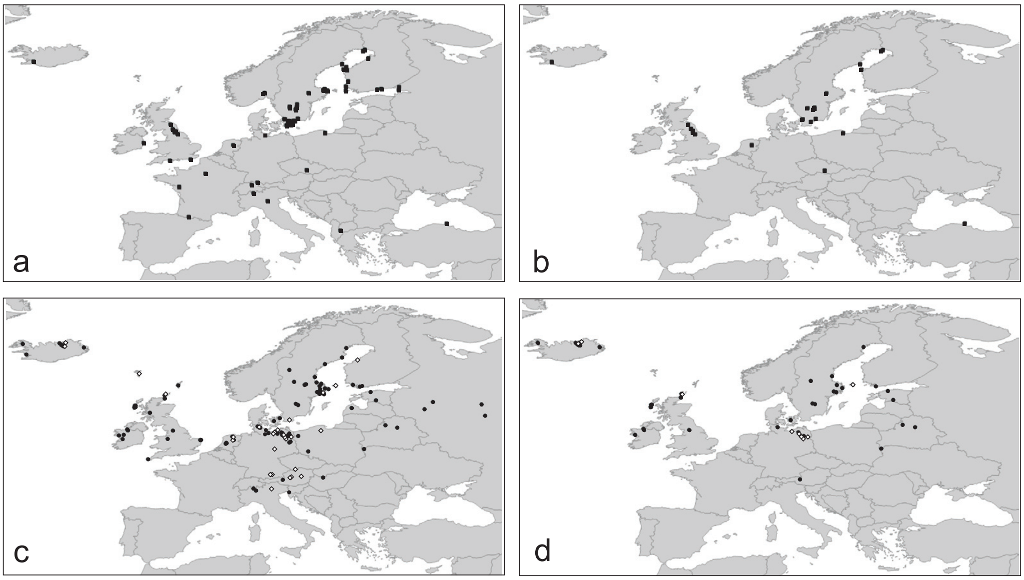

Figure 4. Direct comparisons of all known and extant locations of *A. linnaei* in Europe, shown separately for attached and unattached growth forms. (a) All known locations of attached populations. (b) Extant locations of attached populations. (c) All known locations of unattached growth forms (black dots: unattached filaments/mats, white dots: balls). (d) Extant locations of unattached growth forms (black dots: unattached filaments/mats, white dots: balls).

Discussion

Evidence for decline of A. linnaei *populations*

Detailed knowledge of the distribution of a species is a prerequisite for (a) assessing the status of that species, (b) monitoring (a decline of) its populations, (c) identifying general threats, and (d) employing most conservation efforts. Typical difficulties in reconstructing the area of distribution are related to incomplete or inaccessible records, patchy local knowledge, and to verifying observations made by third parties. Many organisms, especially among the algae, must be regarded as underrecorded, and are frequently misidentified. This is most likely also the case with *A. linnaei*, despite all efforts to assemble as complete a data set as possible. Even though this species has for a long time intrigued scientists (*e.g.,* Brand 1902, Wesenberg-Lund 1903, Acton 1916, Kindle 1934, Waern 1952), very few reports have provided anything more than scanty lists of findings (Palik 1963, van den Hoek 1963, Pankow 1965, Getzen 1967). All biogeographic reconstructions are hampered by incomplete distribution records, and for most algal species, the knowledge of the global distribution is poor and based on many assumptions. Although some regions of the world are well sampled, such as (western) Europe, the largest parts of the globe are not. The absence of *A. linnaei* in northern North America and Siberia (Fig. 3a) could be an artifact of undersampling, despite our efforts to locate records from those areas. Even though it seems unlikely that *A. linnaei* would occur in South America, suitable habitats are potentially abundant in the Andes region, but nobody has looked.

The studies on the extinction or decline of *A. linnaei* cited above are all restricted to a confined area, mostly one individual location. We investigated whether a general trend of declining populations could be revealed by analyzing as many historical and recent findings as possible. Nevertheless, our record of *A. linnaei* is most likely incomplete, despite the fact that the occurrence of the enigmatic balls in a lake is frequently recorded or commented upon, as illustrated by a wealth of historical scientific and amateur literature. Thus, we assume underrecording is less of a problem for *A. linnaei* than for most other algal species, at least for its unattached growth forms. Even though the observed decline in herbarium specimens does probably also reflect a decline in the submission of voucher specimens, the herbarium collections show the same declining trend as the more comprehensive survey, which also includes data from the literature and communicated field observations (Figs. 2d, 3). Extant populations are known for less than half of the original locations (see Fig. 3a). The inferred decline might be affected by another methodological problem, that is, for some regions (*e.g.,* Russia), old literature is available but no or few personal contacts exist, and recent literature is difficult to obtain (see Table 1).

Differences in the degree of decline were found between attached and unattached growth forms, with the loss of unattached forms being more pronounced (Fig. 2d). Unattached forms usually float just above the sediment, and therefore occur only in shallow lakes or shallow parts of a deeper lake, where the euphotic zone reaches the bottom. It seems that the stronger decline of unattached growth forms is not caused by a higher sensitivity to environmental changes compared with the attached forms, but rather by a higher threat to their shallow habitats. The attached form most commonly occurs on submerged rocks or freshwater bivalves (Luther 1951, Waern 1952, Pankow & Bolbrinker 1984, Wakana *et al.* 2001b), and has been found in depths of 20 meters (m; maximum depth 50 m) in deep lakes (Brand 1902, Kindle 1915), as well as in a number of rivers (Holmes & Whitton 1975, 1977, 1981a, Gardavský 1986, Whitton *et al.* 1998). The attached form might be able to escape greater sedimentation and better cope with decreasing underwater light availability by colonizing substrates closer to the surface, and therefore increasing chances of survival in disturbed habitats. In addition, the ability to survive in deep and steep lakes increases the absolute number of potential habitats for the attached form, and those lakes are generally less sensitive to anthropogenic changes than shallow lowland lakes (*e.g.,* Taranu & Gregory-Eaves 2008). The attached form is distributed continuously only in the low-salinity areas of the northern Baltic Sea (Fig. 2b), where species numbers and competition are low. *Aegagropila linnaei* forms dense carpets on rocks in the Bay and Gulf of Bothnia, and can be the dominant macroalga in areas with a salinity below 4 practical salinity units (Bergström & Bergström 1999). However, the number of recent records from that area indicates a declining population as well (Figs. 3, 4). The decline of unattached growth forms is most obvious in central Sweden, northeastern Germany, and the subalpine region of the European Alps (Figs. 4c, d).

Eutrophication as the main cause for the decline of Aegagropila linnaei

Human activities increases and accelerates the external supply of nutrients to aquatic ecosystems worldwide (*e.g.,* Smith *et al.* 1999, MEA 2005). Freshwater habitats especially face increasing threats from physical alteration, changes in water level and salinity, overexploitation, introduction of nonnative species, herbicide and other biocide runoff, airborne pollution, and nutrient loading (MEA 2005, Revenga *et al.* 2005). Eutrophication of aquatic ecosystems is a common process worldwide and leads to the loss of unique habitats and a reduction in biodiversity (*e.g.,* Bayly & Williams 1973, Smith *et al.* 1999).

Enrichment of nitrogen and phosphorus loadings selects for fast-growing algae (phytoplankton, macroalgae such as *Ulva* or *Cladophora* species) at the expense of slower-growing species (Duarte 1995), such as some charophytes, for example, or *A. linnaei*. Shallow lakes are especially prone to the regime shift from aquatic macrophytes to phytoplankton dominance. The decline of several freshwater algal groups has been attributed to eutrophication effects; such groups include macroscopic *Nostoc* species (Mollenhauer 1998, Mollenhauer *et al.* 1999), desmids (Coesel *et al.* 1978, Geissler 1988), and charophytes (Geissler 1988, Blindow 1992, Nagasaka *et al.* 2002). A number of reports on the local decline or extinction of populations of *A. linnaei* mention eutrophication as a responsible factor (Pankow 1985, Wakana *et al.* 2001b, 2005, 2006, Boedeker & Immers 2009).

Table 2. Trophic conditions of lakes where *Aegagropila linnaei* occurred historically or still occurs.

Lakes with information available on the pristine state (n=74)	**Percentage**
Oligotrophic (including dystrophic)	37%
Oligo-mesotrophic	8%
Mesotrophic	47%
Slightly eutrophic	7%
Eutrophic	1%
Lakes with information available on both the pristine and current state (n=61)[a]	
Pristine state oligotrophic (including dystrophic and oligo-mesotrophic)	39%
Pristine state mesotrophic	53%
Pristine state eutrophic (including slightly eutrophic)	8%
Current state oligotrophic	20%
Current state mesotrophic	23%
Current eutrophic	57%
Lakes with information available on the current state (n-80)[a]	
Current state oligotrophic	16%
Current state mesotrophic	18%
Current eutrophic	66%
Aegagropila linnae still present	39%

[a] If a lake had a history of eutrophication but has been restored to a lower nutrient load it is filed under the most severe stage of eutrophication in its history.

Our literature study showed that *A. linnaei* occurs in several different lake types, but most typically in shallow, oligo-mesotrophic, glaciofluvial lakes with reed stands, dense charophyte vegetation, a pH greater than 7, and moderate to high calcium levels. However, the ecological preferences or requirements of *A. linnaei* had never been fully characterized, and conflicting views can be found in the literature with regard to nutrient levels. In a classic volume on European *Cladophora* species, it is stated that *A. linnaei* (as *Cladophora aegagropila*) "seems to occur only in more or less eutrophic water" (van den Hoek 1963). In this study, we collected information on trophic level and changes therein for a considerable number of lakes where *A. linnaei* occurred historically or still occurs (summarized in Table 2). For 74 lakes, information on the pristine state (*i.e.,* the trophic level of a water body without anthropogenic influences) was available (Table 2). Ninety-two percent of these lakes were either oligo- or mesotrophic in their pristine state, indicating the typical habitats of *A. linnaei* with regard to the trophic level that contrast with van den Hoek's statement (1963). For 61 lakes, information on both the pristine state and the current trophic level could be compared. Of these lakes, 92% were originally oligo- or mesotrophic, but more than half of those 61 lakes (57%) are currently eutrophic, thus dramatic changes have occurred in many habitats of *A. linnaei* in the recent past. When looking at all lakes with information on the current trophic state (n = 80), the numbers are even more dramatic. Sixty-six percent of the lakes are now eutrophic, or had been in the recent past, and *A. linnaei* still occurs in only 39% of those 80 lakes. These numbers correspond to the general findings presented in Fig. 2, and strongly suggest that *A. linnaei* occurs mainly in oligo-mesotrophic habitats and that eutrophication is correlated with the observed decline of *A. linnaei* populations.

Seemingly contrary to this inferred habitat preference, extant populations of *A. linnaei* have been found in 15 eutrophic locations (each addressed below). However, in five of these locations (Takkobu marsh, Pon swamp, Kimoma swamp, Lake Akan, and Boven Wijde; see Appendices S3 and S4) a strong decline in population size has been observed (Wakana *et al.* 2001b, 2005, 2006, Boedeker & Immers 2009, respectively), and the future of these populations must be regarded as uncertain. Generally, most eutrophic lakes are turbid and have a poor underwater light climate, but eutrophic, clear-water lakes also exist. Shallow lakes, with abundant submerged macrophyte vegetation, may have very clear water with sparse phytoplankton despite relatively high nutrient loadings in lowland areas with soft rock (Phillips *et al.* 1978). Biological interactions in clearwater lakes differ markedly from "regular" eutrophic lakes (*e.g.,* Jeppesen *et al.* 1999). The eutrophic clearwater lake Mývatn (Iceland) is well known for its population of *A. linnaei* balls (Einarsson *et al.* 2004). In Germany, *A. linnaei* has been found in a couple of eutrophic clear-water lakes (Neuklostersee and Teterower See). In Lake Biwa and Lake Kawaguchi (both in Japan), only restricted parts of the lake are eutrophic, while large parts are still mesotrophic (Nagasaka *et al.* 2002). Two brackish locations of *A. linnaei* are classified as (slightly) eutrophic (Pojo Bay, Finland, and Lake Mälaren, Sweden), as well as one river (River Wear, Scotland). The only "regular" eutrophic lakes where *A. linnaei* was found are Lake Ülemiste (Estonia) and Lake Tiefwarensee (Germany), but the latter had been recently restored to mesotrophic conditions. Even though the evidence is correlative, these numbers strongly suggest that *A. linnaei* occurs mainly in oligo-mesotrophic habitats but can persist in eutrophic, clear-water lakes, and that indirect effects of eutrophication caused the observed loss of *A. linnaei* populations.

The possible role of lake acidification in the decline of Aegagropila linnaei

Other factors besides eutrophication may play a role in the decline of *A. linnaei*, such as the widespread and serious problem of lake acidification. Acidification is mainly caused by the transfer of airborne pollutants by wind from heavily industrialized centers to distant areas where they precipitate as acid rain. Especially in areas where lakes have a low buffering capacity, the effects of acid rain can be drastic, with pH values dropping below 5. Acidification can be a primary and serious problem resulting from airborne dispersal of acidifying pollutants in habitats in remote areas that are not affected by agricultural eutrophication. Many lakes in eastern North America, Great Britain and Ireland, and Scandinavia (except southern Sweden) are characterized by low calcium values and thus a low buffering capacity, which makes them very sensitive to acidification (Jørgensen 1997, Arts 2002). The vegetation of those softwater lakes is highly endangered, and 90% of the habitats of softwater vegetation in the Atlantic region have already disappeared (Brouwer *et al.* 2002). Typical softwater environments are oligotrophic, with a pH between 5.5 and 7, and have a low buffering capacity (Arts 2002). However, *A. linnaei* is not a typical softwater organism; instead, the vast majority of habitats are well buffered and characterized by moderate to high calcium levels and an alkaline pH between 7 and 9. No records exist from known acidic habitats. Generally, even less information is available on (historical) acidity than on trophic levels. In the course of this study, only five habitats of *A. linnaei* were found with a pH below 7 (the lowest known pH is 6.3; historical data for Lake Bültsee, Germany), and only eight lakes were reported to have low calcium levels. This indicates that *A. linnaei* typically occurs in habitats that are not strongly affected by acidification. Furthermore, most lakes in Scandinavia have naturally low conductivity, low calcium content, and low pH (Henriksen *et al.* 1988, Jørgensen 1997), which could possibly explain the absence of *A. linnaei* in Norway and Finland (Fig. 3b).

Acidification has been shown to be a serious problem in remote mountain lakes that are otherwise unaffected by direct pollution (Curtis *et al.* 2005). Although several lakes in the Alps region in which *A. linnaei* was historically found were clearly subject to eutrophication (*e.g.,* Ammersee, Lago di Como, Lago Maggiore, Lago di Varese, Starnberger See), it cannot be excluded that acidification might pose an additional threat in more remote mountain areas in noncalcareous parts of the Alps, the Pyrenees, or central Asia. Likewise, acidification might negatively affect *A. linnaei* populations in otherwise unpolluted lakes elsewhere, and thus pose an additional threat of eutrophication in some regions. However, no conclusive data are currently available.

Which effects of eutrophiction might cause the observed decline?

Competitive exclusion in the face of increased nutrient loading appears to be the main mechanism responsible for the local decline or even extinction of species in temperate freshwater ecosystems (Barker *et al.* 2008); that is, species better adapted to high trophic levels outcompete others when nutrient loads increase. It had been proposed that high levels of phosphorus have a toxic effect on charophytes (Forsberg 1965), but this was not confirmed in other studies (Blindow 1992). Even though direct deleterious effects of high nutrient availability are discussed in terrestrial ecosystems, changes in the light climate of aquatic systems seem to be the most important (Smith *et al.* 1999). The finding of *A. linnaei* in several eutrophic clearwater lakes (see above) could be an indicator that the increase of nutrient levels itself does not negatively affect *A. linnaei*. Shading by increased phytoplankton densities or by opportunistic microalgal epiphytes might be especially significant for *A. linnaei* as a very slow-growing species (Acton 1916, van den Hoek 1963). However, this species survives relatively long and dark winters under the ice in high-latitude lakes such as in Iceland (Jónnson 1992, Einarsson *et al*. 2004), and healthy balls of *A. linnaei* were found below the shallow photic zone in Lake Teterow, Germany (Pankow & Bolbrinker 1984). This illustrates the capacity of this species to cope with low-light conditions, suggesting that other factors than light limitation might play a more important role in the decline of *A. linnaei* populations.

The attached growth form has been found in several eutrophicated environments such as the Seine in Paris (van den Hoek 1963), Lake Boven Wijde in the Netherlands (Boedeker & Immers 2009), and several Japanese swamp lakes (Wakana *et al*. 2001b, 2005). It is possible for the attached form to survive if it grows on rocks or bivalves close enough to the surface to still receive sufficient light while the water body becomes more turbid. In Lake Myvatn in Iceland, it has been proposed that variation in the size of submerged *A. linnaei* mats is negatively correlated with (shading) blooms of the cyanobacterium *Anabaena flos-aquae* (Einarsson *et al*. 2004). Toxins released by blooming cyanobacteria could also be responsible for or contribute to the decline of *A. linnaei* in eutrophicated lakes. Other phenomena correlated with eutrophication that could be linked to the decline of *A. linnaei* are (a) increased feeding of herbivorous fish such as bream, carp, and roach (ten Winkel and Meulemans 1984); (b) local benthic anoxia; (c) higher levels of herbicides and pesticides; (d) greater organic sedimentation; and (e) increased mechanical damage and turbidity caused by recreational boats.

Threat assessment and conservation

Although the exact effects of eutrophication that cause a decline in populations of *A. linnaei* are unclear and require further experimental studies, it seems evident that the decline is correlated with eutrophication. This of course is a general problem that is not easily solved, since the process is closely linked to agriculture, tourism, and human demand and population growth, and is therefore linked to politics and economies. In addition, the populations of *A. linnaei* in several regions and habitats may face specific local problems; for instance, the acid rain degradation of unpolluted, remote habitats. The most famous location of lake balls, Lake Akan, in Japan, is struggling not only with eutrophication and general pollution but also the collection of *Aegagropila* balls as souvenirs. This habit had become so popular that in 1977 the species was classified as critically endangered, and the government asked people to return collected balls to the lake (Soejima *et al*. 2008, TMLA 2009). Since the populations from the Shatsk lakes in Ukraine seem to be the sole source of the popular *A. linnaei* balls for the entire aquarium trade, these populations must be under considerable, if not extreme, harvesting pressure, especially considering the slow growth rates and longevity of the species (see van den Hoek 1963). In this context it is interesting to note that a Canadian study on nonindigenous plants showed *A. linnaei* to be one of the most common species in the Saint Lawrence Seaway (Cohen *et al*. 2007), most likely anthropogenically introduced balls originating from amateur aquaria.

Very few molecular studies on *A. linnaei* exist to date. Recently, it has been shown that Lake Akan contains most of the genetic diversity of *A. linnaei* in Japan, as inferred by isozyme studies; therefore Lake Akan is the most important target of conservation efforts (Soejima *et al*. 2008). Loss of genetic diversity might have occurred already through habitat degradation and extinction of local populations, and further reduction in genetic diversity is to be expected. In many areas, formerly eutrophicated water bodies have been restored to more-or-less natural conditions (van Liere & Gulati 1992, de Bernardo & Giussani 1995). Even after restoration, ecosystems do not necessarily return to their original diversity and community structure (Entwisle 1997). Because of the absence of desiccation-resistant stages in *A. linnaei*, in combination with the species' slow growth and the rarity of spore formation (Leliaert & Boedeker 2007), this species must be regarded as a poor disperser and a poor competitor. Therefore, recolonization of restored habitats might not be very likely. Once a habitat is successfully recolonized by *A. linnaei*, it might take decades before populations are large enough to develop into the typical unattached mats or lake balls. Even if a restored habitat were recolonized, genetic diversity might still be lost. Most extant populations must be regarded as threatened by eutrophication, except in some remote locations. The widespread attached form of *A. linnaei* in the northern Baltic Sea seems to be a stable component of the local algal flora, even though eutrophication has also taken place in this habitat. It is possible that reduced competition in brackish environments plays a role in maintaining *A. linnaei* populations.

One hopes that the popularity of lake balls will help with the conservation of this species and raise awareness of threatened freshwater algae. In addition, we would like to encourage the collection and submission of specimens to accessible collections, not just of *A. linnaei*, but also of species that are less showy and that currently receive less attention.

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Biogeography of *Aegagropila linnaei* (Cladophorales, Chlorophyta): a widespread freshwater alga with low effective dispersal potential shows a glacial imprint in its distribution

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Abstract

Aegagropila linnaei is a freshwater macroalga that is generally regarded as a rare species. It is apparently absent from large but seemingly suitable areas of the Northern Hemisphere, implying a limited dispersal potential and an imprint of Pleistocene glaciations in its biogeography. However, despite the popularity of its enigmatic lake ball-form, detailed biogeographical studies of *A. linnaei* have never been conducted. The main means of reproduction of *A. linnaei* is fragmentation and akinetes are not formed, supporting the assumption of limited dispersal capacity. The aim of this study was to reconstruct the biogeography of *A. linnaei*, and to identify possible refugia during glaciations, as well as to evaluate dispersal potential by quantitative desiccation experiments and ribotyping of populations. The current distribution of *A. linnaei* was inferred from herbarium specimens, literature data and recent field observations. All herbarium specimens were morphologically re-examined. Desiccation experiments were performed with vegetative filaments of three isolates of *A. linnaei*, as no specialized resistant stages are known. For comparison, the widespread freshwater algae *Cladophora glomerata* and *Rhizoclonium* sp. were included. ITS ribosomal DNA sequences were generated for 34 specimens and a ribotype network was constructed. *Aegagropila linnaei* was recorded from 283 locations in freshwater and brackish environments. The majority of locations are situated in previously glaciated areas of Central and Northern Europe. Desiccation experiments showed that *A. linnaei* is very susceptible to desiccation. Five different ribotypes were identified, of which four had a restricted distribution. *A. linnaei* represents a single species with little genetic variation (0.1-0.5%). This is the most comprehensive study of this species so far, reporting many new locations and tackling several taxonomic problems. Few additional finds were made from North America, and the origin of *A. linnaei* is inferred to be in Asia.
The highest density of its present-day locations is in previously glaciated areas in Europe, where glacial ice-dammed lakes might have functioned as refugia. Low effective long-distance dispersal capacity is inferred, based on high susceptibility to desiccation and modes of dispersal.

Introduction

"Biogeography has never been very popular among freshwater phycologists" (Vyverman 1996, p. 107). Generally, freshwater microalgal species are assumed to be geographically widespread due to the ease of passive dispersal, mostly achieved by the production of desiccation-resistant resting stages (*e.g.,* Coleman 1996). This also applies to many freshwater macroalgae, in which the production of restung stages such as akinetes is common (e.g., *Cladophora*, *Microspora*, *Oedogonium*, *Pithophora* or *Spirogyra*). Species of the green algal genera *Cladophora* and *Rhizoclonium* are among the globally most widespread freshwater macroalgae (Whitton 1970, Nienhuis 1975, Dodds & Gudder 1992, Marks & Cummings 1996). For instance, *Cladophora glomerata* (Müller ex Vahl) Kützing is distributed almost worldwide in habitats ranging from streams and lakes to polluted water bodies, mountain lakes and estuaries (van den Hoek 1963, Whitton 1970, Dodds & Gudder 1992). In contrast, a number of desmids (Zygnematales, Chlorophyta) have very specific ecological demands, being restricted in their distribution by the occurrence of suitable habitats rather than by their dispersal ability (Coesel 1996). In cyanobacteria, generally assumed to have a cosmopolitan distribution, it has been shown that many widespread morphospecies represent cryptic species with specialised ecology and a restricted distribution (Komárek 1985, Hoffmann 1996, Joyner *et al.* 2008).

A restricted distribution can also be the result of a limited dispersal capacity. One of the most important dispersal vectors for freshwater algae is migrating birds, which transport spores and thallus fragments, both internally and externally (Schlichting 1960, Green *et al.* 2002). Desiccation-resistant stages of algae such as thick-walled resting spores or akinetes are important for survival during long-distance dispersal events. Even though the lack of fossils for most groups of algae makes it difficult to reconstruct their evolutionary history, biogeographic patterns and dispersal can be inferred using genetic data. However, dispersal also obscures palaeogeographic patterns and it is easier to reconstruct the historical biogeography of organisms with poor dispersal ability (Ball 1975, Hausdorf 2000).

Aegagropila linnaei Kützing is a representative of the *Aegagropila* lineage, an assemblage of species-poor, predominantly freshwater, genera, which is sister to the mainly marine orders Siphonocladales and Cladophorales *sensu stricto* (Hanyuda *et al.* 2002, Yoshii *et al.* 2004). *Aegagropila linnaei* occurs in both freshwater and brackish environments. It is best known for its intriguing unattached ball-form ('lake balls', '*Cladophora* balls', or 'Marimo' in Japan), which can develop under certain hydrographic and topographic conditions (Wesenberg-Lund 1903, Acton 1916, van den Hoek 1963, Kurogi 1980, Niiyama 1989). This spherical growth form (Figs. 1a–b) has led to considerable popularity: the lake balls adorn postal stamps of Japan and Iceland, and they have been designated a 'special natural monument' in Japan (Kurogi 1980) where a diverse range of 'Marimo merchandise' exists. Lake balls have also become very popular in the aquarium trade in recent years. The more inconspicuous attached growth form is widespread in the brackish northern Baltic Sea below 6 psu (Nielsen *et al.* 1995, Bergström & Bergström 1999). Even though *A. linnaei* occurs predominantly in freshwater lakes, the northern Baltic Sea is the only area where it is the dominant macroalgal species, continuously distributed over large areas.

Substantial collections of this species exist in herbaria, and herbarium specimens can easily be re-identified. While superficially resembling species of *Cladophora*, *A.*

linnaei has several distinct morphological features (Fig. 1c; van den Hoek 1963, Leliaert & Boedeker 2007), valuable for biogeographic studies. In addition, the presumably asexual species *A. linnaei* reproduces mainly or entirely vegetatively by means of fragmentation (Brand 1902, van den Hoek 1963). Even though dispersal via vegetative fragmentation can be very effective for fast-growing species, for example in the genus *Caulerpa* (Ceccherelli & Cinelli 1999, Smith & Walters 1999), it is not an effective strategy in slow-growing organisms. *A. linnaei* has slow growth rates, with balls increasing in diameter by less than 1 cm per year (Acton 1916, van den Hoek 1963), making fragmentation events infrequent. Successful establishment in a new habitat after dispersal of a thallus fragment is facilitated by subsequent spore or gamete release, however, formation of asexual zoospores by *A. linnaei* has only been reported four times (Nishimura & Kanno 1927, Palik 1963, Yabu 1975, Burrows 1991). These features imply poor dispersal thus making *A. linnaei* a good candidate for reconstructing biogeographic history.

Fig. 1. a Habit of the ball-form of *Aegagropila linnaei*. **b** Herbarium specimen of the ball-form of *A. linnaei*. **c** Filaments of *A. linnaei* showing characteristic subterminal insertion of branches, indicated by arrows. **d** Resoaked filaments of *A. linnaei* from a herbarium specimen showing characteristic irregular basal cells.

Despite the popularity of *A. linnaei* balls and their long-standing scientific attraction (*e.g.,* Brand 1902, Wesenberg-Lund 1903, Acton 1916, Schröder 1920, Wasmund 1929, Niiyama 1989, Hanyuda *et al.* 2002), only scanty biogeographical accounts are available (Palik 1963, van den Hoek 1963, Pankow 1965, Getzen 1967). Detailed knowledge of the distribution of a species is important both for evolutionary studies and with respect to conservation. Several studies have reported declining or extinct populations of *A. linnaei* in connection with eutrophication (Pankow 1985, Wakana *et al.* 2001b, 2005, 2006, Einarsson *et al.* 2004, Boedeker & Immers 2009, Boedeker et al. 2010a). In this study, a comprehensive overview of the distribution of *A. linnaei* is given, based on herbarium specimens, available literature data, and recent field observations. The modes of dispersal are assessed, and possible glacial refugia are discussed, complemented by nuclear internal transcribed spacer (ITS) ribosomal DNA (rDNA) sequences from a range of locations.

Materials and Methods

Location survey

A worldwide survey of herbarium collections was undertaken to produce detailed distribution maps of *Aegagropila linnaei*. Fifty-nine herbaria responded to enquiries, of which 28 had collections of *A. linnaei* (see Appendix S1 in Supplementary Materials for a list of synonyms of *A. linnaei*, and Appendix S2 for complete list of herbaria). Collections were loaned or visited, and the material was morphologically identified using a light microscope (Olympus BH2, Olympus Europe, Hamburg, Germany) after resoaking fragments in water (Fig. 1d). In total, 1200 herbarium specimens of *A. linnaei* were checked, about half of which gave location details. The resulting dataset was amended by an extensive literature survey and direct inquiries with local and national water monitoring organizations or limnological departments in countries where *A. linnaei* was known or suspected to occur. This dataset was reduced to a digest listing all locations (Appendix S3), and the locations were georeferenced. Excluded specimens, locations, and synonyms are listed in Appendices S1 and S3. Maps were created using the Manifold GIS software (Manifold Net Ltd., Carson City, NV, USA) and data were displayed using the World Geodetic 1984 projection. Several records from the same lake, *i.e.,* in different years or in different areas of the same lake, were treated as one location. Since individual lakes represent the locations in freshwater environments, but almost one fifth of the locations are from the Baltic Sea, the latter were grouped as Baltic proper, Gulf of Bothnia West, Gulf of Bothnia East, Gulf of Finland and Bay of Bothnia, each region counting as one location.

Desiccation experiments

Since *A. linnaei* does not produce desiccation-resistant akinetes unlike many freshwater algae, the desiccation tolerance of vegetative cells was tested to elucidate the likelihood of survival of individuals during long-distance transport, *e.g.,* by birds. Filaments of *A. linnaei* from three different locations (isolates C01, N36 and L69; details are given in Table 1) were used in the desiccation experiments, together with the related widespread freshwater algae *Cladophora glomerata* and *Rhizoclonium* sp. (collected from the lake system 'De Wieden', The Netherlands) for comparison. *A. linnaei* isolate L69 differs in its ITS ribotype from the other two tested isolates (Table 1). All algal material had been kept in culture in D11 medium (Andersen 2005) for at least six months under low irradiance conditions (10 µmol photons m^{-2} s^{-1}) at 10ºC and a 12:12 h light:dark cycle. For *A. linnaei* and *C. glomerata*, three to four healthy filaments per isolate and treatment were mounted separately on one glass slide each, and all cells (minimum 230 cells in total) of these filaments were counted using a light microscope (Olympus BH2). For *Rhizoclonium* sp., more filaments were used, and 50 cells each were inspected in ten random filaments (n = 500 cells) per treatment. As *A. linnaei* reproduces mainly by vegetative fragmentation and many or all filaments in a sample (or even at a location) might represent clones, the independence of filaments is generally impossible to assess. Even though the sampling method did not ensure independence of random sampling, the counted cells were pooled per isolate and treatment and treated as independent replicates in the statistical analysis. The coverslips were subsequently removed and the filaments carefully blotted dry with absorbent paper. The filaments of the five isolates were then exposed to desiccation

Table 1. List of specimens of *Aegagropila linnaei* for which ITS rDNA sequences have been generated, with collection information and GenBank accession numbers. Asterisks indicate brackish locations, bold numbers in brackets refer to the isolates tested in the desiccation experiments, n.d. = no data, n.s. = not submitted (identical sequence).

Location	Country	Latitude	Longitude	Date	Growth form	GenBank no.	ITS genotype
Sällvik, Pojo Bay, Gulf of Finland (**L69**)*	Finland	60.030	23.500	2007	floating	GU325819	A
Lake Myvatn	Iceland	65.600	-17.000	2002	balls	n.s.	A
Lake Akan, Hokkaido	Japan	43.451	144.099	1994-2001	n.d.	n.s.	A
Lake Ogawara, Honshu	Japan	40.783	141.333	2001	attached	n.s.	A
Pond Takkobu/Takkobu marsh, Hokkaido	Japan	43.100	144.483	1996	attached	n.s.	A
Lake Saiko, Honshu	Japan	35.498	138.685	1994	n.d.	n.s.	A
Lake Kawaguchi, Honshu	Japan	35.517	138.750	1993	floating, balls	n.s.	A
Pond Sakyo, Honshu	Japan	41.257	141.398	1996	n.d.	n.s.	A
Pond Tamogi, Honshu	Japan	40.936	141.347	1996	n.d.	n.s.	A
Lake Yamanaka, Honshu	Japan	35.417	138.867	1993	n.d.	n.s.	A
Pond Ane, Honshu	Japan	40.936	141.347	1996	n.d.	n.s.	A
Pond Ichiyanagi, Honshu	Japan	40.936	141.347	1999	n.d.	n.s.	A
Pond Uchi, Honshu	Japan	40.936	141.347	1999	n.d.	n.s.	A
Lake Bemidji	USA	47.474	-94.880	2001	balls	n.s.	A
Lake Akan (Jagaiwa), Hokkaido	Japan	43.451	144.099	2001	n.d.	GU325820	B
Lake Chimikeppu, Hokkaido	Japan	43.633	143.883	1994	n.d.	n.s.	B
Lake Shirarutoro, Hokkaido	Japan	43.179	144.500	1995	floating	n.s.	B
Lake Toro, Hokkaido	Japan	43.144	144.540	1996	attached	n.s.	B
Lake Panke, Hokkaido	Japan	45.031	141.722	2001	n.d.	n.s.	B
Lake Toba, Sakhalin	Russia	46.744	143.190	1993	n.d.	n.s.	B
Loch Watten (**N36**)	Scotland	58.486	-3.315	2008	balls	GU325821	C
aquarium shop, cf. Lake Svityaz (**C01**)	(Ukraine)	51.501	23.851	2006	balls	n.s.	C
Lake Zeller	Austria	47.326	12.806	1999	attached	n.s.	C
Lake Öisu	Estonia	58.209	25.513	2000	balls	n.s.	C
Lake Valgjarv	Estonia	58.090	26.638	2000	n.d.	n.s.	C
Pärnu river	Estonia	58.492	24.833	2000	n.d.	n.s.	C
Ramsholmen, Pojo Bay, Gulf of Finland*	Finland	60.047	23.480	2007	floating	n.s.	C
Tiefwarensee, Mecklenburg-Vorpommern	Germany	53.526	12.691	2008	floating	n.s.	C
Lake Dannemora	Sweden	60.180	17.840	2000	balls	n.s.	C
Lake Erken	Sweden	59.847	18.579	2000	balls	n.s.	C
Holmön, Northern Quark, Gulf of Bothnia*	Sweden	63.830	20.940	2004	attached	n.s.	C
Lake Biwa, Honshu	Japan	35.250	136.083	1997	attached	GU325822	D
Boven Wijde, Overijssel	Netherlands	52.724	6.103	2007	attached	GU325823	E
Kleiner Lankesee, Brandenburg	Germany	52.910	13.225	2006	unattached	n.s.	E

treatments of one hour and six hours aerial exposure, respectively, at 10ºC and low-light conditions (10 µmol photons m^{-2} s^{-1}). After the desiccation periods, culture medium was added and all cells were counted again within each isolate. Two categories "healthy cells" and "plasmolysed cells" were scored. Subsequently, the filaments were re-submerged and transferred to the original culture conditions and the number of plasmolysed or damaged cells was counted again after seven days (termed 'recovery'). A non-parametric binomial test was applied and one-tailed significances were calculated (SPSS 15.0 for Windows, SPSS Inc., Chicago, IL, USA) to compare the proportion of plasmolysed cells between the 'one hour desiccation treatment' and the following one-week recovery, and between the 'six hours desiccation treatment' and the following one week recovery for each isolate, as well as between all isolates within desiccation treatments (including between isolates after recovery). As five isolates were compared, ten pairwise comparisons were carried out for each desiccation treatment. The type 1-error was adjusted for each comparison according to the Bonferroni procedure, *i.e.,* significant differences between pairs were concluded at $P<0.005$ which amounts to an overall maximal type 1-error of 0.05. Any surviving cells were checked after four weeks for possible new cell divisions.

DNA sequence analysis

The complete ribosomal ITS1-5.8S-ITS2 region was sequenced for most samples, while for eight specimens only ITS2 data could be obtained. Sample and collection information, and GenBank accession numbers, are given in Table 1. DNA was extracted from fresh material and specimens that had been desiccated in silica gel after collection (Chase & Hills 1991), or from recent herbarium specimens. Total genomic DNA was isolated using the Chelex method (Goff & Moon 1993). Polymerase chain reaction (PCR) amplifications were performed in a Biomed thermocycler 60 (Biotrade, Vienna, Austria) with an initial denaturation step of 94°C for 3 min followed by 32 cycles of 30 s at 94°C, 30 s at 53°C, and 30 s at 72°C, with a final extension step of 3 min at 72°C. The reaction volume was 25 µL and consisted of approximately 0.1-0.4 µg genomic DNA, 1.25 nmol of each dNTP, 6 pmol of each primer, 2.5 µL of 1x reaction buffer containing 1.5 mM $MgCl_2$ (Qiagen Benelux B.V., Venlo, The Netherlands), 1 µL BSA (2.5%), 17.7 µL H_2O and one unit of *Taq* polymerase (Qiagen). The first approximately 430 bp including the complete ITS1 region were amplified using the universal primers ITS5 forward (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS2 reverse (5′-GCTGCGTTCTTCATCGATGC-3′); the second approximately 540 bp containing most of the 5.8S rDNA gene and the complete ITS2 region were amplified using the universal primers ITS3 forward (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4 reverse (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al.* 1990). Amplifications were checked for correct size by electrophoresis on 1% agarose gels and subsequent staining with ethidium bromide. For samples displaying multiple bands on the agarose gels, the entire PCR product was run in a subsequent gel electrophoresis and bands of the correct size were carefully excised from the gel. PCR products and gel slices were purified with the Promega Wizard clean-up system (Promega Benelux, Leiden, The Netherlands) following the manufacturer's protocols. Cleaned PCR products were sent to Macrogen Inc., Seoul, South Korea, for sequencing. The final consensus sequences were constructed with Sequencher 4.0.5 software (GeneCodes, Ann Arbor, MI, USA), forward and reverse chromatograms were carefully inspected, and sequences were subsequently aligned by eye in Se-Al v2.0a11 (Rambaut 2007) and submitted to GenBank (see Table 1 for accession numbers). A 95% statistical parsimony network of the ITS rDNA sequences was constructed with TCS version 1.21 (Clement *et al.* 2000), with gaps treated as fifth base and indels being recoded.

Results

Location survey

In total, *Aegagropila linnaei* had been recorded from 283 locations (listed in Appendix S3), or 233 locations when corrected for the continuous coastline of the Baltic Sea. The country with most locations is Sweden (51), followed by Germany, Britain and Ireland, Japan and Russia.

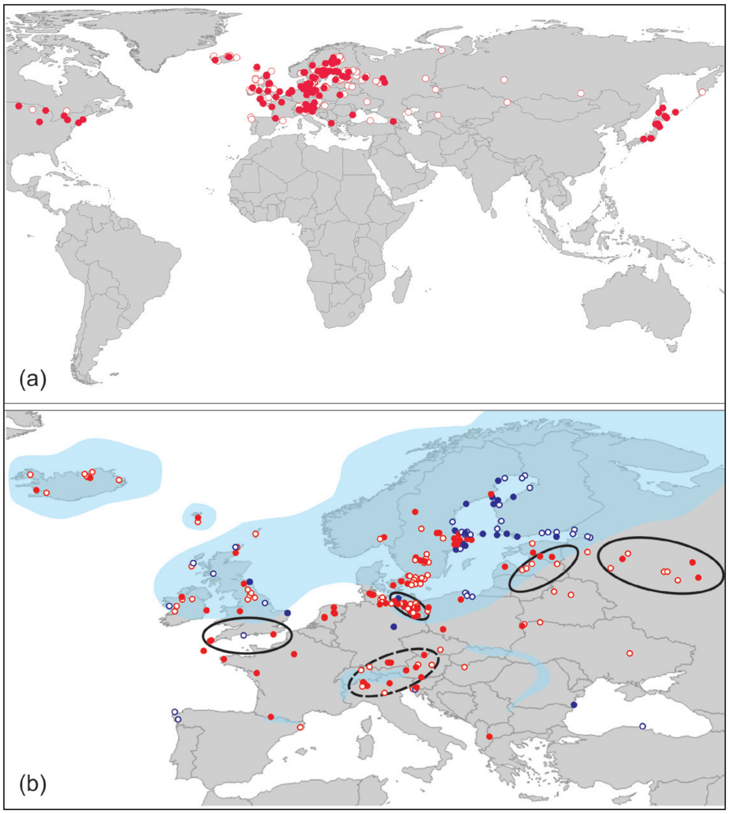

Fig. 2.

a Worldwide distribution of *Aegagropila linnaei* as reconstructed from herbarium collections, literature data and field observations. Filled red circles represent morphologically verified records, open dots represent unverified records.

b Distribution of *A. linnaei* in Europe. Filled circles represent morphologically verified records, open dots represent unverified records. Brackish locations are marked in blue, freshwater locations are indicated in red, The maximum extent of glaciations in Europe (Scandinavian Ice Sheet and mountain glaciers) during the Last Glacial Maximum (20 ka), Late Weichselian, is shaded in blue (redrawn from Mangerud *et al.*, 2004). Potential refugia, *i.e.*, areas with high numbers of records, are encircled.

The worldwide distribution of *A. linnaei* as reconstructed from the data in Appendix S3 is shown in Fig. 2a. The vast majority of locations are situated in central and northern Europe. The most northerly locations are in Iceland (65-66°N) and western Siberia (68°N). The few records in North America were limited to the eastern parts of the northern United States and southern Canada up to 48°N. The southernmost locations in Europe and North America are at 41°N, while the range extends southwards to 35°N in Japan. While being known from more than 20 locations in Japan (Appendix S3), there are only a very few scattered reports from Central and East Asia. Despite efforts to locate specimens from central or northern Canada, China, and Siberia, no records could be found for these regions. This resulted in the inferred disjunct distribution between Japan and Europe, with only three literature records for the Asian continent east of the Urals. A more detailed overview of the locations in Europe, with brackish locations indicated, is given in Fig. 2b. The general distribution of *A. linnaei* in freshwater lakes spans most of Europe, and it becomes rarer in the west and the south-east. However, this species is also widespread in the brackish waters along the coast of the central and northern Baltic Sea at salinities of 6 psu and below. Additionally, *Aegagropila* balls have been recorded from a number of other brackish locations, especially in Britain and Ireland, and also from the Black and Caspian Seas. Furthermore it was reported from one saline inland location in Germany (Salziger See (Mansfelder Seen); near Halle).

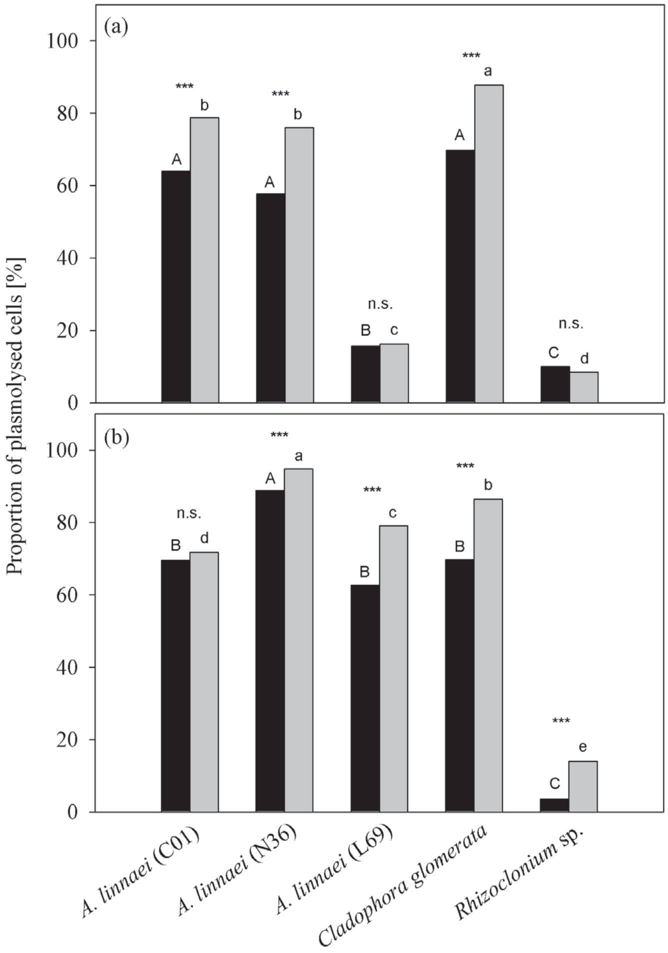

Fig. 3.

Effects of desiccation on three isolates of *Aegagropila linnaei* (C01, N36, L69; see Table 1 for details) and two widespread freshwater representatives of the Cladophorales, *Cladophora glomerata* and *Rhizoclonium* sp.

a 1-hour desiccation treatment.
b 6-hour desiccation treatment.

Proportions of plasmolysed cells are shown for 235-500 counted cells per isolate and treatment. Different upper-case and lower-case letters indicate statistically significant differences between isolates directly after desiccation stress (black bars) and subsequent one week recovery (grey bars), respectively. Stars represent significant ($p<0.005$) differences within each isolate between desiccation stress and subsequent recovery, n.s. indicates differences being non-significant.

Desiccation experiments

The isolates C01 and N36 of *A. linnaei* showed the same proportion of plasmolysed cells in the one-hour desiccation treatment (64% and 58%, respectively) (Fig. 3a), and did not differ from each other after one week of recovery (79% and 76% damaged cells, respectively). The effects were very similar to those in *Cladophora glomerata* (70% plasmolysed cells after one hour desiccation), for which the proportion of damaged cells significantly increased further within one week of recovery (88% damaged cells). The isolate L69 of *A. linnaei* differed significantly from isolates C01 and N36, with only 16% of plasmolysed cells in the one-hour treatment and no further changes within one week of recovery. *Rhizoclonium* sp. was least affected by desiccation, with only about 10% of the cells undergoing plasmolysis. Apart from a generally higher proportion of damaged cells, the results of the six-hour desiccation treatment differed mainly with regard to isolate L69 (63% plasmolysis, 79% after one week recovery), which was as strongly affected as isolate C01 and *C. glomerata* (69% and 70% of plasmolysed cells, respectively) (Fig. 3b). *A. linnaei* isolate N36 was damaged more than the other two isolates, with 89% plasmolysed cells (increasing to 95% in the following week of recovery). *Rhizoclonium* sp. was again the least affected (4-14% plasmolysed cells). The amount of damaged cells was never lower after one week's recovery and in most instances observed damage increased over time (Figs. 3a–b). None of the undamaged cells of the three *A. linnaei* isolates underwent cell division within one month after the experiments, whereas new cell divisions were observed in *Rhizoclonium* sp.

DNA sequence analysis

Complete sequences of the nuclear ribosomal ITS1-5.8S-ITS2 region were obtained for 26 samples of *Aegagropila linnaei*, while for eight samples only the ITS2 region was sequenced. We observed no intra-individual polymorphism in the ITS rDNA sequences. Within those 34 sequences, five ribotypes were identified (labelled A-E, Table 1). The geographic distribution of the individual ribotypes and the ribotype network are shown in Figs. 4a–b. None of the variable nucleotide sites was parsimony-informative. The amount of sequence divergence between ribotypes ranged from 0.1-0.5%, with ribotype D from ancient Lake Biwa (Japan) being the most divergent. Only ribotype D had any mutations in the ITS1 region. Ribotypes B, C and D had one point mutation each in the ITS2 region compared to ribotype A, while ribotype E displayed a 15 bp indel. Ribotype A is inferred to be the ancestral type (Fig. 4b). Ribotype A was also the most common (14 samples) and the only widespread ribotype (Fig. 4a). Eleven records were from Japan, while the three remaining specimens came from Finland, Iceland and the United States. Ribotype C was the second most common and restricted to Europe, present in eleven samples from Austria, Estonia, Finland, Germany, Sweden, and probably the Ukraine (sample from an aquarium shop, Table 1). Ribotype E was also restricted to Europe, but only found twice, from the Netherlands and north-eastern Germany. Ribotype B was recovered from six samples, all originating from northern Japan (Hokkaido) and its vicinity (one sample from southern Sakhalin, Russia). Ribotype D was only found in a specimen from ancient Lake Biwa, Japan. While ribotype D was only detected in attached material from one single location, the other ribotypes (A, B, C, E) were found in attached and unattached growth forms. In one location (brackish Pojo Bay, southern Finland) two different ribotypes (A and C) were found in two different samples (Table 1). It appears that ribotype C is most frequent within individuals from Europe, while types A and B are the most frequent in Japan, with type B being restricted to Hokkaido, and type D being found only once from Lake Biwa (Honshu).

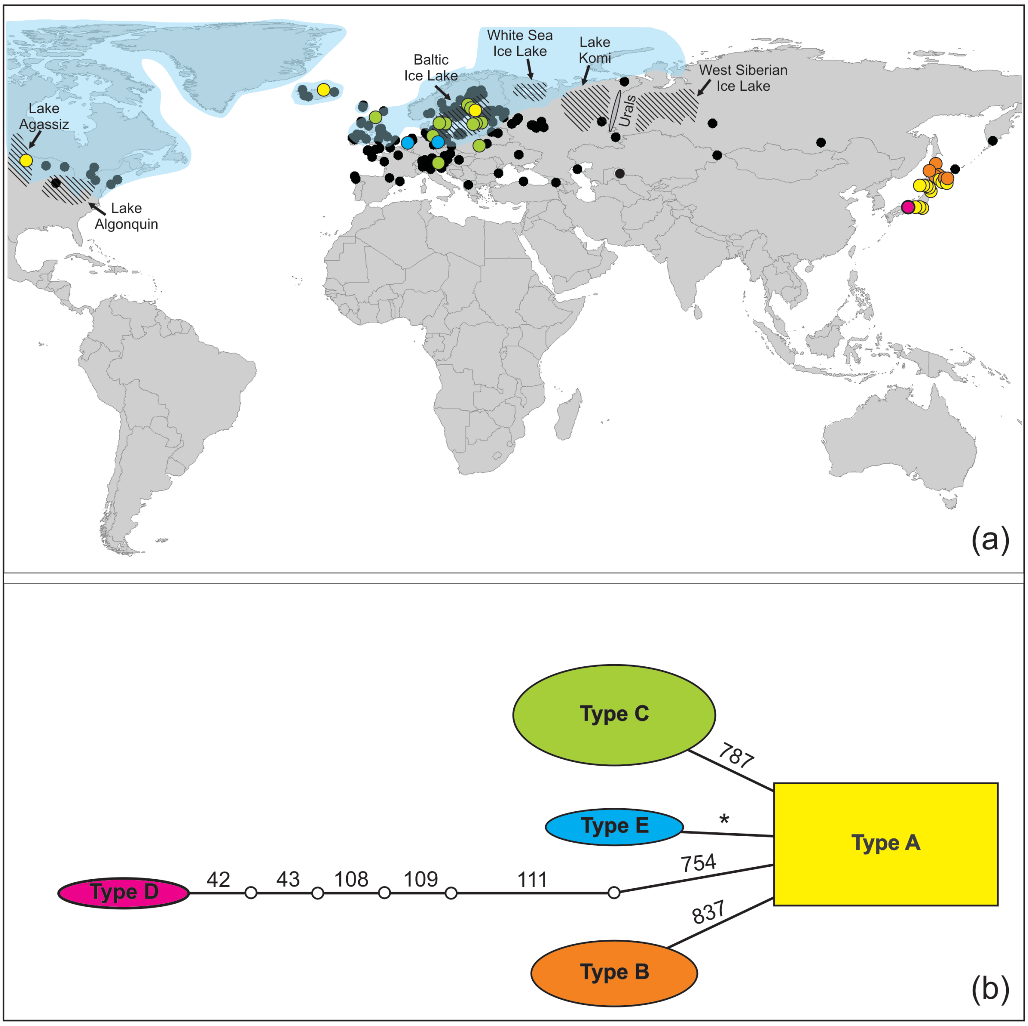

Fig. 4. a Global distribution (black dots) and distribution of ITS ribotypes of *Aegagropila linnaei*. Five ITS ribotypes were discovered among 34 samples, shown as large coloured circles. Hatched areas represent large ice-dammed lakes during Weichselian glaciations; blue shows the maximum extent of the Early Weichselian glaciation (90-80 ka, redrawn from Mangerud *et al.* 2004). **b** Network of the five ITS ribotypes constructed with TCS (Clement *et al.*, 2000), shown in the same colours as in **a**. The letter coding of the ribotypes (A–E) refers to that in Table 1. On the branches are the alignment positions; the asterisk indicates a 15 bp deletion. The area of the circles/squares represents the number of individuals of that particular haplotype.

Discussion

This comprehensive study of the distribution of *Aegagropila linnaei* increases our knowledge of this enigmatic freshwater alga considerably, with most collections originating from Europe and Japan, and only a few found in North America. Many new locations were discovered, and all findings have been critically evaluated including addressing taxonomic issues (Appendices S1 and S3). We found little genetic variation among samples, indicating that *A. linnaei* represents a single species with a wide distribution within the Holarctic (with very few Nearctic finds). However, the species is only known from a few locations in most countries. Among the five ITS ribotypes recovered, only one is widespread, and our laboratory experiments indicate that *A. linnaei* is very susceptible to desiccation stress. Thus, we present a case of a widespread freshwater macroalga with a low dispersal capacity.

Molecular data

The low divergence among ITS sequences (< 0.5%) from distant populations of *A. linnaei* generally suggests recent dispersal from a source population. In the marine green algal genera *Phyllodictyon* and *Boodlea* ITS sequence data showed intraspecific variation of up to 4%, and between-clade divergence of 7-29% (Leliaert *et al*. 2008, 2009b). Considerably higher intraspecific ITS sequence diversity of 18-33% was found in the marine species *Cladophora albida* and *C. vagabunda* (Bakker *et al*. 1992, 1995, Marks & Cummings 1996), however, both species are likely comprised of several cryptic species. In contrast, all samples of the common and easily dispersed freshwater alga *C. glomerata* have identical ITS sequences (Marks & Cummings 1996, Ross 2006). The ITS ribotype network of *A. linnaei* shows ribotype A to be the hypothesized ancestral type and indicates an Asian origin (Fig. 4b). Ribotype A is the only widespread ribotype (Japan, Europe, Iceland, USA), while the other four ribotypes are locally restricted in their distribution. Thus, successful dispersal to areas outside of their site of origin must be rare. Additionally, settlement of new arrivals in regions that are already colonized by conspecifics might have a low success rate as hard substrates are scarce in many shallow lakes and substrate availability might play a role.

Dispersal potential

The main means of reproduction of *A. linnaei* seems to be dispersal via fragmentation (Brand 1902, van den Hoek 1963). Fragmentation takes place by mechanical breaking and by axial cells dying off, releasing the branches above them (Acton 1916, Nishimura & Kanno 1927). Akinetes have only been mentioned once for *A. linnaei,* formed by filaments of decaying lake balls that had been in culture for eight years (as 'hypnospores', Acton 1916). These desiccation-resistant stages are commonly formed by many widespread freshwater macroalgae such as *Cladophora*, *Microspora*, *Oedogonium*, *Pithophora* and *Spirogyra* (Handa 1928, Evans 1958, van den Hoek 1963, Agrawal & Singh 2000, John *et al*. 2002) and are the main agents for long-distance dispersal by birds (*e.g.,* Schlichting, 1960, Green *et al*. 2002). However, individual thick-walled cells in old vegetative filaments of *A. linnaei* might function as resting stages (Kjellman 1898, Brand 1902, Waern 1952), and Acton (1916) illustrated an isolated old cell from the main axis producing new branches. Furthermore, fragmented vegetative filaments themselves might function as dispersal agents. Cell walls in the basal parts are up to 20 µm thick and therefore possibly quite desiccation-resistant. In contrast, Terumoto (1959) showed rapid plasmolysis of vegetative cells exposed to air. Our quantitative desiccation experiments with isolates of *A. linnaei* from different locations show the same trend, with high levels of plasmolysed cells after air exposure. However, we observed variation between isolates, especially after one hour aerial exposure. In isolates C01 and N36 almost 80% of the cells were plasmolysed after one hour of desiccation (Fig. 3a), while in isolate L69 the proportion of plasmolysed cells was much lower (16%). This observation might possibly point to a genetic component in the desiccation tolerance, as isolate L69 represents ITS ribotype A (versus C for the other isolates tested). Ribotype A is also the only widespread ribotype (Japan, USA, Iceland, Finland), which may reflect its increased dispersal capacity.

The other two freshwater algae tested in the desiccation experiment have a much wider distribution than *A. linnaei*. While *C. glomerata* filaments also have low desiccation resistance, this species is easily dispersed via desiccation-resistant akinetes (van den Hoek 1963). Like *A. linnaei*, *Rhizoclonium* does not form any specialized thick-walled resting stages, but its vegetative cells seem to be extremely desiccation-resistant (Fig. 3). This illustrates that *A. linnaei* has a comparatively much lower dispersal capacity than most other widespread freshwater algae. However, our experiments show that some cells of *A. linnaei* can survive desiccation, therefore long-distance transport cannot be ruled out, but those events might be rare. Additionally, successful establishment in a new environment after dispersal of a thallus fragment is facilitated by subsequent spore or gamete release, but spore formation seems to be absent or at least very rare in *A. linnaei*.

The occurrence of *A. linnaei* on islands glaciated during the Last Glacial Maximum (LGM, 20 ka), such as Iceland**,** the Faroe Islands, the Shetland Islands and the Orkney Islands, is most likely explained by bird dispersal taking place after the LGM, indicating that mid-range distances can be bridged. Also the finds of *A. linnaei* in relatively high European mountain lakes, such as Altausseer See, Erlaufsee, Zeller See, Lac Bleu and Lago di Piné (all between 750 and 2000 m a.s.l., Appendix S3) imply dispersal of *A. linnaei*, possibly by birds (Schlichting 1960).

Distribution, dispersal and the role of glaciations

Aegagropila linnaei has a mainly Palaearctic distribution, with very few records from eastern North America. No records from Siberia or northern China could be traced, resulting in the inferred disjunct distribution between Japan and Europe. The sampling of locations within Europe was good, as the latest collections from European herbaria did not yield additional locations. However, coverage for south-eastern Europe was less extensive, raising the possibility of additional locations for *A. linnaei* in that region. Within Europe, the absence of *A. linnaei* from the numerous lakes of Finland and (southern) Norway is striking. The nearly complete absence of *A. linnaei* in Norway and Finland might be explained by most lakes having low conductivity, low calcium content and low pH (Henriksen *et al*. 1988, Jørgensen 1997), conditions not suitable for *A. linnaei* (Boedeker *et al*. 2010a). Furthermore, the species might have been overlooked in suitable locations.

The highest density of records is located in central and northern Europe, especially in previously glaciated areas. Glaciation events seem to have played a considerable role in shaping the current distribution of *A. linnaei*. Records from ice-free locations of Western Europe are few, and are scattered over the south of England, The Netherlands, France, and Spain. Locations in Central and Eastern Europe that remained ice-free are situated in Belarus, Czech Republic, Poland, Ukraine, and some locations in western Russia. Russia west of the Urals, southern Norway, the Atlantic south of the British Isles, and the southern and eastern Baltic Basin are well-known refugia for freshwater animals (*e.g.,* Makhrov & Bolotov 2006). Inferred from the highest density of locations in the distribution of *A. linnaei*, the latter three regions seem to be the most likely refugia for *A. linnaei*. In addition, the extensive glacial ice lakes might have functioned as refugia (indicated in Fig. 4a). *A.*

linnaei can withstand extremely low temperatures down to -15ºC (Terumoto 1959, 1962, 1964) making this species an excellent candidate to survive in those glacial refugia. Many of these resulting water bodies were extensive and connected through drainage systems and by a network of aquatic habitats, and might have functioned as colonization pathways for *A. linnaei* into previously glaciated areas of Europe. Also ice lakes along the margins of mountain glaciers may also have served as refugial habitats for *A. linnaei*, explaining the cluster of records in the Alps region. On the American continent, *A. linnaei* is found near the edge of the maximum glacial extent in areas where large ice-dammed lakes similar to those in Europe and western Russia have existed (Dyke 2004, Pascucci *et al.* 2009). Pleistocene glaciations played a role in shaping the current biogeographic distribution of many freshwater animals (*e.g.,* Segerstråle 1962, Salemaa & Heino 1990, Østbye *et al.* 2005, Makhrov & Bolotov 2006). With respect to algae/cyanobacteria, one of the few known examples includes the macroscopic cyanobacterium *Nostoc pruniforme*. Like *A. linnaei*, it lacks desiccation-resistant stages and its distribution is thought to have been at least partly shaped by glaciations (Mollenhauer 1970, Komárek 1985, Mollenhauer *et al.* 1999), but unfortunately molecular phylogeographic studies are lacking.

Despite our efforts to locate records from northern North America and Siberia, the species seems to be absent from these regions. If this distribution scenario were real and not an artefact of undersampling, it raises interesting questions. The absence of a species from large parts of the same climatic zone can generally not be explained on the basis of unsuitable habitats (Hoffmann 1996). Why was the area north of the few locations in North America not colonized despite abundant suitable habitats located along a major bird migration pathway? Was *A. linnaei* once continuously distributed in the Palaearctic and became extinct in Siberia? Or was it dispersed from Japan to Europe despite bird migration following a north–south direction, and despite the potentially limited long-distance dispersal capacity? With respect to North America, the lack of records from Canada and Alaska suggests that *A. linnaei* did not migrate northwards following the retreating glaciers, unlike in Europe (discussed below). In combination with the finding of ribotype A in the northern USA, this might indicate a relatively recent colonization after the last glaciation, possibly by long-distance dispersal. Since the earliest records from North America date back to the late 19th century (records in CANA & F; Holmgren *et al.* 1990 for abbreviations), the possibility of a human introduction of balls originating from the recently flourishing hobby aquarium trade can be disregarded.

The disjunct distribution between Japan and Europe could be the result of extinction on the Asian continent east of the Urals. Such an extinction event would most likely be linked to one of the Pleistocene glaciations. The Siberian landmass was never glaciated during the Weichselian glaciations due to its dry continental climate. Permafrost was widespread and year-round, leaving few or no open water bodies, and high mountain ranges are located further south. Accordingly, Siberia was lacking widespread refugial habitats for *A. linnaei,* possibly leading to extinction in this area, in contrast to glaciated Europe, which had abundant refugia in the south and west of the ice sheet and in the form of extensive ice-dammed lakes (Svendsen *et al.* 1999, Mangerud *et al.* 2004). This scenario rather than long-distance disperal from Japan to Europe is tentatively supported by ITS ribotypes present in Europe that are missing in Japan, therefore suggesting isolation with accompanying genetic divergence. However, instead of the outlined vicariant scenario this could also be explained by an old dispersal event. These questions will only be answered with additional samples and markers, and some form of molecular dating.

Salinity tolerance

Aegagropila linnaei has a remarkably broad salinity tolerance and is found in a range of far-flung brackish water habitats, in the British Isles, the northern Baltic Sea, the Black Sea, Caspian Sea, and one saline inland location in Germany. Several of the locations in the British Isles are characterized by large salinity fluctuations. Waern (1952) demonstrated that material from the brackish Öregrund (Baltic Sea) could be grown for 10 years in fresh water. Conversely we have found that *Aegagropila* balls from freshwater environments survived for at least two years after transfer to a range of salinities, including full marine medium. In the light of this capacity for osmotic acclimation, the high degree of plasmolysis after short periods of desiccation was rather surprising. Terumoto (1959, 1962, 1964) reported that potassium ions play a pronounced role in preventing freezing of intracellular water in this species. *Aegagropila linnaei* also synthesizes the osmolyte glycine betaine (C. Boedeker, unpublished data) to regulate its intracellular water potential during osmotic (salinity) and matric (desiccation, freezing) stress. This compound is found in a large variety of microorganisms, higher plants and animals (Rhodes & Hanson 1993), and has been reported for some intertidal marine *Cladophora* species *e.g., Cladophora rupestris* (L.) Kützing (Wiencke *et al.* 1992). Interestingly, glycine betaine has not been found in freshwater *Cladophora* species (C. Boedeker, unpublished data).

In a small number of algal checklists *A. linnaei* is listed for fully marine environments in the Mediterranean such as Sicily (Giaccone *et al.* 1985), Sardinia (Brambati *et al.*, 1980), the Ionian Sea (Cecere *et al.* 1996), and the Adriatic Sea (Giaccone 1978, Cormaci *et al.* 2000). All herbarium specimens of *A. linnaei* from the Mediterranean that were checked in the course of this study were re-identified as different species, mostly as *Cladophora coelothrix* Kützing or *Cladophora echinus* (Biasoletto) Kützing. Even though *A. linnaei* can cope with high salinity in culture, it seems not to occur in fully marine environments. For the time being, the literature reports of *A. linnaei* from marine environments in the Mediterranean have not been included in the distribution maps shown in this study.

Relatives, taxonomic considerations and Asian origin

The ITS ribotype network indicates that Japan was the ancestral area. The closest relatives of *A. linnaei* inferred from molecular data are an undescribed species with a *Cladophora*-like morphology from Japan ('*Cladophora* sp. Tateyama'), and the (sub)tropical genus *Pithophora* (Hanyuda *et al.* 2002, Yoshii *et al.* 2004). Thus, it is more likely that *Aegagropila* has an origin in (sub)tropical Asia than in Europe. A group of morphologically closely related species and potential members of the genus *Aegagropila* are the species from ancient Lake Baikal. Eight species of *Aegagropila*/*Cladophora* have been described from Lake Baikal (Meyer 1926, 1927, 1930, Skabichevsky 1976, Zagorenko & Izhboldina 1977, Izhboldina 2007). Their morphological features, though unique, suggest a close relationship to *A. linnaei* within the *Aegagropila*-lineage. Meyer (1926) noted that the *Aegagropila* species from Lake Baikal are sharply separated from the European ones by their ability to form zoospores.

Aegagropila linnaei or its ancestor is assumed to have dispersed throughout the Palaearctic (or the Holarctic) from Central or East Asia. A similar scenario has also been proposed for several freshwater animals found as glacial relicts in Fennoscandian lakes and the brackish parts of the Baltic Sea as well as in some scattered Siberian locations, with ancestors in Lake Baikal (Segerstråle 1962). Molecular data for the morphological relatives of *A. linnaei* from Lake Baikal would strongly add to our understanding of the biogeographic patterns and the age of this unusual and fascinating group of green algae.

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Cladophoropsis brachyartra from southern South America is a synonym of *Wittrockiella lyallii* (Cladophorales, Chlorophyta), previously regarded as endemic to New Zealand

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Abstract

Wittrockiella lyallii is a poorly known green macroalga that has been regarded as endemic to the south of New Zealand. A herbarium specimen from southern Chile was discovered that conforms to the description of *W. lyallii*. Subsequently, it was realised that the South American species *Cladophoropsis brachyartra* is morphologically and ecologically equivalent to *W. lyallii*. Sequences of three ribosomal genes, including the internal transcribed spacer (ITS), from material collected in Chile and New Zealand are identical, suggesting recent long-distance dispersal from New Zealand to South America. The synonymisation of *C. brachyartra* with *W. lyallii* is presented here. Furthermore, the resulting disjunct distribution of the species is discussed in the light of sea surface temperatures, habitat availability in the southern ocean and winter sea ice extent during the last glacial maximum.

Introduction

Wittrockiella lyallii (Harvey) C. Hoek, Ducker & Womersley (Cladophorales, Ulvophyceae) is a poorly known macroalga consisting of a prostrate system of filaments that form green cushions in the high intertidal, typically in marine areas subject to freshwater influx (Adams 1994, Nelson *et al*. 2002). Currently, *W. lyallii* is regarded as endemic to southern New Zealand. The knowledge of the distributions and relationships of seaweed species in the southern ocean is still relatively poor despite some recent advances (Hommersand 2007, Broom *et al*. 2010, Nelson & Broom 2010). This is especially true for members of the Cladophorales and related groups, illustrated for example by the unknown affinities of taxa such as *Lola irregularis* Zaneveld, *Rama* V.J. Chapman, *Siphonocladus voluticola* (Harvey) Børgesen and several supposedly endemic *Chaetomorpha* species, such as for example *Ch. dubyana* Kützing, *Ch. kerguelensis* Levring, or *Ch. mawsonii* Lucas, and *Cladophora* species, such as for example *C. aucklandica* Rabenhorst, *C. confusa* Hariot, *C. incompta* (Hooker f. et Harvey) Hooker f. et Harvey, *C. magellanica* Ardissone, or *C. verticillata* Hooker f. et Harvey.

In New Zealand, *W. lyallii* has been recorded from several locations in Fiordland in the southwest of the South Island (Laing 1927, Nelson *et al*. 2002), Stewart Island (Harvey 1855, Laing 1899, Chapman 1956, Adams *et al*. 1974), the Chatham Islands (Adams 1994, Nelson *et al*. 1991), and the Snares Islands (Hay *et al*. 1985), but it has not been collected from the other New Zealand subantarctic islands. In the Chatham Islands, the cold temperate-subantarctic species *W. lyallii* is part of a mixed flora consisting of both cold and warm water elements (South 1978, Nelson 1994). The species was originally described by Harvey (1855) based on material from Stewart Island as *Cladophora lyallii*, transferred to the genus *Cladophoropsis* (as *C. lyallii*) by Chapman (1956), before being placed in the genus *Wittrockiella* (van den Hoek *et al*. 1984).

Wittrockiella lyallii is distinguished morphologically from the two other species currently placed in the genus *Wittrockiella* (*W. paradoxa* Wille and *W. salina* V.J. Chapman) by the much coarser filaments and larger cell dimensions (cell diameter 200-400 µm), and its cold-temperate to subantarctic southern distribution. The range of *W. lyallii* and *W. salina* overlap on both the Chatham Islands and Stewart Island (Adams 1994). The type of the genus *Wittrockiella* is *W. paradoxa* (Wille 1909), the smallest of the three species (cell diameter < 100 µm), which is known from northwestern Europe (Polderman 1976), and from the northwestern (South 1981) and the eastern USA (Collins 1909). *Wittrockiella paradoxa* occurs in brackish habitats, as endophytes in saltmarsh plants, aerophytically on moist soil, or forming mats on soft sediments. Based on morphological similarities, it has been proposed that *W. paradoxa* might be closely related to the warm-temperate to tropical species *Cladophorella calcicola* Fritsch (van den Hoek *et al*. 1984), which is known from Australia, Bangladesh, and tropical hothouses in Europe (Fritsch 1944, Islam 1964, Cribb 1965, Ettl & Gärtner 1995, Skinner & Entwisle 2004), and typically occurs semi-terrestrially on moist limestone. The slightly bigger *W. salina* (cell diameter < 200 µm) is distributed in southern and eastern Australia, Tasmania, New Zealand, and possibly the Caribbean (Chapman 1949, van den Hoek *et al*. 1984). *Cladophorella marina* V.J.Chapman, described as an endemic species from New Zealand, is regarded as a synonym of *W. salina* (Beanland & Woelkerling 1982, Womersley 1984, Adams 1994). *Wittrockiella salina* occurs in the high intertidal, often attached on mangrove roots or forming cushions on rock in the high intertidal, but it is also known as unattached ball-shaped growth forms from one location in Australia (van den Hoek *et al.* 1984). It frequently occurs in estuarine environments with a lowered salinity (van den Hoek *et al.* 1984).

Early treatments that placed *Wittrockiella* in close proximity but outside the Cladophoraceae (Fritsch 1935), and that proposed close relationships to the genus *Aegagropila* (van den Hoek *et al.* 1984), have been confirmed by molecular studies (Hanyuda *et al.* 2002). The brackish water genus *Wittrockiella* is a member of the *Aegagropila*-lineage, an assemblage of species-poor, mainly freshwater genera such as *Aegagropila*, *Arnoldiella*, *Basicladia*, and *Pithophora* (Hanyuda *et al.* 2002, Yoshii *et al.* 2004). The placement of *W. paradoxa* and *W. lyallii* within the same genus has been shown by molecular analyses (Yoshii *et al.* 2004).

Adaptations of *Wittrockiella* to habitats with strongly fluctuating salinities include the formation of a thick mucilage cover produced by swelling of the outer cell wall layers in *W. paradoxa* (Wille 1909), an endophytic habitat in *W. paradoxa* (Polderman 1976), the presence of haematochrome/oil droplets in *W. paradoxa* and *W. salina* (Wille 1909, van den Hoek *et al.* 1984, respectively), and a cushion-like growth form to preserve moisture during exposure to air in *W. salina* and *W. lyallii*. Both *W. paradoxa* and *W. salina* can produce long septate hairs from cells in the apical parts (Wille 1909, van den Hoek *et al.* 1984), but are not always present. In addition, elongated exit tubes that ensure the release of akinetes or aplanospores to the outside of the mucilage matrix or the tissue of the host halophyte can be mistaken for hairs (C. O'Kelly - pers. comm.). In *W. lyallii*, neither hairs nor akinetes or sporangia have been observed. Very little information is available about the biology of this species, and its distribution, ecology, life history, and dispersal mechanisms are poorly known.

New Zealand has a high proportion of endemic species, *e.g.,* all native reptiles, amphibians and bats are endemic, as well as most birds and freshwater fish (Gibbs 2006), all gymnosperm trees and shrubs, and 87% of grasses (Rogers & Walker 2005). Among the benthic marine algae of New Zealand more than 40% of the species are endemic taxa, and among the Chlorophyta about 38% of the currently recognised ca. 165 species are regarded as endemic (Parsons 1985). Undoubtedly, New Zealand has a unique seaweed flora due to its long history of isolation, its position in the southern Pacific, and the range of ecological niches between the subtropical north and the subantarctic south. However, the high degree of endemism is at least partly an artifact of taxonomic inflation and reflects the lack of monographic studies across the flora. Here we present the finding of the presumed New Zealand endemic *W. lyallii* from southernmost South America, and discuss its distribution in the light of the proposed synonymy with the cold temperate-subantarctic South American species *Cladophoropsis brachyartra* (Svedelius) Wille.

Materials & Methods

A specimen from southern Chile labeled '*Cladophora Brownii*, Harv. ?' was discovered in the herbarium of the Natural History Museum London (BM) in the algal collections (voucher number BM000779086). *Cladophora brownii* Harvey is a synonym of *Aegagropila linnaei* Kützing, a temperate holarctic species, which does not occur in the southern hemisphere. In addition to the geographic location, the marine collection site and the cushion-like growth form consisting of very coarse filaments (Fig. 1) led to the suspicion that this specimen represented misidentified material of *Wittrockiella lyallii*. The specimen was collected in 1867 at Port Gallant (Brunswick Peninsula, Strait of Magellan, southern Chile) by Robert Oliver Cunningham, a naturalist onboard the HMS *Nassau* during an expedition to the Strait of Magellan 1866–69. A small part of this specimen, and herbarium material of *W. lyallii* from New Zealand (Bradshaw Sound, Fiordland, voucher WELT A023866 and Cathedral Caves, Catlins, voucher WELT A023867) for morphological comparison and imaging, were resoaked in water and brought to the boil with a drop of synthetic detergent to allow for full reconstitution of collapsed cells (van den Hoek 1963). Images were taken with a digital camera (ColorView Illu, Soft Imaging Systems) connected to a stereo-microscope (Olympus SZX10).

The suspected occurrence of *W. lyallii* in previously unknown locations in southern cold-temperate and subantarctic waters led to an extensive survey of both original publications of the main (sub)antarctic expeditions and contemporary literature in search of any possible records that could represent *W. lyallii*.

The South American species *Cladophoropsis brachyartra* (Svedelius) Wille was further investigated, and silica gel preserved material was obtained (collected 02 February 2002 by M. E. Ramírez from Isla Dring, Los Chonos archipelago, Aysén, Chile) for sequencing of nuclear ribosomal genes. The small subunit (SSU) rRNA gene, the partial large subunit (LSU) rRNA gene, and the ITS2 region were sequenced for *C. brachyartra*. For *W. lyallii*, SSU and partial LSU sequences were downloaded from GenBank, and the ITS2 region of two samples was sequenced. Collection details and GenBank accession numbers are given in Table 1. DNA extraction, PCR amplification and sequencing were performed as in Boedeker & Immers (2009), modified for the SSU and the ITS2 region as follows: the complete SSU rRNA gene was amplified using the primer pairs SR1-SS11H and SSU897-18SC2 (Leliaert *et al.* 2007a), the 540 bp containing most of the 5.8S rRNA gene and the complete ITS2 region were amplified using the primer pair ITS3-ITS4 (White *et al.* 1990) with an annealing temperature of 53°C.

Table 1. Specimens for which rDNA sequences were compared, collection data (location, collector, date of collection and voucher information) and GenBank accession numbers (newly generated sequences in bold).

Species	**Collection and voucher information**	**SSU rDNA**	**partial LSU rDNA**	**ITS2 rDNA**
Cladophoropsis brachyartra (Svedelius) Wille	high intertidal, Dring Island, Los Chonos archipelago, Aysén, Chile (M.E. Ramírez & D. M. John, 02 Feb. 2002, N61, SGO No. 158361[1])	**GU198502**	**GU198503**	**GU198504**
Wittrockiella lyallii (Harvey) C. Hoek, Ducker et Womersley	high intertidal, Rum River estuary, Bradshaw Sound, Fiordland, New Zealand (S. Heesch, 30 Sep. 2005, H67, UPN626, WELT A023866[1])	AB062717	FN257512	**GU220712**
Wittrockiella lyallii (Harvey) C. Hoek, Ducker et Womersley	high intertidal at cave entrance with mosses (freshwater runoff), Cathedral Caves, Catlins, New Zealand (S. Heesch & R. Dewdney, 25 Mar, 2006, K76, UPN1002, WELT A023867[1])	/	/	**GU936796**

[1] herbarium acronyms according to Holmgren *et al.* 1990.

Maps in Mercator-projection (Figs. 11-12) were created with the online program OMC (not available anymore). The information shown in Fig. 13 on the positions of the subantarctic front (SAF) and the subtropical convergence (STC), and the maximum winter sea ice extent during the last glacial maximum (LGM-WSI, grey area) are based on Bergstrom & Chown (1999) and Gersonde *et al.* (2005).

Results

Molecular analyses

The length of the amplified fragments was 1691 bp, 580 bp, and 516-527 bp for the SSU rRNA gene, the partial LSU rRNA gene, and the ITS2 with flanking regions, respectively. The length of the ITS2 region was 355 bp. None of the sequences contained ambiguous positions. The sequences of these three ribosomal markers of *C. brachyartra* from Chile and *W. lyallii* from New Zealand were identical in all positions (GenBank accession numbers are given in Table 1).

Morphological investigations

'*Cladophora Brownii* Harv.?' - The specimen from southern Chile (BM000779086 - Fig. 1) was identified as *W. lyallii* based on morphological agreement with the original description and all later treatments of the species. The more or less heterotrichous filaments, with cells up to 500 µm in diameter, are typically two to four times as long as broad. Branching can be sparse (Figs. 2-3) or secund (Fig. 4) or irregular, branches are typically inserted subterminally (Figs. 2 & 4). After sprouting a branch, cell wall formation is often delayed (Fig. 4). Apical cells, when not growing into a secondary rhizoid (Figs. 2 & 4), are rounded and typically slightly shorter than the rest.

Wittrockiella lyallii - Resoaked herbarium material of *W. lyallii* from New Zealand is shown in Figs. 5-6 (voucher WELT A023866). The original drawing by Harvey (1855) of the type material of *W. lyallii* (Fig. 7) does not show the most characteristic features of the species, which are clearly depicted in drawings of the type material in Hoek *et al.* (1984) (reproduced in Fig. 8). One specimen (voucher WELT A023867, Table 2) was collected in the Catlins in southeastern New Zealand, a previously unpublished location of *W. lyallii*. This specimen had the broadest cell diameter of all investigated material (up to 740 µm).

Cladophoropsis brachyartra - This species was originally described as *Siphonocladus brachyartrus* by Svedelius (1900) based on material from Puerto Angosto, Isla Desolación (Strait of Magellan, southern Chile), with the following morphological features: forming cushions, consisting of coarse, stiff, brittle filaments with a cell diameter of 200-300 µm, cells being 2-3 times as long as broad, filaments heterotrichous, without primary but with many secondary rhizoids, branch insertion subterminal with delayed cell wall formation, branching irregular or secund, filaments sterile (Fig. 9). The habitat of the type of *S. brachyartrus* is recorded as the high intertidal in a brackish bay with almost freshwater character. Fig. 10 shows *C. brachyartra* in its natural habitat, which corresponds to the habitat of *W. lyallii*.

The location of the type specimen of *S. brachyartrus* seems to be untraceable (Leliaert & Coppejans 2006), but original drawings of the type material are published in Svedelius

Table 2. Examined herbarium specimens of *Wittrockiella lyallii*, with voucher numbers[1] and collection details.

Specimens	**Vouchers**	**Country**	**Location**	**Collection information**
'*Cladophora Brownii* Harv.?'	BM000779086 (L50)	Chile	Port Gallant, Brunswick Peninsula, Strait of Magellan	R. O. Cunningham, 11 Mar. 1867
Cladophoropsis brachyartra	SGO No. 158360	Chile	Traiguén Island, Los Chonos archipelago, Aysén	unknown
Cladophoropsis brachyartra	SGO No. 158361 (N61)	Chile	high intertidal, Dring Island, Los Chonos archipelago, Aysén	M. E. Ramírez & D. M. John, 02 Feb. 2002
Cladophoropsis brachyartra	SGO No. 158362	Chile	Puerto Merino Jarpa, Katalalixar, Aysén	M. E. Ramírez, 05 Feb. 2001
Cladophora lyallii (TYPE)[2]	TCD (lectotype), BM000515828 & BM000515827 (isotypes)	New Zealand	South Island (= Stewart Island)[3]	D. Lyall, 1839-1843
Cladophoropsis lyallii	L385039, WELT A011248	New Zealand	Pryse Peak, Paterson's Inlet, Stewart Island	L. M. Jones, 28 Feb. 1935
Cladophoropsis lyallii	WELT A001053 (*Algae Novae-Zealandiae Exsiccatae* No. 253)	New Zealand	Paterson's Inlet, Stewart Island	collector unknown, February 1947
Cladophoropsis lyallii	L385043, WELT A011114)	New Zealand	Ringaringa Beach, Paterson Inlet, Stewart Island	I. B. Warnock, 01 Mar. 1935
Cladophoropsis lyallii	L385120, WELT A011107	New Zealand	Ocean Beach, Stewart Island	I. B. Warnock, 26 Feb. 1935
Cladophoropsis lyallii	L385037, WELT A011236	New Zealand	Half Moon Bay, Stewart Island	I. B. Warnock, 19 Feb. 1935
Wittrockiella lyallii	WELT A023866 (UPN626; H67)	New Zealand	high intertidal, Rum River estuary, Bradshaw Sound, Fiordland, South Island	S. Heesch, 30 Nov. 2005
Wittrockiella lyallii	WELT A013201	New Zealand	Milford Sound, Fiordland, South Island	J. Hunt & P. Anderson, 12 Jul. 1982
Wittrockiella lyallii	WELT A009968	New Zealand	Pickersgill Harbour, Dusky Sound, Fiordland, South Island	C. H. Hay, 08 Dec. 1977
Wittrockiella lyallii	WELT A023867 (UPN1002; K76)	New Zealand	high intertidal rocks (freshwater runoff), Cathedral Caves, Catlins, South Island	S. Heesch & R. Dewdney, 25 Mar. 2006
Wittrockiella lyallii	WELT A018583	New Zealand	high intertidal rocks, Point Durham, Chatham Islands	W. A. Nelson, 04 Mar. 1987
Wittrockiella lyallii	WELT A016130	New Zealand	high intertidal rocks, Boat Harbour, The Snares Islands	G. Hardy, December 1984

[1] herbarium acronyms according to Holmgren *et al.*, 1990, additional labeling of the same specimen in brackets.

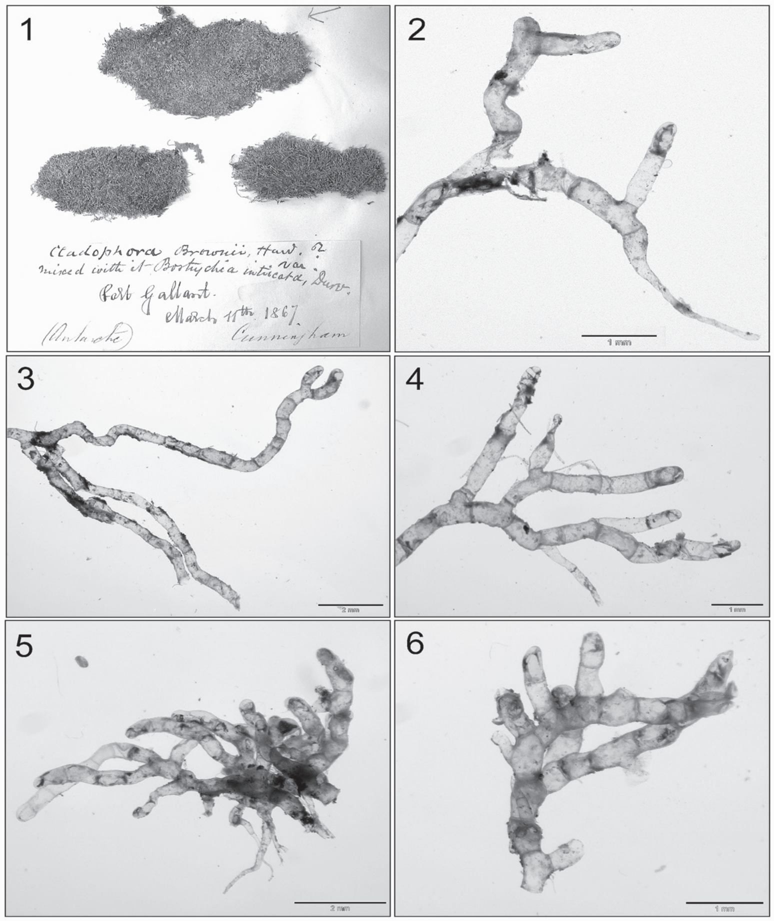
[2] The material in BM was received from TCD (in 1900) – the TCD material should be regarded as the type, the BM material as an isotype.

[3] Lyall referred to what is now known as the South Island as Middle Island.

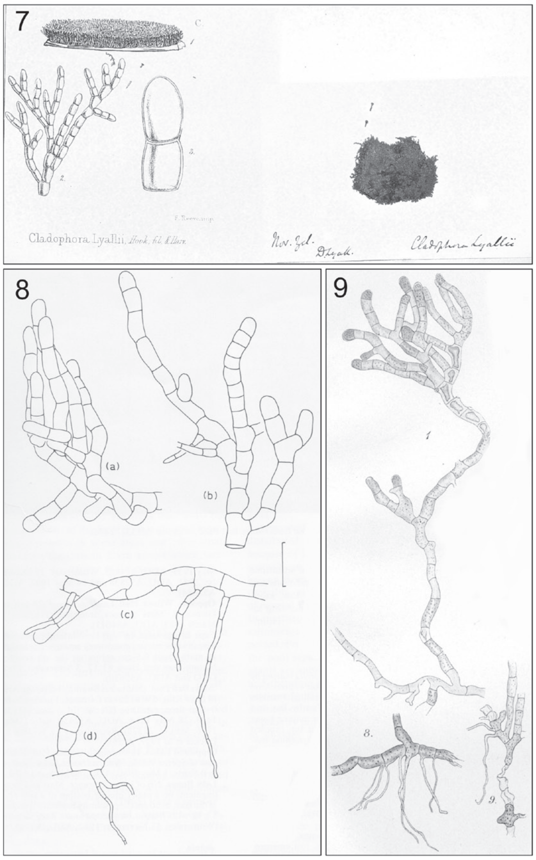
(1900) and are reproduced in Fig. 9.

Distribution of taxa

In addition to the type location, *C. brachyartra* has been reported from Reloncaví estuary, Bahía Sotomó (Puerto Montt, Chile) by Levring (1960); from Chiloé Island, western and southern Patagonia by Hylmö (1938) and Skottsberg (1941); from 'southern Chile and Patagonia' (Ramírez & Santelices 1991); from the Elefantes gulf and estuary, Laguna San Rafael National Park, Aysén (John *et al.* 2003); and from Tierra del Fuego Argentina (Borasode Zaixso 2004). In addition, specimens housed in the Museo Nacional de Historia Natural (Santiago, Chile) are from Quinchao Island (Chiloé Island), from Dring Island and from Traiguén Island (Los Chonos Archipelago, Aysén), and from Puerto Merino Jarpa (Katalalixar, Aysén) (all examined specimens are listed in Table 2). All currently known locations of *W. lyallii* (including *C. brachyartra*) are shown in Figs. 11-12.



Figs. 1-6. *Wittrockiella lyallii* from South America (Figs 1-4, voucher BM000779086) and New Zealand (Figs 5-6, voucher WELT A023866). **1** Herbarium specimen consisting of cushions of coarse filaments that were re-identified as *W. lyallii*. **2** Sparsely branched filament with a secondary rhizoid formed by the apical cell. **3** Sparsely branched filament. **4** Filament showing secund branching, subterminal insertion of branches and apical rhizoid formation. **5** Heterotrichous filament with secondary rhizoids. **6** Filament showing secund branching and rounded apical cells.



Figs. 7-9. Comparison of drawings of the type material of *Wittrockiella lyallii* (Figs 7-8) and *Cladophoropsis brachyartra* (Fig. 9). **7** Type material (isolectotype, BM 000515827) and original drawing by Harvey (1855) of *Cladophora lyallii*. **8** Drawings of the type material from Hoek *et al.* (1984), showing irregular branching, subterminal insertion of branches, rounded apical cells and secondary rhizoids. Scalebar = 1000 µm. **9** Drawings of the type material of *Siphonocladus brachyartrus* by Svedelius (1900), showing essentially the same morphological characters as in Fig. 8.

Neither *W. lyallii* nor *C. brachyartra* nor any other morphologically similar species has been mentioned in algal floras or species lists of temperate or subantarctic islands in the South Pacific (except for *W. lyallii* on the Snares Islands), such as Tasmania, the New Zealand subantarctic islands, Macquarie Island, Gough Island, Tristan da Cunha, the Juan Fernández archipelago, the Antarctic Peninsula, Kerguelen Islands, Falkland Islands, South Georgia, South Orkney Islands, South Shetland Islands, Crozet Islands, St. Paul Island, Prince Edward & Marion Islands, and Heard Island.

As a result of the molecular and morphological investigations presented here we conclude that *C. brachyartra* and *W. lyallii* are conspecific and therefore *C. brachyartra* is synonymised with *W. lyallii*.

Wittrockiella lyallii (Harvey) C. Hoek, Ducker & Womersley 1984: 45
holotype: Stewart Island, New Zealand, collector D. Lyall, TCD (isolectotypes in BM).
basionym: *Cladophora lyallii* Harvey 1855: 262
synonyms: *Siphonocladus brachyartrus* Svedelius 1900: 304, *Cladophoropsis brachyartra* (Svedelius) Wille 1910: 116, *Cladophoropsis lyallii* (Harvey) V.J. Chapman 1956: 471.

Discussion

Conspecificity of C. brachyartra *and* W. lyallii

It had already been noted earlier that the placement of *C. brachyartra* in the genus *Cladophoropsis* is not correct (Leliaert & Coppejans 2006), but no alternative affiliation had been suggested. Chapman (1956) gives a maximum filament diameter of up to 400 µm for *W. lyallii*, while filaments of *C. brachyartra* can be up to 660 µm in diameter (Hylmö 1938). However, our measurements of specimens of *W. lyallii* from New Zealand extend the range to 740 µm. Generally, morphological data of *W. lyallii* is based on few collections and the entire morphological range is unlikely to have been fully assessed. All other morphological features of specimens from New Zealand and South America are congruent, and the fact that all three sequenced ribosomal markers including the variable ITS2 are identical, lead us to conclude that these taxa are conspecific.

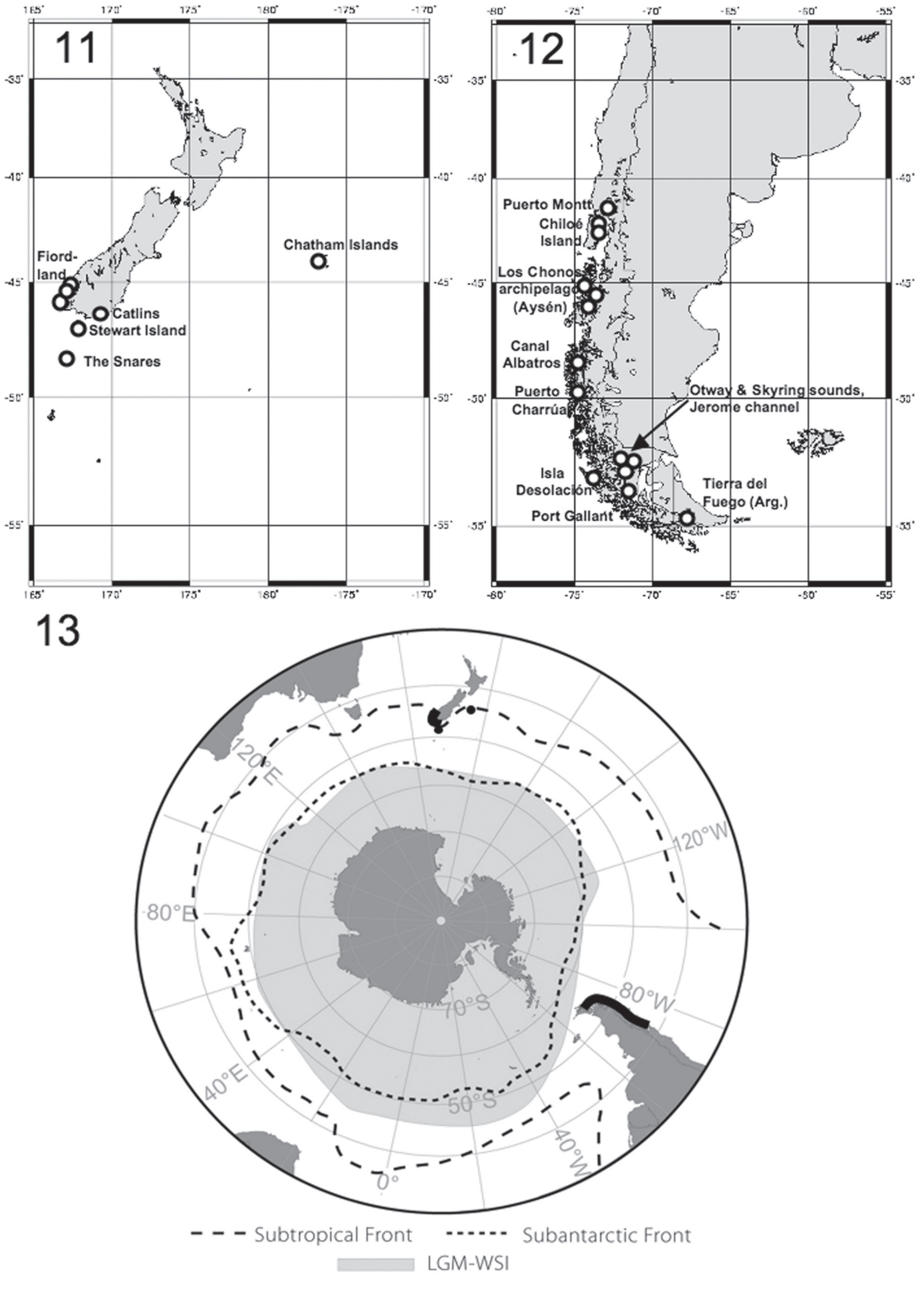
Why did Svedelius not consider the morphological similarity of these taxa when he described *S. brachyartrus* as a separate species? *Wittrockiella lyallii* was originally described as a *Cladophora* species, a possible taxonomic affinity that Svedelius (1900) only mentions briefly when discussing the generic placement of the material from Isla Desolación (Chile). Instead he saw stronger evidence in the delayed cell wall formation for inclusion in the genus *Siphonocladus* and possibly he was misled by Harvey's uninformative drawing of the type of *Cladophora lyallii* (Fig. 7).

Both *C. brachyartra* and *W. lyallii* occur in specialized habitats, typically in the intertidal in areas with freshwater seepage. Freshwater influence is explicitly mentioned for the collection sites in the Argentinian part of Tierra del Fuego (Boraso de Zaixso 2004), and most of the Chilean locations (Hylmö 1938, Skottsberg 1941, Levring 1960, John *et al*. 2003) have a reduced salinity as well (*e.g.,* Otway Sound at the river mouth, Skyring Sound, Jerome channel, Golfo and Estero Elefantes, Canal Albatross, Reloncaví estuary (Bahía Sotomó), and the Quemchi region in northeastern Chiloé Island; see Fig. 12 for locations). The situation in eastern Chiloé (Dávila *et al*. 2002) seems to be quite similar to the marine environments in the Fiordland region of New Zealand, with a low salinity surface layer more than one meter thick forming above more saline water due to intense rainfall and mountain run-off (Gibbs 2001, Nelson *et al*. 2002). In Chile, *C. brachyartra* occurs from the intertidal spray zone to rockpools in the lowermost intertidal, on beach rocks, vertical cliffs, and logs at water's edge in areas with a salinity of 25-34 psu, often in shaded locations (Skottsberg 1941, John *et al*. 2003), similar habitats to those of *W. lyallii* in New Zealand (Adams 1994).

Fig. 10. *Cladophoropsis brachyartra* (=*Wittrockiella lyallii*) in its natural habitat: a patch ca. 50 cm in diameter on vertical and shaded rocks, high intertidal, Chequián/Achao, Isla de Quinchao, Chiloé, Chile (27 January 2002).

Extended distribution of W. lyallii

The inclusion of *C. brachyartra* in *W. lyallii* extends the range of this species considerably, and results in a widely separated disjunct distribution pattern (Figs. 11-13). Populations from New Zealand and South America are separated by ca. 9000 km of Pacific Ocean. The northern distribution limits are the Chatham Islands (43°53'S, New Zealand) and Puerto Montt (41°64'S, Chile), differing only by approximately two degrees of latitude (Figs. 11-12). Records of *C. brachyartra* from northern locations (Valparaíso) of the southern Chilean coast (Etcheverry 1986) are misidentifications (M. E. Ramírez, pers. obs.). Along the coast of South America the southern distribution extends about 800 km further south than in New Zealand: *W. lyallii* does not seem to occur on the New Zealand subantarctic islands south of the Snares Islands. The locations in New Zealand are roughly situated within the sub-



Figs. 11-13. Amended distribution of *Wittrockiella lyallii* in the southern Pacific (Figs 11-12) and oceanographic features of the southern ocean (Fig. 13). **11** Map of New Zealand and its subantarctic islands with the known locations of *W. lyallii*. **12** Map of southernmost South America with the known locations of *W. lyallii* (as *Cladophoropsis brachyartra*). **13** Map of the southern ocean indicating the positions of the subantarctic front (SAF), the subtropical convergence (STC), the maximum winter sea ice extent during the last glacial maximum (LGM-WSI, grey area) (after Bergstrom & Chown 1999; Gersonde *et al.* 2005). Thick black lines and black dots show the distribution of *W. lyallii*.

tropical front (STF), whereas the South American populations are located south of the STF and north of the subantarctic front (Heath 1985, Belkin & Gordon 1996). Accordingly, southern South American populations occur at considerably lower sea surface temperatures (SSTs). Annual SSTs in New Zealand locations range from 11°C in the Snares islands to 13-14°C in Fiordland (Chown *et al.* 1998, Uddstrom & Oien 1999), while the SSTs in South America range from 13°C (Chiloé) to 7.5°C in southwestern Patagonia and Tierra del Fuego (Sepúlveda *et al.* 2009). The northern distribution limit in New Zealand and South America is similar both geographically and with regards to SSTs, and corresponds roughly to the northern edge of the west wind drift (WWD) (Strub *et al.* 1998). This major oceanic current coming from the west splits into the northward flowing Peru Chile Current and the southward flowing Cape Horn Current in the region around 41-42°S on the South American west coast, and this is a typical border in the distribution for many taxa, including seaweeds (*e.g.,* Santelices & Meneses 2000, Hernández *et al.* 2005). The high percentage of subantarctic species (ca. 35%) in the algal flora of Chile (Santelices & Marquet 1998) might be linked to the connection of subantarctic regions via the WWD. Whereas the northern distribution limit of *W. lyallii* might well be explained by SSTs, the absence from the subantarctic islands of New Zealand cannot be explained by current SSTs being too low, since the species occurs at comparable or even lower temperatures in South America.

Why does W. lyallii *not occur on subantarctic islands?*

The availability of suitable habitats is a decisive factor influencing the presence or absence of a species in a particular location or region. *W. lyallii* is restricted to shaded habitats with freshwater influence, and does not occur on very exposed rocky shores. This preference makes establishment on the exposed, rocky and largely unvegetated New Zealand subantarctic Bounty Islands and Antipodes quite unlikely (*e.g.,* Hay *et al.* 1985). Only future collection trips targeting the specific habitats will tell whether *W. lyallii* has been previously overlooked on the larger New Zealand subantarctic Auckland and Campbell Islands. These islands have a much wider range of habitats available for colonization. In particular there may be appropriate habitats in Carnley Harbour (Auckland Islands) where there is some shelter, freshwater input and overhanging vegetation.

Taking the observed annual SST range of ca. 7-13°C as a guideline (and excluding other possibly restricting factors), potentially suitable locations for *W. lyallii* in the southern ocean are the Falkland Islands, Gough Island, and the subantarctic islands of New Zealand (Chown *et al.* 1998). Amsterdam Island, St. Paul, Tristan da Cunha and the Juan Fernández Archipelago have higher annual SSTs (15-19°C) than what may be the upper limiting temperature for *W. lyallii*. South Georgia, Marion, Crozet, Kerguelen, Heard and Macquarie Islands have annual SSTs of 1.5-6°C, however, there is no indication that the lower temperature limit of *W. lyallii* was reached at 7.5°C in the very south of South America. The latter islands have been subject to extensive winter sea ice (WSI) scour though, which might prevent settlement or survival of *W. lyallii* populations in the intertidal.

The past extent of WSI plays a role in shaping the current distributions of intertidal species in the southern ocean. In a recent study on the phylogeography of the circumpolar intertidal species *Durvillaea antarctica* (Chamisso) Hariot (bullkelp), one dominant haplotype was found on all subantarctic islands that had been sampled, except on the New Zealand subantarctic islands (Fraser *et al.* 2009). The proposed explanation for a single widespread haplotype was extinction by ice scour during the last glacial maximum

(LGM) and subsequent rapid recolonisation of those islands, facilitated by the high rafting ability and the long fertility period of *D. antarctica*. The amount of floating thalli of *D. antarctica* is enormous: it has been estimated that 70 million rafts of *D. antarctica* are present in the southern ocean at any given time (Smith 2002). From the recolonisation pattern of *D. antarctica*, it was inferred that during the LGM the WSI extended further than previously estimated based on microfossil records (Gersonde *et al*. 2005). It seems probable that an intertidal species such as *W. lyallii* would have been affected by ice scour in a similar way to the hypothesized impacts on *D. antarctica*. The only offshore islands where *W. lyallii* has been found are Stewart Island, the Snares Islands and the Chatham Islands in the New Zealand region. Gough Island and the subantarctic islands of New Zealand, the Falkland Islands, and possibly Macquarie Island, were inferred to not have been affected by LGM-WSI (Fraser *et al* 2009), and thus could have functioned as refugia for *W. lyallii* (Fig. 13). However, *W. lyallii* has not been recorded from these locations (except the Snares Islands) despite considerable collection activity (Gain 1912, Cotton 1915, Taylor 1939, Skottsberg 1941, Chamberlain 1965, Hay *et al*. 1985, Ricker 1987). Stewart Island and the South Island are separated by only 30 km of water and have been repeatedly connected during glacial periods. The Snares Islands are situated only 200 km south of the South Island. The Chatham Islands are located in the STC ca. 800 km to the east of New Zealand (or rather 1400 km from the nearest extant population of *W. lyallii* on Stewart Island), but in a position favourable for migration from the mainland via the main oceanic currents (South 1978). However, these distances are much less than the ca. 9000 km between New Zealand and Chile, a voyage that would take 1-2 years traveling in the ocean current (Smith 2002, C. Law - pers. comm.). *W. lyallii* does not have any special floating ability, and would appear to be a poor disperser. Reproduction and dispersal in *W. lyallii* seem to take place only vegetatively via fragmentation of filaments. Successful establishment in a new environment after dispersal of a thallus fragment would be facilitated by subsequent spore or gamete release, but spore formation has never been observed in *W. lyallii*. A more likely long-distance dispersal mechanism for *W. lyallii* than direct transport of thallus fragments is the transport for example via floating tree logs. *W. lyallii* has been found growing on trees at the high water mark, and trees from the dense coastal forests of Fiordland or Stewart Island could have reached the ocean as a consequence of coastal erosion (see Thiel & Gutow 2004 for a review).

Long-distance dispersal vs. *vicariance*

The distribution of *W. lyallii* raises the question whether the disjunction between New Zealand and South America is explained by vicariance rather than by long-distance dispersal. In recent years, molecular data have led to a resurrection of trans-oceanic dispersal theories (*e.g.,* de Queiroz 2005, McGlone 2005), and many examples of successful long-distance dispersal have been identified (*e.g.,* Cook & Crisp 2005, Waters 2008, Fraser *et al.* 2009). Wallis & Trewick (2009) presented evidence that most of the New Zealand terrestrial flora derives from long-distance dispersal. If a Gondwanan origin and absence of long-distance dispersal are assumed for *W. lyallii*, the absence from potentially suitable subantarctic islands could be explained by the young geological age of Gough Island and the New Zealand subantarctic islands (Chown *et al.* 1998). However, the fact that ITS2 sequences of *W. lyallii* specimens from New Zealand and Chile are identical strongly implies recent dispersal from a source population. This variable marker provides good resolution at the species level in the Cladophorales (*e.g.,* Bakker *et al*. 1992, 1995, Leliaert *et al.* 2008, 2009b). Generally, ancient separations of taxa that date back to the breakup of Gondwana are reflected by high genetic divergence (*e.g.,* De Wever *et al*. 2009). Among the three species of *Wittrockiella*, the sequence divergence in the ITS2 region is ca. 20-30%, but the sequences are difficult to align (Boedeker, unpublished data). Thus, identical ITS sequences strongly indicate conspecificity of the Chilean and New Zealand populations of *W. lyallii*. Since the WWD is the dominant oceanic current in the southern ocean, the most likely dispersal direction for *W. lyallii* would have been from New Zealand to South America. The absence from subantarctic islands suggests that long-distance dispersal might be a rare event.

Future work including fieldwork targeting specific habitats in the subantarctic islands, culture experiments on the dispersal capacity and life history of *W. lyallii*, detailed phylogeographic studies of all species of *Wittrockiella*, and dated chronograms of the *Aegagropila*-lineage will shed more light on these interesting distribution patterns in the southern ocean.

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Nuclear rDNA sequences of *Wittrockiella amphibia* (Collins) comb. nov. (Cladophorales, Chlorophyta) and morphological characterization of the mat-like growth form

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Abstract

Cladophora amphibia was found in Yaquina Bay (Oregon, USA) for the first time since the type collection from California in 1903. Vegetative plants were buried in the top centimeter of intertidal mud, partially covered by mats of *Rhizoclonium* and *Chaetomorpha*. *C. amphibia* plants had densely pigmented upright branches penetrating the mud surface and overlying algal mats. Morphological and habitat characteristics of *C. amphibia* strongly overlap with those of *Wittrockiella paradoxa* Wille, and molecular sequences of ribosomal genes, including the variable ITS2 region, indicate very high genetic similarity between these two taxa. The seven point mutations in the ITS2 DNA sequences are regarded as low intraspecific variation. Since *C. amphibia* had been described earlier, *W. paradoxa* Wille becomes a taxonomic synonym of *C. amphibia*, and the new binomial *Wittrockiella amphibia* (Collins) comb. nov. is required. As had been previously reported for *W. paradoxa*, the studied Pacific plants were 'cladophoroid' in form (upright, branched thalli with cylindrical cells), in contrast to the stunted and poorly branched 'rhizoidal' plants mainly found in Europe. The heterotrichous mode of growth is further characterized.

Introduction

Cladophora amphibia Collins 1907 is known only from its type collection from a saltmarsh in central California (Collins 1907, Setchell & Gardner 1920), and it has not been collected or recorded again in more than 100 years. It was described as forming a thin layer on the sediment in the highest intertidal in a manner similar to the heterotrichous system of matted filaments reported for *Wittrockiella paradoxa* Wille 1909 by South (1981). The thallus of *C. amphibia* consists of matted lower parts with irregular to subcylindrical cells (40-70 µm in diameter) and more regular erect branches (30-50 µm in diameter), resembling a *Vaucheria* thallus in habit (Collins 1907). Some cells were reported to be swollen in the middle to 100 µm in diameter. The cross-walls that cut off branches from the mother cell occur at some distance from the base as in *Cladophoropsis*. Overall, this species appears to be morphologically very similar to members of the genus *Wittrockiella* and to *W. paradoxa* in particular.

The brackish-water genus *Wittrockiella* is a small group of ulvophycean green algae. It is a member of the *Aegagropila*-lineage, an assemblage of species-poor, mainly freshwater genera such as *Aegagropila*, *Arnoldiella*, *Basicladia*, and *Pithophora* (Hanyuda *et al.* 2002, Yoshii *et al.* 2004). This lineage is one of the four lineages recognized within the Cladophorales (Leliaert *et al.* 2009a). *W. paradoxa* is the type species of the genus (Wille 1909), the other two species in the genus are *W. lyallii* (Harvey) C. Hoek, Ducker *et* Womersley and *W. salina* V.J. Chapman. The placement of *W. paradoxa* and *W. lyallii* within the same genus has been verified by molecular data (Yoshii *et al.* 2004). Moreover, there is speculation that *W. paradoxa* might be related to *Cladophorella calcicola* Fritsch (van den Hoek *et al.* 1984), a morphologically similar warm-temperate to tropical species that typically occurs semi-terrestrially on moist limestone and has been found in Australia, Bangladesh, and tropical hothouses in Europe (Fritsch 1944, Islam 1964, Cribb 1965).

Wittrockiella paradoxa was originally described from a brackish saltmarsh in southwestern Norway as forming a cartilaginous mucilage-capsule in a cyanobacterial crust. The filaments were short, poorly branched and had very irregular to roundish cells. Cells of both *W. paradoxa* and *W. salina* are known to produce long septate hairs (Wille 1909, van den Hoek *et al.* 1984), but these are often absent. Akinetes or aplanospores can form singly or in short chains within the filaments (Wille 1909).

Additional morphological observations have broadened interpretation of the species, and more densely branched growth forms have been described (Polderman 1976, South 1981). The short, poorly branched filaments consisting of round or irregular cells have been termed the ‘rhizoidal growth form’; the more densely branched and upright plants consisting of irregular to cylindrical cells have been termed the ‘cladophoroid growth form’ (Polderman 1976, South 1981, South 1989). Cell sizes range from 8-20 µm in diameter and 10-100 µm in length in the rhizoidal form, and 20-80 µm in diameter and 80-250 µm in length in the cladophoroid form (Polderman 1976, South 1981).

In addition to the crust-like habit described for the type material, *Wittrockiella paradoxa* can form mats on soft estuarine sediments or occur as as epi- and/or endophytes in saltmarsh plants (Polderman 1976, South 1981, 1989). *W. paradoxa* has a wide distribution in the Northern Hemisphere: records are known from the Wadden Sea (Germany and the Netherlands; Polderman 1976), southern and southwestern England (Polderman 1976), the Atlantic coast of France (Dizerbo & Herpe 2007), southwestern Norway (Wille 1909), northwestern Spain (Calvo *et al.* 1999), northeastern America (Collins *et al.* 1910, South

1989), and the Pacific coast of northwestern America (South 1981). It most likely is more widespread, but it could easily have been overlooked due to its unobtrusive habit. In the Atlantic locations, the rhizoidal form seems to dominate. In the Pacific, the cladophoroid form was mostly reported, occurring as a 'greenish fur' on the surface of mud, but the rhizoidal form was also found (South 1981). Through culture work, it has been shown that the rhizoidal form can develop into the cladophoroid form (Polderman 1976, South 1981).

During surveys of the algal flora of Oregon, patches of *Cladophora amphibia* mats were discovered in the Yaquina Bay estuary. The mats are morphologically and ecologically characterized here. In addition, sequences of nuclear ribosomal genes, including the variable internally transcribed spacer 2 region, have been generated for the Oregon material of *C. amphibia* and for a cultured Washington isolate of *Wittrockiella paradoxa*. Due to our findings, the synonymy of *W. paradoxa* with *C. amphibia* is proposed.

Materials and Methods

The following specimens were investigated morphologically:

(1) Field-collected *Cladophora amphibia* (Leiden L0793284, L0793286). Loc. USA, Oregon, Yaquina Bay, Sally's Bend (44.6288N 124.024W); Leg. G.I. Hansen, 07 May 2006 (L0793286) and 20 November 2009 (L0793284). Both were collected from the surface layer of mud under *Chaetomorpha aerea* (Dillwyn) Kützing/*C. linum* (O.F. Müller) Kützing and *Rhizoclonium* sp. mats in an intertidal estuarine habitat (Figs. 1-2) further characterised by Cyanobacteria and *Vaucheria* spp.

(2) Two pressed isotypes of *Cladophora amphibia* (Leiden L1284, L2989). Loc. USA, San Francisco Bay, Alameda; Leg. W.J.V. Osterhout and N.L. Gardner, 26 September 1903. Both were collected from damp soil among *Salicornia* sp. in a saltmarsh. Vouchers in the Leiden herbarium were distributed as Exsiccatae Phycotheca Boreali-Americana No. 1284 (Collins *et al.* 1905).

(3) Cultures of *Wittrockiella paradoxa* (CCMP1674 culture). Loc. USA, Washington, San Juan Islands, Lopez Island (48.420N 122.900W), 1977. Leg. C. O'Kelly. Original material was endophytic in *Zostera marina* Linnaeus leaves.

For morphological comparison and imaging, parts of dried specimens were re-soaked in water and brought to a boil with a drop of synthetic detergent to allow for full reconstitution of the collapsed cells (van den Hoek 1963). Images were taken with a digital camera (ColorView Illu, Olympus Soft Imaging Systems, Münster, Germany) connected to a Olympus SZX10 stereo-microscope and a Olympus BH2 light microscope (Olympus Optical Co. GmbH, Hamburg, Germany). Fresh collections of *C. amphibia* from Oregon were photographed with a Leica DFC 290 digital camera (Leica Camera AG, Solms, Germany) attached to Zeiss Axioskope and Stemi SR microscopes (Carl Zeiss Jena GmbH, Jena, Germany).

We sequenced the small subunit (SSU) rRNA gene (GenBank GU384872), the partial large subunit (LSU) rRNA gene (GenBank GU384873), and the ITS2 region (GenBank GU384875) of *Cladophora amphibia* (specimen L0793284). For *Wittrockiella paradoxa* (CCMP1674), the SSU sequence was downloaded from GenBank (AB078732), and the partial LSU (GenBank GU384874) and the ITS2 (GenBank GU384876) sequences were newly generated. DNA extraction, PCR amplification and sequencing were performed following Boedeker and Immers (2009), but modified for the SSU and the ITS2 region as follows: the complete SSU rDNA gene was amplified using the primer pairs SR1-SS11H and SSU897-18SC2 (Leliaert *et al.* 2007a), the ca. 530 bp containing most of the 5.8S rRNA gene and the complete ITS2 region were amplified using the primer pair ITS3-ITS4 (White *et al.* 1990) at an annealing temperature of 53°C. The ITS2 sequence of *C. amphibia* resulting from direct sequencing contained several ambiguous positions, therefore the PCR product was cloned into the pCR®II vector using the TOPO® TA Cloning® Kit (Invitrogen Ltd., Carlsbad, USA) according to the manufacturer's protocol and re-sequenced.

Results

The material from Oregon (specimens L0793284 and L0793286) consisted of a buried prostrate system with erect branches penetrating the surface of the mud and covering algae (Figs. 3-4). The creeping main axes were densely branched with upright shoots (Figs. 3-5) and produced few rhizoidal filaments (Fig. 5). Reproductive structures were not observed, except one instance of possible beginning akinete formation (Fig. 5). The erect shoots were poorly branched, and were more densely pigmented than the buried prostrate filaments, especially the apical cells that penetrated the mud surface (Figs. 6-8). Erect branches developed with delayed cross-wall formation at more or less right angles to the prostrate supporting filament (Fig. 8). Typically, filaments ranged from 48 µm to 80 µm in diameter, but rarely they were only 28 µm. The material was morphologically identified as *Cladophora amphibia* Collins.

The long-term culture of *Wittrockiella paradoxa* obtained from the CCMP culture collection (strain 1674) contained only rare erect branches and did not show the pronounced differentiation of a prostrate and an upright system. Instead many cells produced outgrowths that were reduced in diameter and were not cut off by cross walls. Occasionally pseudodichotomies were formed. Furthermore, many akinetes and aplanosporangia (Fig. 9) were present. Although quite different from these cultures, the material from Oregon was similar to field collections of *W. paradoxa* from the same location as the CCMP culture (South 1981).

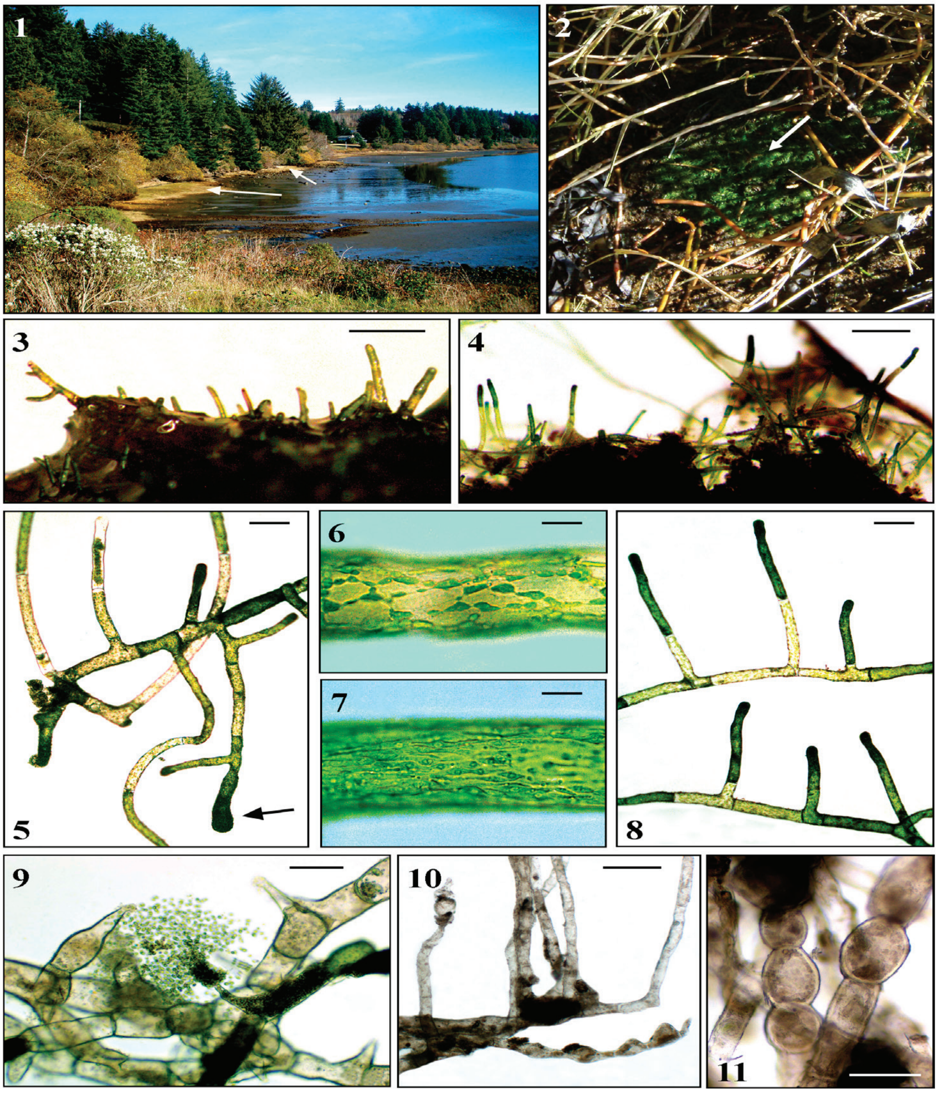
The reconstituted type material of *Cladophora amphibia* consisted of heterotrichous filaments similar to the material from Oregon. Erect branches were formed at right angles and cut off by cross-walls at some distance from the base (Fig. 10). Akinetes up to 95 µm in diameter were present singly and in short chains (Fig. 11).

The SSU and LSU sequences of *C. amphibia* from Oregon and of *W. paradoxa* (CCMP1674) from Washington were identical. In the 364 bp-long ITS2 region, seven point mutations were found between the two specimens, corresponding to less than two percent pairwise differences.

In addition to their morphological similarity, the low sequence divergence in this variable marker indicates that the two species should be considered conspecific. This requires the new combination *Wittrockiella amphibia* (Collins) comb. nov., since the older name *Cladophora amphibia* Collins 1907 has priority over *W. paradoxa* Wille (1909). The holotype of *W. paradoxa* (Lyngør, Norway, Leg. N. Wille, 1907, in L) remains the type specimen for the genus *Wittrockiella*.

***Wittrockiella amphibia* (Collins) Boedeker et Hansen**

Isotypes: Alameda, San Francisco Bay, California, USA, Leg. W.J.V. Osterhout and N.L. Gardner, 26 September 1903, in L, voucher numbers L1284 and L2989, L1284 distributed



Figs 1-11. Habitat, heterotrichous mats and morphological features of *Wittrockiella amphibia* (Collins) comb. nov. from the Pacific. **Figs. 1-2.** Habitat of *Wittrockiella amphibia* (Collins) comb. nov. **1** Estuarine habitat: Sally's Bend, Yaquina Bay, Oregon, USA. Arrows indicate collection sites. **2** *Chaetomorpha* mat (ca. 10 cm in diameter, arrow) between saltmarsh plants covering *W. amphibia* mats. **Figs. 3-11.** Habit and morphological features of *W. amphibia* (Collins) comb. nov. from the northwestern Pacific. **Figs. 3-8.** Material from Sally's Bend, Yaquina Bay, Oregon, USA, November 2009 (specimen L0793284). **3** Prostrate system buried in mud, upright branches penetrating the surface. Scalebar = 500 μm. **4** *W. amphibia*-mat with most of the sediment washed away, showing erect branches with densely pigmented apical cells and parts of the prostrate system. Scalebar = 500 μm. **5** Rhizome-like filament of the prostrate system with upright branches, a rhizoidal filament, and possibly beginning akinete-formation (arrow). Scalebar = 200 μm. **6** Sparsely pigmented cell of the prostrate system, showing the reticulate chloroplast. Scalebar = 10 μm. **7** Densely pigmented apical cell of an erect branch, pyrenoids visible. Scalebar = 20 μm. **8** Upright branches with densely pigmented apical cells, sprouting at right angles and cut off at some distance from the base. Scalebar = 200 μm. **Fig. 9.** Cultured filaments (as *W. paradoxa* (CCMP1674)) showing a sporangium releasing numerous aplanospores through an elongated exit tube. Scalebar = 100 μm. **Figs. 10-11.** Type material of *C. amphibia*, from Alameda, San Francisco Bay, California, USA, 26 September 1903 (voucher number in Leiden herbarium L1284). **10** Heterotrichous growth with erect branches sprouting at right angles from the prostrate filaments. Note one prostrate filament with beginning branch formation ("bumps"). Scalebar = 200 μm. **11** Round and ellipsoid akinetes arranged singly and in short chains. Scalebar = 100 μm.

as Exsiccatae Phycotheca Boreali-Americana (PB-A) No. 1284 (Collins *et al.* 1905). Additional isotypes distributed as No. 1284 of the PB-A to AHFH, BM, FH, PC, TRH, UC and WIS (visit http://sweetgum.nybg.org/ih) for herbarium acronyms), among others. We select the specimen L1284 as the lectotype.

Basionym: *Cladophora amphibia* Collins 1907: 200.
Synonym: *Wittrockiella paradoxa* Wille 1909: 220-221.

Discussion

The initial purpose of our study was to identify the partially buried, heterotrichous mats of green cladophoroid filaments from Oregon. We were able to morphologically compare these filaments with the type description and type specimens of *Cladophora amphibia* from California and with published reports and cultured material of *Wittrockiella paradoxa*. In addition to showing morphological similarity between all of these specimens, we generated molecular data that showed little variation between *C. amphibia* and *W. paradoxa*, leading to the synonymy of these species and to the new combination *Wittrockiella amphibia*.

The current concept of *Wittrockiella paradoxa* already differed substantially from the original description of the species, due to later findings of a greater range in morphology and growth forms. In Wille's description (1909), *W. paradoxa* had no cylindrical cells, no upright system and was very poorly branched. The typical right angled branches and the delayed cell wall formation had not yet been observed. Fritsch (1935) commented on the 'apparent lack of a prostrate system', which was only later discovered to develop in culture (Polderman 1976) and in the field (South 1981). Other later morphological findings included the ability of the thallus to convert in culture from the rhizoidal to the cladophoroid form (Polderman 1976, South 1981), and the discovery of heterotrichous mats in the field (South 1981). These findings, together with the morphological match of *Cladophora amphibia* (Collins 1907) and the cladophoroid form of *W. paradoxa*, and the high genetic similarity between *C. amphibia*-like plants and *W. paradoxa*, make us consider them conspecific. Molecular sequences are increasingly leading to the discovery of taxonomic synonymy of separately described taxa (*e.g.,* Leliaert *et al.* 2008, West & Zuccarello 2009). It had previously been speculated by Silva (1979) that *Cladophora amphibia* may represent a species of *Spongomorpha*, but the present study shows an affiliation with the genus *Wittrockiella* instead.

Since all available sequences originate from the Pacific, there is the possibility that *Wittrockiella amphibia* represents a separate cryptic species of *Wittrockiella* in the Pacifc, with *W. paradoxa* only occurring in the Atlantic. The new name *W. amphibia* would still remain stable for the Pacific species in that case. The existence of one additional undescribed species of *Wittrockiella* that only occurs endophytically in *Zostera* leaves has been mentioned for the northeastern Pacific (Gabrielson *et al.* 2006), and preliminary molecular data suggest that it is a distinct species, not *W. amphibia* (C. O'Kelly - pers. comm.).

Unfortunately, no Atlantic specimens could be obtained for our molecular analyses. The sequence divergence in the ITS2 region between *Wittrockiella amphibia*, *W. salina* and *W. lyallii* is about 20-30%, but the sequences are difficult to align (Boedeker, unpublished data). Thus, the seven point mutations in the ca. 360 bp long ITS2 sequences between material from Washington and Oregon are regarded as low intraspecific variation. The synonymy of the Californian *Cladophora amphibia* with *W. paradoxa* extends the distributional range of the species in the North Pacific by approximately 1000 km further to the south. The species is possibly more widespread in temperate regions of the Northern Hemisphere than is currently known.

The densely pigmented assimilatory cells that penetrate upward through the mud and the covering mats of filamentous green algae contrast greatly with the weakly pigmented cells of the prostrate system. This heterotrichous growth form in mud might be an ecological adaptation to unstable environmental conditions in the estuarine habitat. Burial in soft sediment would help to retain moisture during low tides. Earlier authors have suggested that the species also has other strategies for protection against desiccation and fluctuating salinities. These include the formation of a thick gelatinous cover produced by swelling of the outer cell wall layers (Wille 1909), an endophytic lifestyle (Polderman 1976), and the presence of haematochrome/oil droplets (Wille 1909).

The rhizoidal growth form has been found both as endo- and epiphytic plants and in crusts on the sediment (Wille 1909, Polderman 1976, South 1989). Whether the rhizoidal or the cladophoroid form occurs or dominates could be either habitat-specific, or induced by physical environmental factors, such as different seasons. The few studies on the species provide only limited information on the vegetative or reproductive seasonality. In northwestern Europe, *Wittrockiella paradoxa* was reported to show no obvious seasonality in the epiphytic growth form while the soil growth form was most abundant in spring (Polderman 1976). In saltmarshes in northwestern Spain, it was found in winter, spring and summer but not in autumn. (Calvo *et al*. 1999). On the Atlantic coast of northeastern North America, aplanosporangia occurred in June and July, but not in May or August (South 1989). In Oregon, it was found in February and November, but no reproductive structures were found, except possibly one instance of akinete formation (Fig. 5). However, since this species is so easily overlooked, it could be present year-round in most locations. The thick-walled akinetes would allow it to persist as propagule banks in the sediment during unfavourable conditions.

Further sampling in estuarine environments and investigation of saltmarsh vegetation for epi-and endophytes will likely lead to more findings of *Wittrockiella amphibia*, and possibly further extend the distributional range in the temperate Northern Hemisphere. Additional molecular sequences will help to clarify the relation between Atlantic and Pacific populations of *W. amphibia*, and also provide insights into the relationships with the morphologically similar, subtropical genus *Cladophorella*.

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Chapter

7

Molecular phylogeny, taxonomy and niche evolution of the *Aegagropila*-clade (Cladophorales, Chlorophyta), including the description of *Aegagropilopsis* gen. nov. and *Pseudocladophora* gen. nov.

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In part submitted

Abstract

The *Aegagropila*-clade is a unique group of cladophoralean algae occurring in brackish or freshwater environments. The clade is sister to the species-rich, primarily marine *Cladophora*- and *Siphonocladus*-lineages. Phylogenetic analyses of partial LSU and SSU nrDNA sequences reveal four main lineages within the *Aegagropila*-clade, and allow clarification of the taxonomy. The earliest diverging lineage consists of two marine '*Cladophora*' species, for which the name *Pseudocladophora* gen. nov. is proposed. The next lineage occurs essentially in brackish and semi-terrestrial habitats, and consists of all known *Wittrockiella* species and *Cladophorella calcicola*. Two other lineages are restricted to freshwater. One of them shows a strong tendency for epizoophytism, and consists of *Basicladia* species and *Arnoldiella conchophila*. The other one includes *Aegagropila linnaei*, the genus *Pithophora* and a small number of tropical *Cladophora* species. These *Cladophora* species are transferred to *Aegagropilopsis* gen. nov. The family name Pithophoraceae is proposed for the *Aegagropila*-clade. Previously, polypyramidal pyrenoids had been suggested to be a character for this lineage, but we show the presence of both polypyramidal and bilenticular pyrenoids in members of the Pithophoraceae. The Pithophoracae show interesting patterns of niche evolution, and generally occur in habitats characterized by unstable environmental conditions where competition is low. The heterotrichous habit of *Wittrockiella* and *Arnoldiella*/*Basicladia* might be an adaptation to these harsh conditions.

Introduction

The Cladophorales (Ulvophyceae) is a species-rich order of green algae with a siphonocladous organisation that is widespread from tropical to polar regions. The classification of the group is highly confused, and the Cladophorales are notorious for taxonomic difficulties at all levels from species to orders. In recent years, molecular data have contributed greatly to develop a better understanding of the evolution of this group. Three main clades have been discovered in molecular phylogenies of the Cladophorales (Fig. 1): 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 consists mainly of freshwater species and is sister to the *Cladophora*- and *Siphonocladus*-clades (Hanyuda *et al.* 2002, Yoshii *et al.* 2004). Members of the *Siphonocladus*-clade (=Siphonocladales s.s.) are distributed mainly in the tropics and are comprised of forms with highly specialized thallus architecture such as pseudoparenchymatic clusters, blades and three-dimensional networks. Members of the *Cladophora*-clade (=Cladophorales s.s or Cladophoraceae sensu Wille) extend their distribution into cold temperate and (ant)arctic waters and consist of branched or unbranched filaments. The *Aegagropila*-clade has not yet received a formal taxonomic rank despite robustness in previous phylogenetic studies (Hanyuda *et al.* 2002, Yoshii *et al.* 2004). Hanyuda *et al.* (2002) suggested that the presence of loraxanthin, chitin and polypyramidal pyrenoids are diagnostic characters of the *Aegagropila*-clade. A fourth lineage, Okellyaceae, is sister to the other three clades (Fig. 1) and includes *Okellya curvata* (Printz) Leliaert & Rueness, an unbranched, marine microfilamentous species occurring in temperate subtidal habitats (Leliaert *et al.* 2009a).

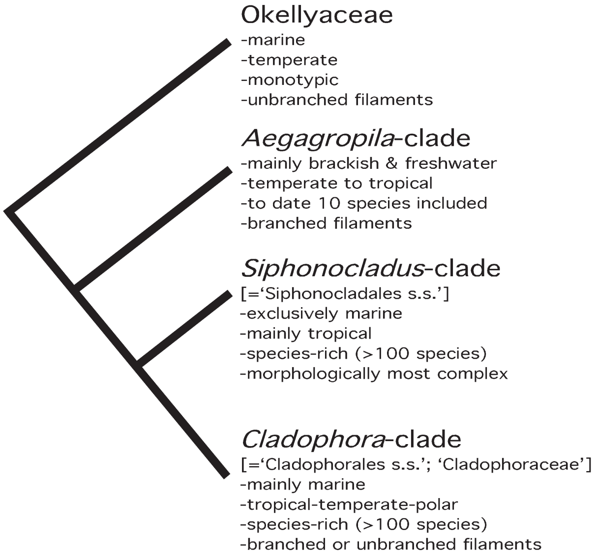

Figure 1. Schematic phylogenetic tree of the Cladophorales with gross information for the four main lineages on habitat, species numbers and morphology.

The *Aegagropila*-clade encompasses six monotypic or species-poor genera and a small number of freshwater *Cladophora* species (Hanyuda *et al.* 2002, Yoshii *et al.* 2004, Rindi *et al.* 2006). Although molecular evidence clearly indicates the polyphyletic nature of the genus *Cladophora*, no nomenclatural changes have been proposed yet based on recent molecular data, except the recent transfer of *C. kosterae* van den Hoek and *C. okamurae* (S. Ueda) van den Hoek to the genus *Basicladia* (Garbary 2010) and the transfer of *Cladophora amphibia* to *Wittrockiella* (Boedeker & Hansen 2010). Species of

Cladophora are distributed in the three main clades of the Cladophorales (Fig. 1). The lectotype species of *Cladophora* is *C. oligoclona* (Kützing) Kützing (Setchell & Gardener 1920, van den Hoek 1963), a synonym of *C. rivularis* (L.) Hoek, which is a member of the *Cladophora*-clade (Leliaert & Boedeker 2007). Thus, taxonomic changes with regards to the *Cladophora* species in the *Aegagropila*-clade and the *Siphonocladus*-clade are required. *Cladophora* species (including earlier synonyms) that are part of the *Aegagropila*-clade, as well as candidate *Cladophora* species for the *Aegagropila*-clade (based on morphological or ecological data) that were shown to have other affiliations by molecular data, are listed in Table 1.

Both from an evolutionary and an ecological perspective this group is very interesting, since it constitutes a freshwater/brackish lineage within the predominantly marine Cladophorales, and most taxa occupy highly specialized niches. Its members occur in narrow niches such as on the carapaces of freshwater turtles (some members of *Basicladia* Hoffmann & Tilden), on freshwater snails and bivalves (monotypic *Arnoldiella* Miller and several *Basicladia* species), on and endophytically in saltmarsh plants and mangrove pneumatophores (some members of *Wittrockiella* Wille), or on marine intertidal snails (*Cladophora conchopheria* Sakai). 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).

The majority of the species in the *Aegagropila-clade* occurs in freshwater environments, the genus *Wittrockiella* occurs in brackish habitats, and *Cladophora horii* van den Hoek (Fig. 2A) and *C. conchopheria* (Fig. 2B) are the only marine species. *Wittrockiella* currently encompasses three species, *W. lyallii* (Harvey) van den Hoek, Ducker & Womersley (Fig. 2C), *W. salina* V.J. Chapman (Fig. 2D) and *W. amphibia* Boedeker & Hansen (Fig. 2F). A close relationship between *W. salina* and the (sub)tropical, semi-terrestrial species *Cladophorella calcicola* Fritsch (Fig. 2E) has been speculated by van den Hoek *et al.* (1984), but no molecular data are available to date. The genus *Basicladia* consists of seven species, mainly occurring on freshwater turtles. Culture experiments have shown that other substrates can be colonized as well (Proctor 1958), and the recently included species *B. okamurae* (S. Ueda) Garbary (Figs. 2G & H) and *B. kosterae* (van de Hoek) Garbary (Figs. 2G & I) are only sporadically encountered on turtles. The diminutive and poorly known *Arnoldiella conchophila* Miller has been found on the shells of freshwater bivalves (Miller 1928), freshwater gastropods (Kargupta 1994, Keshri & Hazra 2009) and a range of other substrates (Cox Downing 1970). More than 35 taxa of *Pithophora* Wittrock, a genus easily recognized by the characteristic akinetes (Figs. 2K & L), have been described, but numbers are most likely inflated due to plastic morphological characters (Ernst 1908, Mothes 1930, Fott 1971, Pankow & Täuscher 1980). This mainly (sub)tropical freshwater genus only occurs unattached in stagnant, nutrient-rich waters and can form extensive floating masses that can be local nuisances (Entwisle & Price 1992, Lembi 2003). The freshwater species *Aegagropila linnaei* Kützing (Fig. 2P) is currently regarded as the sole member of its genus (van den Hoek 1963, Hanyuda *et al.* 2002), but a number of potentially closely related species has been identified (Boedeker *et al.* 2010b). *Aegagropila linnaei* is the best known representative of the lineage and gained considerable scientific, cultural (in Japan) and economic (aquarium trade) fame due to the peculiar lake balls formed under specific conditions (Kurogi 1980, Niiyama 1989, Boedeker *et al.* 2010a).

Table 1. List of (former) *Cladophora* species that are members of the *Aegagropila*-clade, and candidate members that were shown by molecular data to have a different systematic affiliation.

former *Cladophora* species that are members of the *Aegagropila*-clade

Cladophora species	*Cladophora* section/subgenus	current name	reference for placement in *Aegagropila*-clade
Cladophora horii C. Hoek & Chihara 2000	section *Rugulosae*[a]	still the same	Leliaert *et al.* 2003
Cladophora conchopheria Sakai 1964	section *Glomeratae*[a]/section *Opacae*[b]	still the same	Hanyuda *et al.* 2002
Cladophora kosterae C. Hoek 1963	section *Basicladia*[c]	*Basicladia kosterae* (C. Hoek) Garbary 2010	Yoshii *et al.* 2004
Cladophora okamurae S. Ueda1932	section *Basicladia*[c]	*Basicladia okamurae* (S. Ueda) Garbary 2010	Hanyuda *et al.* 2002 (as *Chaetomorpha okamurae*)
Cladophora aegagropila (L.) Rabenhorst 1868	section *Aegagropila*[c]/subgenus *Aegagropila*[b]	*Aegagropila linnaei* Kützing 1843	Hanyuda *et al.* 2002
Cladophora sp. ('Tateyama-Marimo')	/	still the same (undescribed species)	Hanyuda *et al.* 2002, Yoshii *et al.* 2004
Cladophora lyallii Harvey	/	*Wittrockiella lyallii* (Harvey) C. Hoek, Ducker & Womersley 1984	Hanyuda *et al.* 2002
Cladophora amphibia Collins 1907	/	*Wittrockiella amphibia* (Collins) Boedeker & Hansen 2010	Yoshii *et al.* 2004 (as *W. paradoxa*)
Cladophora oedogonia (Montagne) Montagne 1856	/	*Pithophora oedogonia* (Montagne) Wittrock 1877[d]	Hanyuda *et al.* 2002 (for the genus *Pithophora*)
Cladophora roettleri (Roth) Kützing 1849	/	*Pithophora roettleri* (Roth) Wittrock 1877[d]	Hanyuda *et al.* 2002 (for the genus *Pithophora*)

candidate members of the *Aegagropila*-clade that were shown by molecular data to have different affiliations

Cladophora species	*Cladophora* section/subgenus	systematic placement	reference
Cladophora catenata (L.) Kützing 1843	section *Aegagropila*[e]/subgenus *Aegagropila*[b, f]	*Siphonocladus*-clade	Hanyuda *et al.* 2002, Leliaert *et al.* 2007a
Cladophora echinus (Biasoloetto) Kützing 1849	section *Aegagropila*[c]	Longi-Articulatae clade (*Cladophora*-clade)	Leliaert *et al.* 2009c
Cladophora patentiramea (Montagne) Kützing[g]	subgenus *Aegagropila*[b]	(*Siphonocladus*-clade)[g]	(Hanyuda *et al.* 2002, Leliaert *et al.* 2007)
Cladophora sibogae Reinbold	subgenus *Aegagropila*[b]	*Siphonocladus*-clade	Leliaert *et al.* 2003, Leliaert *et al.* 2007
Cladophora socialis Kützing	subgenus *Aegagropila*[b]	*Siphonocladus*-clade	Hanyuda *et al.* 2002, Leliaert *et al.* 2007
Cladophora battersii C. Hoek 1963	section *Rupestres*[c]	*Cladophora*-clade	Leliaert *et al.* 2009c
Cladophora pygmaea Reinke 1888	section *Chamaethamnion*[c]	*Cladophora*-clade	Leliaert *et al.* 2009c
classic freshwater *Cladophora* species[h]	sections *Glomeratae* & *Cladophora*[c]	*Cladophora*-clade	Marks & Cummings 1996, Hanyuda *et al.* 2002

[a] van den Hoek & Chihara 2000, [b] Sakai 1964, [c] van den Hoek 1963, [d] Pankow & Täuscher (1980) reduced *Pithophora* to two species, however disagreement over the number of species exists. Originally, Wittrock (1877) had also transferred *Cladophora acrosperma* Kützing, *C. sumatrana* van Martens and *C. zelleri* van Martens to *Pithophora* in addition to the two species listed here, [e] van den Hoek 1982, [f] as *C. fuliginosa* Kützing, [g] probably a synonym of *C. coelothrix* Kützing (see van den Hoek & Chihara 2000), [h] *C. fracta* (Müller ex Vahl) Kützing, *C. globulina* (Kützing) Kützing, *C. glomerata* (L.) Kützing, *C. rivularis* (L.) C. Hoek.

Figure 2. Morphological variety in the *Aegagropila*-clade. **A**: *Cladophora horii* (D78). Scalebar=200 µm. **B**: *Cladophora conchopheria* (N71), filaments on shell of the marine gastropd *Lunella coronata*. Scalebar=100 µm. **C**: *Wittrockiella lyalli* (H67), heterotichous filaments with secondary rhizoids. Scalebar=1 mm. **D**: *Wittrockiella salina* (B92), heterotichous filaments in culture. Scalebar=100 µm. **E**: *Cladophorella calcicola* (K92), clump of heterotrichous filaments in culture. Scalebar=200 µm. **F**: *Wittrockiella amphibia* (N73), heterotrichous filaments with densely pigmented upright shoots. Scalebar=200 µm. **G**: *Cladophora kosterae* (J79, branched plant on left) and *Cladophora okamurae* (J78, unbranched filaments on right), growing together on dead wood. Scalebar=2 mm. **H**: *Cladophora okamurae* (J78), rhizoidal stratum inside surface layer of wood substrate giving rise to upright shoots. Scalebar=200 µm. **I**: *Cladophora kosterae* (J56), filaments on shell of the freshwater bivalve *Anodonta anatina*. Scalebar=1 mm. **J**: *Basicladia ramulosa* (J85), unbranched basal parts of filaments with very long cells, growing on carapax of the freshwater turtle *Chelodina longicollis*. Scalebar=200 µm. **K**: *Pithophora oedogonia* (K01), filaments with intercalary and terminal akinetes. Scalebar=200 µm. **L**: *Pithophora* cf. *roettleri* (K93), two filaments consisting mainly of germinating akinetes diving rise to new branches. Scalebar=200 µm. **M**: *Pithophora* cf. *polymorpha* (K97), sterile filament without akinetes showing branches being subterminally inserted and delayed cell wall formation. Scalebar=200 µm. **N**: *Cladophora sterrocladia* (G91), filaments with unbranched basal parts and opposite branches. Scalebar=2 mm. **O**: *Cladophora clavuligera* (L70), filament showing serial insertion of branches. Scalebar=100 µm. **P**: *Aegagropila linnaei* (C01), filaments showing subterimal insertion of branches. Scalebar=200 µm.

Previous molecular phylogenetic studies, based on SSU rDNA sequence data, only partly resolved the relationships within the *Aeagropila*-clade and suffered from low taxon sampling (Hanyuda *et al.* 2002, Yoshii *et al.* 2004). These studies showed that

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. kosterae* is allied to a species of *Basicladia*, and that *Aegagropila* and *Pithophora* have a close relationship. The relation of *Arnoldiella conchophila* and *C. okamurae* (as *Chaetomorpha okamurae*) to the other taxa was not resolved. The aerophytic unicellular alga *Spongiochrysis hawaiiensis* was recovered on a basal polytomy with *C. conchopheria* (Rindi *et al*. 2006).

In order to gain a better understanding of the evolution of morphology and specialized habitat preferences in the *Aegagropila*-clade, and to re-assess the taxonomy of the group, the phylogenetic relationships need to be resolved. This study extends the previous phylogenies by increasing taxon sampling and by combining SSU and LSU sequence data, which has been shown to lead to better resolved phylogenies in the Cladophorales (Leliaert *et al*. 2007a). The validity of pyrenoid ultrastructure as a diagnostic character for the lineage was tested with a wide range of taxa.

Materials and Methods

Taxon sampling and morphological analysis

Thirty specimens from the *Aegagropila*-clade were sampled in various habitats from a broad geographical range (Tables 2 & 4). The type species of the following of the currently recognized genera in the *Aegagropila*-clade have been included in the phylogenetic analyses: *Aegagropila* (*A. linnaei*), *Arnoldiella* (*A. conchophila*), *Cladophorella* (*C. calcicola*), and *Wittrockiella* (*W. amphibia*). Wittrock (1877) did not designate a type species for *Pithophora*, the sole genus included in Pithophoraceae at its inception, and we have found no prior lectotypication. We here select *P. kewensis* Wittrock as the type species (isotypes in BM (incl. K) & L, we choose the specimen from L (no. 938112 639) as the lectotype) for the genus *Pithophora* and the family Pithophoraceae (=*Aegagropila*-clade, see discussion), because it is the species that first attracted his attention, as Wittrock (1877) himself acknowledged in the introduction to his monograph, and it is the most thoroughly described and illustrated of the eight that make up his account of the genus. Furthermore, the type specimen of *P. kewensis* is fertile and displays the typical terminal and intercalary akinetes of the genus *Pithophora*, unlike the types of several other *Pithophora* species. *Basicladia crassa* Hoffmann & Tilden is the type species of the genus (Hoffmann & Tilden 1930), but no sequence data are available for this species. However, *B. crassa* is morphologically similar to *B. chelonum*, with intermediate forms frequently encountered (Proctor 1958), and both species are often found growing together. The recognition of *B. chelonum* and *B. crassa* as distinct species has been questioned (Proctor 1958, Garbary *et al*. 2007). It is therefore likely that *B. crassa* is closely related to *B. chelonum* and the other species of *Basicladia* included in this study. We excluded *Spongiochrysis hawaiiensis* (which has been proposed to be a member of the *Aegagropila*-clade by Rindi *et al*. 2006) from our analyses due to data conflict. We obtained partial LSU and partial SSU rDNA sequences of living material of *S. hawaiiensis* from the type locality. Our SSU sequence is identical to the ones published by Rindi *et al*. (2006) (GenBank accession nos. DQ077805, -806), and groups on a long, separate branch with the marine species *Cladophora horii* and *C. conchopheria* in our phylogenetic trees. However, our LSU sequence groups with members of the Trentepohliales in phylogenetic trees. For confirmation we re-extracted, amplified and sequenced the partial LSU rDNA three times, always with the same result. In conclusion, the placement of *S. hawaiiensis* in the Cladophorales could not unequivocally be established, and further study of this organism is clearly necessary. We also did not include the published sequences of the *Basicladia* cultures UTEX LB810 and LB811 (University of Texas Culture Collection of Algae; GenBank accession nos. AB078726, -727; published in Yoshii *et al.* 2004), since our own SSU rDNA sequences of the same cultures differed by 10-20 bp. *Cladophora clavuligera* Grunow and *C. sterrocladia* Skuja were also included based on preliminary sequence data that indicated an affiliation with the *Aegagropila*-clade.

Fresh algal material was preserved in silica gel for DNA extraction, and vouchers were prepared from the same sample as herbarium sheets or preserved in a 5% formalin solution and deposited in L (herbarium abbreviations follow Holmgren *et al.* 1990). Specimens, either fresh, formalin-preserved or reconstituted from herbarium material, were examined with a Olympus SZX10 stereo-microscope and a Olympus BH2 light microscope (Olympus Optical Co. GmbH, Hamburg, Germany), and images were taken with a connected digital camera (ColorView Illu, Olympus Soft Imaging Systems, Münster, Germany). Polypyramidal pyrenoids had been proposed to be a diagnostic character for the *Aegagropila*-clade (Hanyuda *et al.* 2002), so we examined the ultrastructure of the pyrenoids of a range of members of this lineage by TEM. Morphological and ultrastructural investigations were performed on specimens also included in the molecular study (Table 2) or that had identical nucleotide sequences (Tables 2 & 4). For TEM, living cells from cultured material were fixed with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), rinsed in buffer, postfixed with 1% OsO_4 in buffer, stained by dehydration in a gradual series of ethanol containing 1% uranylacetate, and finally embedded in Epon. Sections were cut with a diamond knife on a LKB-3 ultratome, mounted on grids and post-stained with 3% uranyl acetate and lead citrate. Preparations were viewed and photographed in a JEAL 1010 electron microscope operated at 60 kV.

Molecular phylogenetic analyses

The specimens used in the phylogenetic analyses are listed in Table 2 (members of the *Aegagropila*-clade) and Appendix S5 (other Cladophorales and outgroups, see Supplementary Materials). A total of 43 specimens were analysed (22 specimens of the *Aegagropila*-clade, eight taxa of the *Cladophora*-clade, ten taxa of the *Siphonocladus*-clade, three outgroup taxa). Molecular phylogenetic analyses were based on nuclear-encoded small subunit (SSU) and partial large subunit (LSU) rDNA sequences. DNA extraction, PCR amplification and sequencing were performed as in Boedeker & Immers (2009), modified for the SSU as follows: the complete SSU rRNA gene was amplified using the primer pairs SR1-SS11H and SSU897-18SC2 (Leliaert *et al.* 2007a). Obtained sequences have been deposited in GenBank (see Table 2 for accession numbers). Taxa for which new sequences were generated are listed in Table 2 (plus *Trentepohlia* sp. in Appendix S5). If multiple identical sequences were available for a species, only two sequences from specimens from different locations were included in the analysis (see Table 2). Sequences were aligned using MUSCLE (Edgar 2004) and subsequently edited by eye in Se-Al v2.0a11 (Rambaut 2007). Two short variable regions of the LSU alignment (33-38 bp in total, depending on outgroups) were ambiguous and excluded from all analyses. Evolutionary models of nucleotide substitution were determined by the Akaike Information Criterion for all

Table 2. Specimens of members of the *Aegagropila*-clade used in this study with collection data (voucher information, indicated in bold). n.i. = no information.

species	voucher[a], culture	no.
Aegagropila linnaei Kützing	L0793580	B54
Aegagropila linnaei	L0793577	C01
Aegagropila linnaei	L0793543	N36
Cladophora sp. 'Tateyama'		
Cladophora clavuligera Grunow	L0793298, L0793299	L70
Cladophora sterrocladia Skuja	L0793287	G91
Pithophora oedogonia (Montagne) Wittrock	L0793288	K01
Pithophora cf. *roettleri* (Roth) Wittrock[b]	L0793289, ACOI997	K93
Wittrockiella amphibia (Collins) Boedeker & Hansen	/ , CCMP1674	N60
Wittrockiella amphibia	L0793284	N73
Wittrockiella salina Chapman	L0793300, L0793301	B92
Wittrockiella lyallii (Harvey) van den Hoek, Ducker & Womersley	WELT A023866/n.i.	LSU: H67
Wittrockiella lyallii	SGO No. 158361	N61
Cladophorella calcicola Fritsch	L0793292, ACOI471	K92
Basicladia kosterae (C. Hoek) Garbary	L0793302, L0793303	J56
Basicladia kosterae	L0793294, CCAP505.6, UTEX LB1485	K06
Basicladia kosterae	L0793295, CCAP505.11	K09
Basicladia kosterae	L0793293	J79
Basicladia cf. *chelonum*[c]	L0793296, UTEX LB811	K98
Basicladia ramulosa Ducker	L0793304, L0793305	J85
Arnoldiella conchophila Miller		
Basicladia okamurae (S. Ueda) Garbary[d]	L0793306, FACHB795	L84
Basicladia okamurae	L0793310-312	J78
Cladophora horii van den Hoek & Chihara	HEC10983/n.i.	LSU: F53
Cladophora horii	L0793316-318	D78
Cladophora conchopheria Sakai	L0793297, UTEX LB2870/n.i.	LSU:K99
Cladophora conchopheria	L0793313, L0793314	N71
Spongiochrysis hawaiiensis Rindi *et al.*[e]	L0793315, GALW015489	LSU: G89

[a] Index Herbariorum: Holmgren *et al.,* 1990

[b] obtained as *P. oedogonia* (Montagne) Wittrock

[c] obtained as *Basicladia* sp.

[d] obtained as *Basicladia chelonum* (Collins) Hoffmann & Tilden

[e] not included in the phylogenetic trees due to data conflict

*not included in the phylogenetic trees, identical sequence to conspecific specimens above location, collector, date of collection) and GenBank accession numbers (sequences generated in this study are

location	**collectors**	**GenBank accession nos.**	
		LSU	**SSU**
Lake Myvatn, Iceland	Á. Einarsson	EU655697	**FR719925**
cf. Lake Svityaz, Ukraine	(online aquarium shop)	EU655698	**FR719926**
Loch Watten, Caithness, Scotland	C. Scanlan	*	
Tateyama, Toyama, Japan	I. Wakana	/	AB062711
tropical aquarium	A. Immers	**FR719939**	**FR719927**
botanical garden, Leiden, Netherlands	C. Boedeker	**FR719940**	**FR719928**
botanical garden, Leiden, Netherlands	C. Boedeker	**FR719941**	**FR719929**
rice field, Montemor-o-Velho, Portugal	A. F. Frias	**FR719942**	**FR719930**
Lopez Island, Washington, USA	C. O'Kelly	GU384874	AB078732
Yaquina Bay, Oregon, USA	G.I. Hansen	GU384873	GU384872
Jawbone Reserve, Victoria, Australia	J. West	**FR719943**	**FR719931**
Bradshaw/Milford Sound, Fiordland, New Zealand	S. Heesch/T. Hanyuda	FN252712	AB062717
Dring Island, Aysén, Chile	M.E. Ramírez, D.M. John	GU198503	GU198502
Lagoa de Óbidos, Portugal	O. Lourenço	**FR719944**	**FR719932**
Kager Plassen, Leiden, Netherlands	C. Boedeker	**FR719945**	**FR719933**
botanical garden, Paris, France	C. van den Hoek	**FR719946**	AB078730
unknown	unknown	*	
canal, Meije/Bodegraven, Netherlands	C. Boedeker	*	
Missouri, USA	V. Proctor	**FR719947**	**FR719934**
Annie creek, Kimberley region, Australia	N. FitzSimmons	**FR719948**	**FR719935**
Lake Panke, Japan	S. Arai	/	AB062712
Wuhan, China	M. Chen	**FR719949**	**FR719936**
canal, Meije/Bodegraven, Netherlands	C. Boedeker	**FR719950**	**FR719937**
KwaZulu-Natal, South Africa/Ishigaki, Okinawa, Japan	E. Coppejans/S. Arai	AJ544728	AB078731
Trafalgar, KwaZulu Natal, South Africa	C. Boedeker	*	
Shimoda/Shirasaki, Ishikawa. Japan	C. v. d. Hoek/T. Hanyuda	**FR719951**	AB062705
Oishi, Sumoto, Awaji-shima, Japan	S. G. A. Draisma	*	
Waimanolo, O’ahu, Hawaiian Islands	A. Sherwood	not included	not included

alignments in PAUP* 4.0b10 /Modeltest v3.7 (Posada & Crandall 1998, Swofford 2002), or in PAUP/MrModeltest 2 v2.3 (Nylander 2004) for the subsequent use of MrBayes (see below). Uncorrected pairwise distances for the members of the *Aegagropila*-clade were calculated in PAUP.

First, the LSU (37 sequences plus *Okellya curvata* as outgroup) and SSU (39 sequences plus *Okellya curvata* as outgroup) datasets were analysed separately.
The I_{SS} statistic, a measure of substitution saturation in molecular phylogenetic datasets, was calculated with DAMBE (Xia & Xie 2001) for the SSU and partial LSU data separately. Congruence between the two genes was tested by conducting the incongruence length difference (ILD) test implemented in PAUP (Farris *et al.* 1995) under parsimony with 100 replicates, and indicated that the SSU and the partial LSU rDNA data were not significantly heterogeneous (P=0.57). The two genes were then combined into concatenated alignments, which were subsequently used for all analyses.

Three different concatenated LSU-SSU alignments were created (named 'test', 'outgroup' and 'ingroup', see Table 3). Initial phylogenetic analyses of the 'test' dataset were performed to evaluate the effect of removal of taxa with partial sequence data, and the effect of model selection on tree topology and branch support (Appendix S6). The 'outgroup' alignment contained two distant outgroups (*Ulva fasciata* and *Trentepohlia* sp.), *Okellya curvata*, and all ingroup sequences except two with missing LSU data (40 sequences in total). This alignment was created to establish whether the *Cladophora*-clade, the *Siphonocladus*-clade and the *Aegagropila*-clade represent monophyletic groups, and to infer the position of the root of the ingroup (=Cladophorales excluding Okellyaceae). Because distant outgroups can influence the inferred relationships of the ingroup taxa (Bergsten 2005), the 'ingroup' alignment was assembled which consisted of all 39 ingroup sequences. In addition to rooting with the three outgroup-taxa, the position of the root was also determined for the 'ingroup' dataset by midpoint rooting of the BI and ML trees and by molecular clock rooting (Appendix S7).

The datasets were analysed with maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). MP analyses were performed with PAUP and consisted of a full heuristic search with 1000 random sequence addition replicates and Tree Bisection Reconnection (TBR) branch swapping. Gaps were treated as missing data. Branches with zero length were collapsed. Robustness of the MP trees was tested by non-parametric bootstrapping (Felsenstein 1985) with 1000 pseudoreplicates. ML analyses were performed using PHYML v3.0 (Guindon & Gascuel 2003), starting with five random neighbour-joining trees and using SPR & NNI tree topology search and 1000 non-parametric bootstrap replicates. BI was performed with MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003). Analyses consisted of two independent, simultaneous runs of one cold and three incrementally heated chains, and 3×10^6 generations with sampling every 100 generations. Posterior probabilities were calculated using a Metropolis-coupled Markov chain Monte Carlo approach. The average standard deviation of the spilt frequencies of the two parallel runs approached zero in all analyses (0.0005-0.0065, depending on alignment), indicating that the tree samples became increasingly similar and that a stationary distribution was reached. The log-files of the runs were checked with Tracer v1.4.1 (Rambaut & Drummond 2007) and a burn-in sample of 4000 trees was removed before calculating the majority rule consensus trees in MrBayes.

Table 4. Voucher and collection information of eight *Pithophora* specimens with identical LSU or SSU sequences. 'ID' indicates our own re-identification result, differing from the original species designation of the culture collections; Ø represents diameter; n.d. = no data.

specimen designation (GenBank accession numbers in brackets)	**voucher**	**no.**	**location**	**collector**	**identical sequences**		**morphological information**
					LSU	**SSU**	
Pithophora oedogonia (Mont.) Wittrock (LSU: FR719941, SSU: FR719929)	L0793288	K01	hothouse, botanical garden, Leiden, Netherlands	C. Boedeker	**x**	**x**	Main axis 40-75µm Ø; branches 38-50µm Ø, opposite or solitary; terminal akinetes 70-100µm Ø, pointy; intercalary akinetes 75-110µm Ø, cask-shaped.
Pithophora oedogonia (**ID**: *P.* cf. *roettleri*) (LSU: FR719942, SSU: FR719929)	L0793289, ACOI997[a]	K93	rice field, Montemor-o-Velho, Portugal	A.F. Frias	**x**	**x**	Main axis 60-100µm Ø; many short branches, 60-70-(110)µm Ø, sometimes opposite, mainly solitary; terminal akinetes ca. 100µm Ø, pointy; intercalary akinetes 100µm Ø, cask-shaped; many helicoid cells.
Pithophora roettleri (Roth) Wittrock	L0793290, CCALA408[a]	K96	thermal spa, Piestany, Slovakia	F. Hindák	**x**	n.d.	Main axis 50-65µm Ø; long branches, 50-60 µm Ø, solitary, widely spaced; terminal akinetes 70-100µm Ø, cylindrical-pointy; intercalary akinetes 125-140µm Ø, cask-shaped/trapezoid, in chains.
Pithophora sp. (**ID**: *P.* cf. *polymorpha)*	L0793291, UTEX LB787[a]	K97	Brooklyn, Indiana, USA	C.J. O'Kelly	**x**[b]	n.d.	Main axis 38-70µm Ø, irregular outline; many short branches, 30-40µm Ø, opposite or solitary; no terminal akinetes; intercalary akinetes 125µm Ø, rare.
Pithophora sp. 'Kosrae'	missing	G99	Kosrae, Micronesia	G. Zuccarello	**x**	n.d.	sterile material (no akinetes)
Pithophora sp. 'Kamigori' (AB062713)	n.d.		Kamigori, Japan	S. Arai	n.d.	**x**	n.d.
Pithophora sp. 'Sano' (AB066646)	n.d.		Sano, Japan	S. Arai	n.d.	**x**	n.d.
Pithophora sp. 'Singu' (AB066647)	n.d.		Singu, Japan	S. Arai	n.d.	**x**	n.d.

[a] culture collection number

[b] this LSU sequence differs in three positions from the others

Table 3. Specification of datasets, summary of models and model parameters obtained.

Alignment name	LSU	SSU
Genes	partial LSU	SSU
Taxa	38	40
Ingroup taxa	37	39
Outgroup taxa	1 (*Okellya curvata*)	1 (*Okellya curvata*)
Alignment length/analysed length	643/610	1690/1690
Variable sites/parsimony informative sites	248/212	285/198
Model estimated[a]	GTR+I+G	GTR+I+G
Estimated base frequencies (A/C/G/T)	0.21/0.25/0.32/0.22	0.26/0.22/0.27/0.25
Estimated substitution frequencies (AC/AG/AT/CG/CT/GT)[a]	0.75/3.18/1.43/0.90/6.14/1	1.36/3.04/2.63/0.87/7.30/1
Among-site variation I/G[b]	0.34/0.58	0.67/0.46

[a] estimated by Akaike Information Criterion (AIC)

[b] proportion of invariable sites (I) and gamma distribution shape parameter (G) as estimated in PAUP/MrModeltest

Results

Datasets and alignments

Details of the different alignments including number of in- and outgroup taxa, alignment length and number of variable sites, as well as estimated parameters of nucleotide substitution are given in Table 3. For all datasets, a general time reversible model of evolution with a proportion of invariable sites and gamma shape (GTR+I+G) was determined. The SSU sequences are nearly three times as long as the partial LSU sequences but they contain comparable amounts of variable and parsimony-informative sites. Additional identical LSU sequences that were excluded from the phylogenetic analysis are listed in Tables 2 and 4. No significant saturation was detected in either the SSU or the partial LSU data, based on the I_{SS} statistic (Xia & Xie 2001).

‘test’	‘outgroup’	‘ingroup’
partial LSU & SSU	partial LSU & SSU	partial LSU & SSU
40	40	39
39	37	39
1 (*Okellya curvata*)	3 (*Ulva fastigia*, *Trentepohlia* sp., *Okellya curvata*)	none
2333/2300	2358/2336	2333/2300
533/410	738/541	484/406
GTR+I+G	GTR+I+G	GTR+I+G
0.24/0.23/0.29/0.24	0.24/0.23/0.29/0.24	0.24/0.23/0.29/0.24
1.08/3.17/1.94/1.04/6.47/1	1.15/3.17/1.83/0.97/6.42/1	1.07/3.34/2.05/1.07/6.92/1
0.58/0.46	0.42/0.37	0.65/0.63

In the trees resulting from separate BI analyses of the SSU and the partial LSU datasets, the relationships among and within genera were largely unresolved (not shown). Topological differences between the SSU and the LSU tree were not supported. The combined SSU-LSU analyses were found to perform better in terms of resolution and support values than the separate SSU/LSU analyses. Analysing the ‘test’ alignment with a simple model of nucleotide substitution such as HKY (and employing phylogenetic methods of inference not utilising evolutionary models such as MP) resulted in slightly different tree topologies and support values compared to using the complex GTR+I+G model (Appendix S6). Excluding the three taxa with missing sequence data had no effect on tree topology or support values.

Outgroup tree

Three ingroup clades were recovered, representing the *Cladophora*-clade, the *Siphonocladus*-clade and the *Aegagropila*-clade (Fig. 3), which together with the monotypic

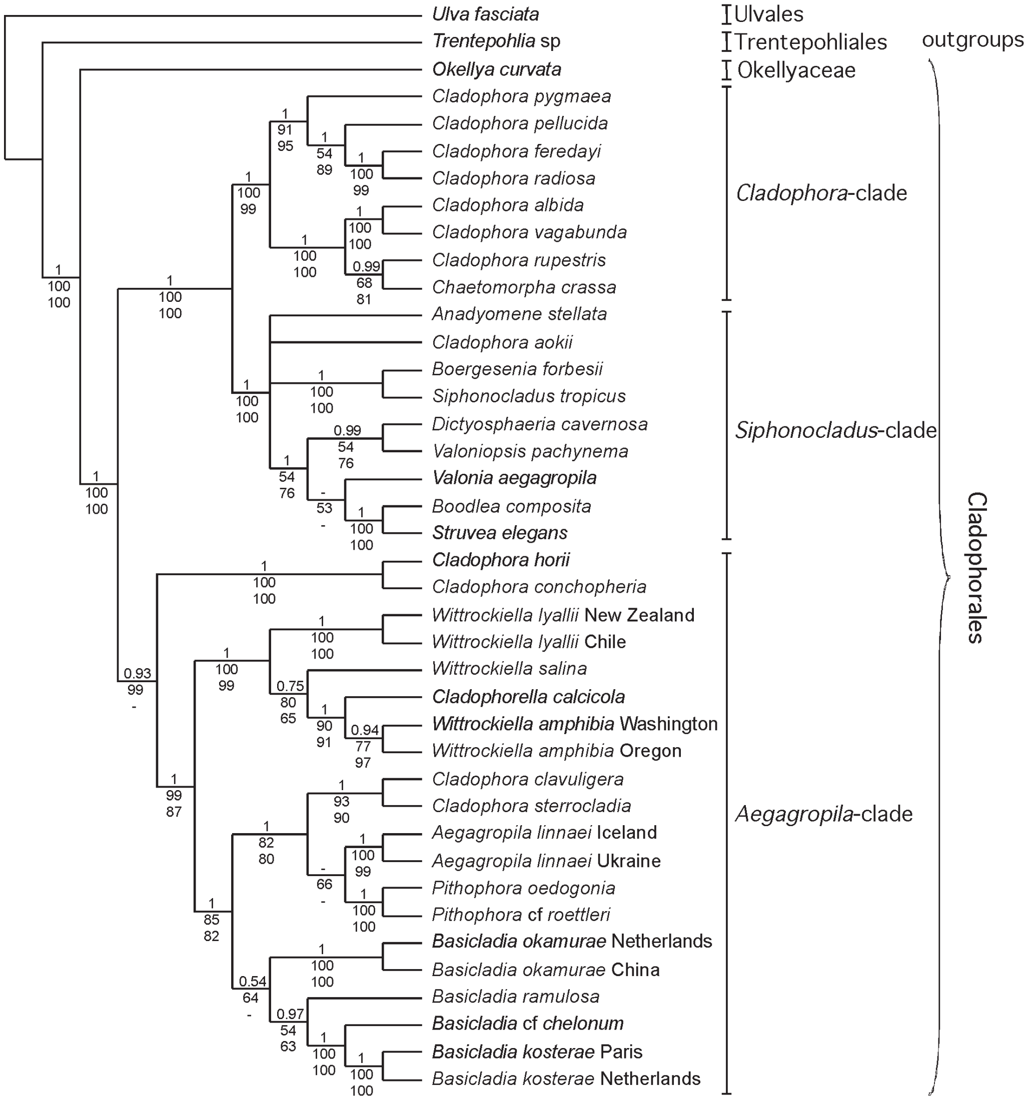

Figure 3. Maximum parsimony (MP) bootstrap consensus tree inferred from concatenated partial large and complete small subunit rDNA sequences with gaps treated as missing data showing the four groups within Cladophorales, rooted with two distant ulvophytes ('outgroup' dataset). Bayesian inference posterior probabilities are indicated above branches, MP and maximum likelihood bootstrap values (1000 replicates) are shown below branches.

Okellyaceae represent all currently known lineages of the Cladophorales. MP, ML and BI yielded similar tree topologies, but differed in levels of support on many branches. The *Aegagropila*-clade received high support in the MP analysis, but only moderate support in the BI analysis (posterior probability 0.93), and no support in the ML analysis. The root of the ingroup (Cladophorales excluding Okellyaceae) was placed on the branch separating the *Aegagropila*-clade from the sister groups *Siphonocladus*-clade and *Cladophora*-clade. The *Aegagropila*-clade is divided into four subclades. The earliest diverging clade consists

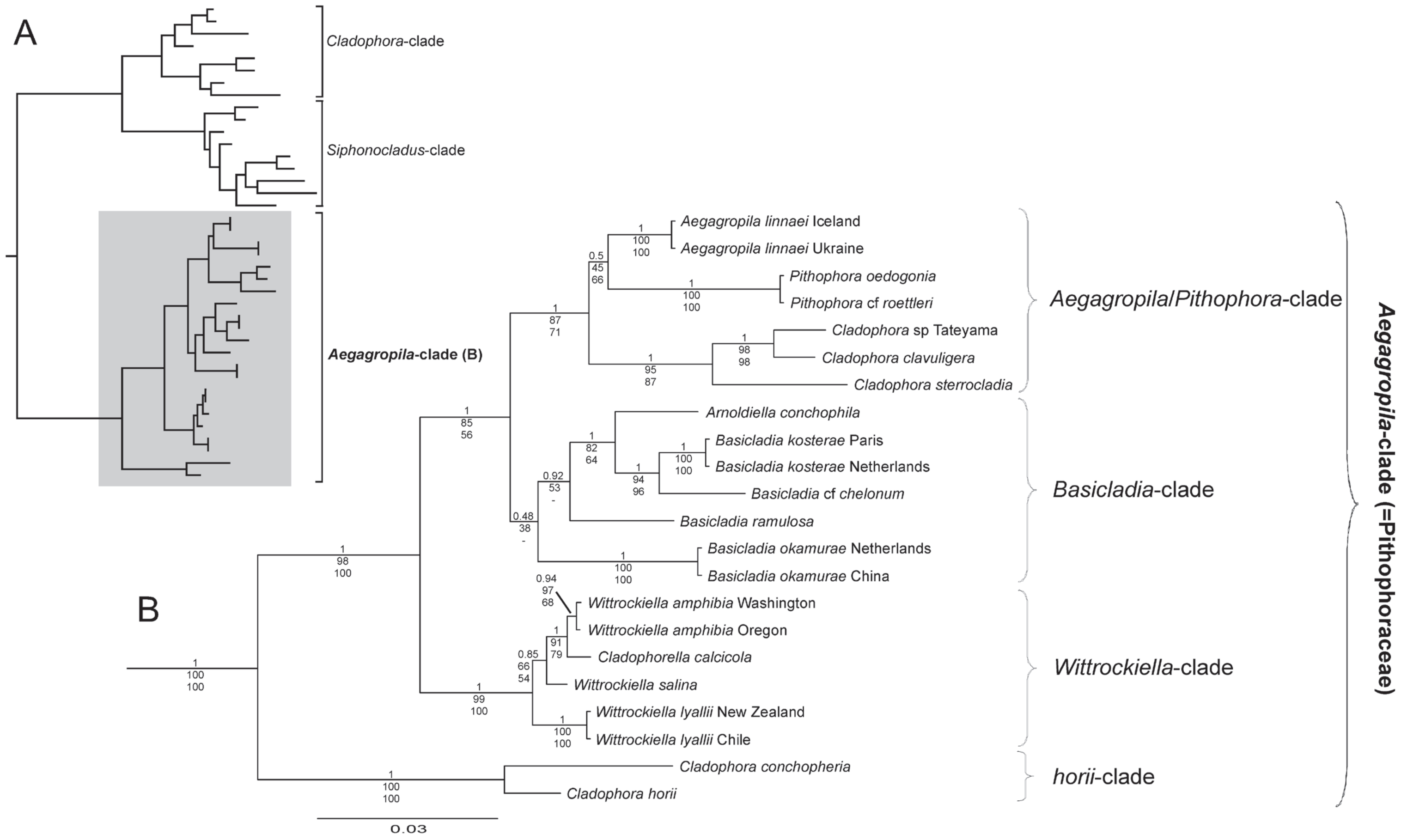

Figure 4. A: Bayesian inference (BI) tree inferred from concatenated partial large and complete small subunit rDNA sequences with gaps treated as missing data showing the three main clades of the Cladophorales ('ingroup' dataset). **B**: Close-up of the *Aegagropila*-clade highlighted in grey in **A**, with genus names indicated on the right. Bayesian inference posterior probabilities are indicated above branches, MP and maximum likelihood bootstrap values (1000 replicates) are shown below branches.

of *C. horii* and *C. conchopheria* (‘*horii*-clade’), the next clade is composed of all *Wittrockiella* species and *Cladophorella calcicola* (‘*Wittrockiella*-clade’), and a clade consisting of all *Basicladia* species (‘*Basicladia*-clade’) is sister to a clade composed of *Aegagropila linnaei*, *Pithophora* and two poorly known *Cladophora* species (‘*Aegagropila/Pithophora*-clade’). The *Wittrockiella*-clade received high support, while support values for the *Aegagropila/Pithophora*-clade were moderate to high. The *Basicladia*-clade was only weakly supported in the MP analysis, and received no support in the BI and ML analyses.

Ingroup tree

The trees constrained with a strict and a relaxed molecular clock and midpoint rooting performed on BI and ML trees of the ‘ingroup’ dataset (Appendix S7) showed the same root position as revealed by outgroup rooting (Fig. 3). An overview of the relationships between the three main ingroup clades and branch length information is shown in Fig. 4A. The *Aegagropila*-clade is enlarged in Fig. 4B. This BI majority-rule phylogram shows the relationships, support values of the three phylogenetic inference methods used and the branch lengths. The *Aegagropila*-clade was recovered as a monophyletic group with high support in all three analyses. The same four main clades as shown in Fig. 3 were found, with the *Aegagropila/Pithophora*-clade further divided into three subclades with long branches.

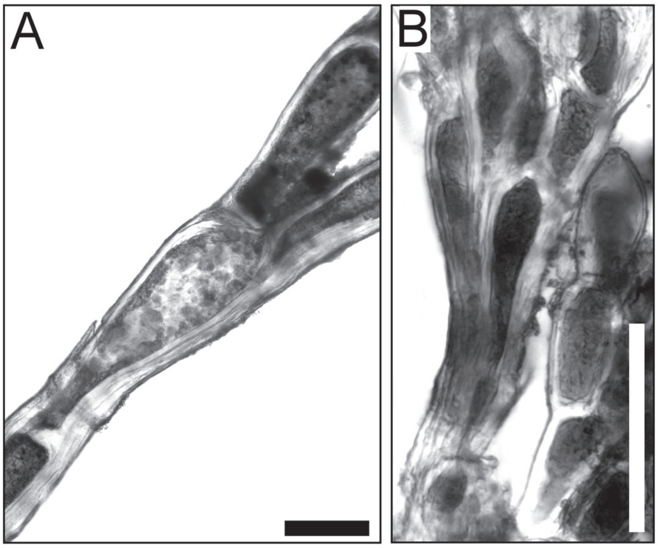

Figure 5.
Stem-like basal branches two members of the *horii*-clade (=*Pseudocladophora* gen. nov.) formed by descending intra- and extracuticular rhizoids that have fused with the cell walls of cells below, leading to a polysiphonous base. **A**: *Cladophora horii* (D78). **B**: *Cladophora conchopheria* (N71). Scalebars=100 µm.

Cladophora horii and *C. conchopheria* form a clade with high support that is sister to the rest of the *Aegagropila*-clade. Additional specimens of both taxa had identical LSU rDNA sequences (Table 2). While the two species differ markedly in habit (*C. horii* forming much larger thalli than *C. conchopheria* (Figs. 2 A, B), they share the characteristic stem-like basal branches that are formed by descending intra- and extracuticular rhizoids that fuse with the cell walls of cells below, leading to a polysiphonous base and very thick, stacked cell walls (Fig. 5).

The *Wittrockiella*-clade is highly supported in all analyses. This clade contains all currently recognized species of *Wittrockiella* plus *Cladophorella calcicola*. *Wittrockiella lyallii* (Fig. 2C) is sister to the rest and is represented by two identical sequences from populations in Chile and New Zealand. The grouping of *W. salina* (Fig. 2D) with *C. calcicola* (Fig. 2E) and *W. amphibia* (Fig. 2F) received only moderate to low support. The sequences of *W. amphibia* from two locations in the northwestern Pacific are identical. The *Wittrockiella*-clade is characterized by short branches in comparison to all other clades in the phylogeny. The members of this clade share a heterotrichous growth form, with creeping main axes that produce relatively short upright filaments (Figs. 2C - F). Adventitious rhizoids can be produced in all cells along of the stolonoid axes (Fig. 2C), and can develop from any part of the cells.

The *Basicladia*-clade consists of four *Basicladia* species plus *Arnoldiella conchophila*. The clade received low or no support in the three analyses (Fig. 3), with *B. okamurae* being frequently recovered on a basal polytomy on the branch connecting the *Basicladia*-clade and the *Aegagropila*/*Pithophora*-clade (Figs. 3 & 4). However, this clade received high support in BI analyses under a HKY model of nucleotide evolution (Appendix Figs. S6B & D) and in the LSU-only analysis (not shown), and received moderate support in the ML analysis of the ‘test’-dataset (Appendix Fig. S7D, bootstrap support=74). The placement of *B. ramulosa* Ducker with the remaining taxa of this clade was only weakly supported, while the grouping of *A. conchophila* with *B. chelonum* and *B. kosterae* was well supported. The four LSU rDNA sequences of *B. kosterae* from the Netherlands and France showed no intra-individual variation (Table 2). The LSU sequences of *B. okamurae* from the Netherlands and China were also identical. The stunted *Arnoldiella conchophila* is nested within *Basicladia*, between the northern Australian endemic *B. ramulosa* and *B. chelonum*-*B. kosterae*. All species in this clade share a heterotrichous growh form (e.g., Fig. 2H). Branched or unbranched upright shoots (Fig. 2G, I) with a characteristic long basal cell (Fig.2J) arise from an extensive prostrate, rhizome-like stratum consisting of coalescent, branched filaments. The upright filaments typically increase in diameter from the base to the apex.

The *Aegagropila*/*Pithophora*-clade is comprised of three subclades which represent the monotypic *Aegagropila* (*A. linnaei*), the genus *Pithophora*, and a subclade consisting of three Asian *Cladophora* species. The clade as a whole is highly supported in Bayesian analyses, but received only moderate bootstrap support in ML and MP analyses. The sister relationship of *Aegagropila* and *Pithophora* is only weakly supported in ML analyses, and unsupported in other inference methods, resulting in a basal polytomy of the three subclades. The sister relationship of *Aegagropila* and *Pithophora* received high support when employing the simpler HKY model of nucleotide evolution (Appendix Fig. S6). Among the three *Cladophora* species, *C. sterrocladia* is sister to both *C. clavuligera* and an undescribed species from Japan (*C.* sp. ‘Tateyama’), and these relationships were highly supported. The two specimens of *A. linnaei* have identical sequences, and sequence variation of maximal 1 bp within this species in partial LSU rDNA sequences had been shown before with specimens from a wide range of locations (Boedeker & Immers 2009). All sequenced *Pithophora* specimens, sampled form different localities and including various morphotypes (Table 4), have identical LSU sequences, except for specimen K97 (*P.* cf. *polymorpha*), which has three point mutations.

The species in the *Aegagropila*/*Pithophora*-clade are morphologically distinct from the *Wittrockiella* and *Basicladia* clades by the absence of a prostrate system, *A. linnaei* and the *Cladophora* species have primary coralloid holdfasts, the genus *Pithophora* is only known unattached. Members of the *Aegagropila*/*Pithophora*-clade are characterized by secondary rhizoids, branches being inserted subterminally, and delayed cross-wall formation in newly produced branches. The secondary rhizoids are frequently very long and seem to play an important additional role in attaching the thallus to the substrate. There is a tendency, particularly in apical parts, for opposite (Figs. 2N - P) or secund branching (Fig. 2M). Both *Pithophora* and *Aegagropila* are characterized by frequent inversion of polarity. A unique feature of the genus *Pithophora* are characteristic terminal or intercalary akinetes which occur either solitary (Fig. 2K), in pairs, or in short chains (Fig. 2L). Sterile plants without akinetes are morphologically similar to the other members of this clade (Fig. 2M). *Aegagropila linnaei*, *C. clavuligera* and *C. sterrocladia* are morphologically similar, and are the only known members of the Cladophorales that can have branches inserted serially (Fig. 2O). *Cladophora sterrocladia* (Fig. 2N) and *C. clavuligera* (Fig. 2O) can be distinguished from *A. linnaei* (Fig. 2P) by several upright shoots arising from the same base, the more sparsely branched basal parts of the main axes composed of regular cylindrical cells (Fig. 2N), while the basal parts of thalli of *A. linnaei* consist of irregular shaped cells with thick walls that can have branches inserted in any position of the mother cell. Furthermore, the arrangement of laterals in the upper parts of *C. clavuligera* is dominantly opposite (sometimes opposite branches in series on one cell), and frequently verticillate in *C. sterrocladia*, with up to 5 laterals per cell.

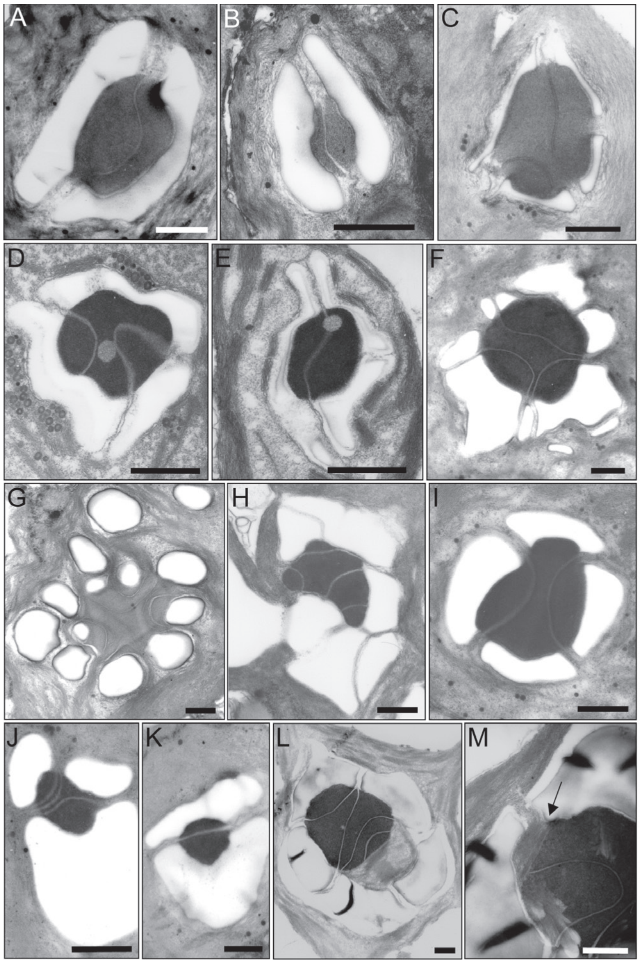

Figure 6.
Transmission electron microscope images of the pyrenoid ultrastructure in members of the *Aegagropila*-clade. Representative examples of all sectioned pyrenoids per species were chosen.
A, **B**: *Wittrockiella salina* (B92), bilenticular pyrenoids. **C**: *Cladophorella calcicola* (K92), polypyramidal pyrenoid. **D**, **E**: *Wittrockiella amphibia* (N60), intraspecific variation in pyrenoid type. **D**: polypyramidal pyrenoid. **E**: bilenticular pyrenoid. All pyrenoids of this species showed a characterstic light spot inside the pyrenoids. **F**: *Basicladia kosterae* (K09), polypyramidal pyrenoid. **G***:* *Basicladia okamurae* (L84), polypyramidal pyrenoid with extremely segregated surrounding starch sheath. **H**: *Pithophora* cf. *roettleri* (K93), polypyramidal pyrenoid. **I**: *Pithophora* cf. *polymorpha* (K97), polypyramidal pyrenoid. **J**, **K**: *Pithophora roettleri* (K96), intraspecific variation in pyrenoid type. **J**: polypyramidal pyrenoid. **K**: bilenticular pyrenoid. **L**, **M**: *Aegagropila linnaei* (N36), polypyramidal pyrenoid. **M**: Close-up of a thylakoid membrane stack associated with the pyrenoid (arrow). All pyrenoids of this species showed this association. All scalebars=1 μm.

Pyrenoid ultrastructure

Pyrenoid ultrastructure was examined by TEM with respect to the number of thylakoid membranes transversing the pyrenoid (bilenticular *vs.* polypyramidal structure) and the arrangement of starch plates. In the *Wittrockiella*-clade, both polypyramidal and bilenticular pyrenoids were observed. All pyrenoids of *W. salina* (specimen B92, Figs. 6A & B, n=17) were bilenticular, with the surrounding starch layer divided into two halves. The majority of pyrenoids in *Cladophorella calcicola* (specimen K92, Fig. 6C, n=21) were polypyramidal with the starch layer divided into several pieces, but 33% of the pyrenoids had a bilenticular structure. Similarly, about half of the pyrenoids in *W. amphibia* was found to be polypyramidal (57%), the other 43% were bilenticular (specimen N60, Figs. 6D & E, n=14). All pyrenoids in *W. amphibia* displayed a small round spot of a different density than the surroundings. All pyrenoids of members of the *Basicladia*-clade were polypyramidal (n=64), with no intra-individual or intraspecific variation in the pyrenoid structure. The surrounding starch layer was highly fragmented (Fig. 6F), with the most extreme form of segregation observed in *B. okamurae* (Fig. 6G). Pyrenoids of the genus *Pithophora* showed variation in their ultrastructure, both intra-individually and between specimens. In specimen K93 (*P*. cf. *roettleri* Wittrock, Fig. 6H, n=31), all pyrenoids were polypyramidal with the surrounding starch layer divided into irregular pieces. In specimen K97 (*P*. cf. *polymorpha* Wittrock), 16% of the pyrenoids were bilenticular and 84% were polypyramidal (Fig. 6I, n=19). Specimen K96 (*P. roettleri*) about two thirds of the studied pyrenoids showed a polypyramidal structure (Fig. 6J, n=11) and about one third of the pyrenoids was bilenticular (36%, Fig. 6K). The starch plates in this specimen were very large (Figs. 6J & K). All pyrenoids in *A. linnaei* were polypyramidal (specimen N36, Fig. 6L, n=9). A unique feature found in all pyrenoids of this species was the association of a stack of thylakoid membranes with the pyrenoid, inside the surrounding starch layer (Fig. 6M).

Discussion

In the present study we aimed at establishing the phylogenetic relationships within the *Aegagropila*-clade with more confidence than previous treatments (Hanyuda *et al.* 2002, Yoshii *et al.* 2004) by increasing both the number of taxa and the number of characters (combining LSU and SSU sequence data). Earlier phylogenies of the *Aegagropila*-clade were based on SSU rRNA gene sequences only, a gene that has been shown in other phylogenetic studies of the Cladophorales to not resolve relationships well. By adding partial sequences of the more variable LSU rRNA gene, the number of informative characters was doubled (Table 3). Another potential benefit of combining genes is the emergence of relationships that are not recovered from individual partition trees (Gontcharov *et al.* 2004). The advantages of combined analyses have been demonstrated in numerous phylogenetic studies (e.g., Murray *et al.* 2005, Feau *et al.* 2006, Leliaert *et al.* 2007a). The relationships inferred in our phylogenetic analyses are in overall agreement with the previously published trees of the *Aegagropila*-clade (Hanuyda *et al.* 2002, Yoshii *et al.* 2004), and are generally well supported. In addition, we show that *Wittrockiella salina* and *Cladophorella calcicola* are part of the *Wittrockiella*-clade, and that all *Basicladia* species plus *Arnoldiella conchophila* are closely related and likely form a clade. We characterize a new subclade closely related to *Aegagropila* and *Pithophora* that contains tropical species with a *Cladophora*-type morphology. For a discussion of slightly different tree topologies and support values recovered when employing simple models of nucleotide substitution, see Appendix S6.

Apomorhies of the Aegagropila-*clade*

Hanyuda *et al.* (2002) suggested that the *Aegagropila*-clade was characterized by a number of unique/derived biochemical and ultrastructural features, including the presence of the carotenoid pigment loraxanthin, the presence of chitin in the cell walls and polypyramidal pyrenoids. In contrast, the other two main lineages of the Cladophorales (the *Cladophora*-clade. and the *Siphonocladus*-clade) are assumed to be characterised by uniformly bilenticular pyrenoids (van den Hoek *et al.* 1995). Polypyramidal pyrenoids have been identified in all members of the *Aegagropila*-clade investigated so far, namely: *Cladophora horii* (van den Hoek & Chihara 2000); *C. conchopheria*, *Wittrockiella lyallii*, *Arnoldiella conchophila*, *B. okamurae*, *Pithophora mooreana* Collins and *Aegagropila linnaei* (Matsuyama *et al.* 1998; Hanyuda *et al.* 2002); as well as in *Basicladia chelonum* (Mrozinska *et al.* 2009). Polypyramid pyrenoids have, however, also been found in *Cladophora catenata* Kützing (Matsuyama *et al.* 1998), a species of the *Siphonocladus*-clade (Hanyuda *et al.* 2002), and *Rhizoclonium tortuosum* (Dillwyn) Kützing (Miyaji 1999), for which no molecular data are available at present but which is assumed to be a member of the *Cladophora*-clade. Furthermore, polypyramid pyrenoids are known for *Dictyospheria cavernosa* Forsskål (Børgesen) (Hori & Ueda 1975), a member of the *Siphonocladus*-clade. In the present study, polypyramidal pyrenoids have been confirmed for *Basicladia okamurae*, *B. kosterae*, one isolate of *Pithophora* (*P.* cf. *roettleri*, K93) and *Aegagropila linnaei*. Both polypyramidal and bilenticular pyrenoids were found in *Wittrockiella amphibia*, *Cladophorella calcicola*, *Pithophora* cf. *polymorpha* and *P. roettleri*. In *W. salina*, only bilenticular pyrenoids were observed, as had been previously mentioned by van den Hoek *et al.* (1984). Thus, the pyrenoid ultrastructure does not seem to be a stable diagnostic character to separate the *Aegagropila*-clade from the rest of the Cladophorales.

The other two suggested characters are also problematic. Loroxanthin, found in all members of the *Aegagropila*-clade studied to date, is also present in some members of the *Cladophora*-clade (Fawley 1991, Yoshii *et al.* 2004) and several orders of green algae (Fawley 1991). *Blastophysa rhizopus*, the closest known relative of the Cladophorales (Cocquyt *et al.* 2010), has siphonoxanthin (O'Kelly 1982), a character that otherwise appears to be derived within Cladophorales (Yoshii *et al.* 2004). Since lutein, loroxanthin, and siphonoxanthin are thought to be successive products in a biosynthetic series (Egeland *et al.* 1997, Yoshii *et al.* 2004), the actual xanthophylls produced may be less informative for phylogenetic purposes than the enzymes responsible for synthesizing them, and especially the genetic factors affecting their expression. Chitin is only known to be present in the cell walls of *Pithophora* species (Pearlmutter and Lembi 1978, 1980), for other members of the *Aegagropila*-clade data are lacking. There are, however, reports of the presence of chitin in the cell walls of *Cladophora glomerata* (Wurdack 1923) and *C. vagabunda* (Jónsson 1962, as *C. expansa*). Both species are members of the *Cladophora*-clade, rendering the presence of chitin invalid as a diagnostic character.

While polypyramidal pyrenoids and the presence of loroxanthin might represent apomorphies of the *Aegagropila*-clade, they do not represent diagnostic characters.

Thus, the *Aegagropila*-clade is currently only defined by molecular data. However, this multinucleate lineage can be characterized by occurring in brackish or freshwater habitats; a tendency for dominant asexual reproduction; extensive development of secondary rhizoids; a tendency for heterotrichous organization: from polysiphonous holdfast clusters (*horii*-clade), to a clear division into a prostrate and an upright system (*Wittrockiella*- and *Basicladia*-clade), to loss of the prostrate system coupled with ease of inversion of polarity or being unattached (*Aegagropila* & *Pithophora*); and subterminal insertion of laterals combined with delayed cell wall formation.

The *Aegagropila*-clade has not received a formal taxonomic rank, but considering its robustness in molecular phylogenetic analyses (Hanyuda *et al*. 2002, Yoshii *et al*. 2004, Rindi *et al*. 2006, present study), the designation of a family name is warranted. This lineage includes type species of three families: Pithophoraceae Wittrock 1877, Wittrockiellaceae Wille 1909 and Arnoldiellaceae Fritsch 1935. *Pithophora* and *Wittrockiella* are the sole genera within their families, while Arnoldiellaceae includes *Arnoldiella*, *Basicladia*, and the monotypic *Cladostroma* Skuja. The name **Pithophoraceae** has priority, and its use for the *Aegagropila*-clade is recommended here. In its new sense, the family includes the genera *Aegagropila*, *Aegagropilopsis*, *Arnoldiella* (including *Basicladia*), *Pithophora*, *Pseudocladophora* and *Wittrockiella*. Although the monotypic genus *Spongiochrysis* has been characterized as a member of the *Aegagropila*-clade (Rindi *et al*. 2006), we tentatively refrain from including it into this lineage because of data conflict between SSU and LSU rDNA data (unpublished data), resulting in an ambiguous phylogenetic position.

Genera of the Pithophoraceae (=**Aegagropila*****-clade)***

horii-clade

Cladophora conchopheria and *C. horii* are the only marine species in the Pithophoraceae, and form the earliest diverging clade. *Cladophora conchopheria* grows exclusively on shells of the marine snail *Lunella coronota,* occurring in South Korea and Japan (Sakai 1964, Matsuyama *et al*. 1999, van den Hoek & Chihara 2000). *Cladophora horii* has been found in shallow subtidal waters and intertidal rockpools, sometimes as an epiphyte on *C. prolifera*, along the east coast of South Africa (Leliaert & Coppejans 2003) and Okinawa, Japan (van den Hoek & Chihara 2000). The robust, broom-like tufts of *C. horii* and the minute turfs of *C. conchopheria* share a polysiphonous holdfast formed by fusion of descending rhizoids with the walls of basal cells. This character was not regarded as synapomorphic in a morphological treatment, in which *C. horii* was placed in the *Cladophora* section *Rugulosae* while *C. conchopheria* was placed in the section *Glomeratae* (van den Hoek & Chihara 2000). Other members of the section *Rugulosae* have extensive secondary rhizoidal development, often with annular constrictions, but the rhizoids do not fuse with the walls of other cells. Coalescent basal stipes are also known from other members of the section *Glomeratae* such as *C. albida* (Nees) Kützing or *C. opaca* Sakai (van den Hoek & Chihara 2000), but these differ in their mode of formation and are not characterized by very thick, layered cell wall wedges (Fig. 5).

Both *C. conchopheria* and *C. horii* are densely branched and show a typical *Cladophora*-like architecture, and the new genus *Pseudocladophora* is proposed here to accommodate the two species. We select *C. conchopheria* as the type species of the new genus.

Pseudocladophora **Boedeker gen. nov.**

Type species: *Pseudocladophora conchopheria* (Sakai) Boedeker comb. nov. (*Cladophora conchopheria* Sakai 1964: 48).

Latin diagnosis

Algae marinae thallis rigidis erectis filamentis uniseriatis compositis divisionibus acropetalis intercalaribusque. Filamenta sparse ramosa in thalli partibus inferioribus, distaliter dense ramosa fere cellulis omnibus laterales uno vel duos rare tres septis obliquis ad ardue inclinatis e ramorum basi. Rami angulos acutos inclinati. Ramorum ordinatio parum acropetala ad opposita vel irregularia. Thalli substratum affixi hapeteronibus rhizoidealibus stipitibus numerosis e basi vulgari radiatis. Cellulae in thalli partibus mediis et inferioribus rhizoidea secundaria descendentia formantes saepe intracuticularia cellulis inferioribus. Rhizoidea adventitia parietibus cellulariis cellularum inferiorum connatescentia structuri caulibus similibus basin polysiphonam formantia. Cellularum parietes crassi partibus apicalibus 1.5-3-(5) µm*, basalibus* 5-15-(30) µm *crassis*.

Description

Marine algae with stiff, erect thalli composed of uniseriate filaments growing by acropetal and intercalary cell divisions. Cells are multinucleate with parietal net of polypyramidal chloroplasts. Filaments sparsely branched in lower parts of the thallus, densely branched in distal parts with almost every cell cutting of one or two (rarely three) laterals by oblique to steeply inclined cross walls at the base of branches. Branches inclined at acute angles. Branching patterns slightly acropetal to opposite to irregular. Thalli attached to the substratum by rhizoidal holdfasts with many stipes radiating from a common base. Cells in the middle and lower parts of the thallus producing descending secondary rhizoids, frequently intracuticular in cells below. The adventitious rhizoids become fused with the cell walls of the lower cells, producing stem-like structures that form a polysiphonous base. Cells cylindrical, apical cells rounded. Cell walls thick, in apical parts 1.5-3-(5) µm, in basal parts 5-15-(30) µm.

Members

Pseudocladophora conchopheria (Sakai) Boedeker comb. nov.
Holotype: Nagahama near Maizuru, Kyoto Prefecture, Japan, collector I. Umezaki, May 1949, SAP (SAP 029140), on shell of the marine gastropod *Lunella coronata* Gmelin.
Basionym: *Cladophora conchopheria* Sakai 1964: 48.

Pseudocladophora horii (van den Hoek & Chihara) Boedeker comb. nov.
Holotype: Sesoko Island, Okinawa, Japan, collectors S. Kamura, C. van den Hoek & T. Hori, April 1990, TNS (TNS-AL-46793).
Basionym: *Cladophora horii* van den Hoek & Chihara 2000: 68.

Wittrockiella-clade

Wittrockiella grows in brackish-water and estuarine environments, and currently contains three species (*W. amphibia*, *W. lyallii* and *W. salina* (including two varieties), which are primarily separated by their cell dimensions. The three species form a highly supported clade which also includes *Cladophorella calcicola*. *Cladophorella calcicola* is most closely related to *W. amphibia*, a relationship that was already proposed based on morphological similarities (van den Hoek *et al*. 1984). *Cladophorella calcicola* is a warm-temperate to tropical semi-terrestrially species reported from moist limestone, bricks and mud in China, Bangladesh, and tropical hothouses in Europe (Fritsch 1944, Islam 1964, Cribb 1965, Ettl & Gärtner 1995, Liu 1999). The sample used in this study was found in an estuarine lagoon in Portugal (Table 2). Since all species of *Wittrockiella* occur in brackish environments (Wille 1909, Polderman 1976, South 1981, van den Hoek *et al*. 1984, Nelson *et al*. 2002), it seems likely that the freshwater/semi-terrestrial species *C. calcicola* evolved from a brackish ancestor (see Fig. 4). No molecular data are available yet for the other described *Cladophorella* species (*C. frischii* Islam, *C. sunderbanensis* Islam, *C. netzhualpilii* Galicia-García & Novelo).

Wittrockiella (1909) has priority over *Cladophorella* (1944), thus the new combination *Wittrockiella calcicola* (Fritsch) Boedeker is proposed (see below). For typification of *W. salina, W. amphibia* and *W. lyallii* see van den Hoek *et al*. (1984), Boedeker & Hansen (2010) and Boedeker *et al*. (2010c), respectively. We here select an epitype for *W. paradoxa*, the type species of the genus, to serve as a complement to the original drawings that represent the holotype.

***Wittrockiella* Wille**
Type species: *Wittrockiella amphibia* (Collins) Boedeker & Hansen (*Wittrockiella paradoxa* Wille 1909: 220-221).
Generitype: original specimen (collected in 1907 by N. Wille, Lyngør, Norway) not traceable, thus the original drawings represent the holotype material (Wille 1909: Tables XI-XIV).
Epitype: Lyngør, southeastern Norway, collector B. Lynge, January 1909, det. N. Wille, O (six iso-epitypes).

Wittrockiella calcicola (Fritsch) Boedeker comb. nov.
Holotype: original specimen (collected by F.E. Fritsch, tropical hothouse, Cambridge Botanical Garden, Britain, 1944, on moist limestone) lost or destroyed (used to be in BM), thus the original drawings represent the holotype material (Fritsch 1944: Figs. 1A-I, 2A-G, 3A-C, 4A-G).
Epitype: Lagoa de Óbidos, Portugal, collector O. Lourenço (sample no. K92; ACOI culture collection 471), 1989, L (L0793292).
Basionym: *Cladophorella calcicola* Fritsch 1944: 157-171.

Basicladia-clade

Seven species of *Basicladia* have been described, a genus that has a reputation to be restricted to freshwater turtles as the only possible substrate (e.g., Edgren *et al*. 1953, Normandin & Taft 1959). DNA sequences from three species could not be obtained for this study, namely *B. crassa*, *B. sinensis* and *B. vivipara*. The genus *Basicladia* was erected to accommodate *B. chelonum* Hoffmann & Tilden and *B. crassa* Hoffmann & Tilden (Hoffmann & Tilden 1930). These two species differ only in their cell dimensions and intermediate forms exist. Both species frequently occur in the same habitat, or even on the same turtle, and it was proposed that they could represent merely different growth forms of a single plastic species, such as a sun and a shade form (Proctor 1958, Garbary *et al*. 2007). *Basicladia sinensis* (Gardner) G.M. Smith is only known from one specimen collected from a Chinese freshwater turtle that was imported into the USA (Gardner 1936), and its habit and cell dimensions are actually in the range of *B. crassa*. The species *B. vivipara* Normandin & Taft is only known from the freshwater gastropod *Viviparus malleatus* Reeve (Normandin & Taft 1959), and is morphologically very similar to *B. chelonum*. Thus, the actual number of species might not have been correctly assessed yet, and molecular data of these taxa would be very interesting. The grouping of *Arnoldiella conchophila* with *B. chelonum* and *B. kosterae* was well supported in our phylogenetic trees. It shares both the heterotrichous growth form of *Basicladia*, in which a dense basal stratum of branching filaments is united into a continuous layer, and a strong preference for epizoophytic freshwater habitats (Miller 1928, Kargupta 1994, Keshri & Hazra 2009).

Previous phylogenies had recovered *B. okamurae* on a basal polytomy on the branch connecting either *Basicladia* sp. or *Arnoldiella conchophila* and the *Aegagropila*/*Pithophora*-clade (Hanyuda *et al*. 2002, Yoshii *et al*. 2004). Our analyses yielded similar results (Figs. 3 & 4, Appendix S6 & S7), thus monophyly of *Basicladia* (including *A. conchophila*) could not be demonstrated with certainty. However, there is strong morphological evidence for keeping *B. okamurae* in the same genus as the other *Basicladia* species such as the formation of a dense basal stratum of branching filaments that unite into a continuous layer, and the very large basal cells of the upright filaments. Since *A. conchophila* is nested within *Basicladia* and has nomenclatorial priority over *Basicladia*, we propose the transfer of all *Basicladia* species used in this study to the emended genus *Arnoldiella*.

***Arnoldiella* V. Miller emend. Boedeker**

Type species: *Arnoldiella conchophila* V. Miller 1928: 20-21.

Description

Thallus differentiated into a prostrate layer consisting of coalescing filaments and a compact system of rigid upright filaments. Cells of the prostrate layer with one to few nuclei, cells of the erect filaments multinucleate. Erect filaments can be densely branched, rarely branched or entirely unbranched. If branched, branches more numerous in apical parts of the thallus. If unbranched, occasional branching can occur directly at the base of the upright filaments. Primary, secondary and tertiary branching can be present in erect filaments. Branches inserted subterminally or cut off by an almost horizontal cross wall resulting in a pseudichotomy. Cells gradually becoming shorter and wider from base to apex. Apical cells rounded or pointed. Thick cell walls, at least in basal parts. Terminal zoosporangia, sometimes formed in chains.

Members

Arnoldiella chelonum (Collins) Boedeker comb. nov.

Holotype: Walnut Lake, Michigan, USA, collector T.L. Hankinson, on turtle carapaces, NY (00887601).

Basionym: *Chaetomorpha chelonum* Collins 1907: 198-200.

Synonym: *Basicladia chelonum* (Collins) Hoffmann & Tilden 1930: 382-383.

Arnoldiella conchophila V. Miller
Holotype:, original specimen (collected by V. Miller, Lake Pereslavl, Vladimir district, Russia, 1921, on shell of freshwater bivalve) untraceable, thus the original drawings represent the holotype material (Miller 1928: Figs. 2-20).

Arnoldiella kosterae (van den Hoek) Boedeker comb. nov.
Holotype: Jardin des Plantes, Paris, France, collector C. van den Hoek, 25 April 1961, L (L 0054830).
Basionym: *Cladophora kosterae* van den Hoek 1963: 37-38.
Synonym: *Basicladia kosterae* (van den Hoek) Garbary 2010: 39.

Arnoldiella okamurae (S. Ueda) Boedeker comb. nov.
Holotype: Shirahama, Tokyo, Japan, collector S. Ueda, Tokyo University of Marine Science and Technology.
Basionym: *Chaetomorpha okamurae* S. Ueda 1932: 23-24.
Synonyms: *Cladophora okamurae* (S. Ueda) van den Hoek 1963: 39.
Basicladia okamurae (S. Ueda) Garbary 2010: 39.

Arnoldiella ramulosa (Ducker) Boedeker comb. nov.
Holotype: Stratford, Victoria, Australia, collector S.C. Ducker, 11 December 1956, on carapace of turtle, MEL. Isotypes in BM & MELU.
Basionym: *Basicladia ramulosa* Ducker 1958: 165-166.

Aegagropila/*Pithophora*-clade

This clade is characterized by a reduced rhizoidal system and the erect system has become the sole thallus. This is most extreme in *Pithophora*, which only occurs unattached. Also *Aegagropila* is commonly found unattached, and both genera display frequent inversion of polarity. Secondary rhizoids are formed by all members of this clade, but no prostrate system is formed.

1. *Aegagropila*

Aegagropila linnaei is currently the only species of the genus. More than 90 synonyms exist for *A. linnaei* (holotype in L, van den Hoek 1963), stemming from over-interpretation of plastic morphological characters. This species produces different growth forms, including attached filaments, free-floating mats and ‘lake balls’. Little genetic variation was found among samples from the entire geographic range, indicating that *A. linnaei* indeed represents a single species (Boedeker *et al.* 2010b).

2. *Pithophora*

Species of *Pithophora* are widespread in the (sub)tropics (Wittrock 1877, Möbius 1895, Fritsch 1907a, Bourrelly 1966), but also widely distributed in the temperate regions of the eastern USA (John 2003). More than 35 taxa of *Pithophora* have been described (Index Nominum Algarum, http://ucjeps.berkeley.edu/INA.html), including a large number of varieties. The extent of phenotypic plasticity in the few morphological characters that had led to an inflation in the number of described species (Ernst 1908, Mothes 1930, also Fott 1971). Identification at the species level became basically impossible due to overlap of character states (Möbius 1895, van Oye 1922). Conflicting with the number of described taxa based on subtle morphological differences, it was shown that akinete formation and germination is controlled by a wide range of environmental conditions (Ernst 1908, Agrawal 1986, Stevens & Neilson 1987), that the size of akinetes is age-dependent (Brand 1904), that akinete and branch formation are the same reversible process (Mothes 1930), and that helicoid formation is inducible as a wounding response (Mothes 1930). Pankow & Täuscher (1980) concluded that species level identifications are not feasible due to the amount of redundant species descriptions and recognized only two species, synonymizing all taxa with either *P. oedogonia* (Montagne) Kützing or with *P. roettleri* (Roth) Wittrock. The only morphological character separating these two species is the shape of intercalary akinetes. However, the distinction between isosporous and heterosporous is not clear at all (see Ernst 1908, van den Hoek 1959, Prescott 1951, Pankow & Täuscher 1980, Skinner & Entwisle 2004).

The lack of genetic variation in the LSU and SSU rDNA sequences (all SSU sequences identical, max. 3 bp differences in the LSU sequence of one out of five samples) of samples of different origin and morphologies (Table 4), hints to the existence of just one widespread, polymorphic species. The frequent formation of desiccation-resistant akinetes in *Pithophora* implies a high long-distance dispersal capacity. Based on morphological and molecular data we thus regard all described *Pithophora* taxa to be conspecific, rendering the genus monotypic. The name *P. roettleri* (Roth) Wittrock has priority (basionym: *Ceramium roettleri* Roth 1806, type in L), and is proposed to be used as the single species name. No type species had been selected for *Pithophora* to date, the sole genus included in Pithophoraceae at its inception. We here select *P. kewensis* Wittrock as the lectotype for the genus *Pithophora* and the family Pithophoraceae (see Materials and Methods), which is assumed to reside with the sequenced *Pithophora* specimens in the *Aegagropila*/*Pithophora* clade.

The sister relationship with the temperate species *A. linnaei* invites for interesting speculation. Both genera are assumed to be asexual (Möbius 1895, Brand 1902, Ernst 1908, Heering 1921, Mothes 1930, Fritsch 1935, van den Hoek 1963, Soejima *et al.* 2009) and polyploid (chromosome counts in *Pithophora*: Geitler 1936, Verma 1979; own unpublished data of C-values for *A. linnaei*), factors that could play a role in the lack of speciation within the genera, the low intraspecific genetic variation and the extensive morphological plasticity.

Pithophora **Wittrock**
Type species: *Pithophora roettleri* (Roth) Wittrock (*Pithophora kewensis* Wittrock 1877: 52-55).
Lectogeneritype: *Pithophora kewensis* Wittrock, tropical aquarium ('Waterlily-house'), Kew Gardens, Britain, collector V.B. Wittrock, August 1872, L (no. 938112 639). Isotypes in BM, L, UPS & S.

Pithophora roettleri (Roth) Wittrock
Holotype: Tranquebar, eastern India, collector Klein, January 1799, L (no. 93825 38). Isotype in UPS.
Basionym: *Ceramium roettleri* Roth 1806: 123.
Synonyms: all described species of *Pithophora* (including all intraspecific taxa) *P. aequalis* Wittrock, *P. affinis* Nordstedt, *P. chinensis* Skworzow, *P. clavifera* Schmidle, *P. cleveana*

Wittrock, *P. kewensis* Wittrock, *P. macrospora* Brand, *P. microspora* Wittrock, *P. mooreana* Collins, *P. oedogonia* (Montagne) Wittrock, *P. pachyderma* Schmidle, *P. pragensis* Sula, *P. polymorpha* Wittrock, *P. radians* W. & W.S. West, *P. reinecki* Schmidle, *P. sumatrana* (Martens) Wittrock, *P. varia* Wille, *P. variabilis* Schmidle, *P. zelleri* (Martens) Wittrock.

3. *Cladophora*-subclade

Three Asian *Cladophora* species are united in this subclade. One of them is still wanting a formal description, even though it has been included in a number of studies under several designations: in Nagai (1988) as *C. sauteri* forma *sauteri*, in Kanda (1991) as *C. sauteri*, in Hanyuda *et al.* (2002) and Yoshii *et al.* (2004) as *Cladophora* sp. 'Tateyama', and in Wakana *et al.* (2001b) as *Aegagropila* sp. nov. ('Tateyama-Marimo'). This species is morphologically very similar to *A. linnaei*, and in addition to epilithic growth forms it occurs also as free-floating tufts (Wakana *et al.* 2001b). Our phylogeny shows a sister relationship with *C. clavuligera*, a poorly-known species that has been reported from shells of a freshwater gastropod from Sri Lanka (Grunow 1868), and from brackish and freshwater gastropods (*Pila globosa* Swainson) and bivalves as well as from wood and stones in India (Verma 1981, Krishnamurthy 2000), but some of these identifications seem doubtful. Morphologically, *C. clavuligera* is very close to *C. yuennanensis* Skuja from China and *C. beneckei* Möbius from Java (see also Table 5). Our identification of *C. clavuligera* must be viewed as tentative too, since the material was collected in a tropical aquarium and is thus of unknown geographic origin. The third member of this subclade is *C. sterrocladia*, sister to both *C. clavuligera* and *Cladophora* sp. 'Tateyama'. It has been described from the shell of a freshwater snail (*Paludina*) from Myanmar (Skuja 1949). In a number of studies, this (sub)tropical species has been confused with the temperate species *A. linnaei* (e.g., Prasad & Misra 1992, Gardavský 1993, Liu 1999, Islam & Irfanullah 2005). As in the genus *Arnoldiella*, there seems to be a tendency in this clade for epizoophytism, especially to colonise freshwater gastropods. In comparison to *Arnoldiella*, the rhizoidal system in the *C. clavuligera/sterrocladia*-subclade is reduced, but long secondary rhizoids are formed.

So far, this subclade is restricted to Asia. Tropical Asian freshwater *Cladophora* species with a morphology similar to *C. clavuligera* and *C. sterrocladia* are for example *C. basicladioides* Jao, C. *beneckei*, *C. codiola* Zeller, *C. exigua* Zeller, *C. glomerata* var. *nana* Wang, *C. shensiensis* Jao, and *C. yuennanensis* Skuja. *Cladophora dusenii* Brand from Cameroon and *C. parvula* Möbius from Australia are probably also closely related to this group of species. However, the freshwater algal floras of both Africa and South America are less well known than of Asia, and the actual number of taxa and their distributions are most likely underestimated. Additional taxon sampling is clearly required, also in Asia (see Table 5).

The species of this subclade need to be transferred from *Cladophora* to a new genus. We decide against the possibility to merge *Aegagropila*, *Pithophora* and the three *Cladophora* species into *Aegagropila*, and erect a new genus to accommodate the three *Cladophora* species. The sequence divergence between the four main clades within the *Aegagropila*-clade is in the range 4.2-6.6%. Maximum intrageneric sequence divergence in *Wittrockiella*, *Pseudocladophora* and *Arnoldiella* emend. is 1.3%, 2.6% and 3.2-3.5%, respectively. In comparison, the sequence divergence between the three lineages in the *Aegagropila/Pithophora*-clade is 2.8-5%, and the recognition of three separate genera does not seem to be exaggerated. We propose the name *Aegagropilopsis* gen. nov., based on the morphological similarity to the genus *Aegagropila*, and select *C. sterrocladia* as the type species.

***Aegagropilopsis* Boedeker gen. nov.**

Type species: *Aegagropilopsis sterrocladia* (Skuja) Boedeker comb. nov. (*Cladophora sterrocladia* Skuja 1949: 94-95).

Latin diagnosis

*Algae aquae dulcis interdum nonnihil salsugineae caespitibus minutis minus quam 1.5 cm altis filamentis erectis unserialibus hapterone coralloideo rhizoideis secundariis longis. Thalli dense ramosi saepe partibus inferioribus sparse ramosis. Rami praecipue oppositi interdum pectinati insertione subterminali interdum seriali angulis acutis septis tarde formantibus ardue inclinatis. Cellulae usque ad cinque laterales saepe verticillatas typice parum ad nodos constrictas partibus apicalibus longis eramosis. Rhizoidea adventitia e thalli omnibus partibus facta. Thalli praecipue epizoophtici ad cochleas aquae dulcis (sub) tropicis (*Pila, Paludina*) sed quoque substratis immobilis affixi inventus*.

Description

Freshwater algae, sometimes penetrating into slightly brackish waters, forming minute tufts or turfs less than 1.5 cm tall, consisting of erect uniseriate filaments, attached by a coralloid holdfast and long secondary rhizoids. Sometimes several shoots arising from the same holdfast, basal cells are short. Thalli densely branched, with lower parts of the thallus frequently sparsely branched. Branches mainly opposite, sometimes pectinate. Insertion of branches subterminally, sometimes serially, at acute angles, with delayed cross wall formation, cross walls steeply inclined. Up to five laterals per cells, frequently forming whirls. Cells typically slightly constricted at nodes. Apical parts long and unbranched. Adventitious rhizoids formed in all parts of the thallus. Cells in main axis 3-6 times as long as broad, cells in branches can be up to 1.5 mm long. Cell shape cylindrical to irregular. Apical cells 20-50 µm in diameter, branches 20-60 µm, basal parts up to 130 µm. Cell walls relatively thin, up to 6 µm in basal parts, 1 µm in apical cells. Zoospore formation by transformation of terminal cells into slightly swollen zooidingia. Thalli mainly epizoophytic on (sub)tropical freshwater snails (*Pila*, *Paludina*), but also found attached to stationary substrates.

Members

Aegagropilopsis sterrocladia (Skuja) Boedeker comb. nov.

Holotype: original specimen (collected by H. Skuja, Burma/Myanmar, on shell of freshwater gastropod) lost (used to be in RIG), thus the original drawings represent the holotype material (Skuja 1949: Plate XXXVII).

Epitype: pond in tropical hothouse, Hortus Botanicus Leiden, The Netherlands, collector C. Boedeker (sample G91), 26 April 2006, attached on mangrove pneumatophores (submerged), L (L0793287).

Basionym: *Cladophora sterrocladia* Skuja 1949: 94-95.

Aegagropilopsis clavuligera (Grunow) Boedeker comb. nov.

Holotype: Ceylon/Sri Lanka (Expedition Novara), collector G. von Frauenfeld, W (2010/2274), on shell of freshwater gastropod.

Basionym: *Cladophora clavuligera* Grunow 1868: 40.

Specialised niches and competition

Almost all species in the Pithophoraceae occur in habitats that can be characterized by fluctuating environmental conditions and reduced competition. Such conditions are found either in brackish environments (*Wittrockiella*, and to some extent *Aegagropila linnaei*) or on the surface of mobile host animals (*Pseudocladophora conchopheria*, *Arnoldiella* and to some extent *Aegagropilopsis*). The environment types and host animals of the members of the Pithophoraceae are indicated in Fig. 7. *Pseudocladophora conchopheria* occurs exclusively on the shells of the intertidal gastropod *Lunella coronota* along the coasts of South Korea and central and southern Japan (Sakai 1964, Matsuyama *et al.* 1999, van den Hoek & Chihara 2000), facing frequent desiccation stress and changes in other abiotic parameters. No other algae have been found to colonise those shells. Members of the genus *Arnoldiella* are most frequently encountered as epizoophytes on aquatic animals, especially on freshwater molluscs and turtles, habitats that have a drastically reduced algal diversity compared to permanent and stationary substrates (e.g. Edgren *et al.* 1953, Belusz & Reed 1968). Due to the basking and burrowing behaviour of many freshwater turtle species, they represent one of the most challenging habitats for freshwater algae. The species of *Arnoldiella* (as *Basicladia*) have a reputation to be restricted to freshwater turtles as the only possible substrate (e.g., Edgren *et al.* 1953, Normandin & Taft 1959). The Australian endemic *A. ramulosa* is actually the only *Arnoldiella* species that is exclusively known from turtles (Ducker 1958, Skinner *et al.* 2008). In North America, *A. chelonum* and *B. crassa* are the most common algae found on the carapaces of a wide range of turtle species (Proctor 1958, Belusz & Reed 1968, Garbary *et al.* 2007). The species *B. vivipara* Normandin & Taft is only known from the freshwater gastropod *Viviparus malleatus* Reeve (Normandin & Taft 1959). *Arnoldiella kosterae* has only rarely been encountered on turtles (Belusz & Reed 1968, also Ernst & Norris 1978 (as *B. crassa*)), but has been found on freshwater bivalves and dead wood (samples J56 and J79, respectively), and on stones and concrete (van den Hoek 1963). Only *A. okamurae*, the earliest diverging member of *Arnoldiella*, has actually never been encountered on turtles, and is the only *Arnoldiella/Basicladia* species that so far has never been encountered on an animal host of any kind (but observations are few). Thus, the tendency for epizoophytism might represent a more derived state and the evolution of the heterotrichous habit, also present in *A. okamurae*, might have been a prerequisite to colonise animal hosts (see also discussion on heterotrichy below). However, we could not show well-supported grouping of *A. okamurae* with the other *Arnoldiella/Basicladia* taxa in our phylogenetic analyses, thus the possibility exists that *A. okamurae* represents a separate unbranched, non-epizoophytic lineage.

It has been demonstrated for *A. chelonum* and *B. crassa* that other substrates can also be colonized in culture (Proctor 1958). These findings indicate competition effects, since these taxa are predominantly encountered on turtles in nature and not on other substrates. While some mutualistic relationships between the turtles and the algae have been considered (Edgren *et al.* 1953, Neill & Allen 1954), it seems more likely that competitive exclusion is at work, and that *Arnoldiella/Basicladia* species found their niche as epizoophytic freshwater algae. Besides *Arnoldiella/Basicladia* and *Dermatophyton radians* Peter, probably a closely related species, very few other macroalgae are found on turtle carapaces (e.g., Edgren *et al.* 1953, Belusz & Reed 1968, Garbary *et al.* 2007). *Dermatophyton radians* is generally considered to consist of merely a prostrate crust, but it was shown in culture that an erect system simlar to *Arnoldiella* spp. can develop (Potter

1888, Feldmann 1936). *Arnoldiella/Basicladia* species are most frequently encountered on the shells and carapaces of freshwater animals, probably because they are well adapted to those habitats by their heterotrichous organization and because competition in those habitats is drastically reduced compared to other solid substrates.

Similarly, brackish environments are generally characterized by low species numbers, and macroalgal diversity declines with decreasing salinity (Munda 1978, Nielsen *et al.* 1995, Middelboe *et al.* 1997). As both marine and freshwater species are at a disadvantage and species diversity is at a minimum (Remane 1934, Den Hartog 1969), competition can be less intense in brackish environments. The species of *Wittrockiella* occur in habitats characterised by strongly reduced biotic interactions. *Wittrockiella calcicola* mainly occurs semi-terrestrially on moist stone (Fritsch 1944, Islam 1964) or on mud (Table 2), while the other members of the genus are found in estuarine or intertidal habitats that are characterized by high fluctuations of environmental parameters and by frequently reduced salinities due to freshwater seepage. Adaptations of *Wittrockiella* to habitats with fluctuating salinities include the formation of a thick mucilage cover in *W. amphibia* (Wille 1909), an endophytic lifestyle in *W. amphibia* (Polderman 1976), the presence of haematochrome/oil droplets in *W. amphibia* and *W. salina* (Wille 1909, van den Hoek *et al.* 1984, respectively), and a cushion-like growth habit to preserve moisture during exposure to air in *W. salina* and *W. lyallii*. Another example for competition possibly shaping the ecological niche is *Aegagropila linnaei*, a species that occurs in both freshwater and brackish environments. *Aegagropila linnaei* is only found in the northern parts of the brackish Baltic Sea where salinity levels are below 6 psu (Boedeker et al 2010b). Northwards of the transition zone between the Bothnian Sea and the Bay of Bothnia where the salinity drops from 5 to 3.5 psu, *A. linnaei* even becomes the dominant macroalga on hard substrates down to a depth of 10 m (Nielsen *et al.*, 1995; Bergström & Bergström, 1999). However, *A. linnaei* can survive several years under fully marine salinities in culture (Boedeker, unpublished data). Since *A. linnaei* disperses via fragmentation, the absence from higher salinities cannot be explained by lower salinity tolerances of spores or gametes. Accordingly, the physiological niche of *A. linnaei* is much broader than its realized niche, and increased biotic interactions might cause the absence of this species in the Baltic Sea in areas with higher salinities than 6 psu. This distribution suggests that *A. linnaei* is a poor competitor and can only establish in areas with low biotic interactions. Similar to *A. linnaei*, it has been shown in culture that the closely related genus *Pithophora* can cope with salinities of up to 20 psu (Mothes 1930), but has only been encountered in brackish environments on very few occasions, possibly washed in from rivers or floodings. *Pithophora* is primarily found in nutrient-rich, stagnant water bodies in the (sub)tropics. These environments are characterized by a less diverse algal flora than similar habitats in the temperate zones due to low oxygen levels in the water (e.g. Fritsch 1907a & b). It appears that the more derived members of the Pithophoraceae have retained a high osmoacclimation potential (the desiccation potential of *Arnoldiella* emend. is physiologically comparable), that has not been lost when the marine or brackish ancestors colonized less saline or even pure freshwater environments (Fig. 7).

Heterotrichy as a morphological adaptation to harsh conditions

In addition to the physiological features discussed above, the heterotrichous growth form of *Wittrockiella* and *Arnoldiella* emend. also represents an adaptation to harsh conditions.

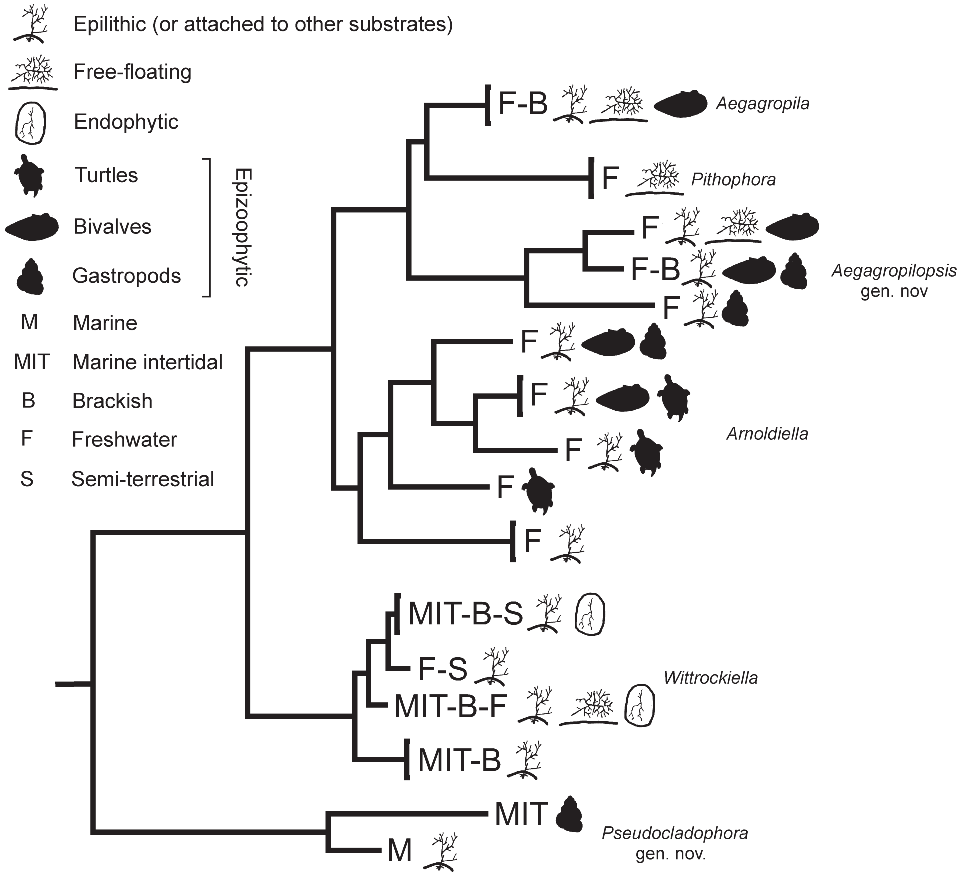

Figure 7. Phylogenetic tree of the *Aegagropila*-lineage (same as Fig. 4B), with letters indicating environment types in which the taxa occur, and stylised symbols indicating known host animals of epizoophytic taxa and growth forms.

Heterotrichy was defined by Fritsch (1935) as a prostrate and an erect system forming the (algal) thallus.

A heterotrichous growth form is generally considered of very significant adaptice importance, and is widespread in frequently disturbed habitats such as the marine intertidal zone (Fritsch 1935, Littler & Kauker 1984). The heterotrichous habit of the aerophytic green algal order Trentepohliales can maintain itself successfully in the extreme terrestrial environment (Fritsch 1945). Fritsch (1944) regarded the heterotrichous habit of *Wittrockiella calcicola* as a direct adaptation to growth on semi-terrestrial rock surfaces. The prostrate system is generally more sheltered from desiccation and abrasion (Seapy & Littler 1982, Littler & Littler 1983, Littler & Kauker 1984). Sand abrasion could be one factor that prevents other algal taxa to establish themselves on freshwater bilvalve shells or the carapaces of burrowing turtles (e.g. Miller 1928, Ducker 1958). Of all species in the Pithophoraceae, *A. conchophila* has the most reduced erect system, which might be either an adaptation to the movements of the host bivalves (*Anodonta* sp. and *Unio* sp.) in and out of the sediment, or an artefact created by the constant abrasion of the developing erect system due to those movements.

Furthermore, the development of a prostrate system allows for retention of space during disturbances and fast recovery from disturbances. It has been shown that *A. ramulosa* perennates as the prostrate system during the hibernation of the host turtle on parts of the carapace that is covered with mud (Ducker 1958). The rhizoids of *Pseudocladophora conchopheria* penetrate the shell of the host gastropod (Matsuyama *et al.* 1999), possibly allowing for regrowth after the loss of the erect filaments. A common feature for heterotrichous species or life-stages is the tendency to reproduce vegetatively (Fritsch 1953, Perrone & Felicini 1988). Prostrate systems typically grow by apical cell divisions and spread outwards, so that a stolon-like growth pattern can develop and subsequently result in fragmentation and vegetative propagation. The frequent secondary rhizoids observed in the Pithophoraceae probably facilitate attachment after fragmentation events. Freshwater representatives of predominantly marine groups often have truncated life histories (Raven 1999). This and the heterotrichous lifestyle in disturbed habitats might explain why sexual reproduction has been observed in so few instances and so few species in the Pithophoraceae.

Our phylogeny indicates that a primary holdfast, present in the earliest diverging genus *Pseudocladophora*, has been lost in *Wittrockiella* and *Arnoldiella* and secondarily gained in *Aegagropila* and *Aegagropilopsis*. Members of the *Aegagropila/Pithophora*-clade are commonly found in quiet waters with less fluctuating environmental conditions, which might have led to the loss of the prostrate system. *Aegagropila linnaei* is even frequently encountered as unattached growth forms, and *Pithophora* is exclusively unattached (Fig. 7). In this context, the Chinese freshwater species *Cladophora rhizobrachialis* Jao is interesting as it displays an intermediate morphology between a prostrate and an erect growth form (Jao 1944). The thallus consists of a main upright filament that is accompanied by several elongate branches arising from nearly every cell of the basal portion, and these branches produce long rhizoids from the basal parts of most cells.

In addition to the colonization of highly specialised niches and the restriction to habitats with reduced competition, the Pithophoraceae represent a fascinating example of a transition from marine (*Okellya curvata* and most other Cladophorales, *Pseudocladophora*) to marine-brackish (*Wittrockiella*) to freshwater organisms (rest of the Pithophoraceae), with some of the most derived taxa penetrating secondarily into brackish habitats again (*Aegagropila linnaei*, *Aegagropilopsis clavuligera*) (Fig. 7). The Cladophorales are part of the essentially marine, species-rich BCD-clade (Bryopsidales-Cladophorales-Dasycladales; Cocquyt *et al*. 2010), and marine-freshwater transitions have only occurred in the Cladophorales: once in the Pithophoraceae, and once in the *Cladophora*-clade (*Cladophora glomerata*-complex and *Rhizoclonium riparium*-complex). One of the earliest diverging members of the Pithophoraceae, *Pseudocladophora horii*, occurs in marine intertidal environments that do not appear to represent a specialised niche or to be characterized by particularly low biotic interactions. However, the physiological capacity for osmoacclimatization is apparently present at the base of the tree and seems to have been retained by all taxa of the Pithophoraceae. The sister species of *P. horii*, *P. conchopheria*, is restricted to a highly specialised niche that seems to be almost competition-free. Thus, the ancestor of the rest of the Pithophoraceae might have evolved a heterotrichous habit and have thrived in estuarine or intertidal environments and subsequently diversified into a brackish lineage (*Wittrockiella*) and a mainly epizoophytic freshwater lineage. Since selection generally operates on suites of traits, changes in morphology are often accompanied by changes in physiology (Pfennig *et al*. 2010). Phenotypic plasticity, very pronounced in the Cladophorales, can facilitate adaptive responses and colonization of novel environments (Pfennig *et al*. 2010), while at the same time phenotypic diversification is promoted by ecological opportunity (Yoder *et al*. 2010).

In many groups of organisms the boundary between marine and freshwater environments is crossed infrequently or not at all, and marine and freshwater taxa are normally not closely related (Round & Sims 1981, see also Logares *et al*. 2007 for additional references). In general, marine-freshwater transitions are rare events in algae. Among the algae, the marine-freshwater interface acts as an effective barrier for most groups of diatoms (Mann 1996), dinoflagellates (Logares *et al*. 2007), and transitions from one realm into the other are rare among Phaeophyceae (e.g., Wehr 2003), Bangiales (e.g., Nelson et al. 2006), trebouxiophytes (Henley *et al*. 2004), cryptophytes (Shalchian-Tabrizi *et al*. 2008) and goniomonads (von der Heyden *et al*. 2004). Differences in the biogeophysical conditions between marine and freshwaters are thought to constitute the blockade for cross-colonization, even though physiological adaptations that facilitate these transitions remain largely unknown. The marine-freshwater boundary represents a selective rather than a physical barrier. Obstacles to evolutionary migration across the salinity spectrum can be due to difficulties in access and dispersal, lack of physiological adaptability, or inability to complete the life cycle (Mann 1996). These do not appear to strongly apply to the brackish and freshwater Pithophoraceae, as their ancestor most likely has been physiologically pre-adapted to intertidal conditions and possibly to an epizoophytic lifestyle. Freshwater taxa of mainly marine groups often have truncated life histories (Raven 1999). Possibly the ability of the Pithophoraceae to reproduce vegetatively enabled them to persist in the novel environments despite disruption of the life cycle. One additional factor that can play a role in making the marine-freshwater interface difficult to cross could be competitive exclusion by adapted residents. Colonizing specialised niches with reduced competition might have enabled the Pithophoraceae to circumvent the barrier and successfully establish lineages in brackish and freshwater environments.

The Pithophoraceae is most likely very old, however, fossils and precisely dated phylogenies are missing. The split of *Cladophora*-clade and the *Siphonocladus*-clade can be deduced at around 200 mya (Cocquyt *et al*. 2010), considering that the siphonous orders are about 600 my old (Verbruggen et al 2009). Based on those estimates, the age of the *Aegagropila-clade* is at least 200 my old. Changes in sea levels during the last 250 my flooded large continental areas (Haq *et al*. 1987), which could have promoted the early invasion of estuarine and continental waters by the Pithophoraceae. Similar scenarios have been proposed for the evolution of freshwater lineages in the diatoms (Sims *et al*. 2006) and the dinoflagellates (Logares *et al*. 2007). The evolution and timing of habitat preferences invites for interesting speculation with regards to the beginning diversification of mollusks starting around 500 mya (Levin 1999) and of turtles starting around 200 mya (Gaffney *et al*. 1987). Early turtles were marsh dwellers, the highly aquatic and terrestrial forms are secondarily derived (Edgren *et al*. 1953), so maybe the common ancestor of the brackish genus *Wittrockiella* and of *Arnoldiella* evolved around that time in brackish estuaries and saltmarshes.

Taxon sampling and diversity of the Pithophoraceae

The whole group must be regarded as undersampled, partly due to their unobtrusive habit, smallness in size, misidentification (e.g. as *Cladophora* spp.), and their occurrence in unusual habitats not regularly targeted in algal surveys. A general problem in inferring local diversity or species distributions is the detectability of the species in question, which leads to the reconstruction of apparent rather than real diversity or distributional range (Kéry *et al.* 2010). While basically nothing is known about the occurrence of members of the Pithophoraceae in Africa and South America, it seems likely that also in Europe interesting discoveries could be made with regard to the systematic position of many algal species of unknown affiliation (see Table 5). Based on the current sampling, the diversity of the Pithophoraceae appears to be highest in Asia (about 75% of the known taxa), and particularly high in Japan (about 50% of the known taxa). Several enigmatic species have not been sampled for molecular analyses but are assumed to shed light on issues such as thallus evolution (e.g., *Chaetonella goetzei* Schmidle, *Cladophora basicladioides* Jao, *Cladophora cornuta* Brand, *Cladophora rhizobrachialis*, *Cladostroma setschwanense* Skuja), niche evolution (*Basicladia vivipara*, *Cladogonium ogishimae* Hirose & Akiyama – epizoophytic on freshwater shrimps), (historical) biogeography (e.g. the Australian *Cladophora parvula*; or the taxa from Lake Baikal, see below). A group of morphologically closely related species and potential members of the genus *Aegagropila* (and of *Arnoldiella*) are some endemic cladophoralean species from ancient Lake Baikal, Russia (see Bourrelly 1966, Izhboldina 2007). *Aegagropila linnaei* or its ancestor is assumed to have dispersed throughout the Palaearctic (or the Holarctic) from Central or East Asia (Boedeker *et al.* 2010b). A similar scenario has also been proposed for several freshwater animals found as glacial relicts in Fennoscandian lakes and the brackish parts of the Baltic Sea as well as in some scattered Siberian locations, with ancestors in Lake Baikal (Segerstråle, 1962). Molecular data for the morphological relatives of *A. linnaei* from Lake Baikal would strongly add to our understanding of the biogeographic patterns and age of the fascinating Pithophoraceae.

Table 5. Inquirendae: list of genera and species that are potential members of the Pithophoraceae (= *Aegagropila*-clade) based on morphology and habitat, or based on a small subunit rDNA sequences in the case of *Spongiochrysis hawaiiensis*.

'Aegagropila' repens var. *antarctica* Gain, 1912

Bolbocoelon jolyi Yamaguishi-Tomita 1970

Chaetocladiella Meyer & Skabichevsky, 1968 (3 species)
    C. microscopica (Meyer) Meyer & Skabichevsky, 1968
    C. pumila (Meyer) Meyer & Skabichevsky, 1968
    C. litoralis (Skabichevsky) Meyer & Skabichevsky, 1968

Chaetomorpha baicalensis Meyer, 1922
Chaetomorpha curta (Skabichevsky) Skabichevsky, 1969
Chaetomorpha moniliformis Skabichevsky, 1936
Chaetomorpha solitaria Skabichevsky, 1931

Chaetonella Schmidle, 1901 (monotypic)
    C. goetzei Schmidle, 1901

Cladogonium Hirose & Akiyama, 1971 (monotypic)
    C. ogishimae Hirose & Akiyama, 1971

Cladophora aegagropiloidea Hoek & Womersley, 1984
Cladophora alpina Brand, 1899
Cladophora basicladioides Jao, 1947
Cladophora beneckei Möbius, 1893
Cladophora codiola Zeller, 1873
Cladophora compacta (Meyer) Skabichevsky, 1976
Cladophora contorta Zeller, 1873
Cladophora cornuta Brand, 1895
Cladophora dusenii Brand, 1902
Cladophora exigua Zeller, 1873
Cladophora floccosa Meyer, 1927 - 2 varieties
Cladophora globulus (Meyer) Skabichevsky, 1976
Cladophora glomerata var. *nana* Wang, 1935
Cladophora humida Brand, 1913
Cladophora intertexta Collins 1901
Cladophora koktschetavensis Sviridenko, 1995
Cladophora kozhowii Zagorenko & Izhboldina, 1977
Cladophora kursanovii (Meyer) Skabichevsky, 1976
Cladophora kusnetzowii Meyer, 1930
Cladophora mamillata Leliaert 2005
Cladophora meyerii (Meyer) Skabichevsky, 1976 – 2 varieties
Cladophora pachyderma (Kjellman) Brand, 1909
Cladophora parvula Möbius, 1895
Cladophora pithophoroides Phinney 1945
Cladophora pulvinata (Meyer) Skabichevsky, 1976
Cladophora rhizobrachialis Jao, 1944
Cladophora shensiensis Jao, 1948
Cladophora yuennanensis Skuja, 1937

Cladophorella fritschii Islam, 1964
Cladophorella netzhualpilii C. Galicia-García & E. Novelo, 2000
Cladophorella sundarbanensis Islam, 1964

Cladostroma Skuja, 1937 (monotypic)
    C. setschwanense Skuja, 1937

Dermatophyton Peter, 1886 (monotypic)
    D. radians Peter, 1886

Gemmiphora Skabichevsky, 1931 (monotypic)
    G. compacta Skabichevsky, 1931

Rhizoclonium lapponicum Brand, 1913

Spongiochrysis Rindi, López-Bautista, Sherwood & Guiry, 2006 (monotypic)
    S. hawaiiensis Rindi *et al.* 2006

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Chapter

8

General Discussion

Are there rare and endangered algae?

Our results suggest that *Aegagropila linnaei* is not only a rare alga in most countries, but that it is also endangered (Chapters 2 & 3). A simple statement like this has connections to a multitude of topics, and is hard to prove, even with the biggest possible effort. Whitford (1983) postulated that there are no rare freshwater algal species, and gave four categories for why some algae are (falsely) considered 'rare': 1) they have been sparsely collected, 2) they have a restricted distribution, 3) they have dormant stages (that might be difficult to experimentally germinate), and 4) they have highly specific habitat requirements. It could be added to this list that they can be easily confused with more common species. The biggest problem is the detectability of the species in question, which leads to the reconstruction of apparent rather than real diversity or distributional range or temporal occurrence (Kéry *et al.* 2010). Similarly, it is very difficult to determine whether a species is already extinct (e.g., Collen *et al.* 2010).

There is a difference between 'rare' in the sense of being seldomly encountered, and 'rare' in a sense of declining populations and the survival of the species being endangered (of course both meanings can also can be applicable simultaneously). Furthermore, 'seldomly encountered' could mean that a species really occurs in small numbers and/or during a short period of time (but still in a way that the survival of the species is maintained, e.g., truly or naturally rare), or that it is an artefact of our observation methods (falsely 'rare', *sensu* Whitford [1983]). Regardless of the definition of "rare", the meaning always remains quite vague, since it is based on some form of human assessment.

Do any of Whitford's four categories apply to *Aegagropila linnaei*? Category 1 does not really apply, since the species has been frequently collected, and especially the ball-form has always drawn particular attention to this species. The idea behind category 2, which questions the existence of locally restricted species and relates the perceived rarity to lack of observations, is probably true for many freshwater algae, however, there are taxa that are not easily dispersed (Chapter 3, see also Coleman 1996). Furthermore, it could be shown that four out of five ribotypes of *A. linnaei* do have restricted distributions. Category 3 proposes that while some species are not encountered in the field, they might still be present as dormant stages in the sediment, waiting for favourable conditions to germinate and give rise to a new population. Resting stages are not known for *A. linnaei* (Chapter 3). To check this observation, sediments from several of the historical collection sites of *A. linnaei* were cultured for more than one year, and observed regularly for cladophoralean germlings. All of these germlings that were checked by DNA sequencing turned out to be the common freshwater species *Cladophora glomerata*, a species known to produce akinetes. Category 4 applies to stenoecious species, however, *A. linnaei* occupies a wide ecological niche, including a wide range of temperature, salinity, nutrients (to some extent), and light conditions. Thus, while *A. linnaei* must also be regarded as under-recorded to some extent, it does not appear to be a case of an alga falsely labelled as rare, even though it is widespread in the Northern Hemisphere.

Many members of the Pithophoraceae appear to be rare, but this would most likely change if an additional collection effort was made, targeting the particular habitats (see discussion in Chapter 7). Some of them seem to have restricted distributions, e.g., *Pseudocladophora conchopheria*, *Wittrockiella lyallii*, *W. salina*, and apparently a number of potentially related taxa (see for example discussion below on Lake Baikal). A large number of Pithophoraceae species appear to be stenoecious, but competitive exclusion might be at work rather than highly specific ecological requirements (Chapter 7). Surely there are also some rare species among well-monitored groups of freshwater algae, in particular among the Characeae. For less conspicuous taxa most likely both is true, that there are many which are not really rare but just appear to be, and also cases that are truly rare.

The above-mentioned problems with deficient observations directly translate into problems with assessing the current status of freshwater algae with regard to being declining or threatened, and in most taxa this is a difficult task. Concerning this matter, *Aegagropila linnaei* and its ability to produce the conspicuous ball-forms represent a fortunate example, with plentiful historical data available and little taxonomic uncertainty involved. But, these difficulties aside, and continuing Whitford's question as to the existence of rare algae, are there endangered algae, and if so, how to protect them? Several examples of rare algae have been documented: see Entwisle (1997) and Brodie *et al.* (2009) for examples and a general discussion. Charophytes (but few other freshwater algal groups) are well-documented on several regional or national red lists (e.g., Ludwig & Schnittler 1996, van Raam 1998, Gärdenfors 2005); see also Chapters 2 & 3, Nielsen et al. 1996, Schories *et al.* 1996, Geissler 1988, or Mollenhauer *et al.* 1999.

One problem with determining whether a species is declining is the quantity, quality and accessibility of historical data, which are required as a reference. In Chapter 3 it was discussed how an observed decline in herbarium collections over time could be an artifact of collection activity, and how in the case of a conspicuous species such as *A. linnaei* this might indicate a real decline (based on additional sources as well). However, an overview of the seaweed collections held at the Natural History Museum in London showed a similar trend, with the vast majority of samples collected in Victorian times (second half of the 19th century, 45% of the specimens, Brodie *et al.* 2008). There has been comparatively little recent acquisition of data for both marine and freshwater algae. It is still very true what Whitford stated in 1983: "If collectors would publish detailed descriptions of the habitats of so-called rare algae, they would be collected more often."

How to protect algae? Clearly, the biggest threat to algae is habitat degradation and destruction. Thus, individual species cannot be protected, only their habitats could be. In Japan, *A. linnaei* is a protected species, has been commercialised and there is widespread public awareness for this species. The Japanese government successfully asked people to return collected balls to the famous site Lake Akan (Soejima et al. 2008, TMLA 2009). This must be seen as a very unique situation in which also local history plays a role, but it also illustrates that giving a species a protective status can help to raise awareness. It appears to be the main benefit of Red Lists that they can be used as a tool for habitat protection. For most algae the available data is highly deficient though, and detailed assessments of the distributional ranges and long-term monitoring and scientific study are clearly required to determine changes in abundance and distribution.

Additional members of the Pithophoraceae and their potential usefulness in understanding the evolution of this group

Now that new data on the phylogeny of the Pithophoraceae is emerging, it becomes clear that the group is undersampled (indicated for example by Table 5 in Chapter 7), and that several taxa are missing from the phylogenies that could shed light on topics such as historical biogeography or niche evolution. Especially the phylogenetic position of the subtropical Asian '*Cladophora*' species such as *C. basicladioides*, *C. rhizobrachialis* (Fig. 1A), *C. shensiensis* Jao, the monotypic genus *Cladostroma* (Fig. 1B) from China, and the cladophoralean species from Lake Baikal (see below) would be very illuminating with regards to the evolution of (sub)tropical (*Aegagropilopsis* and *Pithophora*) and temperate lineages (*Aegagropila*). Species representing potential relatives of *Aegagropilopsis clavuligera* and *A. sterrocladia* have been described and recorded from the Andaman Islands (Prasad & Misra 1992), Bangladesh (Islam & Irfanullah 2005), China (Wang 1935, Skuja 1937, Jao 1948), India (Verma 1981), Indonesia (Möbius 1893), Myanmar (Grunow 1868), Sri Lanka (Zeller 1873, Skuja 1949), subtropical Australia (Möbius 1895), and Cameroon (Brand 1902). Some of these have been proposed as synonyms of *Aegagropila linnaei*, but in fact they seem to represent separate species within or close to the genus *Aegagropilopsis*. Potential temperate relatives of *Aegagropila linnaei* are *Cladophora alpina* Brand, *C. cornuta* Brand, *C. humida* Brand, *C. pachyderma* (Kjellman) Brand and several taxa from Lake Baikal (see below).

A group of closely related species and potential members of the genera *Aegagropila* and *Arnoldiella* are the endemic Cladophorales from ancient Lake Baikal. Fifteen endemic species have been described from Lake Baikal in the genera *Aegagropila*/*Cladophora*, *Chaetocladiella* (Fig. 1C), and *Gemmiphora* (Fig. 1D) (plus some additional intraspecific ranks, see Izhboldina 2007). Most of these species were described in the 1930s (e.g., Meyer 1926, 1927, 1930), and no molecular data are available to date. However, these taxa appear to be crucial to our understanding of the evolution of the whole lineage. Lake Baikal is an ancient lake with an estimated age of 20-30 my (Rossiter & Kawanabe 2000), and thus could provide a calibration point for dating molecular phylogenies in the absence of fossils (see below). Typical features of ancient lakes are species flocks and large numbers of closely related taxa deriving from a single ancestor. The best known examples are the cichlid fishes in the East African Rift valley lakes and the amphipods of Lake Baikal (Rossiter & Kawanabe 2000). The green algal order Chaetophorales is represented in Lake Baikal by a large number of closely related endemics, in particular *Draparnaldia* and allied genera (e.g., Izhboldina 2007). Eight species of *Aegagropila*/*Cladophora* have been described from Lake Baikal (Meyer 1926, 1927, 1930, Skabichevsky 1976, Zagorenko & Izhboldina 1977, Izhboldina 2007), making it the only place in the world where sympatric speciation appears to have happened in the Cladophorales. Their morphological features, though unique, suggest a close relationship to *A. linnaei* within the Pithophoraceae. Most of these assumed *Aegagropila*-relatives form very short (< 2 cm, mostly < 5mm) turfs or hemiglobose tufts attached to rocks in several meters depth, while three species are exclusively known as tiny unattached balls (like *Pithophora*).

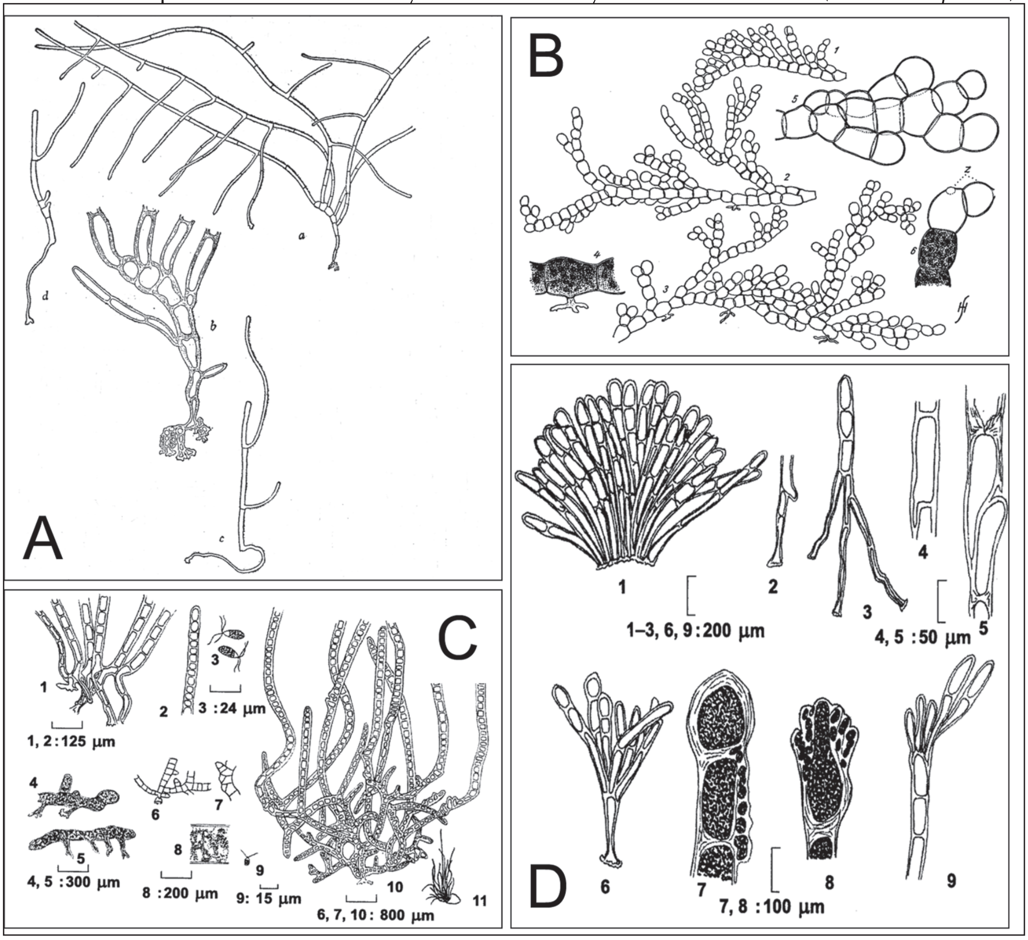

Fig. 1. Morphological relatives of *Aegagropila* and *Arnoldiella* from Asia that are likely to be missing links with regards to thallus evolution. **A.** *Cladophora rhizobrachialis* Jao from China (reproduced from Jao 1944). **B.** *Cladostroma setschwanense* Skuja from China (reproduced from Skuja 1937). **C.** *Chaetocladiella* Skabichevsky from Lake Baikal (reproduced from Izhboldina 2007). **D.** *Gemmiphora compacta* Skabichevsky from Lake Baikal (reproduced from Izhboldina 2007).

Meyer (1926) noted that the *Aegagropila* species from Lake Baikal are sharply separated from the European ones by their ability to form zoospores. Zoospores have been observed in seven of the eight species described from Lake Baikal. The *Cladophora* section *Pulvinata* Skabichevsky was erected to accommodate the Baikalian species *Cladophora* (*Aegagropila*) *compacta*, *globulus* and *pulvinata* (Skabichevsky 1976). A closely related species is *C. koktschetavensis* Sviridenko from Lake Sczuczje in Northern Kazakhstan (Sviridenko 1995), very similar to the Baikalian species *C. kusnetzowii* Meyer. The presumed Baikalian endemics *Cladophora floccosa* Meyer and *C. kozhovii* Zagorenko & Izhboldina have also been found in Lake Khubsugul in Mongolia (Kozhova *et al.* 1994). It is noteworthy that Lake Khubsugul is also one of the world's 17 ancient lakes, estimated to be around 20 my old (Rossiterm & Watanabe 2000). Perhaps the 'Asian lineage' (*Aegagropilopsis* and

Pithophora) evolved from a Baikalian ancestor during the early Miocene (ca. 20 mya) when Lake Baikal had a subtropical climate (Sherstyankin & Kuimova 2006) and spread southwards into the Asian (sub)tropics. *Aegagropila linnaei* (or its ancestor) is assumed to have dispersed throughout the palaearctic (or maybe the entire holarctic) from (central or east) Asia. An Asian origin had been inferred for *A. linnaei* (Chapter 4), and the most diverging genotype comes from ancient Lake Biwa in Japan (ca. 4 my old). *Aegagropila linnaei* has been found in three other ancient lakes (Appendix S3), namely Lake Aral (ca. 5 my old), the Caspian Sea (ca. 5-6 my old) and Lake Ohrid (ca. 5 my old). DNA sequences from these locations would be very interesting to include in a haplotype network and to compare for example to the sequences from Lake Biwa.

The endemic Baikalian genus *Chaetocladiella* (Fig. 1C) occurs as thin layers on rocks and is characterised by the differentiation of a prostrate and an erect system of filaments (see Izhboldina 2007), which implies an affinity to *Arnoldiella*, in particular *A. okamurae*. We could not show well-supported grouping of *A. okamurae* with the other *Arnoldiella/Basicladia* taxa in our phylogenetic analyses (Chapter 7), thus the possibility exists that *A. okamurae* represents a separate unbranched, non-epizoophytic lineage that includes *Chaetocladiella*. Based on morphological features alone, Bourrelly (1966) stated that *Chaetocladiella* could be regarded as non-epizoophytic *Basicladia* species. The species of *Chaetocladiella* also show a morphological affinity to *Cladostroma* (Fig. 1D).

In conclusion, to fully understand the historical biogeography and niche evolution within the Pithophoraceae, additional taxon sampling and molecular analyses are clearly required.

Long-distance dispersal* vs. *ancient vicariance

The split of the *Cladophora*-clade and *Siphonocladus*-clade can be deduced at around 200 mya (Cocquyt *et al.* 2010), thus, the split of the Pithophoraceae and the *Cladophora-Siphonocladus*-clade must necessarily be older. *Wittrockiella lyallii* (and the genus *Wittrockiella* as a whole), and to some extent also *Aegagropila linnaei*, have distribution patterns that imply ancient vicariance events, but on the other hand the low genetic divergence between distant populations suggests recent long-distance dispersal (Chapters 4 & 5). It seems warranted to study the intraspecific genetic variation in *W. lyallii* in more detail, and molecular data from the subantarctic islands of New Zealand, the Chatham Islands and the very south of Chile would be particularly insightful.

There are only a few examples of seaweed species restricted to mainland New Zealand and the coasts of south-western South America (Parsons 1985). Several examples of retained remnant biota in New Zealand exist that predate the Oligocene (23-34 mya) or even date back to Gondwana, such as the frogs of the genus *Leiopelma* (Roelants & Bossuyt 2005), the famous reptile tuatara (*Sphenodon*) (Rest *et al*. 2003), the marine mollusc *Pulvinites exempla* Hedley (Marshall 1998), some Onychophora (Allwood *et al.* 2010), the gigantic kauri trees (*Agathis* spp., Stöckler *et al*. 2002), and an ancient origin in eastern Gondwana has also been proposed for the red algae *Minerva aenigmata* W.A. Nelson and *Dione arcuata* W.A. Nelson (Broom *et al.* 2004, Nelson *et al.* 2005). On the other hand, it has been proposed that much or all of terrestrial New Zealand is actually significantly younger, and only emerged (again) from the sea about 25 mya after the 'Oligocene drowning' (e.g., Trewick *et al*. 2007).

While vicariance was once the explanation for many disjunct distributions (Nelson

1979, Wiley 1988, Morrone & Crisci 1995, De Queiroz 2005), dispersal has become the prevailing view in explaining those biogeographic scenarios (De Queiroz 2005, McGlone 2005, Trewick *et al*. 2007). New Zealand's biota have played a key role in explaining southern hemisphere distributions and were thought to contain Gondwanan remnants (Craw 1988, Bellamy *et al*. 1990, Baker *et al*. 2005), but this view has been challenged (Pole 2001, Briggs 2003, Sanmartín & Ronquist 2004, Campbell & Hutching 2007, Trewick *et al*. 2007, Wallis & Trewick 2009, Biffin *et al*. 2010). Molecular data indicate that even 'iconic' vicariant distributions of classic Gondwanan taxa are due to long-distance dispersal (Cooper *et al*. 2001, Briggs 2003, Knapp *et al*. 2005, McGlone 2006). However, vicariant Gondwanan patterns could be shown in some freshwater organisms (e.g., Toon *et al*. 2010).

Long-distance dispersal scenarios are in stark contrast with the assumed low dispersal capacity of the Pithophoraceae (except in *Pithophora* due to the frequent akinete formation). An alternative explanation for the lack of genetic divergence could be provided by very low evolutionary rates. Similar to *W. lyallii*, several bryophyte species with a disjunct distribution between New Zealand and southern South America, such as *Lopidium concinnum* (Hook.) Wils., *Hypopterygium didictyon* Müll. Hal., *Weymouthia cochleariifolia* (Hedw.) Broth., or *Polytrichadelphus magellanicus* (Hedw.) Mitt., show very low or no genetic divergence between the disjunct areas and also have a low dispersal potential (Frey *et al*. 1999, Pfeiffer 2000, Quandt *et al*. 2001, Stech *et al*. 2002). These patterns have not been interpreted as recent dispersal events, but rather as cases of ancient Gondwanan vicariance with strongly reduced rates of morphological and molecular evolution, for which the term 'stenoevolution' has been coined (Frey *et al*. 1999). Rates of molecular evolution are known to be highly variable in different groups of organisms (e.g., Bromham & Penny 2003, De Clerck *et al*. 2005, Stenøien 2008, Korall *et al*. 2010). Although the mechanisms leading to strongly reduced rates of molecular evolution are not known, it has been shown that bryophytes have lower evolutionary rates in nuclear, chloroplast and mitochondrial markers than vascular plants (Stenøien 2008). It has been shown that evolutionary rates are related to generation time in animals, with longer generation time resulting in lower rates of evolution (e.g., Wu & Li 1985). In higher plants, annuals have faster rates than perennials and herbaceous species have faster rates than arborescent species (e.g., Smith & Donoghue 2008). Recently, it was shown that the evolution of arborescence in ferns is coinciding with drastically decelerated molecular evolution (Korall *et al*. 2010). However, the described scenario of stenoevolution is controversial, and many intercontinental ranges of bryophyte taxa have been shown to be a result of dispersal rather than vicariance (e.g., Devos & Vanderpoorten 2009, Heinrichs *et al*. 2009). Furthermore, cases of dispersal events superimposed on ancient Gondwanan distributions have been identified (e.g., Upchurch *et al*. 2002, Waters *et al*. 2000). Thus, even though long-distance dispersal from New Zealand to South America seems likely for *W. lyallii*, it is still difficult to definitively conclude whether the disjunct pattern represents a case of long-distance dispersal or vicariance, and the mechanisms governing evolutionary rates are still poorly understood. As a presumably clonal species (sexual reproduction, or any form of spore formation unknown) with resulting indefinite generation times, *W. lyallii* might represent a promising candidate to study the possibility of stenoevolution by increasing intraspecific sampling for genotyping and by culture experiments to evaluate dispersal capacities. The length of branches in the *Wittrockiella*-clade are considerably shorter than in other clades of the Cladophorales phylogeny (Fig. 4A in Chapter 7), indicating lowered rates of evolution. Alternatively, dated phylogenies would also allow for testing whether the disjunct distributions are young enough to be explained by the origin of bird migration during the Pliocene (ca. 5 mya, Wolfson 1948).

Asexuality, polyploidy and species concepts

There seems to be a tendency for dominant vegetative reproduction in the Pithophoraceae. Sexual reproduction has so far only been observed in the early-diverging species *Pseudocladophora conchopheria* (Wang & Sakai 1986), and in three *Arnoldiella/Basicladia* species (Hamilton 1948, Hirose 1954). In particular the monotypic genera *Aegagropila* and *Pithophora* are believed to be strictly asexual (Möbius 1895, Brand 1902, Ernst 1908, Mothes 1930, Fritsch 1935, Soejima *et al.* 2008) and polyploid (*Pithophora*: Geitler 1936, Verma 1979; own unpublished data of C-values for *A. linnaei*). Phenotypic plasticity and polyploidy may play a role in overcoming the limitations of asexuality (Maynard-Smith 1978, 1986, Crow 1992, Rice & Friberg 2007, Pfennig *et al.* 2010). Extreme physiological plasticity of *A. linnaei* has been observed under a range of culture conditions (Boedeker, unpubl. data), and both *A. linnaei* and *Pithophora* display a considerable morphological plasticity (see General Introduction and Chapter 7). Confirming the assumed asexuality would challenge the assumption that recombination is essential to produce the required variation for long-term survival (McPhee & Robertson 1970, Maynard-Smith 1978, 1986, Crow 1992). One of the few existing examples are the asexual bdelloid rotifers which diversified into distinct evolutionary lineages (Fontaneto *et al.* 2007). Long-term asexuality is a rare phenomenon that has been confirmed in a diverse range of organisms (Farrar 1990, Poinar & Ricci 1992, Butlin & Griffiths 1993, Remy *et al.* 1994, Normark *et al.* 2003), but is poorly explored in green algae. If asexuality in the sister taxa *Aegagropila* (temperate) and *Pithophora* (tropical) could be confirmed (by molecular data), this system would represent an important showcase for persistence in the absence of sex and for asexual adaptation to different environments.

The scarcity of observations of sexual reproduction in the Pithophoraceae has additional, taxonomic ramifications. As a result, the biological species (Mayr 1963, 1992) concept is not applicable, since it cannot really be tested in praxis. The biological species concept, which introduced reproductive isolation as the criterion to recognise distinct species, is theoretically appealing, but is problematic when there is either too little or too much sex (Templeton 1989). The oldest and (still) most widely used species concept is the morphological species concept, in which morphological discontinuities are regarded as species boundaries. While appropriate in well-studied organisms marked by defined morphological differences, it has proven useless in groups with simple morphologies or extensive phenotypic plasticity (see General Introduction for an illustration on the taxonomic confusion in the taxonomy of *Pithophora* resulting from applying the morphological species concept to a very plastic organism). Traditional species concepts hamper the identification and interpretation of species boundaries in morphologically simple organisms, of cryptic species, and of ecotypes (e.g. different ecotypes of one species *vs*. interspecific differences in the traits of interest). Endless debates (with little empirical data) over what species are have been held, with no widely accepted conclusion reached (e.g., Hey 2001, 2006). More than 20 different ‘species concepts’ have been developed (Mayden 1997). The rise of cladistics has challenged the supremacy of the biological species concept, and has led to increasing application of cladistic or phylogenetic species concepts, i.e., concepts based on evolutionary descent as the sole criterion for classification (Hennig 1950, Cracraft 1989). Recently, important conceptual progress has been made in thinking about species concepts (de Queiroz 1998, 2007). Most evolutionary biologists now accept that species are lineages. These phylogenetic approaches resulted in DNA taxonomy and barcoding (e.g., Templeton 2001, Hebert *et al.* 2003, de Queiroz 2007, Wiens 2007), methods that have also been applied to algae in recent years (e.g. Saunders 2005, Lilly *et al*. 2007, Verbruggen *et al*. 2007, Leliaert *et al*. 2009b, Le Gall & Saunders 2010). Molecular approaches that view species as lineages have been very successful in solving such questions, and allow for a more sophisticated study of speciation mechanisms (e.g. allopatric and sympatric evolution of ecotypes, isolation by distance, etc.). Molecular phylogenies of the Cladophorales have in recent years overthrown most of the proposed earlier relationships based on morphology, and in return provide a powerful tool to study character evolution.

Ribosomal DNA and chloroplast markers

Green algal phylogenies have relied heavily on ribosomal markers, especially in the Cladophorales. Actually, **all** sequence-based phylogenetic reconstructions of the Cladophorales are based on rDNA sequences **only**. The 18S rDNA gene is the most widely used marker in algal phylogenies, but is of limited use at the species level. The 28S rDNA, coding for the large ribosomal subunit, was found to be more variable and more useful for species level phylogenies (Rousseau *et al.* 1997, Leliaert *et al*. 2003, Leliaert *et al*. 2007a). However, several studies using the variable, non-coding ITS regions of the ribosomal cistron have identified cryptic species that were not recovered by 28S or 18S rDNA sequences (Bakker *et al*. 1992, 1995, Marks & Cummings 1996, van der Strate *et al*. 2002, Leliaert *et al*. 2008, 2009b). An independent marker from the chloroplast genome is painfully missing. Not a single chloroplast gene (e.g., *rbc*L, UCPs [Provan *et al*. 2004], *tuf*A, *psa*A-*psa*B spacer, *cod*A) has ever been able to be amplified in the Cladophorales, despite considerable efforts in at least three different labs (Leiden University, Gent University, University of Louisiana at Lafayette). The presence of high molecular weight DNA in cladophoralean chloroplasts has been shown using DAPI fluorescence staining (La Claire & Wang 2000). Early evidence suggests that the chloroplast genomes of the Cladophorales are anomalous compared to their sister groups (Ebert *et al*. 1985, Pombert *et al*. 2005, 2006, Zuccarello *et al*. 2009). Plasmid-like DNA has been found to be a component of the chloroplast DNA in *Ernodesmis verticillata* and *Ventricaria ventricosa* (*Siphonocladus*-clade) and the plasmids were shown to contain chloroplast gene homologs (La Claire *et al*. 1997, 1998, La Claire & Wang 2000). In dinoflagellates (Chromalveolata), the entire plastid genome is reduced to single gene minicircles (Zhang *et al.* 1999). This raises questions as to where which chloroplast genes are located in the Cladophorales, whether they have been transferred to the nuclear genome, which role the plasmid-like DNA might play, and how much homology to other green algal genomes exists. Sequencing whole chloroplast genomes and/or cloning and sequencing of the plasmids will enable to answer these questions and to finally generate chloroplast markers useful for phylogenetic reconstructions. Generally, green algal genomes are much less homogeneous than those of land plants, which average 140 kbp and 110 genes (Simpson & Stern 2002). Among the green algae, the most extensive variation in chloroplast genomes is found in the phylum Chlorophyta (Pombert *et al*. 2006). The Chlorophyta consists of the four classes Prasinophyceae, Trebouxiophyceae, Chlorophyceae and Ulvophyceae. The size of green algal chloroplast genomes ranges from 37 kbp in the parasitic, non-photosynthetic trebouxiophyte *Helicosporidium* (Tartar & Boucias 2004) to ca. 1500 kbp in the ulvophyte *Acetabularia* (Simpson & Stern 2002). One of the smallest known chloroplast genomes in photosynthetic green algae is that of *Codium fragile* (Bryopsidales), which is estimated to be about 89 kbp and lacks large inverted repeat elements (Manhart *et al*. 1989). Thus, the chloroplast genome structure within the ulvophytes is highly variable and very interesting from an evolutionary perspective, especially in the light of several unsettled taxonomic debates about relationships within the class. Of the 33 sequenced chlorophytan chloroplast genomes, the majority stems from representatives of the Prasinophyceae and Chlorophyceae, and only two complete ulvophycean chloroplast genomes are available. These two genomes are from unusual members of the Ulvophyceae, namely the very early diverging *Pseudendoclonium* (Pombert *et al*. 2005), and the unicellular *Oltmannsiellopsis* (Pombert *et al*. 2006), and thus might not be very representative for this group of mainly marine macroalgae. However, these ulvophyte genomes revealed marked differences to other green algal chloroplast genomes, such as a distinct quadripartite architecture with a large inverted repeat (IR) containing an inverted ribosomal operon, several genes normally found in the large single copy region have been transferred to the small single copy region, and the IR contains only ribosomal genes (Pombert *et al*. 2005, 2006).

In addition to evolutionary insights of chloroplast genome evolution, chloroplast markers will provide important insights into the phylogeny of the Ulvophyceae, more specifically the phylogenetic relationships between the orders Bryopsidales, Dasycladales, Trentepohliales, *Blastophysa rhizopus* and Cladophorales (BCD and TBCD clades, see Cocquyt *et al*. 2010). Moreover, independent molecular markers from the plastid genome would be particularly informative in the presumed asexuals *Aegagropila* and *Pithophora*.

The confused taxonomy of the *Aegagropila*-lineage was clarified using methods of molecular phylogenetic inference, resulting in the re-instatement of the Pithophoraceae, descriptions of two new genera and several nomenclatural changes. Phylogenetic analyses led to an understanding of the evolution of the heterotrichous habit as an adaptation to changeable environmental conditions. In selected taxa, topics such as phylogeography and dispersal abilities, historical biogeography and glacial refugia, and ecology and declining populations were studied in detail.

Chapter 2

The situation of *Aegagropila linnaei* in the Netherlands

The freshwater alga *Aegagropila linnaei*, best known for the ball-shaped growth form, occurred in the Netherlands in eight locations before the 1970s. After 1967, this enigmatic species has not been recorded anymore. All historical locations were searched by snorkeling, SCUBA diving, dredging and shore surveys. All habitats have drastically changed during the last 50 years and were severely affected by eutrophication. Only in two of the eight locations could the species be found. Sampled filaments were identified by morphology and by rDNA sequences. Only one healthy population still exists (Zuideindigerwiede) in the Netherlands. Habitats that had been severely eutrophicated but restored to mesotrophic conditions in recent years had not been re-colonised. In conclusion, *Aegagropila linnaei* is regarded as a threatened species in the Netherlands.

Chapter 3

Global decline of and threats to *Aegagropila linnaei*, with special reference to the lake balls habit

The worldwide distribution of *Aegagropila linnaei* was reconstructed based on more than 1200 herbarium specimens from 28 herbaria, an extensive literature survey and field observations. The species was found in 283 locations, with the majority located in central and northern Europe, and only very few in North America. The preferred habitat is inferred to be oligo-mesotrophic lakes with moderate to high calcium levels. In more than 50% of the known locations, *A. linnaei* has not been found in the last 30 years, and this decline is correlated with changes in trophic conditions in those lakes. The unattached growth forms show a stronger decline than attached populations as a result of the more restricted habitat types where they can occur.

Chapter 4

Biogeography of *Aegagropila linnaei*: a widespread freshwater alga with low effective dispersal potential shows a glacial imprint in its distribution

Aegagropila linnaei is generally regarded as a rare species, and the species is apparently absent from large but seemingly suitable areas of the Northern Hemisphere. The main means of reproduction of *A. linnaei* is vegetative fragmentation and desiccation-resistant akinetes are not formed. The dispersal potential of the species was examined in desiccation experiments with vegetative filaments, compared to other widespread freshwater Cladophorales. In addition, ITS rDNA sequences were generated from populations throughout the distribution range. Four of the five recovered genotypes have locally restricted distributions. These findings, together with the inferred low desiccation-tolerance, imply a limited dispersal potential. The only widespread genotype was found to be significantly more desiccation-tolerant than the ones with restricted distributions. The majority of the locations of *A. linnaei* in Europe are situated in previously glaciated areas, and the distribution patterns of the species are discussed with respect to glacial events, possible refugia and modes of dispersal.

Chapter 5

Nuclear rDNA sequences of *Wittrockiella amphibia* (Collins) comb. nov. and morphological characterisation of the mat-like growth form

The poorly known species *Cladophora amphibia* was found for the first time since the type collection from California in 1903. It appears that morphological and ecological characteristics strongly overlap with those of *Wittrockiella paradoxa*. rDNA sequences, including the variable ITS2 region, of the two taxa indicate very high genetic similarity. The seven point mutation in the ITS2 rDNA sequences between the two taxa are regarded as intraspecific variation within one species. Since *C. amphibia* had been described earlier, *W. paradoxa* becomes a taxonomic synonym of *C. amphibia*, and the new binomial *Wittrockiella amphibia* (Collins) comb. nov. is required. The heterotrichous mode of growth is further characterised.

Chapter 6

Cladophoropsis brachyartra from southern South America is a synonym of *Wittrockiella lyallii*, previously regarded as endemic to New Zealand

Wittrockiella lyallii is a poorly known green macroalga that has been regarded as endemic to the south of New Zealand. A herbarium specimen from southern Chile was discovered that conforms to the description of *W. lyallii*. Subsequently, it was realised that the South American species *Cladophoropsis brachyartra* is morphologically and ecologically equivalent to *W. lyallii*. Sequences of three ribosomal genes, including the internal transcribed spacer (ITS), from material collected in Chile and New Zealand are identical, suggesting recent long-distance dispersal from New Zealand to South America. The synonymisation of *C. brachyartra* with *W. lyallii* is presented. Furthermore, the resulting disjunct distribution of the species is discussed in the light of sea surface temperatures, habitat availability in the southern ocean and winter sea ice extent during the last glacial maximum.

Chapter 7

Molecular phylogeny, taxonomy and niche evolution of the *Aegagropila*-clade (Cladophorales, Chlorophyta), including the description of *Aegagropilopsis* gen. nov. and *Pseudocladophora* gen. nov.

The *Aegagropila*-lineage was discovered in recent years based on molecular methods as sister to the species-rich, mainly marine *Cladophora-Siphonocladus*-clade within the Cladophorales. The members of this clade mainly occur in freshwater and brackish environments. Taxon sampling had been limited so far, and no taxonomic changes had been proposed yet based on the novel phylogenetic relationships. This study is the first multi-gene phylogeny of this group, and the 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. In the phylogenetic analyses, the *Aegagropila*-lineage was recovered as a monophyletic group. A new genus, *Pseudocladophora* gen. nov., is erected for the two taxa (*Cladophora horii* and *C. conchopheria*), the only marine members of this group. It was found that *Cladophorella calcicola* is nested within the genus *Wittrockiella*, and the species is transferred to that genus. The monotypic genus *Arnoldiella* was nested within the mainly epizooic genus *Basicladia*, and because of priority rules all *Basicladia* species are transferred to *Arnoldiella*. The monotypic temperate genus *Aegagropila* was found to be closely related to the unattached (sub)tropical genus *Pithophora*, and the morphospecies-rich genus *Pithophora* represents probably just one single phenotypically plastic species. A third subclade consisting of tropical members of the polyphyletic genus *Cladophora* is related to *Aegagropila* and *Pithophora*, and the new genus *Aegagropilopsis* gen. nov. is proposed for those taxa. All previously proposed diagnostic characters for the *Aegagropila*-lineage could be rejected, including pyrenoid ultrastructural features. The family Pithophoraceae is re-instated to serve as a name for the *Aegagropila*-lineage, and is currently solely defined by molecular characters. Basically all members of the Pithophoraceae occur in habitats characterised by strongly fluctuating environmental conditions and reduced competition. In addition to the wide physiological range of these organisms, the heterotrichous habit of the genera *Wittrockiella* (including *Cladophorella*) and *Arnoldiella* (including *Basicladia*) has been inferred to also represent an adaptation to changeable and harsh conditions. Primary holdfasts, present in *Pseudocladophora*, have been lost in *Wittrockiella* and *Arnoldiella*, and secondarily been gained in the stagnant-water genera *Aegagropila* and *Aegagropilopsis*.

In dit proefschrift wordt de complexe taxonomie van de *Aegagropila*-clade opgehelderd aan de hand van morfologische en moleculair-fylogenetische methodes. Deze studie resulteerde in een nieuwe omschrijving van de familie Pithophoraceae, de beschrijving van twee nieuwe genera en verschillende nieuwe combinaties. Fylogenetische analyses hebben verder geleid tot een beter begrip van morfo-ecologische evolutie binnen de groep, zoals de evolutie van een heterotriche groeivorm als adaptatie aan veranderlijke milieuomstandigheden. Geselecteerde taxa werden in detail bestudeerd. Zo werd de historische biogeografie van *Aegagropila linnaei* onderzocht in functie van verspreidingscapaciteit en glaciale refugia. Bij deze soort werd ook de ecologie van achteruitgaande en bedreigde populaties bestudeerd.

Hoofdstuk 2
Het voorkomen van *Aegagropila linnaei* in Nederland

De zoetwateralg *Aegagropila linnaei* is het best bekend als bolvormende planten op de bodem van meren. In Nederland kwam deze soort voor in acht locaties tot in de jaren 1960. Na 1967 is de alg niet meer waargenomen. In deze studie werd het voorkomen van *A. linnaei* in Nederland onderzocht door alle historische locaties te bemonsteren. De onderzochte habitats bleken drastisch veranderd te zijn in de afgelopen 50 jaar en waren sterk geëutrofieerd. Slechts in twee van de acht locaties werd de soort teruggevonden. De identiteit van de bemonsterde filamenten werd geverifiëerd aan de hand van morfologie en rDNA-sequentiebepaling. Slechts in één locatie (Zuideindigerwiede) werd een gezonde populatie aangetroffen. Sterk geëutrofieerde habitats die recent hersteld werden tot mesotrofe habitats waren niet opnieuw gekoloniseerd. Als gevolg daarvan wordt *Aegagropila linnaei* beschouwd als een bedreigde soort in Nederland.

Hoofdstuk 3
Globale achteruitgang en bedreigingen van *Aegagropila linnaei*, met bijzondere aandacht voor de bolvormende groeivorm in meren

De wereldwijde verspreiding van *Aegagropila linnaei* werd onderzocht op basis van ruim 1200 specimens uit 28 herbaria, literatuurgegevens en veldwaarnemingen. De soort werd aangetroffen in 283 locaties, voornamelijk in Midden- en Noord-Europa, en een kleine minderheid in Noord-Amerika. De soort komt voornamelijk voor in oligo-mesotrofe meren met een matig tot hoog calciumgehalte. In meer dan de helft van de locaties werd *A. linnaei* niet meer aangetroffen in de afgelopen 30 jaar. Deze achteruitgang is gecorreleerd met veranderingen in de trofische staat van de meren. De niet-vastgehechte groeivormen vertonen een sterkere achteruitgang dan vastgehechte populaties, mogelijk als gevolg van de specifiekere habitats waarin ze voorkomen.

Hoofdstuk 4

Biogeografie van *Aegagropila linnaei*: een wijdverspreide zoetwateralg met een beperkte dispersiecapaciteit vertoont een glaciale imprint in de distributie

Aegagropila linnaei wordt algemeen beschouwd als een zeldzame soort. De soort komt niet voor in een groot aantal, schijnbaar geschikte habitats van het noordelijk halfrond. *Aegagropila linnaei* plant zich voort door middel van vegetatieve fragmentatie en droogte-resistente akineten worden niet gevormd, wat een lage dispersiecapaciteit doet vermoeden. De dispersiecapaciteit van de soort werd verder onderzocht door middel van uitdrogingsexperimenten van vegetatieve filamenten en deze resultaten werden vergeleken met andere wijdverspreide zoetwater Cladophorales. Ribosomaal DNA sequenties van populaties uit het volledige verspreidingsgebied werden geanalyseerd. Vier van de vijf genotypes hebben beperkte, locale geografische distributies. Deze sequentiegegevens en de lage uitdrogingstolerantie impliceert een beperkte dispersiecapaciteit. Het ene genotype met een wijde verspreiding bleek beduidend toleranter te zijn voor uitdroging dan de genotypes met beperktere distributies. Het merendeel van de Europese locaties van *A. linnaei* is gelegen in gebieden die met ijs bedekt waren tijdens de laatste ijstijd. Het huidige distributiepatroon van de soort moet dan ook bekeken worden als functie van ijstijden, mogelijke refugia en lage dispersiecapaciteit.

Hoofdstuk 5

Nucleair ribosomaal DNA sequenties van *Wittrockiella amphibia* (Collins) comb. nov. en morfologische karakterisering van de matvormige groeivorm

De slecht bekende soort *Cladophora amphibia* werd voor het eerst teruggevonden sinds de aanleg van de type collectie uit Californië in 1903. Morfologische en ecologische kenmerken van deze soort overlappen sterk met die van *Wittrockiella paradoxa*. Ribosomaal DNA sequenties (inclusief de variabele ITS2 regio) van de twee taxa lijken heel sterk op elkaar. De zeven puntmutaties in de ITS2 regio tussen de twee taxa worden beschouwd als intraspecifieke variatie binnen eenzelfde soort. Omdat *C. amphibia* eerder werd beschreven, wordt *W. paradoxa* een taxonomisch synonym van *C. amphibia*, en de nieuwe combinatie *Wittrockiella amphibia* (Collins) comb. nov. is vereist. Ten slotte werd de heterotriche groeivorm van deze soort in detail gekarakteriseerd.

Hoofdstuk 6

Cladophoropsis brachyartra uit het zuiden van Zuid-Amerika is een synoniem van *Wittrockiella lyallii*, een soort die voordien beschouwd werd als endemisch voor Nieuw-Zeeland

Wittrockiella lyallii is een slecht bekende soort en wordt beschouwd als endemisch voor het zuiden van Nieuw-Zeeland. Een herbariumexemplaar uit het zuiden van Chili komt overeen met de beschrijving van *W. lyallii*. Verder onderzoek wees uit dat de Zuid-Amerikaanse soort *Cladophoropsis brachyartra* morfologisch en ecologisch zeer sterk op *W. lyallii* lijkt. Ribosomaal DNA sequenties (inclusief de “internal transcribed spacer” [ITS]), van Chileens en Nieuw Zeelands materiaal zijn identiek, wat kan wijzen op recente lange afstands-verspreiding van Nieuw-Zeeland naar Zuid-Amerika. Op basis van deze morfologische, ecologische en moleculaire data wordt *Cladophora* *brachyartrus* gesynonymiseerd met *W. lyallii*. Ten slotte wordt de disjuncte verspreiding van de soort besproken in functie van zeewatertemperatuur, habitatbeschikbaarheid in de Zuidelijke Oceaan en uitbreiding van het zeeijs tijdens de laatste ijstijd.

Hoofdstuk 7

Moleculaire fylogenie, taxonomie en niche evolutie van de *Aegagropila*-clade (Cladophorales, Chlorophyta), en de beschrijving van *Aegagropilopsis* gen. nov. en *Pseudocladophora* gen. nov.

De *Aegagropila*-clade werd recent gekarakteriseerd op basis van DNA sequentiegegevens. Deze clade vormt een zustergroep met de twee andere, voornamelijk mariene Cladophorales clades (de *Cladophora*- en *Siphonocladus*-clade). In tegenstelling tot deze clades komen soorten van de *Aegagropila*-clade voornamelijk voor in zoet- en brakwater habitats. Tot voor kort waren DNA sequenties slechts voor enkele soorten voorhanden en taxonomische wijzigingen binnen de clade bleven beperkt. In deze studie worden fylogenetische relaties binnen de *Aegagropila*-clade onderzocht aan de hand van DNA sequenties van twee ribosomale genen (SSU en LSU rDNA) van een groot aantal soorten. Fylogenetische relaties tonen aan dat enkele taxonomische wijzigingen noodzakelijk zijn. Een nieuw genus, *Pseudocladophora*, wordt opgericht voor de enige mariene soorten uit de groep, *Cladophora horii* en *C. conchopheria*, die samen een zusterclade met de rest van de *Aegagropila*-clade vormen. *Cladophorella calcicola* blijkt binnen de *Wittrockiella*-clade te vallen, en de soort wordt naar dit genus overgeplaatst. Het monotypische genus *Arnoldiella* valt binnen het epizoöfytische genus *Basicladia*, en door de prioriteitsregel worden alle *Basicladia* soorten overgeplaatst naar *Arnoldiella*. Het monotypische genus *Aegagropila* uit gematigde streken bleek nauw verwant aan het (sub)tropische genus *Pithophora*, dat waarschijnlijk slechts een enkele fenotypisch plastische soort vertegenwoordigt. Een subclade die nauw verwant is aan *Aegagropila* en *Pithophora* bestaat uit tropische soorten met een *Cladophora*-achtige morfologie. Voor deze subclade wordt het nieuwe genus *Aegagropilopsis* voorgesteld.

Verschillende morfologische, ultrastructurele en biochemische diagnostische kenmerken werden eerder gesuggereerd voor de *Aegagropila*-clade. Reëvaluatie van deze kenmerken, met inbegrip van nieuwe gegevens over pyrenoïd ultrastructuur, wijzen uit dat geen enkel van deze kenmerken uniek is voor de clade. De oude familienaam *Pithophoraceae* wordt opnieuw opgenomen als naam voor de *Aegagropila*-clade, en dit taxon wordt momenteel uitsluitend omschreven door de DNA sequentiegegevens.

De meeste soorten Pithophoraceae komen voor in habitats met sterk wisselende milieu-omstandigheden en verminderde competitie. De brede fysiologische range van deze organismen en de heterotriche groeivorm van *Wittrockiella* en *Arnoldiella* zijn waarschijnlijk adaptaties aan deze veranderlijke en vaak barre milieuomstandigheden.

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Appendix S1. Synonyms of *Aegagropila linnaei* and a list of described *Aegagropila* species not referring to *Aegagropila linnaei*. [**Chapters 3 & 4**]

>>> also available online at:
www.nationaalherbarium.nl/supplements/boedeker/BioScience_2010

Appendix S2. List of herbaria contacted in this study, including all inspected collections of *Aegagropila linnaei*. [**Chapters 3 & 4**]

>>> also available online at:
www.nationaalherbarium.nl/supplements/boedeker/BioScience_2010

Appendix S3. List of all known locations of *Aegagropila linnaei*, with a list of locations that were excluded because of doubtful species identification. [**Chapters 3 & 4**]

>>> also available online at:
www.nationaalherbarium.nl/supplements/boedeker/BioScience_2010

Appendix S4. List of all known extant populations of *Aegagropila linnaei* (observations in the last 30 years). [**Chapters 3 & 4**]

>>> also available online at:
www.nationaalherbarium.nl/supplements/boedeker/BioScience_2010

Appendix S5. List of outgroup taxa and their GenBank accession numbers. [**Chapter 7**].

Appendix S6. Effects of model choice and missing data on tree topologies and support. [**Chapter 7**]

Appendix S7. Molecular clock and midpoint rooting of the ingroup. [**Chapter 7**]

Appendix S8. List of all nomenclatorial changes proposed in this thesis. **[Chapters 4 - 7]**

Appendix S1. Synonyms of *Aegagropila linnaei* and a list of described *Aegagropila* species not referring to *Aegagropila linnaei*

Synonyms of *Aegagropila linnaei* Kützing, 1843

Aegagropila	**reference**
Aegagropila armeniaca (Wittrock) Heering, 1921	van den Hoek 1963*
Aegagropila brownii (Dillwyn) Kützing, 1854	van den Hoek 1963*
Aegagropila bulnheimii Rabenhorst, 1859	van den Hoek 1963*
Aegagropila canescens Kjellmann, 1898	van den Hoek 1963*
Aegagropila coactilis (Sauter) Nees von Esenbeck, 1824	Zahlbruckner 1901
Aegagropila froelichiana Kützing, 1845 (non *Cladophora froelichiana* Kützing = *C. sericea*)	van den Hoek 1963*
Aegagropila holsatica (Kützing) Kützing, 1854	van den Hoek 1963*
Aegagropila kannoi Tokida, 1954	Sakai 1964, Niiyama 1989*
Aegagropila kurilensis Nagai, 1940	Sakai 1964, Niiyama 1989*
Aegagropila lagerheimii Brand, 1906	van den Hoek 1963*
Aegagropila martensii (Meneghini ex Kützing) Kützing, 1854	van den Hoek 1963*
Aegagropila minima Okada, 1957	Sakai 1964
Aegagropila muscoides Meneghini ex Kützing, 1854	van den Hoek 1963*
Aegagropila muscoides var. *armeniaca* Wittrock et Nordstedt, 1878	van den Hoek 1963*
Aegagropila profunda (Brand) Heering, 1921	van den Hoek 1963*
Aegagropila sauteri Nees von Esenbeck ex Kützing, 1843	van den Hoek 1963*
Aegagropila sauteri var. *borgeana* (Brand) Heering, 1921	van den Hoek 1963*
Aegagropila sauteri var. *daldinii* Cesati & De Notaris ex Bertolini, 1862	Brand 1902
Aegagropila sauteri var. *minima* Okada, 1953	Sakai 1964, Niiyama 1989*
Aegagropila sauteri var. *sauteri* f. *profunda* (Brand) Heering, 1921	Niiyama 1989*
Aegagropila sauteri var. *yamanakaensis* Okada, 1957	Sakai 1964, Niiyama 1989*
Aegagropila sauteri f. *profunda* Brand, 1906	van den Hoek 1963*
Aegagropila sauteri f. *kurilensis* (Nagai) Sakai, 1964	Niiyama 1989*
Ceramium	
Ceramium aegagropilum (L.) de Lamarck & De Candolle, 1805	van den Hoek 1963*
Chloronitum	
Chloronitum aegagropila (L.) Gaillon, 1833	van den Hoek 1963*
Cladophora	
Cladophora Aegagropila daldinii Cesati & De Notaris ex Bertolini, 1862	van den Hoek 1963*
Cladophora Aegagropila brownii (Dillwyn) Harveyin Kützing, 1849	van den Hoek 1963*
Cladophora Aegagropila holsatica Kützing, 1849	van den Hoek 1963*
Cladophora (Aegagropila) lagerheimii Brand, 1906	van den Hoek 1963*
Cladophora Aegagropila linnaei (Kützing) Kützing, 1849	van den Hoek 1963*
Cladophora Aegagropila martensii Meneghini ex Kützing, 1849	van den Hoek 1963*
Cladophora Aegagropila muscoides Meneghini ex Kützing, 1849	van den Hoek 1963*
Cladophora Aegagropila sauteri (Nees von Esenbeck ex Kützing) Kützing, 1849	van den Hoek 1963*
Cladophora aegagropila (L.) Trevisan, 1845	see Silva, Basson & Moe 1996

Cladophora aegagropila (L.) Rabenhorst, 1868	van den Hoek 1963*
Cladophora aegagropila var. *brownii* (Dillwyn) Rabenhorst, 1868	van den Hoek 1963*
Cladophora aegagropila var. *bulnheimii* (Rabenhorst) Rabenhorst, 1869	van den Hoek 1963*
Cladophora aegagropila var. *daldinii* (Cesati & De Notaris ex Bertolini) Rabenhorst, 1868	van den Hoek 1963*
Cladophora aegagropila var. *holsatica* (Kützing) Rabenhorst, 1868	van den Hoek 1963*
Cladophora aegagropila var. *linnaei* (Kützing) Rabenhorst, 1868	van den Hoek 1963*
Cladophora aegagropila var. *martensii* (Meneghini ex Kützing) Rabenhorst, 1868	van den Hoek 1963*
Cladophora aegagropila var. *muscoides* (Meneghini ex Kützing) Rabenhorst, 1868	van den Hoek 1963*
Cladophora aegagropila var. *sauteri* (Nees von Esenbeck ex Kützing) Rabenhorst, 1868	van den Hoek 1963*
Cladophora armeniaca (Wittrock) Brand, 1902	van den Hoek 1963*
Cladophora brownii (Dillwyn) Harvey, 1846	van den Hoek 1963*
Cladophora bulnheimii (Rabenhorst) Rabenhorst, 1863	van den Hoek 1963*
Cladophora holsatica Kützing, 1849	Brand, 1902
Cladophora linnaei (Kützing) Kützing, 1849	Brand 1902, van den Hoek 1963*
Cladophora martensii Meneghini ex Kützing, 1849	van den Hoek 1963*
Cladophora moravica (Dvořák) Gardavský, 1986	Gardavský 1986
Cladophora muscoides Meneghini ex Kützing, 1849	Brand, 1902*
Cladophora okadae Sakai et Yoshida, 1977	Sakai 1980
Cladophora okadae f. *crassa* (Sakai) Sakai et Yoshida, 1977	Sakai 1980
Cladophora profunda Brand, 1895	van den Hoek 1963*
Cladophora profunda var. *nordstedtiana* Brand, 1902	van den Hoek 1963*
Cladophora sauteri (Nees von Esenbeck ex Kützing) Kützing	Brand 1902
Cladophora sauteri var. *borgeana* Brand, 1902	van den Hoek 1963*
Cladophora sauteri var. *daldinii* (Cesati & De Notaris ex Bertolini) Rabenhorst, 1868	Brand, 1902
Cladophora sauteri f. *sauteri* Sakai, 1964	Sakai 1964
Cladophora sauteri [var. *sauteri*] f. *kurilensis* (Nagai) Sakai, 1964	Sakai 1964
Cladophora sauteri [var. *sauteri*] f. *kannoi* (Tokida) Sakai, 1964	Sakai 1964
Cladophora sauteri f. *profunda* (Brand) Heering, 1921	Kanda 1982
Cladophora sauteri [var. *sauteri*] f. *toroensis* Kanda, 1982	Kanda 1982
Conferva	
Conferva aegagropila L., 1753	van den Hoek 1963*
Conferva aegagropila var. *adnata* C. Agardh, 1824	van den Hoek 1963*
Conferva brownii Dillwyn, 1809	van den Hoek 1963*
Conferva coactilis Sauter ex Nees von Esenbeck, 1824 (non *Conferva coactilis* Mertens ex Jürgens)	van den Hoek 1963*
Conferva froelichiana (Kützing) Rabenhorst, 1847	van den Hoek 1963*
Conferva pusilla Rabenhorst, 1847 (non *Conferva pusilla* Bory de St Vincent; non *C. pusilla* Lyngbye)	van den Hoek 1963*
Conferva sauteri Nees van Esenbeck ex Kützing, 1843	Zahlbruckner 1901
Conferva sauteri f. *suecica* Areschoug, 1862	van den Hoek 1963*
Oscillatoria	
Oscillatoria aegagropila (L.) Corda, 1836	van den Hoek 1963*

* synonymity already proposed in this publication

Appendix S1 (continued). Synonyms of *Aegagropila linnaei*, invalid names, and a list of described *Aegagropila* species not referring to *Aegagropila linnaei.*

Illegitimate, unpublished, and otherwise invalid synonyms of *A. linnaei*	**reference**	**comments**
Aegagropila froelichii	van den Hoek 1963*	unpublished name
Aegagropila lagerheimii Kanno (non Nordstedt), 1934	Sakai 1964, Niiyama 1989*	illegitimate
Aegagropila lagerheimii Nordstedt	Sakai, 1964	probably from exsiccatae
Aegagropila sauteri var. *borgeana* Kanno (non Nordstedt), 1934	Sakai 1964, Niiyama 1989*	illegitimate
Cladophora Aegagropila lauenburgensis Kützing (in litt.)	van den Hoek 1963*	unpublished
Cladophora aegagropila f. *brownii*	van den Hoek 1963*	inedit.
Cladophora aegagropila f. *linnaei*	van den Hoek 1963*	inedit.
Cladophora coactilis (Sauter) Nees von Esenbeck, 1824	Zahlbruckner 1901	illegitimate
Cladophora holsatica f. *adnata* Wittrock et Nordstedt (exsicc. 212)	Brand 1902	nomen nudum
Cladophora holsatica f. *fluitans* Wittrock et Nordstedt (exsicc. 35)	Brand 1902	nomen nudum
Cladophora mansfeldensis Kützing (in litt.)	van den Hoek 1963*	unpublished
Cladophora minima (Okada) Sakai, 1964	Sakai 1964, Niiyama 1989*	illegitimate
Cladophora minima f. minima Sakai	Sakai 1964	illegitimate
Cladophora minima f. crassa Sakai	Sakai 1964	illegitimate
Cladophora minima [var. *minima*] f. minima Sakai	Niiyama 1989*	illegitimate
Cladophora minima [var. *minima*] f. crassa Sakai	Niiyama 1989*	illegitimate
Cladophora muscoides Hansgirg	Brand 1902	inedit.
Cladophora valderamosa Kützing (in litt.)	van den Hoek 1963*	unpublished
Conferva gigantea Sauter	van den Hoek 1963*	unpublished
Conferva verbana Martens	van den Hoek 1963*	unpublished

* synonymity already proposed in this publication

Appendix S1 (continued). Synonyms of *Aegagropila linnaei*, invalid names, and a list of described *Aegagropila* species not referring to *Aegagropila linnaei.*

Aegagropila* species not referring to *Aegagropila linnaei	
(Index Nominum Algarum and other sources)	**current name**
Aegagropila agardhii Kützing, 1843	*Cladophora fracta* (O.F. Müller ex Vahl) Kützing, 1843
Cladophora (Aegagropila) amphibia Collins, 1907	*Cladophora amphibia* Collins, 1907
Aegagropila biasolettiana Kützing, 1845	*Cladophora vadorum* (J.E. Areschoug) Kützing, 1849
Aegagropila biformis Wittrock, 1878	*Cladophora rupestris* (Linnaeus) Kützing, 1843
Aegagropila breviarticulata Reinbold, 1905	*Cladophora* cf. *coelothrix* Kützing, 1843
Aegagropila coelothrix (Kützing) Kützing, 1854	*Cladophora coelothrix* Kützing, 1843
Aegagropila compacta Meyer, 1926	*Cladophora compacta* (Meyer) Skabichevsky, 1976
Aegagropila composita (Harvey) Kützing, 1854	*Boodlea composita* (Harvey) Brand, 1904
Aegagropila conglobata (Kützing) Kützing, 1854	*Cladophora dalmatica* Kützing, 1843
Aegagropila cornea Kützing, 1843	*Cladophora echinus* (Biasoletto) Kützing, 1849

Aegagropila cornea f. *verticillata* Kützing, 1845	*Cladophora echinus* (Biasoletto) Kützing, 1849
Aegagropila cornuta (Brand) Heering, 1921	*Cladophora cornuta* Brand, 1895
Aegagropila corynarthra (Kützing) Kützing, 1854	*Cladophora vadorum* (J.E. Areschoug) Kützing, 1849
Aegagropila echinus (Biasolett) Kützing, 1845	*Cladophora echinus* (Biasoletto) Kützing, 1849
Cladophora (Aegagropila) elegans Möbius, 1883	cf. *Cladophora vagabunda* (Linnaeus) Hoek, 1963
Aegagropila enormis (Montagne) Kützing, 1854	*Bryopsis*? (Chile), Canary Islands: *Cladophora lehmanniana* (Lindenberg) Kützing, 1843
Aegagropila flaccida (Kützing) Kützing, 1854	*Cladophora vagabunda* (Linnaeus) van den Hoek, 1963
Aegagropila forskalii (Kützing) Kützing, 1845	*Siphonocladus forsskalii* (Kützing) Bornet ex De Toni 1889
Aegagropila fuliginosa (Kützing) Kützing, 1854	*Cladophora catenata* (Linnaeus) Kützing, 1843
Aegagropila globulus Meyer, 1927	*Cladophora globulus* (Meyer) Skabichevsky, 1976
Aegagropila herpestica (Montagne) Kützing, 1854	*Cladophora herpestica* (Montagne) Kützing, 1849
Aegagropila javanica Kützing, 1847 (in litt.)	*Cladophora herpestica* (Montagne) Kützing, 1849
Aegagropila leprieurii (Kützing) Kützing, 1854	not *A. linnaei* (see Brand, 1902)
Aegagropila membranacea (C. Agardh) Kützing, 1854	*Cladophoropsis membranacea* (Hofman Bang ex C. Agardh) Børgesen, 1905
Aegagropila meneghiniana Kützing, 1845	*Cladophora laetevirens* (Dillwyn) Kützing, 1843
Aegagropila modonensis (Kützing) Kützing, 1854	*Cladophora coelothrix* Kützing, 1843
Aegagropila montagneana (Kützing) Kützing, 1854	*Cladophora catenata* (Linnaeus) Kützing, 1843
Aegagropila montagnei Kützing var. *waianaeana* Brand, 1904	*Cladophora catenata* (Linnaeus) Kützing, 1843 or *Cladophora luxurians* Abbott & Huisman, 2003
Aegagropila patentiramea (Montagne) Kützing, 1854	*Cladophora coelothrix* Kützing, 1843
Aegagropila pulvinata Meyer, 1926	*Cladophora pulvinata* (Meyer) Skabichevsky, 1976
Aegagropila pusilla Suhr (in litt.)	INA: published as *Conferva pusilla* Rabenhorst, 1847
Aegagropila pygmaea Meyer, 1927	*Cladophora kursanovii* (Meyer) Skabichevsky, 1976
Aegagropila repens (J. Agardh) Kützing, 1854	*Cladophora coelothrix* Kützing, 1843
Aegagropila repens meneghiniana (see van den Hoek, 1963)	*Cladophora socialis* Kützing, 1849
Aegagropila repens var. *gaditana* Bornet (in litt.)	*Cladophora socialis* Kützing, 1849
Aegagropila repens f. *antarctica* Gain, 1912	*Aegagropila repens* f. *antarctica* Gain, 1912 (marine, unknown affinity)
Aegagropila rudolphiana (Agardh) Kützing, 1845	*Cladophora sericea* (Hudson) Kützing, 1843
Cladophora (*Aegagropila*) *senta* Brand, 1904	? - tropical marine species
Aegagropila sibogae Reinbold, 1905	*Cladophora coelothrix* Kützing, 1843
Aegagropila simplex Lenormand ms. (not published)	INA: pro syn. *Cladophora repens* J. Agardh, 1849 (Harvey phyc. Brit. 2: pl. CCXXXVI)
Aegagropila socialis (Kützing) Kützing, 1854	*Cladophora socialis* Kützing, 1849
Cladophora (Aegagropila) socialis Kützing var. *hawaiiana* Brand, 1904	cf. *Cladophora socialis* Kützing, 1849
Cladophora (Aegagropila) socialis Kützing var. *sandwicensis* Brand, 1904	cf. *Cladophora socialis* Kützing, 1849
Aegagropila subtilis Kützing, 1854	*Cladophora dalmatica* Kützing, 1843
Cladophora (Aegagropila) subtilis Kützing var. *oahuana* Brand, 1904	? - tropical marine species (*Cladophora socialis* Kützing, 1849?)
Aegagropila trichotoma (C. Agardh) Kützing, 1854	*Cladophora pellucida* (Hudson) Kützing, 1843
Aegagropila zollingeri (Kützing) Kützing, 1854	*Cladophora herpestica* (Montagne) Kützing, 1849

refused synonyms	comments
Cladophora aegagropila var. *thermalis* Wolle, 1885	description suggests different affinity (*Cladophora sp.*)
Cladophora sterrocladia Skuja, 1949	synonymity had been proposed by Liu Guoxiang, 1999
Cladophora yuennanensis Skuja, 1937	synonymity had been proposed by van den Hoek, 1963
Cladophora shensiensis Jao, 1948	synonymity had been proposed by Liu Guoxiang, 1999
Cladophora glomerata var. *nana* Wang, 1935	synonymity had been proposed by Liu Guoxiang, 1999
Cladophora parvula Möbius, 1895	synonymity had been considered by Skinner & Entwisle, 2004
Cladophora dusenii Brand, 1902	possible synonymity had been discussed by Brand, 1902

INA = Index Nominum Algorum (www.ucjeps.berkeley.edu/INA.html)

Appendix S2 List of herbaria contacted in this study, including all inspected collections of *Aegagropila linnaei*

No.	Institution *Aegagropila linnaei* collections checked	Acronym[a]
1	Botanischer Garten und Botanisches Museum Berlin-Dahlem, Germany	B
2	Ulster Museum, Belfast, Northern Ireland	BEL
3	The Natural History Museum, London, United Kingdom	BM
4	Hungarian Natural History Museum, Budapest, Hungary	BP
5	National Botanic Garden of Belgium, Meise, Belgium	BR
6	Masaryk University, Brno, Czech Republic	BRNU
7	University of Copenhagen, Copenhagen, Denmark	C
8	Canadian Museum of Nature, Ottawa, Canada	CANA
9	Kharkov State University, Kharkov, Ukraine	CWB
10	National Botanic Gardens, Glasnevin, Dublin, Ireland	DBN
11	Field Museum of Natural History, Chicago, USA	F
12	Museo di Storia Naturale dell'Università, Florence, Italy	FI
13	Institute of Hydrobiology, Wuhan, China (re-identified as *Cladophora sterrocladia*)	HBI
14	National Academy of Sciences of Ukraine, Kiev, Ukraine	KW
15	Nationaal Herbarium Nederland, Leiden, The Netherlands	L
16	Botanical Museum, Lund, Sweden	LD
17	V. L. Komarov Botanical Institute, St. Petersburg, Russia	LE
18	Botanische Staatssammlung München, Munich, Germany	M
19	New York Botanical Garden, New York, USA	NY
20	Botanical Museum, Natural History Museum, Oslo, Norway	O
21	Botanical Museum, University of Oulu, Finland	OULU
22	Muséum National d'Histoire Naturelle, Paris, France	PC
23	Université Laval, Quebec, Canada	QFA
24	Swedish Museum of Natural History, Stockholm, Sweden	S
25	Hokkaido University, Sapporo, Japan	SAP
26	Trinity College, Dublin, Ireland	TCD
27	University of British Columbia, Vancouver, Canada	UBC
28	Natural History Museum, Vienna, Austria	W
	no *Aegagropila linnaei* specimens	
29	Universität Zürich, Zurich, Switzerland	Z
30	University of Alberta, Edmonton, Canada	ALTA
31	Hugo de Vries-Laboratory, University of Amsterdam, Amstedam, The Netherlands	AMD
32	Chingan State Nature Reserve, Archara, Russia	ARKH
33	Dalhousie University, Halifax, Canada	DAL
34	Xishuangbanna Tropical Botanical Garden, Academia Sinica, China	HITBC
35	Irkutsk State University, Irkutsk, Russia	IRKU
36	Polish Academy of Sciences, Kraków, Poland	KRAM
37	Lódz University, Lódz, Poland	LOD
38	Main Botanical Garden, Moscow, Russia	MHA
39	I. I. Mecynikov State University of Odessa, Odessa, Ukraine	MSUD
40	Provincial Museum of Newfoundland and Labrador, St. John's, Canada	NFM
41	Nova Scotia Museum of Natural History, Halifax, Canada	NSPM
42	Royal Botanic Gardens, Sydney, Australia	NSW

43	Royal Alberta Museum, Edmonton, Canada	PMAE
44	National Museum, Prague, Czech Republic	PR
45	Charles University, Prague, Czech Republic	PRC
46	Queen's University, Kingston, Canada	QK
47	Singapore Botanic Gardens, Singapore	SING
48	University of New Brunswick, Fredericton, Canada	UNB
49	Herbarium of the Urals Pedagogical University, Ekaterinburg, Russia	URP*
50	University of Toronto Culture Collection, Toronto, Canada	UTTC
51	Warsaw University, Warsaw, Poland	WA
52	Omsk State Pedagogical University, Omsk, Russia*	
53	Herbarium of the Zeya Nature Reserve, Zeya, Russia*	
	no algal collections	
54	South China Botanical Garden, Guangzhou, China	IBSC
55	Royal Botanic Gardens, Kew, United Kingdom	K
56	Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China	KUN
57	Lakeland College, Vermillion	LCVA
58	University of Calgary, Calgary, Canada	UAC
59	Devonian Botanic Garden, University of Alberta, Edmonton, Canada	UAMH
60	Gdansk University, Gdansk, Poland	UGDA
	no answer	
61	Altai State University, Barnaul, Russia	ALTB
62	Chelyabinsk State Pedagogical University, Chelyabinsk, Russia	CHPU
63	Far Eastern University, Vladivostok, Russia	FENU*
64	Siberian Institute of Plant Physiology and Biochemistry, Irkutsk, Russia	IRK
65	Krasnoyarsk State Pedagogical University, Krasnoyarsk, Russia	KRAS
66	Real Jardín Botánico, Madrid, Spain	MA
67	University of Massachusetts, Amherst, USA	MASS
68	National Academy of Sciences of Belarus, Minsk, Belarus	MSK
69	Belarusian State University, Minsk, Belarus	MSKU
70	Moscow State University, Moscow, Russia	MW
71	Memorial University of Newfoundland, St. John's, Canada	NFLD
72	Siberian Central Botanical Garden, Novosibirsk, Russia	NS
73	Institute of Plant and Animal Ecology, Ekaterinburg, Russia	SVER
74	Royal Ontario Museum, Toronto, Canada	TRT
75	Institute of Biology and Soil Science, Far Eastern Branch, Vladivostok, Russia	VLA
76	Museum of Natural History, Wroclaw, Poland	WRSL
77	Yakutsk State University, Yakutsk, Russia*	
78	Urals State University, Ekaterinburg, Russia*	

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[a] Index Herbariorum: Holmgren *et al.,* 1990

*Index Herbariorum Rossicum: www.binran.ru/projects/herbaria

Appendix S3. List of all known locations of *Aegagropila linnaei*, with a list of locations that were excluded because of doubt-
verified). ITS genotypes are given (A-E), and brackish locations are indicated.

no.	COUNTRY	LOCATION	LAT	LONG	HERB	
1	Albania/ Macedonia	Lake Ohrid	41.048	20.715	X	
2	Austria	Almsee, near Grünau	47.753	13.957		
3	Austria	Altausseer See, Steiermark	47.640	13.785	X	
4	Austria	Erlaufsee, near Mariazell	47.792	15.275		
5	Austria	Klagenfurt	46.625	14.307	X	
6	Austria	Lake Zeller, Pinzgau	47.326	12.806	X	
7	Belarus	Lake Naroch, Myadzyel district	54.850	26.776		
8	Belarus	Lake, Lahoysk district	54.519487	28.433		
9	Canada	Lake Doré, Ontario	45.618	-77.108		
10	Canada	Lake Ontario	43.771	-78.036	X	
11	Czech Republic	Kaplice (as Kaplitz), Okres Český Krumlov	48.741	14.488	X	
12	Czech Republic	river Oslava	49.283	16.067		
13	Denmark	Furesøen	55.801	12.419	X	
14	Denmark	Sorø sø	55.426	11.548	X	
15	England	Heigham Sound, Norfolk	52.727	1.606	X	
16	England	Hickling Broad, Norfolk	52.739	1.580		
17	England	Holywell, west of Eastbourne	50.747	0.258	X	
18	England	Land's End, Cornwall coast	50.067	-5.700	X	
19	England	Malham Tarn, Yorkshire	54.097	-2.167		
20	England	Mousehole cave, Cornwall	50.133	-5.550	X	
21	England	River Swale	54.298	-1.507		
22	England	River Tees	54.527	-1.857		
23	England	River Tyne	54.980	-2.099		
24	England	River Wear	54.719	-1.853		
25	England	Spittal, Northumberland	55.760	-1.995	X	
26	England	The Fleet, Dorset	50.616	-2.518		
27	England	Walling Fen Moor, west of Hull	53.717	-0.548		
28	England	Whitemere, Shropshire	52.890	-2.872	X	
29	Estonia	Lake Öisu	58.209	25.513	X	
30	Estonia	Lake Ülemiste, near Tallinn	59.400	24.767		
31	Estonia	Lake Valgjärv	58.090	26.638	X	
32	Estonia	Pärnu river	58.492	24.833	X	
33	Faeroe Islands	Sandsvatn, Sondoy	61.852	-6.814	X	
34	Faeroe Islands	Vaag (Vágur), Suðuroy	61.469	-6.833		
35	Finland	Brahestad (Raahe), Bay of Bothnia	64.687	24.472		
36	Finland	Houtskari, Archipelago Sea	60.217	21.383	X	

- ful species identification. “HERB” indicates morphologically verified specimens, “LIT” indicates literature data (not

LIT	ITS type	brack?	REFERENCES
X			Brand 1902, Pankow 1965
X			Brand 1902, Pankow 1965
			Brand 1902, Pankow 1965
	C		Pankow 1965, van den Hoek 1963
X			MINIPRIRODA 2006
X			MINIPRIRODA 2006
X			Kindle 1934
			Kindle 1915
X			Gardavský 1986
			van den Hoek 1963
			Wesenberg-Lund 1903, van den Hoek 1963
		X	
X		X	West & Fritsch 1927
X			Lund 1961, van den Hoek 1963, Pentecost 1999
			van den Hoek 1963
X			Holmes & Whitton 1977
X			Holmes & Whitton 1981a
X			Holmes & Whitton 1981b
X			Whitton *et al*. 1998
		X	van den Hoek 1963
X		X	Burrows 1991
X		X	West & West 1901
			van den Hoek 1963
	C		Einarsson *et al*. 2004
X			Trei & Pedusaar 2006
	C		
	C		Mölder 1944, van den Hoek 1963
			Brand 1902, van den Hoek 1963
X			van den Hoek 1963
X		X	Waern 1952, van den Hoek 1963
		X	

no.	COUNTRY	LOCATION	LAT	LONG	HERB	
37	Finland	Loviisa (Lovisa), Gulf of Finland	60.450	26.233		
38	Finland	near Solf (Sulva), Vaasa (as Glofjärden in Solv near Wasa), Gulf of Bothnia	63.047	21.600		
39	Finland	Northern Quark, Gulf of Bothnia	63.105	21.116	X	
40	Finland	Nyland, Tenala	60.057	23.295	X	
41	Finland	Parainen, Gulf of Bothnia	60.305	22.305	X	
42	Finland	Pojo Bay, Gulf of Finland	60.052	26.987	X	
43	Finland	Pori (Björneborg), Gulf of Bothnia	61.483	21.783		
44	Finland	Porvoo (Borgå), Gulf of Finland	60.377	25.664		
45	Finland	Ritgrund, near Vaasa, Gulf of Bothnia	63.427	21.509	X	
46	Finland	Saltvik, Gulf of Bothnia	60.279	20.036	X	
47	Finland	Uusikaupunki- (Nystad-) archipelago	60.800	21.417		
48	Finland	Kaskhusfjärden, Kruunupyy, gulf of Bothnia	63.776	22.981	X	
49	France	Brest, Bretagne	48.390	-4.444	X	
50	France	Lac Bleu, Bagnères-de-Bigorre, Hautes-Pyrénées	42.937	0.079	X	
51	France	river Moine, Clisson	47.095	-1.285	X	
52	France	river Seine, Paris	48.854	2.284	X	
53	Georgia	Lake Tabatskuri (Tabiščur)	41.650	43.617	X	
54	Germany	Ammersee, near München	48.031	11.120	X	
55	Germany	Bültsee, Schleswig-Holstein	54.500	9.754	X	
56	Germany	Diecksee, Schleswig-Holstein	54.166	10.503		
57	Germany	Dobbertiner See, Mecklenburg-Vorpommern	53.615	12.084	X	
58	Germany	Dreetz See, Feldberg, Mecklenburg-Vorpommern	53.289	13.434		
59	Germany	Galenbecker See, Mecklenburg-Vorpommern	53.633	13.719		
60	Germany	Großensee, Trittau	53.626	10.351	X	
61	Germany	Grosser Dreisee, Uckermark	53.222	13.352	X	
62	Germany	Grosser Plöner See, Schleswig-Holstein	54.140	10.417		
63	Germany	Jakobsdorfer See, Uckermark	53.132	13.892	X	
64	Germany	Kellersee, Schleswig-Holstein	54.173	10.599		
65	Germany	Kleiner Lankesee, Brandenburg	52.910	13.225	X	
66	Germany	Krakower See, Mecklenburg-Vorpommern	53.664	12.292		
67	Germany	Krummsee, Schleswig-Holstein	54.194	10.603		
68	Germany	Lake Constance (as Bodensee)	47.630	9.380		
69	Germany	Lake Müritz, Mecklenburg-Vorpommern	53.435	12.678	X	
70	Germany	Mechower See, near Ratzeburg	53.717	10.826	X	

LIT	ITS type	brack?	REFERENCES
X		X	Waern 1952, van den Hoek 1963
X		X	Waern 1952
		X	Bergström & Bergström 1999
		X	
		X	
	L68: C, L69: A	X	Luther 1951, Waern 1952
X		X	Waern 1952, van den Hoek 1963
X		X	Waern 1952, van den Hoek 1963
		X	van den Hoek 1963
		X	
X		X	Waern 1952, van den Hoek 1963
		X	
			van den Hoek 1963
			van den Hoek 1963
			van den Hoek 1963
			Brand 1902, van den Hoek 1963
			Brand 1902
			Jöns 1934
X			Kann 1940, van den Hoek 1963
			Pankow & Bolbrinker 1984
X			Pankow & Bolbrinker 1984
X			Pankow 1965, Pankow & Bolbrinker 1984
			van den Hoek 1963
			Bolbrinker 2000
X			Kann 1940, Waern, 1952, van den Hoek 1963
X			Kann, 1940, van den Hoek 1963
	E		
X			Pankow & Bolbrinker 1984
X			Brand 1902, Kann 1945
X			van den Hoek 1963
			van den Hoek 1963, Pankow & Bolbrinker 1984
			van den Hoek 1963, Pankow 1965

no.	COUNTRY	LOCATION	LAT	LONG	HERB	
71	Germany	Müggelsee, Berlin	52.437	13.649	X	
72	Germany	Neuklostersee, Mecklenburg-Vorpommern	53.852	11.702	X	
73	Germany	Salziger See (Mansfelder Seen), near Halle	51.483	11.658	X	
74	Germany	Sankelmarker See, Flensburg	54.713	9.427	X	
75	Germany	Schleswig, Schleswig-Holstein	54.517	9.567	X	
76	Germany	Schweriner See, Mecklenburg-Vorpommern	53.660	11.465		
77	Germany	Starnberger See (as Würmsee), near München	48.000	11.350	X	
78	Germany	Stechlinsee, Brandenburg	53.154	13.031		
79	Germany	Stienitzsee, Berlin	52.508	13.828	X	
80	Germany	Teterower See, Mecklenburg-Vorpommern	53.794	12.611	X	
81	Germany	Tiefwarensee, near Waren, Mecklenburg-Vorpommern	53.526	12.691	X	
82	Germany	Tollensesee, Uckermark	53.526	13.225		
83	Germany	Unteruckersee, Prenzlau	53.284	13.861	X	
84	Germany	Wandlitzsee, Brandenburg	52.761	13.455		
85	Germany	Warnemünde, Baltic Sea	54.167	12.083	X	
86	Germany	Zansen, Feldberg, Mecklenburg-Vorpommern	53.330	13.477		
87	Hungary	Fényes spring, Tata	47.646	18.327		
88	Iceland	Laugabólsvatn	65.939	-22.681		
89	Iceland	Miklavatn	65.965	-17.603		
90	Iceland	Vestmannsvatn	65.795	-17.416		
91	Iceland	Másvatn	65.637	-17.239		
92	Iceland	Kringluvatn	65.765	-17.228		
93	Iceland	Vikingavatn	66.107	-16.826		
94	Iceland	Snjóölduvötn	65.399	-14.348		
95	Iceland	Lake Myvatn	65.600	-17.000	X	
96	Iceland	Lake Thingvallavatn	64.168	-21.127		
97	Iceland	Myrasysla, Alftanes	64.467	-22.150	X	
98	Ireland	Loch an tSaile, near Screebe, Co. Galway	53.385	-9.555		
99	Ireland	Loch Raha, Co. Clare, Corofin	53.433	-8.850		
100	Ireland	Lough Ballycullinan, Co. Clare	52.919	-9.056		
101	Ireland	Lough Gill, near Sligo, Co. Sligo	54.253	-8.384	X	
102	Ireland	River Unshin, Lough Arrow, Co. Sligo	54.068	-8.332		
103	Ireland	Wicklow, Co. Wicklow	52.993	-6.053	X	
104	Italy	Lake Como, Lombardy	45.871	9.144	X	
105	Italy	Lake near Baselga di Piné, Tirol	46.115	11.226	X	

LIT	ITS type	brack?	REFERENCES
			Umweltministerium Mecklenburg-Vorpommern 2004
		X	van den Hoek 1963
			van den Hoek 1963
X			Brand 1906, Pankow & Bolbrinker 1984
			Brand 1902, van den Hoek 1963
X			
			van den Hoek 1963, Pankow 1965
			Pankow & Bolbrinker 1984
	C		
X			Pankow & Bolbrinker 1984
X			Pankow 1965
		X	Rabenhorst 1847, van den Hoek 1963
X			Pankow & Bolbrinker 1984
X			Palik 1963
X			p.c. Á. Einarrsson
X			p.c. Á. Einarrsson
X			p.c. Á. Einarrsson
X			p.c. Á. Einarrsson
X			p.c. Á. Einarrsson
X			p.c. Á. Einarrsson
X			Hallgrímsson 2002
	A		Einarsson *et al.* 2004
X			Jónsson 1992
X		X	Roden 1998
X			
X			van den Hoek 1963
X			John *et al*. 2002
			Burrows 1991
			Brand 1902, Pankow 1965

no.	COUNTRY	LOCATION	LAT	LONG	HERB	
106	Italy	Lake Varese, Lombardy	45.811	8.736		
107	Italy	Mantua lakes (Mantova)	45.150	10.800		
108	Italy	Trieste	45.649	13.780	X	
109	Japan	Kamoi swamp, Pref. Hokkaido	45.310	142.185		
110	Japan	Kamuito swamp, Pref. Hokkaido	45.310	142.185		
111	Japan	Kimoma swamp, Pref. Hokkaido	45.310	142.185		
112	Japan	Lake Akan, Pref. Kushiro	43.451	144.099	X	
113	Japan	Lake Biwa, Pref. Shiga	35.250	136.083	X	
114	Japan	Lake Chimikeppu	43.633	143.883	X	
115	Japan	Lake Kawaguchi, Pref. Yamanashi	35.517	138.750	X	
116	Japan	Lake Ogawara, Pref. Aomori	40.783	141.333	X	
117	Japan	Lake Panke, Pref. Hokkaido	45.031	141.722	X	
118	Japan	Lake Saiko, Pref. Yamanashi	35.498	138.685	X	
119	Japan	Lake Shirarutoro, Pref. Hokkaido	43.179	144.500	X	
120	Japan	Lake Toro, Pref. Hokkaido	43.144	144.540	X	
121	Japan	Lake Yamanaka, Pref. Yamanashi	35.417	138.867	X	
122	Japan	Naebo-numa, Etorofu (Iturup) Island, Kuriles	45.031	147.714	X	
123	Japan	Pon swamp, Pref. Hokkaido	45.310	142.185		
124	Japan	Pond Ane	40.936	141.347	X	
125	Japan	Pond Ichiyanagi	40.936	141.347	X	
126	Japan	Pond Sakyo	41.257	141.398	X	
127	Japan	Pond Takkobu/Takkobu marsh, Pref. Hokkaido	43.100	144.483	X	
128	Japan	Pond Tamogi	40.936	141.347	X	
129	Japan	Pond Uchi	40.936	141.347	X	
130	Kazakhstan	Lake Markha-Kul' (Markakol)	48.750	85.750		
131	Latvia	Babites Ezers, near Riga	56.925	23.758		
132	Latvia	Lake Aluksnes	57.447	27.079		
133	Latvia	Lake Kisezers, near Riga	57.017	24.184		
134	Latvia	Lake Limbažu	57.484	24.701		
135	Latvia	Lake Usmas	57.205	22.177	X	
136	Netherlands	Beulaker Wijde	52.704	6.054		
137	Netherlands	Boven Wijde	52.724	6.103	X	
138	Netherlands	Duinigermeer	52.722	6.002		
139	Netherlands	Molengat	52.745	6.089	X	
140	Netherlands	Naardermeer	52.297	5.125	X	
141	Netherlands	Wijde blik, Loosdrechtse Plassen	52.227	5.055	X	
142	Netherlands	Zuideindiger Wijde	52.702	6.081	X	
143	Netherlands	Zwarte Broek	53.251	5.943	X	

LIT	ITS type	brack?	REFERENCES
X			
X			van den Hoek 1963
			no data
X			Wakana *et al*. 2001b
X			Wakana *et al*. 2001b
X			Wakana *et al*. 2001b
	A & B		Kurogi 1980, Niiyama 1989
	D		Soejima *et al*. 2008
	B		Wakana *et al*. 2003
	A		Kurogi 1980, Sakai 1980
	A		Soejima *et al*. 2008
	B		Wakana *et al*. 2001a
	A		Soejima *et al*. 2008
	B		Sakai 1964, Kanda 1979, Niiyama 1989
	B		Kanno 1934, Kanda 1982, Niiyama 1989
	A		Sakai 1964, Niiyama 1989
			Sakai 1964, Niiyama 1989
X			Wakana *et al*. 2001b
	A		Wakana *et al*. 2001a
	A		Wakana *et al*. 2001a
	A		Niiyama 1989
	A		Wakana *et al*. 2005
	A		Wakana *et al*. 2001a
	A		Wakana *et al*. 2001a
X			Ergashev & Taubaev 1967
X			Skuja 1928
X			Skuja 1927
X			Skuja 1928
X			Skuja 1928
			Skuja 1927
X			Koster 1959
	E		Boedeker & Immers 2009
X			Segal & Groenhart 1967
			Koster 1959, van den Hoek 1963
			Koster 1959
			Koster 1959
			Segal & Groenhart 1967
			Kops et al. 1911, Koster 1959

no.	COUNTRY	LOCATION	LAT	LONG	HERB	
144	Northern Ireland	small lake west of Clones	54.148	-7.405		
145	Norway	Semsvand, Akershus	59.858	10.422		
146	Norway	river Akerselven	59.957	10.766	X	
147	Poland	Gulf of Puck (Zatoka Pucka), Baltic Sea	54.721	18.445		
148	Poland	Jantar, Baltic Sea	54.347	19.070		
149	Poland	Kościerzyna (as Berent), Pomorze Wschodnie	54.123	17.977	X	
150	Poland	Lake Miedwie (as Madüsee), near Szczecin	53.264	14.882	X	
151	Poland	Lake Tatarak (as Pansdorfer See), near Legnica (as Liegnitz)	51.221	16.311	X	
152	Poland	Lake Wierzbnickie (as Soldiner See), near Myślibórz	52.917	14.867		
153	Poland	Rewa, Baltic Sea	54.633	18.500		
154	Poland	Sopot, Baltic Sea	54.444	18.583		
155	Romania	Lake Techirghiol, near Constanta, Black Sea	44.048	28.627	X	
156	Russia	Bol'shoy Berezovyy [Ber’ozovyj ostrov (Björkö)], Gulf of Finland	60.317	28.582		
157	Russia	coast of St. Petersburg (as Leningrad)	59.965	30.221		
158	Russia	Hogland (Suursaari), Gulf of Finland	60.480	26.980		
159	Russia	Lake Arakhlei, Transbaikalia	52.200	112.867		
160	Russia	Lake Cheremenetskoye (Čermeneckoje), Leningrad oblast	58.616	29.967		
161	Russia	Lake Lino, Novgorod oblast	58.450	33.750		
162	Russia	Lake near Noginsk (as Bogorodsk), Moscow oblast	55.881	38.489		
163	Russia	Lake Poimo, Taldom (as Leninsk), Moscow oblast - cf. Dubna wetlands	56.729	37.535		
164	Russia	Lake Sabolotje (Zabolot'ye), Tver oblast	57.033	32.105		
165	Russia	Lake Sabolotskoje (Zabolockoje), Moscow/Vladimir oblast - cf. Dubna wetlands	56.757	37.142		
166	Russia	Lake Valdaj (Valdajskoye), Novgorod oblast	57.976	33.291	X	
167	Russia	near Vladimir city, Vladimir oblast	56.129	40.410	X	
168	Russia	near Yaroslavl city, Yaroslavl oblast	57.631	39.864	X	
169	Russia	Sestrorezk (Systerbäck), Gulf of Finland	60.081	29.960		
170	Russia	Toba marsh, Sakhalin	46.744	143.190	X	
171	Russia	Vyborgskij zaliv (Gulf of Vyborg), Gulf of Finland	60.715	28.699		
172	Russia	Caspian Sea	45.120	49.219		
173	Russia	Vashutkiny Lakes, Nenetsia	68.026	61.526		
174	Russia	Kamchatka peninsula	52.613	157.668		

LIT	ITS type	brack?	REFERENCES
X			van den Hoek 1963
X			Wille 1901
X		X	Waern 1952, van den Hoek 1963
X		X	Pliński & Jóźwiak 2004
			Pankow 1965
X			Pankow 1965
X		X	Pliński & Jóźwiak 2004
X		X	Pliński & Jóźwiak 2004
		X	
X		X	Waern 1952, van den Hoek 1963
X		X	Waern 1952, Pankow 1965
X		X	van den Hoek 1963
X			Matafonov *et al*. 2005
X			van den Hoek 1963
X			van den Hoek 1963
X			Pankow 1965
X			Pankow 1965
X			Brand 1902
X			van den Hoek 1963, Pankow 1965
			van den Hoek 1963
X		X	Waern 1952
	B		Tokida 1954
X		X	Waern 1952, van den Hoek 1963
X		X	Getzen 1967, Caspian Sea Biodiversity Project
X			Getzen 1967
X			Getzen 1967

no.	COUNTRY	LOCATION	LAT	LONG	HERB
175	Russia	Magnitogorsk area, Chelyabinsk oblast	53.579	59.189	
176	Russia	near Perm, Perm Krai	57.569	55.602	
177	Russia	near Krasnyy Yar (not Krasnoyarsk), Tomsk oblast	57.100	84.488	
178	Scotland	Appin, Argyll	56.563	-5.353	
179	Scotland	Loch Kildona, Outer Hebrides	57.223	-7.407	
180	Scotland	Loch Leodosay, Outer Hebrides	57.546	-7.334	
181	Scotland	Loch of Harray, Orkney Islands	59.014	-3.226	X
182	Scotland	Loch of Stenness, Orkney Islands	58.995	-3.248	
183	Scotland	Loch Watten, Caithness	58.486	-3.315	X
184	Scotland	Oban nam Fiadh, Outer Hebrides	57.545	-7.273	
185	Scotland	River Tweed	55.595	-2.481	X
186	Scotland	Shetland Islands	60.271	-1.431	
187	Scotland	West Loch Ollay, Outer Hebrides	57.261	-7.388	
188	Slovenia	Piran	45.528	13.571	
189	Slovenia	Debeli rtič, Adriatic	45.587	13.715	
190	Slovenia	near Koper, Adriatic	45.548	13.701	
191	Spain	Corrubedo saltmarsh, Galicia	43.367	-9.046	
192	Spain	Ría de Arosa, Galicia	42.657	-8.749	
193	Spain	river Onar, Girona	41.973	2.835	
194	Sweden	Ängsskär, Grepen, Öregrund archipelago	60.483	18.068	
195	Sweden	Barkerydsjön/Ribbingsnäsjön, near Nässjö, Småland	57.727	14.555	X
196	Sweden	Bilhamn, Älvkarleby, Uppland	60.634	17.440	X
197	Sweden	Bornsjön, Botkyrka near Stockholm	59.233	17.747	
198	Sweden	Börringesjön, Skåne	55.482	13.316	
199	Sweden	Brosjön, near Stockhom	59.175	17.790	
200	Sweden	Bygdeå, Västerbotten	64.067	20.850	X
201	Sweden	Dalarö, near Stockholm, Öregrund archipelago	59.133	18.406	X
202	Sweden	Dalbyö, Baltic Sea	58.865	17.493	X
203	Sweden	Djurgården, Stockholm	59.317	18.117	X
204	Sweden	Fårösundet, Piteå, Bay of Bothnia	65.338	21.484	X
205	Sweden	Fibysjön, Uppsala	59.895	17.350	X
206	Sweden	Finjasjön, Skåne	56.131	13.708	
207	Sweden	Fläcksjön, Västmanland	59.868	16.315	
208	Sweden	Fluttuskär, Öregrund archipelago	60.475	18.544	
209	Sweden	Granfjärden, Öregrund archipelago	60.304	18.332	
210	Sweden	Gravtjärn near Orsa, Dalarna	60.777	14.303	

LIT	ITS type	brack?	REFERENCES
X			Getzen 1967
X			Getzen 1967
X			Getzen 1967
X		X	van den Hoek 1963
X			Acton 1916, van den Hoek 1963, John *et al.* 2002
X		X	BMT Cordah Limited 2004
		X	International Centre for Island Technology 2004
X		X	International Centre for Island Technology 2004
	C		
X		X	BMT Cordah Limited 2004
			John *et al.* 2002
X			van den Hoek 1963
X			John *et al.* 2002
X			Lazar 1975, Battelli 1997
X		X	Battelli 1997, Turk & Vukovic 2000
X		X	Battelli 1997
X		X	Calvo *et al.* 1999
X		X	Donze 1968, Calvo & Bárbara 2002
X			Pena Haitz 1980
X		X	Waern 1952
			van den Hoek 1963
		X	Waern 1952
X			Sjöö et al. 2005
X			Lundh 1951
X			Giegold & Tutturen 1996
		no data	
		X	
		X	van den Hoek 1963
		X	
		X	
X			Lundh 1951
X			p.c. R. Bengtsson
X		X	Waern 1952
X		X	Waern 1952
X			p.c. I. Lennmark

no.	COUNTRY	LOCATION	LAT	LONG	HERB
211	Sweden	Grindsjön, Södertörn	59.086	17.871	
212	Sweden	Gyllebosjön, Skåne	55.596	14.191	
213	Sweden	Halsaren, Gulf of Bothnia	60.221	18.910	
214	Sweden	Hammarbysjön, Stockholm	59.308	18.101	X
215	Sweden	Hammarsjön, Skåne	55.983	14.225	
216	Sweden	Hästsjön, Jönköping county, Småland	57.736	14.679	
217	Sweden	Havgårdssjön, Skåne	55.484	13.359	
218	Sweden	Kansjön, Jönköping county, Småland	57.639	14.535	
219	Sweden	Krankesjön, Skåne	55.699	13.484	
220	Sweden	Kvesarumssjön, Skåne	55.941	13.658	
221	Sweden	Lake Aspen, Botkyrka near Stockholm	59.222	17.810	X
222	Sweden	Lake Dannemora, Uppsala	60.180	17.840	X
223	Sweden	Lake Erken, Uppsala	59.847	18.579	X
224	Sweden	Lake Hedeviken, Uppland	62.399	13.712	X
225	Sweden	Lake Hönsan, Dala-Husby, Dalarna	60.390	15.948	X
226	Sweden	Lake Mälaren	59.347	17.456	X
227	Sweden	Lake Mien, Kronoberg county, Småland	56.416	14.856	
228	Sweden	Lake Säbysjön, Järfälla, Uppland	59.438	17.868	X
229	Sweden	Lake Tåkern, Östergötland	58.351	14.774	
230	Sweden	Lake Tullan, near Södertälje, Södermanland	59.217	17.689	
231	Sweden	Lake Valloxen, Uppland	59.741	17.858	X
232	Sweden	Lake Vättern, Jönköping, Småland	57.783	14.183	X
233	Sweden	Launinkari, Haparanda, Bay of Bothnia	65.789264	24.039	
234	Sweden	Levrasjön, Skåne	56.103	14.489	
235	Sweden	Lidingö, Stockholm	59.367	18.133	
236	Sweden	Lillsjön, Stockholm	59.341	17.957	X
237	Sweden	Lötaviken, Nabbo (Rådmansö), Norrtälje	59.750	18.998	X
238	Sweden	Luleå, Bothnian Gulf	65.583	22.150	
239	Sweden	Malmö	55.600	13.000	X
240	Sweden	Måskobben, Gulf of Bothnia	60.198	18.855	
241	Sweden	Näske, Gulf of Bothnia	63.155	18.521	X
242	Sweden	Northern Quark, Gulf of Bothnia	63.830	20.940	X
243	Sweden	Nynäshamn, Baltic Sea	58.901	17.954	
244	Sweden	Oppmannasjön, Skåne	56.099	14.324	
245	Sweden	Öregrund, Öregrund archipelago	60.340	18.429	X
246	Sweden	Östhammar, near Stockholm	60.267	18.373	

LIT	ITS type	brack?	REFERENCES
X			Giegold & Tutturen 1996
X			Lundh 1951
X		X	Waern 1952
		X	
X			p.c. R. Bengtsson
X			p.c. R. Bengtsson
X			Lundh 1951
X			p.c. R. Bengtsson
X			Lundh 1951
X			Lundh 1951
	C		Hanyuda *et al*. 2002, Yoshii *et al*. 2004
	C		Kahlert *et al*. 2002
			van den Hoek 1963, Pankow 1965
			Brand 1902, van den Hoek 1963
X			p.c. R. Bengtsson
X			Waern 1939, 1952, van den Hoek 1963
X			Giegold & Tutturen 1996
			van den Hoek 1963
X		X	Tolstoy & Österlund 2003
X			Lundh 1951
X		X	van den Hoek 1963
			Brand 1902, Pankow 1965
			Brand 1902, van den Hoek 1963, Pankow 1965
X		X	van den Hoek 1963
		no data	
X		X	Waern 1952
		X	Waern 1952, van den Hoek 1963
	C	X	
X		X	Bardun & Ljungberg 2001
X			Lundh 1951
		X	Waern 1952, van den Hoek 1963
X		X	Waern 1952

no.	COUNTRY	LOCATION	LAT	LONG	HERB	
247	Sweden	Östra Småskären, Överenhörna, Sörmland	59.309	17.371		
248	Sweden	Råbelövssjön, Skåne	56.105	14.234		
249	Sweden	Ringsjön, Skåne	55.865	13.557		
250	Sweden	Rössjön, Skåne	56.316	13.104		
251	Sweden	Sandhemssjön, Västergötland	57.995	13.781	X	
252	Sweden	Sandskär, Haparanda, Bay of Bothnia	65.577	23.741		
253	Sweden	Säter (Ljustern?), Dalarna	60.345	15.757	X	
254	Sweden	Skutskär, near Gävle	60.641	17.402		
255	Sweden	Snogeholmssjön, Skåne	55.562	13.726		
256	Sweden	Södra Vixen, near Nässjö, Småland	57.620	14.883		
257	Sweden	Sötåsasjön, Jönköping county, Småland	57.944	14.687		
258	Sweden	Sövdesjön, Skåne	55.574	13.666		
259	Sweden	Stora Korssten, Gulf of Bothnia	60.203	18.842		
260	Sweden	Tjörnarpssjön, Skåne	55.994	13.613		
261	Sweden	Vallsjön, Jönköping county, Småland	57.845	13.729		
262	Sweden	Vässarö Garpen, Gulf of Bothnia	60.251	18.701		
263	Sweden	Västersjön, Skåne	56.321	13.044		
264	Sweden	Vombsjön, Skåne	55.687	13.572		
265	Sweden	Waldemarsudden (Saltsjön), Stockholm	59.304	18.227	X	
266	Sweden	Yttra Storhamn, Långvind, Gävleborg, Gulf of Bothnia	61.420	17.172		
267	Switzerland	Lake Zürich	47.336	8.556		
268	Switzerland	River Sasso near Locarno	46.167	8.800	X	
269	Switzerland	San Pancrazio, Lago Maggiore	46.132	8.736	X	
270	Switzerland	Södra Wixen SE of Nässjö in Småland county, 1991	46.164	8.806	X	
271	Turkey	Sinop, Black Sea	42.027	35.158		
272	Ukraine	Lake Svityaz, Shatsk National Park	51.501	23.851	X	
273	Ukraine	river Dnjpr (Dnipro), Poltava Oblast	48.930	33.904		
274	Ukraine	Lake Bile, Pripyat marshes, Rovenskyi region	51.839	25.499		
275	Ukraine	Lake Pisochne, Shatsk National Park	51.573	23.919		
276	United States	Cayuta Lake, New York	42.369	-76.735	X	
277	United States	Lake Bemidji, Minnesota	47.474	-94.880	X	
278	United States	Lake Quannapowitt, Massachusetts	42.516	-71.079	X	
279	United States	Long Lake, Indiana	41.524	-87.050	X	
280	United States	Millcoguins lake, Mackinac (Michigan?)	45.771	-84.760	X	
281	United States	Thimble Islands, Long Island Sound, Connecticut	41.264	-72.753	X	

LIT	ITS type	brack?	REFERENCES
X			Giegold & Tutturen 1996
X			Lundh 1951
X			Lundh 1951
X			Lundh 1951
			Brand 1902, Pankow 1965
X		X	p.c. A. Tolstoy
X		X	Waern 1952
X			Lundh 1951
X			p.c. I. Lennmark
X			p.c. R. Bengtsson
X			Lundh 1951
X		X	Waern 1952
X			Lundh 1951
X			p.c. R. Bengtsson
X		X	Waern 1952
X			Lundh 1951
X			Lundh 1951
		X	Waern 1952
X		X	p.c. H. Schreiber
X			Messikommer 1954
			van den Hoek 1963
			van den Hoek 1963
X		X	Aysel *et al*. 2004
	C		
X			p.c. O. Gerasymova
X			p.c. O. Gerasymova
X			p.c. O. Gerasymova
			Clovis 1955
	A		Wakana *et al*. 2001b
			(Collins 1909, Smith 1950)
		X	

no.	COUNTRY	LOCATION	LAT	LONG	HERB	
282	United States	Trout Lake, Wisconsin	46.036	-89.673		
283	Uzbekistan	Lake Aral	43.945	60.095		
283	Uzbekistan	Lake Aral	43.945	60.095		

Appendix S3 continued. List of locations of *Aegagropila linnaei* that were excluded from our study.

EXCLUDED LOCATIONS	references/collections*
diff. tax. affinity suggested/synomity refused	
USA (Florida?)	Wolle 1887
Iraq (Shatt al-Arab region)	Hinton & Maulood 1983
Libya (Ain Cherchara (Scersciara), Tarhuna)	Nizzamuddin & Gerloff 1982
Pakistan (Mahmood Booti; Mureedke; Neelum Valley)	Zarina *et al*. 2006
Sri Lanka	Durairatnam 1961, Durairatnam & Ragunathamuthaliar 1976
Sri Lanka	Grunow 1868
Myanmar, Pegu district (Irrawaddi, Balachoung (?), Tonghoo)	Zeller 1873
Bangladesh (Baraoora Lake, Srimangal)	Islam & Irfanullah 2005
India (Andamans and Nicobar Islands)	Prasad & Misra 1992
China (Lake Donghu, Wuhan)	HBI
China (Manhoa springs, Yunnan)	Skuja 1937, van den Hoek 1963
Padua, Italy (botanical garden, hothouse)	P; van den Hoek 1963
Halle, Germany (zool. garden, hothouse)	W
Lake Baikal	Izhboldina 2007
Mediterranean locations	references
Mediterranean (Sardinia & Sicily)	Báez *et al*. 2005
Mediterranean (Corsica/Sardinia, Sicily, Adriatic)	Gallardo *et al*. 1993
Mediterranean (Adriatic)	Furnari *et al*. 1999
Mediterranean (Sardinia)	Cossu 1992
Mediterranean (Ionian Sea)	Cecere *et al*. 1996
Mediterranean (Adriatic)	Cormaci *et al*. 2000
Mediterranean (Sardinia)	Brambati *et al*. 1980
Mediterranean (Sicily)	Giaccone *et al.* 1985
Mediterranean (Gulf of Venice, Trieste?)	Giaccone 1978
Mediterranean (Sea of Marmara/Aegean Sea? Lake Iznik?)	Getzen 1967
placenames not located	collections*
Muckawer See, Germany	L
Vestergarn, Skane, Sweden	BR, M,PC, S
river Monie, Ireland	TCD
Oregon, USA (no further information given)	BM

* herbarium acronyms follow Holmgren et al. 1990

LIT	ITS type	brack?	REFERENCES
X			Prescott 1951
X			Getzen 1967
X			Getzen 1967

	notes
	freshwater record, information suggesting different *Cladophora* species
	freshwater record, information suggesting different *Cladophora* species
	one freshwater record (Muschler 1910), no further information given
	freshwater record, drawing suggests diff. affinity (*Cladophora sp.*)
	marine records - cf. *Cladophora coelothrix* or *Boodlea vanbosseae*
	Cladophora clavuligera, on freshwater snails
	Cladophora codiola, *Cladophora contorta*, *Cladophora exigua* (on freshwater snails)
	freshwater record (on snail), drawing suggests *Cladophora sterrocladia*
	freshwater record, drawing suggests *Cladophora sterrocladia*
	re-identified as *Cladophora sterrocladia*
	Cladophora yuennanensis
	hothouse, cf. *Cladophora sterrocladia*
	hothouse, cf. *Cladophora sterrocladia*
	A. linnaei only listed in this book, not in the original publications
	notes
	quotes Gallardo *et al.* 1993, Furnari *et al.* 1999 (see below)
	quotes Cossu 1992, Giaccone 1978, Giaccone *et al*. 1985 (see below)
	quotes to Cormaci *et al*. 2000, Giaccone 1978 (see below)
	quotes Brambati *et al.* 1980 (see below)
	fully marine record
	fully marine record
	fully marine record
	fully marine record
	location unknown
	location unknown

Appendix S4. List of all known extant populations of *Aegagropila linnaei* (observations in the last 30 years).

no.	LOCATION	COUNTRY	LAT[a]	LONG[a]
1	Lake Zeller	Austria	47.326	12.806
2	Lake Naroch, Myadzyel district	Belarus	54.850	26.776
3	Lake, Lahoysk district	Belarus	54.519	28.433
4	Caspian Sea		45.120	49.219
5	Oslava River (as *Cl. moravica*)	Czech Republic	49.283	16.067
6	Sorø sø	Denmark	55.426	11.548
7	River Wear	England	54.719	-1.853
8	River Tees	England	54.527	-1.857
9	Malham Tarn, Yorkshire	England	54.097	-2.167
10	River Tyne	England	54.980	-2.099
11	River Swale	England	54.298	-1.507
12	Lake Ülemiste	Estonia	59.400	24.767
13	Lake Öisu	Estonia	58.209	25.513
14	Lake Valgjarv	Estonia	58.090	26.638
15	Pärnu river	Estonia	58.492	24.833
16	Northern Quark, Gulf of Bothnia	Finland	63.105	21.116
17	Saltvik, Gulf of Bothnia	Finland	60.279	20.036
18	Pojo Bay, Gulf of Finland	Finland	60.046	23.505
19	Neuklostersee	Germany	53.852	11.702
20	Grosser Dreisee	Germany	53.222	13.352
21	Teterower See	Germany	53.794	12.611
22	Kleiner Lankesee	Germany	52.910	13.225
23	Jakobsdorfer See	Germany	53.132	13.892
24	Tiefwarensee	Germany	53.526	12.691
25	Stechlinsee	Germany	53.154	13.031
26	Bültsee	Germany	54.500	9.754
27	Lake Myvatn	Iceland	65.600	-17.000
28	Lake Thingvallavatn	Iceland	64.168	-21.127
29	Laugabólsvatn	Iceland	65.939	-22.681
30	Miklavatn	Iceland	65.965	-17.603
31	Vestmannsvatn	Iceland	65.795	-17.416
32	Másvatn	Iceland	65.637	-17.239
33	Kringluvatn	Iceland	65.765	-17.228
34	Vikingavatn	Iceland	66.107	-16.826
35	Snjóölduvötn	Iceland	65.399	-14.348
36	Lough Gill	Ireland	54.253	-8.384

	SOURCE[b]	Most recent coll. date	GROWTH FORM
	Kann & Sauer 1982, Wakana *et al*. 2001a	1999	attached
	http://redbook.minpriroda.by/plantsinfo.html	1993	balls
	http://redbook.minpriroda.by/plantsinfo.html	1993	balls
	Caspian Sea Biodiversity Project.	?? (but recent)	n.d.
	Gardavsky 1986	1978[c]	attached
	C, No. 41, coll. Krasiln.	1977[c]	attached & floating, balls
	Whitton *et al*. 1998	1996	attached
	Holmes & Whitton 1981	1977[c]	attached
	Pentecost 1999, http://www.allphotography.free-online.co.uk/malham_web	2005	balls
	Holmes & Whitton 1975, 1981	1975[c]	attached
	Holmes & Whitton 1977	1976[c]	attached
	Trei & Pedusaar 2006	2000	balls
	Wakana *et al*. 2001a	2000	balls
	Wakana *et al*. 2001a	2000	n.d.
	Wakana *et al*. 2001a	2000	n.d.
	Bergström & Bergström 1999	1996	attached
	C, No. 34-1, coll. H. Mathiesen	1993	floating
	L 0793578 & 79, coll. R. Munsterhjelm	2007	floating
	Umweltministerium Mecklenburg-Vorpommern 2004, p.c. T. Kabus	2007	floating
	Bolbrinker 2000, p.c. P. Bolbrinker	2004	balls
	Pankow & Bolbrinker 1984, p.c. P. Bolbrinker	2003	floating
	p.c. T. Kabus	2006	unattached
	p.c. T. Kabus	2008	floating
	L 0793546, coll. T. Kabus	2008	floating
	p.c. T. Kabus, M. Teppke	2007	floating
	p.c. M. Bahnwart	2008	balls
	L 0793580, coll. A. Einarsson	2002	balls
	Jónsson 1992	1992	attached
	p.c. A. Einarsson	2005	balls
	p.c. A. Einarsson	2000	balls
	p.c. A. Einarsson	2000	balls
	p.c. A. Einarsson	2000	floating
	p.c. A. Einarsson	1990s	balls
	p.c. A. Einarsson	2000	floating
	Hallgrímsson 2002	2002	balls
	DBN, G7335, coll. J. J. Campbell	1987	balls

37	Loch an tSaile (near Screebe)	Ireland	53.385	-9.555
38	River Unshin, Lough Arrow	Ireland	54.068	-8.332
39	Lake Akan	Japan	43.451	144.099
40	Lake Chimikeppu	Japan	43.633	143.883
41	Lake Ogawara	Japan	40.783	141.333
42	Lake Biwa	Japan	35.250	136.083
43	Pond Takkobu/Takkobu marsh	Japan	43.100	144.483
44	Lake Saiko	Japan	35.498	138.685
45	Lake Shirarutoro	Japan	43.179	144.500
46	Lake Toro	Japan	43.144	144.540
47	Lake Kawaguchi	Japan	35.517	138.750
48	Pond Sakyo	Japan	40.804	140.441
49	Kimoma swamp	Japan	45.269	142.234
50	Kamuito swamp	Japan	45.269	142.234
51	Kamoi swamp	Japan	45.269	142.234
52	Pon swamp	Japan	45.269	142.234
53	Lake Panke	Japan	45.031	141.722
54	Pond Tamogi	Japan	40.750	141.000
55	Lake Yamanaka	Japan	35.417	138.867
56	Pond Ane	Japan	39.683	141.983
57	Pond Ichiyanagi	Japan	40.900	141.350
58	Pond Uchi	Japan	40.750	141.000
59	Boven Wijde	Netherlands	52.724	6.103
60	Jantar, Baltic Sea	Poland	54.347	19.070
61	Rewa, Baltic Sea	Poland	54.633	18.500
62	Sopot, Baltic Sea	Poland	54.444	18.583
63	Lake Arakhlei	Russia	52.200	112.867
64	Lake Toba, Sakhalin	Russia	46.744	143.190
65	Loch of Harray, Orkney Islands	Scotland	59.014	-3.226
66	Loch of Stenness, Orkney Islands	Scotland	58.995	-3.248
67	Oban nam Fiadh, Outer Hebrides	Scotland	57.545	-7.273
68	Loch Leodosay, Outer Hebrides	Scotland	57.546	-7.334
69	West Loch Ollay, Outer Hebrides	Scotland	57.261	-7.388
70	Loch Kildona, Outer Hebrides	Scotland	57.223	-7.407
71	River Tweed	Scotland	55.595	-2.481
72	Loch Watten	Scotland	58.486	-3.315
73	Debeli rtič, Adriatic	Slovenia	45.587	13.715
74	near Koper, Adriatic	Slovenia	45.548	13.701
75	Corrubedo saltmarsh, Galicia	Spain	43.367	-9.046

	Roden 1998	1992	balls
	John et al. 2002, p.c. N.H. Holmes	1977[c]	balls
	Soejima *et al*. 2008	2002	attached & floating, balls
	Soejima *et al*. 2008	2002	attached & floating
	Soejima *et al*. 2008	2001	attached
	Soejima *et al*. 2008	2001	attached
	Wakana *et al*. 2005	2004	attached
	Soejima *et al*. 2008	2002	attached & floating
	Niiyama 1989	ca. 1987	floating
	Kanda 1982, Niiyama 1989	ca. 1987	attached
	Niiyama 1989	ca. 1987	floating, balls
	Niiyama 1989	ca. 1987	attached & floating
	Wakana *et al*. 2001b	1999	attached
	Wakana *et al*. 2001b	1999	attached & floating
	Wakana *et al*. 2001b	1999	attached
	Wakana *et al*. 2001b	1999	attached
	Wakana *et al*. 2001a	2001	n.d.
	Wakana *et al*. 2001a	1996	n.d.
	Wakana *et al*. 2001a	1993	n.d.
	Wakana *et al*. 2001a	1996	n.d.
	Wakana *et al*. 2001a	1999	n.d.
	Wakana *et al*. 2001a	1999	n.d.
	Boedeker & Immers 2009	2007	attached
	Pliński & Jóźwiak 2004	2000	n.d.
	Pliński & Jóźwiak 2004	1998	n.d.
	Pliński & Jóźwiak 2004	2000	n.d.
	Matafonov *et al*. 2005	2001	attached & floating
	Wakana *et al*. 2001a	1993	n.d.
	International Centre for Island Technology 2004	2002	floating
	International Centre for Island Technology 2004	2002	balls
	BMT Cordah Limited 2004	2002	balls
	BMT Cordah Limited 2004	2002	balls
	John *et al*. 2002, p.c. B. A. Whitton	ca. 1998	balls
	John *et al*. 2002	1980s (?)	balls
	John *et al*. 2002, p.c. B. A. Whitton	1977	attached
	L 0793543, coll. C. Scanlan	2008	balls
	Battelli 1997, Turk & Vukovic 2000	1999	attached
	Battelli 1997	ca. 1996	attached
	Calvo *et al*. 1999	1996-1998	n.d.

76	Brosjön	Sweden	59.175	17.790
77	Grindsjön	Sweden	59.086	17.871
78	Bornsjön	Sweden	59.233	17.747
79	Lake Tullan	Sweden	59.217	17.689
80	Östra Småskären	Sweden	59.309	17.371
81	Lake Dannemora	Sweden	60.180	17.840
82	Lake Erken	Sweden	59.847	18.579
83	Piteå, Bay of Bothnia	Sweden	65.338	21.484
84	Lake Mälaren	Sweden	59.347	17.456
85	Nynäshamn, Gulf of Bothnia	Sweden	58.901	17.954
86	Öregrund, Gulf of Bothnia	Sweden	60.340	18.429
87	Gravtjärn near Orsa, Dalarna	Sweden	60.777	14.303
88	Södra Vixen , near Nässjö, Småland	Sweden	57.620	14.883
89	Northern Quark, Gulf of Bothnia	Sweden	63.830	20.940
90	Barkerydsjön/Ribbingsnäsjön, near Nässjö, Småland	Sweden	57.727	14.555
91	Sötåsasjön, Jönköping county, Småland	Sweden	57.945	14.687
92	Vallsjön, Jönköping county, Småland	Sweden	57.845	13.729
93	Kansjön, Jönköping county, Småland	Sweden	57.640	14.535
94	Hästsjön, Jönköping county, Småland	Sweden	57.737	14.679
95	Lake Mien, Kronoberg county, Småland	Sweden	56.417	14.856
96	Fläcksjön, Västmanland	Sweden	59.868	16.315
97	Hammarsjön, Skåne	Sweden	55.983	14.225
98	Rössjön, Skåne	Sweden	56.317	13.104
99	Västersjön, Skåne	Sweden	56.321	13.044
100	Launinkari, Haparanda, Bay of Bothnia	Sweden	65.789	24.039
101	Sandskär, Haparanda, Bay of Bothnia	Sweden	65.578	23.741
102	Yttra Storhamn, Långvind, Gävleborg, Gulf of Bothnia	Sweden	61.420	17.172
103	Sinop, Black Sea	Turkey	42.027	35.158
104	Lake Svityaz, Shatsky (Shatsk)	Ukraine	51.501	23.851
105	Lake Bile, Pripyat marshes, Rovenskyi region	Ukraine	51.839	25.499
106	Lake Pisochne, Shatsk National Park	Ukraine	51.573	23.919
107	Lake Bemidji	USA	47.474	-94.880

	Giegold & Tutturen 1996	1995	n.d.
	Giegold & Tutturen 1996	1995	n.d.
	Sjöö *et al.* 2005	2005	balls
	Giegold & Tutturen 1996	1995	n.d.
	Giegold & Tutturen 1996	1995	n.d.
	Yoshii *et al.* 2004	2000	balls
	Kahlert *et al.* 2002, Wakana *et al.* 2001a	2000	balls
	S, A3976, I.-G. Förare	1977[c]	balls
	S, A3978, coll. J. Rydström, Bardun & Ljungberg 2001	1998/1999	balls
	Bardun & Ljungberg 2001	1998/1999	n.d.
	C, No.'s 31-1, 32-1, 33-1, coll. H. & L. Mathiesen, M. Waern	1982	n.d.
	p.c. I. Lennmark	1985	balls
	p.c. I. Lennmark	1991	balls
	L 0793571, coll. L. Bergstroem & J. Ask	2004	attached
	p.c. R. Bengtsson	2006	balls
	p.c. R. Bengtsson	2006	attached
	p.c. R. Bengtsson	2006	attached
	p.c. R. Bengtsson	2006	attached
	p.c. R. Bengtsson	2006	attached
	p.c. R. Bengtsson	ca. 1998	attached
	p.c. R. Bengtsson	2006	attached
	p.c. R. Bengtsson	2006	attached
	p.c. R. Bengtsson	2006	attached
	p.c. R. Bengtsson	2006	attached
	Tolstoy & Österlund 2003	1996	attached
	p.c. A. Tolstoy	1996	attached
	p.c. H. Schreiber	> 2000	balls
	Aysel et al. 2004	< 2003	attached
	KW, coll. P. M. Tsarenko	2006	balls
	p.c. O. Gerasymova	1980s (?)	n.d.
	p.c. O. Gerasymova	recent	balls
	Wakana *et al.* 2001a	2001	balls

[a] http://www.gorissen.info/Pierre/maps/googleMapLocation.php

[b] herbarium acronyms (bold letters) follow Holmgren et al. 1990

[c] last record older than 30 years, but population probably still present

Appendix S5. List of outgroup taxa and their respective GenBank accession numbers for the LSU and SSU rDNA sequences (sequences generated in this study are indcated in bold).

outgroup taxa	**GenBank accession nos.**	
	LSU	SSU
Trentepohlia sp.	**FR719952**	**FR719938**
Ulva fasciata Delile	AJ544726	AB425964
Okellya curvata (Printz) Leliaert & Rueness	FN257507	FN257508
Cladophora coelothrix Kützing	AM503443	AM498749
Anadyomene stellata (Wulfen) C. Agardh	AJ544746	AF510147
Boergesenia forbesii (Harvey) J. Feldmann	AJ544742	AF510164
Cladophora aokii Yamada	AM503434	AM498747
Siphonocladus tropicus (P. Crouan & H. Crouan) J. Agardh	AJ544744	AM498761
Boodlea composita (Harvey) Brand	AJ544731	AF510157
Struvea elegans Børgesen	AJ544737	AF510149
Dictyosphaeria cavernosa (Forsskål) Børgesen	AJ544745	AM498756
Valonia aegagropila C. Agardh	AJ544748	AM498762
Valoniopsis pachynema (G. Martens) Børgesen	AJ544741	AM498765
Cladophora pygmaea Reinke	FM205040	FM205051
Cladophora feredayi Harvey	FM205030	FM205049
Cladophora pellucida (Hudson) Kützing	FM205037	Z35314
Cladophora radiosa (Suhr) Kützing	FM205042	FM205052
Cladophora albida (Nees) Kützing	AM503433	Z35317
Cladophora vagabunda (Linnaeus) van den Hoek	AJ544760	AB062710
Cladophora rupestris (Linnaeus) Kützing	AJ544764	Z35319
Chaetomorpha crassa (C. Agardh) Kützing	AJ544767	AB062701

Appendix S6

Effects of model choice and missing data on tree topologies and support

The 'test' alignment was assembled to test for the effects of using a complex *vs.* a simple evolutionary model both with and without outgroup and consisted of 39 ingroup taxa (see Table 3 in Chapter 7). Since the phylogenetic position of the early-diverging clade consisting of *Cladophora horii* and *C. conchopheria* was not stable in preliminary BI and MP analyses performed with the 'test' alignment, the effects of using a complex (GTR+I+G, determined by Modeltest) *vs.* a simple (HKY) model of nucleotide substitution on the tree topology was evaluated in BI analyses, both with (Figs. S6 A & B) and without *Okellya curvata* as outgroup (Figs. S6 C&D). In both cases, the trees resulting from the analysis with the simple HKY model were better resolved and had higher support for several branches. Main differences in the topologies under the two different models were the placement of *Cladophora horii* and *C. conchopheria* (Figs. S6 A & B), the monophyly of the *Basicladia*-clade (Figs. S6 A & B, C & D), and the overall resolution within the *Aegagropila/Pithophora* clade. When an outgroup was used, the clade consisting of *C. horii* and *C. conchopheria* grouped without support with the *Siphonocladus*-clade and the *Cladophora*-clade under the GTR+I+G model (Fig. S6 A). Under the HKY model, this clade grouped with high support with the *Aegagropila*-clade (Fig. S6 B). Without ougroup, this clade grouped with high support with the *Aegagropila*-clade under both models (Figs. S6 C & D). The *Basicladia*-clade was only monophyletic with high support when a simple model (HKY) was used instead of a more complex model (GTR+I+G) that was estimated to have the best fit for the data, or in MP analyses. Under a GTR+I+G model *B. okamurae* was recovered on a polytomy. MP analysis is considerd as one of the least reliable methods, especially if sites are not evolving independently as in the rRNA genes, since no model of nucleotide evolution is employed (Schöniger & von Haeseler 1995). Similarly, only under the HKY model the relationships within the *Aegagropila/Pithophora* clade were resolved, while the three subclades formed a polytomy under a complex model. Both with and without using outgroup sequences, the trees resulting from the analysis with the simple HKY model were better resolved and had higher support for several branches. However, it is incorrect to regard better resolved or better supported trees as superior since there is no *a priori* knowledge of the true phylogeny and support does not necessarily correlate with accuracy (Gontcharov *et al*. 2004). Employing simple models of evolution for complex datasets can possibly lead to the recovery of wrong topologies (Posada & Crandall 2001). Only the trees obtained under the inferred complex model are shown in the main text. Possible explanations for the failure to unequivocally establish the phylogenetic placement of *B. okamurae* with high support could be a higher proportion of homoplasic characters in *B. okamurae*, long branch attraction (Graham *et al*. 2001) or insufficient taxon sampling.

The 'test' alignment was also used to test for the effects of missing sequence data (not shown). Taxa with missing LSU sequences (two taxa: *Cladophora* sp. 'Tateyama' and *Arnoldiella conchophila*) and with missing partial SSU sequences (one taxon: *Cladophora sterrocladia*) were excluded from this alignment, and we subsequently compared the resulting tree topologies from BI analyses (not shown). Excluding the three taxa with missing sequence data had no effect on the tree topology. The model choice (GTR+I+G *vs.* HKY) had the same effects on datasets without missing data as outlined above.

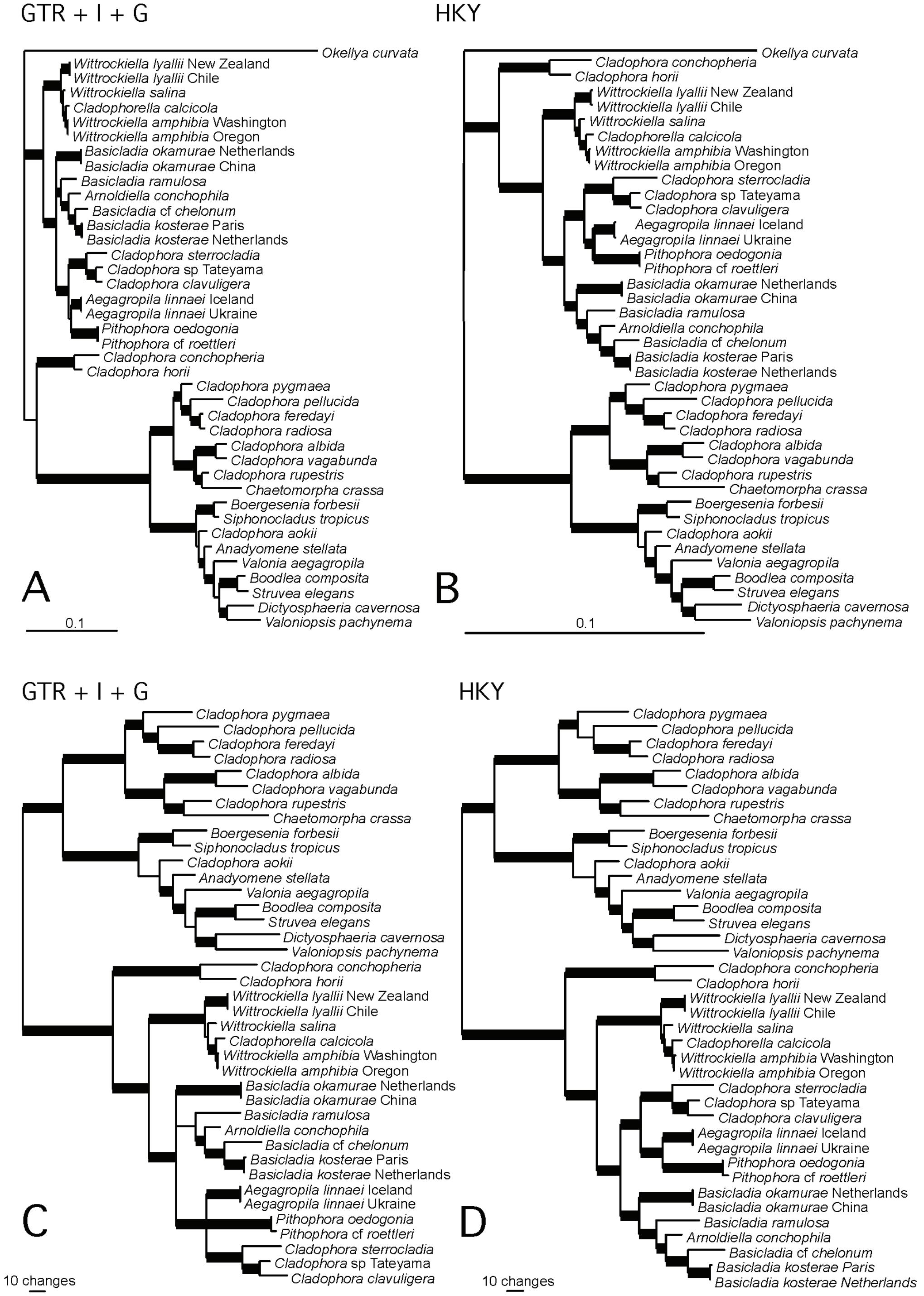

Fig. S6. Comparison of model performance (GTR+I+G *vs*. HKY) used in Bayesian Inference analysing the combined SSU_LSU dataset. **A**: LSU_SSU dataset with outgroup under a GTR+I+G model. **B**: SSU_LSU dataset with outgroup under a HKY model. **C**: SSU_LSU dataset without outgroup under a GTR+I+G model. **D**: SSU_LSU dataset without outgroup under a HKY model. Thick branches indicate posterior probabilities > 0.95.

Appendix S7

Molecular clock and midpoint rooting of the ingroup

In addition to rooting with the three outgroup-taxa, the position of the root was determined for the 'ingroup' dataset (see Table 3 in Chapter 7) by molecular clock rooting using BEAST v1.5.2 (Rambaut & Drummond 2007b) and by midpoint rooting of the BI and ML trees (Fig. S7). The molecular clock analysis automatically roots the tree along its oldest branch. The likelihood ratio tests of the LSU and SSU alignments separately and combined rejected rate constancy in all cases. However, molecular clock rooting is robust to moderate degrees of rate heterogeneity (Huelsenbeck *et al*. 2002), so it is still possible to accurately root trees with a (relaxed) molecular clock when local clock deviations exist. The dataset was analysed under both a relaxed (Drummond *et al*. 2006) and a strict molecular clock assumption n BEAST. For all analyses, two independent runs of 50 x 10^6 generations with a fixed mean substitution rate, default priors and a Yule model as the tree prior were performed at the BioHPC cluster (http://cbsuapps.tc.cornell.edu/beast.aspx), which were subsequently combined with LogCombiner (part of the BEAST package). A lognormal relaxed clock was utilized. Trees were sampled every 5000 generations, and a burnin of 1000 trees was removed before generating consensus trees with TreeAnnotator (part of the BEAST package). The log-files were checked with the program Tracer.

The trees constrained with a strict and a relaxed molecular clock (Figs. S7 A & B, respectively) both show the same root position as the outgroup rooting results (Fig. 3 in Chapter 7), separating the *Aegagropila*-clade as a monophyletic clade (including the *horii*-clade) with high support from the sister groups *Siphonocladus*-clade and *Cladophora*-clade. Additional midpoint rooting performed on BI and ML trees of the 'ingroup' dataset (Figs. S7 C & D, respectively) showed the same root position as revealed by outgroup rooting and molecular clock rooting.

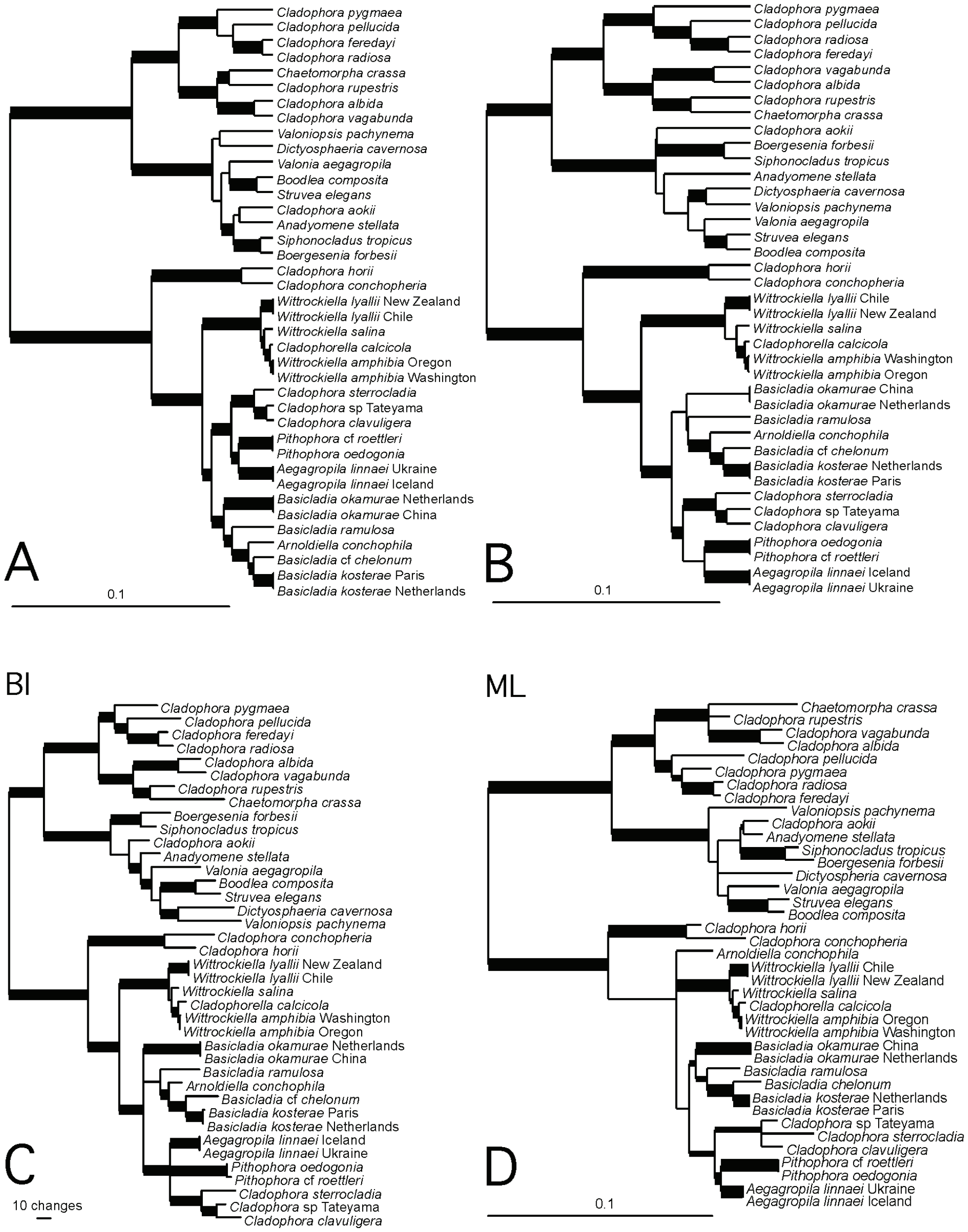

Fig S7: Ingroup_only analysis of the SSU_LSU dataset under different phylogenetic inference methods (all with GTR+I+G as substitution model). Molecular clock rooting was performed in BEAST under a strict (**A**) and a relaxed (**B**) molecular clock algorithm. The Bayesian tree (**C**) and the Maximum Likelihood tree (**D**) were rooted using midpoint. Taxa of the *Cladophora*-clade and the *Siphonocladus*-clade are included to evaluate root placement. Thick branches indicate posterior probabilities > 0.95 in **A**-**C**, while in **D** medium-thickness branches reflect bootstrap values over 70 and thick branches reflect bootstrap values over 90 (1000 replicates).

Appendix S8

List of all nomenclatorial and taxonomic changes proposed in this thesis

Nomenclatorial-taxonomic changes proposed in this thesis are underlined.

New descriptions, new combinations, new synonymizations

***Aegagropilopsis* Boedeker gen. nov.**
Type species: *Aegagropilopsis sterrocladia* (Skuja) Boedeker comb. nov. (*Cladophora sterrocladia* Skuja 1949: 94-95).

***Aegagropilopsis clavuligera* (Grunow) Boedeker comb. nov.**
Holotype: Ceylon/Sri Lanka (Expedition Novara), collector G. von Frauenfeld, W (2010/2274), on shell of freshwater gastropod.
Basionym: *Cladophora clavuligera* Grunow 1868: 40.

***Aegagropilopsis sterrocladia* (Skuja) Boedeker comb. nov.**
Holotype: original specimen (collected by H. Skuja, Burma/Myanmar, on shell of freshwater gastropod) lost (used to be in RIG), thus the original drawings represent the holotype material (Skuja 1949: Plate XXXVII).
Epitype: pond in tropical hothouse, Hortus Botanicus Leiden, The Netherlands, collector C. Boedeker (sample G91), 26 April 2006, attached on mangrove pneumatophores (submerged), L (L0793287).
Basionym: *Cladophora sterrocladia* Skuja 1949: 94-95.

***Arnoldiella* V. Miller emend. Boedeker**
Type species: *Arnoldiella conchophila* V. Miller 1928: 20-21.

***Arnoldiella chelonum* (Collins) Boedeker comb. nov.**
Holotype: Walnut Lake, Michigan, USA, collector T.L. Hankinson, on turtle carapaces, NY (00887601).
Basionym: *Chaetomorpha chelonum* Collins 1907: 198-200.
Synonym: *Basicladia chelonum* (Collins) Hoffmann & Tilden 1930: 382-383.

***Arnoldiella conchophila* V. Miller**
Holotype:, original specimen (collected by V. Miller, Lake Pereslavl, Vladimir district, Russia, 1921, on shell of freshwater bivalve) untraceable, thus the original drawings represent the holotype material (Miller 1928: Figs. 2-20).

***Arnoldiella kosterae* (van den Hoek) Boedeker comb. nov.**
Holotype: Jardin des Plantes, Paris, France, collector C. van den Hoek, 25 April 1961, L (L 0054830).

Basionym: *Cladophora kosterae* van den Hoek 1963: 37-38.
Synonym: *Basicladia kosterae* (van den Hoek) Garbary 2010: 39.
Arnoldiella okamurae **(S. Ueda) Boedeker comb. nov.**
Holotype: Shirahama, Tokyo, Japan, collector S. Ueda, Tokyo University of Marine Science and Technology.
Basionym: *Chaetomorpha okamurae* S. Ueda 1932: 23-24.
Synonyms: *Cladophora okamurae* (S. Ueda) van den Hoek 1963: 39.
Basicladia okamurae (S. Ueda) Garbary 2010: 39.

Arnoldiella ramulosa **(Ducker) Boedeker comb. nov.**
Holotype: Stratford, Victoria, Australia, collector S.C. Ducker, 11 December 1956, on carapace of turtle, MEL. Isotypes in BM & MELU.
Basionym: *Basicladia ramulosa* Ducker 1958: 165-166.

Pithophora **Wittrock**
Type species: *Pithophora roettleri* (Roth) Wittrock (*Pithophora kewensis* Wittrock 1877: 52-55).
Lectogeneritype: *Pithophora kewensis* Wittrock, tropical aquarium (‘Waterlily-house’), Kew Gardens, Britain, collector V.B. Wittrock, August 1872, L (no. 938112 639). Isotypes in BM, L, UPS & S.

Pithophora roettleri **(Roth) Wittrock**
Holotype: Tranquebar, eastern India, collector Klein, January 1799, L (no. 93825 38). Isotype in UPS.
Basionym: *Ceramium roettleri* Roth 1806: 123.
Synonyms: all described species of *Pithophora* (including all intraspecific taxa) *P. aequalis* Wittrock, *P. affinis* Nordstedt, *P. chinensis* Skworzow, *P. clavifera* Schmidle, *P. cleveana* Wittrock, *P. kewensis* Wittrock, *P. macrospora* Brand, *P. microspora* Wittrock, *P. mooreana* Collins, *P. oedogonia* (Montagne) Wittrock, *P. pachyderma* Schmidle, *P. pragensis* Sula, *P. polymorpha* Wittrock, *P. radians* W. & W.S. West, *P. reinecki* Schmidle, *P. sumatrana* (Martens) Wittrock, *P. varia* Wille, *P. variabilis* Schmidle, *P. zelleri* (Martens) Wittrock.

Pseudocladophora **Boedeker gen. nov.**
Type species: *Pseudocladophora conchopheria* (Sakai) Boedeker comb. nov. (*Cladophora conchopheria* Sakai 1964: 48).

Pseudocladophora conchopheria **(Sakai) Boedeker comb. nov.**
Holotype: Nagahama near Maizuru, Kyoto Prefecture, Japan, collector I. Umezaki, May 1949, SAP (SAP 029140), on shell of the marine gastropod *Lunella coronata* Gmelin.
Basionym: *Cladophora conchopheria* Sakai 1964: 48.

Pseudocladophora horii **(van den Hoek & Chihara) Boedeker comb. nov.**
Holotype: Sesoko Island, Okinawa, Japan, collectors S. Kamura, C. van den Hoek & T. Hori, April 1990, TNS (TNS-AL-46793).
Basionym: *Cladophora horii* van den Hoek & Chihara 2000: 68.

***Wittrockiella* Wille**
Type species: *Wittrockiella amphibia* (Collins) Boedeker & Hansen (*Wittrockiella paradoxa* Wille 1909: 220-221).
Generitype: original specimen (collected in 1907 by N. Wille, Lyngør, Norway) untraceable, thus the original drawings represent the holotype material (Wille 1909: Plates XI-XIV).
Epitype: Lyngør, southeastern Norway, collector B. Lynge, January 1909, det. N. Wille, O (six iso-epitypes).

***Wittrockiella amphibia* (Collins) Boedeker et Hansen comb. nov.**
Isotypes: Alameda, San Francisco Bay, California, USA, Leg. W.J.V. Osterhout and N.L. Gardner, 26 September 1903, in L, voucher numbers L1284 and L2989, L1284 distributed as Exsiccatae Phycotheca Boreali-Americana (PB-A) No. 1284 (Collins *et al.* 1905). Additional isotypes distributed as No. 1284 of the PB-A to AHFH, BM, FH, PC, TRH, UC and WIS, among others. We select the specimen L1284 as the lectotype.
Basionym: *Cladophora amphibia* Collins 1907: 200.
Synonym: *Wittrockiella paradoxa* Wille 1909: 220-221.

***Wittrockiella calcicola* (Fritsch) Boedeker comb. nov.**
Holotype: original specimen (collected by F.E. Fritsch, tropical hothouse, Cambridge Botanical Garden, Britain, 1944, on moist limestone) lost or destroyed (used to be in BM), thus the original drawings represent the holotype material (Fritsch 1944: Figs. 1A-I, 2A-G, 3A-C, 4A-G).
Epitype: Lagoa de Óbidos, Portugal, collector O. Lourenço (sample no. K92; ACOI culture collection 471), 1989, L (L0793292).
Basionym: *Cladophorella calcicola* Fritsch 1944: 157-171.

***Wittrockiella lyallii* (Harvey) C. Hoek, Ducker & Womersley 1984: 45**
Holotype: Stewart Island, New Zealand, collector D. Lyall, TCD (isolectotypes in BM).
Basionym: *Cladophora lyallii* Harvey 1855: 262
Synonyms: *Siphonocladus brachyartrus* Svedelius 1900: 304, *Cladophoropsis brachyartra* (Svedelius) Wille 1910: 116, *Cladophoropsis lyallii* (Harvey) V.J. Chapman 1956: 471.

Doubtful, erroneous, and excluded names

Refused synonyms of *Aegagropila linnaei* Kützing (Chapter 4, Appendix S1)
Cladophora aegagropila var. *thermalis* Wolle 1885
> description suggests different affinity (*Cladophora sp.*)
Cladophora sterrocladia Skuja 1949
> synonymity had been proposed by Liu (1999)
Cladophora yuennanensis Skuja 1937
> synonymity had been proposed by van den Hoek (1963)
Cladophora shensiensis Jao 1948
> synonymity had been proposed by Liu (1999)
Cladophora glomerata var. *nana* Wang 1935
> synonymity had been proposed by Liu (1999)
Cladophora parvula Möbius 1895

> synonymity had been considered by Skinner & Entwisle (2000)

Cladophora dusenii Brand 1902

> possible synonymity had been discussed by Brand (1902)

Christian Bödeker was born on the 27th of August 1976 in Münster, Germany. He obtained his highschool diploma in 1996 and subsequently performed his civil service in Münster. In 1997, Christian started studying biology at the University of Göttingen, Germany. After the intermediate examinations in general biology in 1999, he switched to the University of Rostock, Germany, to specialise in marine biology as a major. Minor subjects were ecology, botany and physical oceanography. While studying in Rostock, Christian worked in numerous projects as a student assistant and took part in several phycological fieldtrips abroad. His term paper on the physiology of red algae under UVR-stress was carried out at the Alfred Wegener Institute for Polar and Marine Research in Bremerhaven, Germany. The practical work for the graduation thesis ('Diplom') was undertaken at the National Institute for Water and Atmospheric Research and Te Papa Tongarewa National Museum of New Zealand in Wellington, New Zealand, and funded by a scholarship of the German Academic Exchange Service (DAAD). The title of the thesis was 'Do morphology and life history reflect phylogeny? Culture experiments with *Bangia* strains (Bangiales, Rhodophyta) from New Zealand', supervised by Dr. Wendy Nelson (Wellington) and Prof. Dr. Ulf Karsten (Rostock). After graduating in December 2003, Christian started his PhD in May 2004 at the National Herbarium of the Netherlands on phylogenetics and systematics of the Cladophorales (Chlorophyta). Originally, the PhD project focussed on the genera *Chaetomorpha*, *Cladophora* and *Rhizoclonium*, but was later changed to studies in the *Aegagropila*-clade. During his PhD, Christian assisted undergraduate classes and supervised a BSc project on the biogeography of the green freshwater alga *Aegagropila linnaei*. Furthermore, he undertook phycological fieldwork in many countries and presented his research at international conferences in Leiden (Netherlands), Durban (South Africa), London (United Kingdom), Hobart (Australia) and Tokyo (Japan).

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And, how could I forget, there's good old Hana. I like.