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

Boedeker, C.

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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

Christian Boedeker, Anja Eggert, Anne Immers & Isamu Wakana

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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 resting 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, Niyama 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 ($10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) at 10°C 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 limnaei* for which ITS rDNA sequences have been generated, with collection information and GenBank accession numbers. Asterisks indicate bracketish 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
Säilvik, 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 Yamnaka, 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 Ichiyanaagi, 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 (Iagaiwa), 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 Shiratoro, 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 Tobu, 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 Oisu	Estonia	58.209	25.513	2000	balls	n.s.	C
Lake Valgiarv	Estonia	58.090	26.638	2000	n.d.	n.s.	C
Pänuu 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
Tietwarensse, Mecklenburg-Vorpommern	Germany	53.526	12.691	2008	floating	n.s.	C
Lake Danemora	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 10°C and low-light conditions (10 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$). 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 recorded.

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.

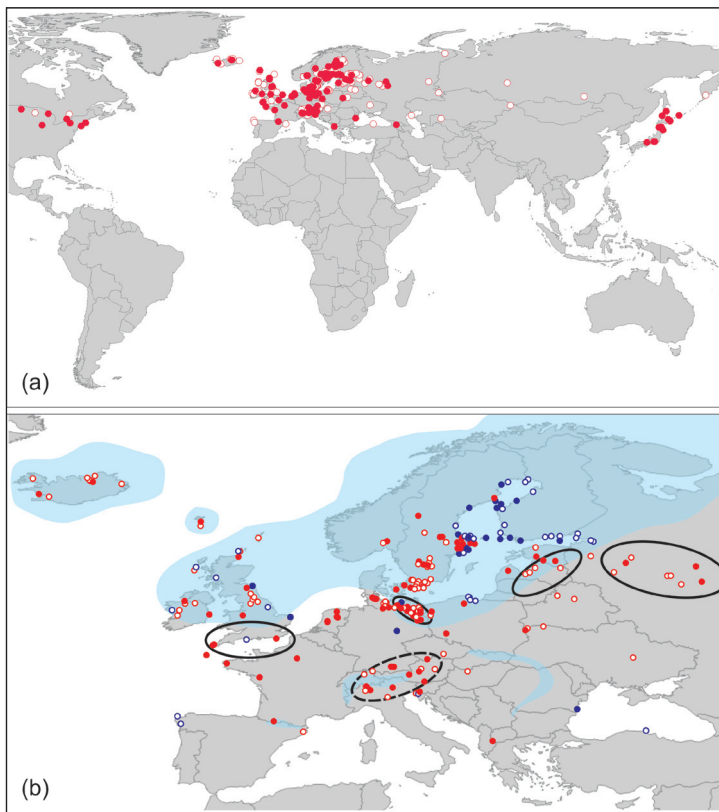


Fig. 2.

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).

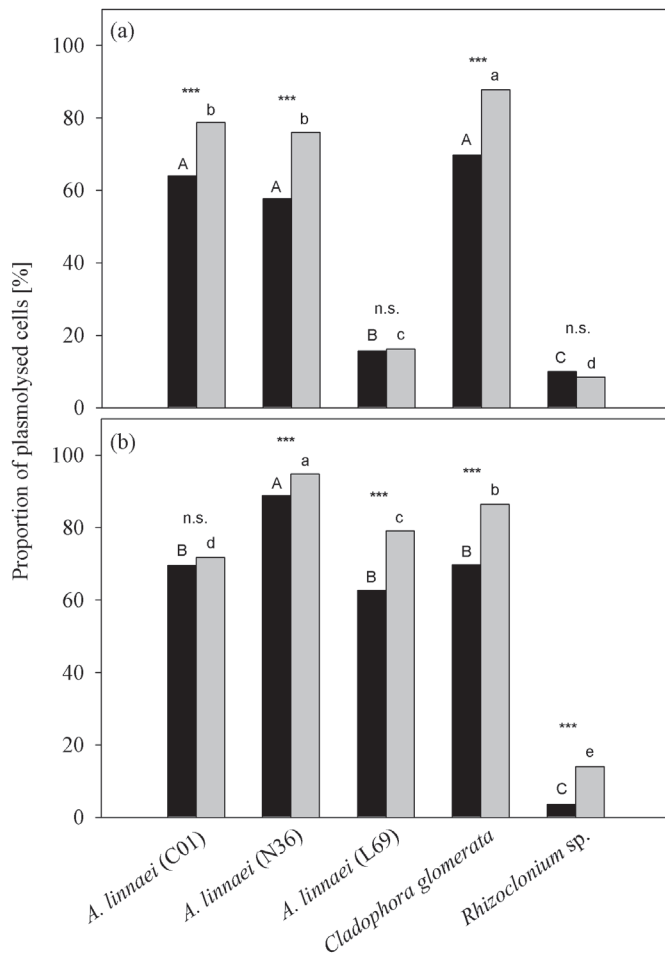


Fig. 3.

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).

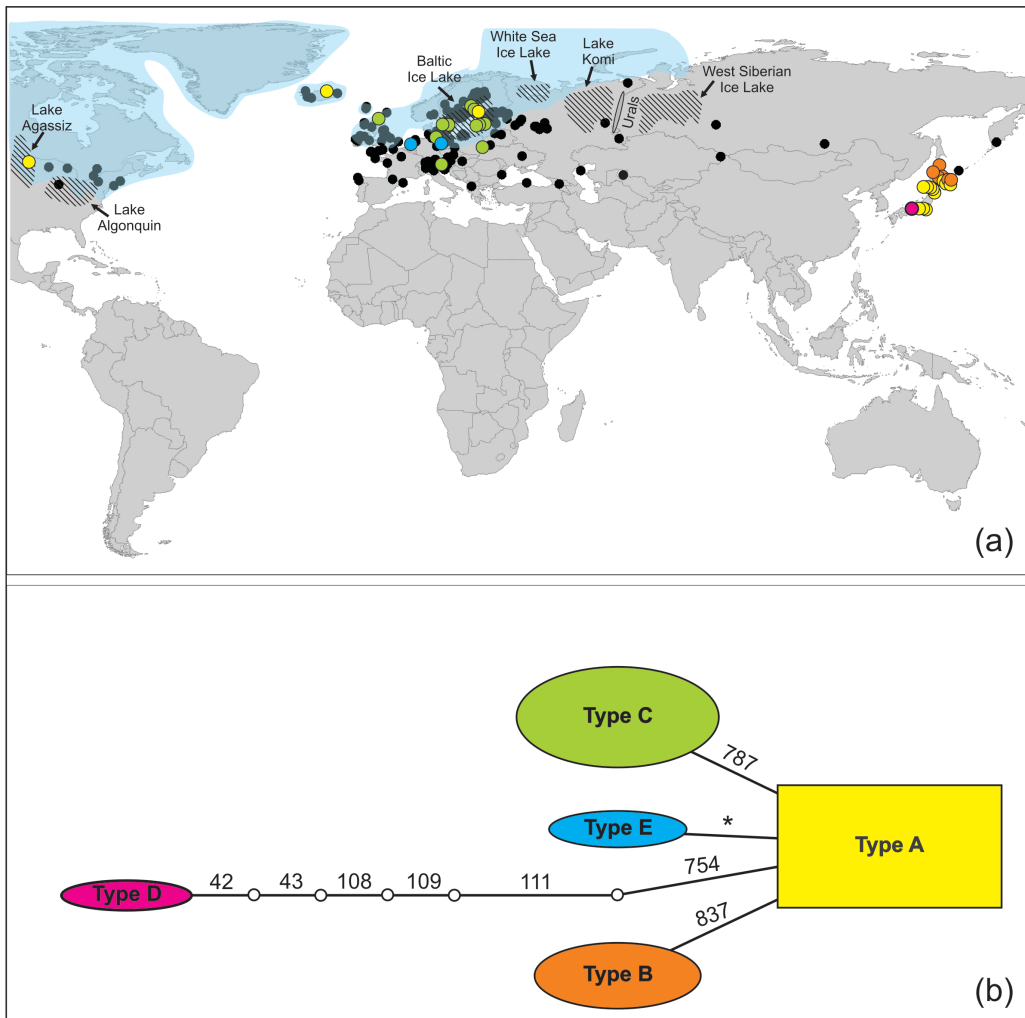


Fig. 4. **a** Global distribution (black dots) and distribution of ITS ribotypes of *Aegagropila linnaei*. Five ITS ribotypes were discovered among 34 samples, shown as large coloured circles. Hatched areas represent large ice-dammed lakes during Weichselian glaciations; blue shows the maximum extent 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 *Phyllocladon* 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 'hypnosporos', 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 -15°C (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 dispersal 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* (Biaioletto) 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 & Izboldina 1977, Izboldina 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 (Seegerstråle 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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