Species differentiation in the genus *Chara* (Charophyceae): considerable phenotypic plasticity occurs within homogenous genetic groups

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Running title

Genetic and morphological variability of *Chara*

Keywords

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Abstract

Charophytes are benthic algae with a complex morphology and high phenotypic plasticity. This has led to ambiguities in species delineation. However, until now genetic studies on *Chara* have been based on samples collected from a restricted geographical range or only included a restricted number of taxa. This may have hindered a general interpretation of the results. We applied barcoding of *matK*, a rapidly evolving coding section of the plastid genome, in 324 *Chara* samples collected from 19 countries, in order to test whether the distribution of barcode haplotypes among individuals was consistent with species boundaries as they are currently understood. The phylogenetic tree grouped the 324 *Chara* individuals, which according to commonly used identification keys represented 29 species, into 12 well-defined groups (i.e. monophyletic morphospecies or groups of morphospecies). Considerable morphological variation occurred within genetically homogeneous groups. This included traits which are commonly used for *Chara* species determination, such as the length and number of spine cells, the length of stipulodes and bract cells, cortication (tylacanthous, isostichous, aulacanthous, and absent cortication), as well as sex differentiation. However, there were also substantial genetic differences among morphologically similar species (e.g. *C. virgata – C. globularis – C. connivens*). No morphological trait consistently reflected genetic differences. This indicates that morphological traits for specific taxa indeed may serve as diagnostic tools for species delimitation, but that they are not generally suitable for inferring genetic differentiation or phylogenetic relationships. We propose that i) *C. virgata* and *C. strigosa*, ii) *C. liljebladii*, *C. horrida* and *C. baltica*, and iii) *C. hispida*, *C. rudis* and *C. polyacantha* are conspecific. Our data also indicate that *C. gymnophylla* should be divided into tylacanthous forms (which are closely related to *C. contraria*) and aulacanthous forms (which are related to *C. vulgaris*).

Introduction

Charophytes, defined as the extant and fossil members of the order Charales plus the members of the extinct orders Sycidiales and Moellerinales (Schneider *et al.*, 2015*a*), are algae with a complex morphology. Species delineation of charophytes is commonly based on morphological traits of the plant thallus. There is, however, considerable overlap in morphological characteristics used to discriminate species such that uncertainties occur in charophyte species delineation (Boegle *et al*., 2007; 2010*a*, 2010*b*). In addition, different flora treatments differ in their description of one and the same species (see e.g. description of *C. hispida* in Wood & Imahori (1965), Moore (1986) and Krause (1997)). Indeed, Proctor (1975) pointed out that "almost no regional studies from Eurasia involving [the *C. hispida* L.] complex agree upon the exact number of species to be recognized or how they are to be distinguished". In spite of these uncertainties, many *Chara* species are reported to have become rare in recent decades (Baastrup-Spohr *et al.*, 2013), they are red-listed in many countries (e.g. Sjøtun *et al.*, 2010; Auderset Joye & Schwarzer, 2012) and they are also frequently used as indicators for ecological status assessment of rivers and lakes (e.g. Stelzer *et al.*, 2005; Penning *et al.*, 2008). Thus, accurate identification of charophyte species is not only critical for understanding their diversity but also for ecosystem assessment. Information about which morphological traits reflect genetic differences may thus be important for ecosystem management.

The most extensive study that compared genetic and morphological characteristics of *Chara* was done by Mannschreck (2003), who used AFLP (Amplified Fragment Length Polymorphism, a genetic fingerprinting technique) to study 213 individuals belonging to 13 *Chara* species from Sweden, Germany, Poland, France and Mexico. She was able to discriminate all species except two pairs: *C. baltica*-*C. intermedia*, and *C. virgata*-*C. strigosa*. Subsequent detailed AFLP studies on the *C. baltica*-*C. intermedia* pair, together with several closely related species, partly differed in which species they were able to separate from each other (Boegle *et al.*, 2007, 2010*a*, 2010*b*; Urbaniak & Combik, 2013). This may be explained with the different and restricted geographical range from where individuals in each of these studies originated. Indeed, the most recent AFLP studies (Boegle *et al.*, 2010*a*; Urbaniak & Combik, 2013) indicated that a continuum may exist within taxa included in this cluster, rather than discreet entities. This is consistent with a recent study by Schneider *et al.* (2015*b*), who, based on barcoding three DNA markers in 91 *Chara* samples belonging to 14 different taxa, showed that eight European taxa within the *C. baltica*-*intermedia*-complex were

identical, and only samples from South-America differed in one base-pair from all other samples in this cluster.

However, AFLP and barcoding studies on *Chara* so far have been based on samples collected from a restricted geographical range, or included a restricted number of taxa. Consequently, there is a risk that genetic or morphological variation of taxa has been underestimated, with implications for interpretation of morphospecies. We also lack knowledge about the relative variation within and among taxa, i.e. whether some morphologically homogeneous taxa are more genetically variable than others, or whether some genetically homogeneous taxa have greater morphological variation than others. In order to fill this knowledge gap, we assembled 324 samples of the genus *Chara* collected from 19 countries, most of them in Europe, but also from North- and South America, Asia, and Africa. Less than 30 % of the samples have been used in a previous study (Schneider *et al.*, 2015*b*), while the remaining samples are reported for the first time. According to commonly used determination keys, our samples were identified as representing 29 species of the genus *Chara* (Table S1 in the supplementary material). We applied barcoding of *matK*, a plastid-encoded protein-coding gene, in order to test if the distribution of barcode haplotypes among individuals is consistent with species boundaries as they are currently understood. *MatK* is one of the most rapidly evolving coding sections of the plastid genome (Hilu & Liang, 1997), is recommended as one of two barcoding regions for plants (CBOL Plant Working Group, 2009) and has recently been shown to match well with other commonly used genetic markers in *Chara* (Schneider *et al.*, 2015*b*).

Material and Methods

Taxon sampling

The study included 327 individuals (324 from the genus *Chara*, two *Lamprothamnium* and one *Nitellopsis*) from 15 countries in Europe, as well as Argentina, Canada, Egypt and Nepal (Table S1). 319 samples were either collected fresh and dried in silica gel shortly after sampling, or from herbaria that are stored at the Norwegian Institute for Water Research, the Natural History Museum (University of Oslo, Norway), or the University of Rostock, Germany. Earlier studies indicated that herbarium specimen and silica gel dried samples were equally suitable for genetic analyses of *Chara* (Schneider *et al.*, 2015*b*). Voucher specimens exist for all samples (see Table S1). Eight charophyte *matK* sequences (six from the genus *Chara*, one *Lamprothamnium* and one *Nitellopsis*) were obtained from GenBank.

Taxonomy

Many *Chara* taxa have been variously recognized as species, varieties, or forms, and there is little consensus about appropriate rank among different flora treatments. The two most widely applied taxonomic concepts are those of Wood & Imahori (1965) and Krause (1997). While the former authors belong to the school of "lumpers" (lumping taxa into broad categories), the latter is a so-called "splitter" (creating many narrowly defined categories). For example, Wood & Imahori (1965) discriminate 19 species world-wide within the genus *Chara*, whereas Krause (1997) recognizes 29 species in Europe alone. In order to be consistent, and to provide data that are as taxonomically informative as possible, our species delineation generally followed that of Krause (1997), with the following exceptions: i) *C. aculeolata* was differentiated by its longer spines and stouter appearance from *C. intermedia*, because there is an ongoing debate as to whether or not these two taxa should be separated; Krause (1997) recognized *C. aculeolata* as "form" within *C. intermedia*; ii) for the same reason, *C. liljebladii* was differentiated by its larger size from *C. baltica*; Wood & Imahori (1965) recognized this taxon as *C. hispida* var. *baltica* f. *liljebladii*; iii) *C. arcadiensis* is a tentative name for a hitherto undescribed taxon; it morphologically resembles *C. contraria*, but is dioecious; using Krause (1997) and Wood & Imahori's (1965) keys led to *C. contraria*, but then mismatched with the species description as monoecious; iv) *C. calveraensis*, *C. corfuensis*, and *C. longifolia* were determined using Wood & Imahori (1965) because the taxa are not listed in Krause (1997)(*C. calveraensis* and *C. longifolia* are described from outside Europe, and the treatment put forth by Krause (1997) only deals with European taxa; the reason why Krause did not list *C. corfuensis* is unknown); Wood & Imahori (1965) recognized these taxa as *C. vulgaris* var. *vulgaris* f. *calveraensis*, *C. hispida* var. *hispida* f. *corfuensis*, and *C. hornemannii* f. *longifolia*, respectively; however, we gave these taxa species rank in order to be consistent with Krause's (1997) taxonomic concept.

Following these principles, our samples were tentatively identified, using the morphological traits described below, as representing 29 species of the genus *Chara* (Table S1). The number of individuals sampled per species ranged from 1 – 38 (Table S1). The material used in this study contains specimens from Wood & Imahori's (1965) subsections Agardhia, Braunia, Chara, Desvauxia, Grovesia, Hartmania, and Wallmania (Table S1).

Morphological traits of Chara

The plant thallus of *Chara* consists of a stem with elongate single-celled multinucleate internodes separated by multicellular nodes. Branchlets (also called branches), with a similar modular structure to the axis, arise from the nodes (see Fig. 1 for an illustration of typical morphological traits of *Chara*). In most, but not all, *Chara* species the internode and branchlet cells are overlaid by a one cell thick layer of lateral cells termed cortex. The stem cortex can be i) haplostichous (number of cortex cell rows corresponds to the number of branchlets), diplostichous (twice as many cortex cell rows as the number of branchlets), or triplostichous (three times as many cortex cell rows as the number of branchlets), and ii) aulacanthous (secondary cortex cell rows more prominent, spines on thinner cortex cells), tylacanthous (primary cortex cell rows more prominent, spines on thicker cortex cells), or isostichous (primary and secondary cortex cells equally prominent). At the axial nodes, stipulodes form a (often double) ring subtending the branchlets. In most *Chara* species the branchlets have a simplified cortex, and bract cells arise at the branchlet nodes (Fig. 1). Gametangia develop at branchlet nodes. Charophytes can be monoecious (antheridia and oogonia on the same plant) or dioecious (antheridia and oogonia on different plants).

DNA extraction, primers, amplification, and sequencing

The genetic analyses presented in this study were performed by three different working groups: a) Norwegian Institute for Water Research, b) University of Rostock, c) Canadian Centre for DNA Barcoding (CCDB). Accordingly, three different DNA extraction methods, sets of primers, and methods for PCR amplification were used. The primers designed by each working group are summarized in Table 1.

a) *Chara* material was incubated for 5 minutes at 100 °C with 600 µL sodium phosphate buffer (pH 8) in 1.5 ml Eppendorf tubes, and then transferred to a 2 ml cryopreservation tube with 0.5 g zirconium beads and 100 µl 25% sodium dodecyl sulfate added. DNA was then extracted according to the protocol in Hagman *et al.* (2015). PCR amplification was performed on a CFX 96 Realtime System (BIORAD, Oslo, Norway) using iProof™ HF Master Mix (BIORAD). PCR was performed with a denaturation step: 98 ºC (30 s), followed by 35 cycles of 98 ºC (10 s), 62 ºC (20 s), and 72 ºC (20 s) with a final elongation step of 72 ºC for 5 min. For each PCR product, both strands were sequenced on an ABI 3130 XL genetic analyzer using the BigDye terminator V.3.1 cycle sequencing kit (Applied Biosystems,

Applera Deutschland GmbH, Darmstadt, Germany) according to the manufacturer's instructions.

b) Total genomic DNA was extracted from silica-dried material using the standard DNeasy Plant Mini Kit (Quiagen, Hilden, Germany). PCR was performed with an initial five-minute 94 °C denaturation step and one minute each of denaturation (94 °C), annealing (55 °C), and polymerisation (72 °C) for 15 cycles, followed by one minute each of denaturation (94 C), annealing (52 °C), and polymerisation (72 °C) for 20 cycles before the final elongation step (10 min). Sequencing was carried out using an Applied Biosystems 3130xl Genetic Analyzer with sequencing primers identical to primers used for PCR reactions.

c) Total genomic DNA was extracted from *Chara* material as described in Schneider *et al.* (2015*b*). Amplification and sequencing of the *matK* region was conducted following the protocols of the CCDB, as detailed and described in Kuzmina *et al.* (2012). Sequence chromatograms were proofed, edited, and contigs assembled using the program CodonCode Aligner version 2.0.6 (CodonCode Co, USA). Contigs were aligned using the MUSCLE multiple sequence alignment algorithm (Edgar, 2004) as implemented in CodonCode Aligner.

Phylogenetic analyses

Barcode data were quality-controlled iteratively throughout data collection to detect potential contamination, misidentification, and alignment error. Voucher specimens of problematic samples were re-examined resulting in the correction of misidentified taxa. Sequences were aligned using Align (version 03/2007) MS Windows-based manual sequence alignment editor (SequentiX - Digital DNA Processing, Klein Raden Germany) to obtain a DNA sequence alignment, which was then corrected manually. Segments with highly variable and ambiguous regions and gaps making proper alignment impossible were excluded from the analyses. A *matK* set containing 518 positions was used. *Chara longifolia* (AY170444), *Chara connivens* (AY170442), *Chara globularis* (AY170443), *Chara polyacantha* (AY170445), *Chara vulgaris* (DQ229102 and NC00803) and *Lamprothamnium macropogon* (AY170446) were obtained from GenBank and included in the study. *Nitellopsis obtusa* (AY170447), was employed as outgroup taxon. Evolutionary substitution models were evaluated in MEGA version 6 (Tamura *et al*., 2013) and GTR+G was selected as best-fitting evolutionary model. A Bayesian analysis was conducted in BEAST 1.82 (Drummond *et al*., 2012). A relaxed lognormal clock model and a coalescent constant size tree prior (Kingman, 1982) were used.

The Monte Carlo Markov chains (MCMC) were set to run three times for 10 million generations each, logging tree parameters every 1,000 generations. Chain mixing and convergence were checked in Tracer v.1.6 (Rambaut *et al*., 2014) to confirm that the estimated sample size (ESS) values for all parameters were >200. The posterior distribution of trees from the three runs were combined after removal of a proportion of each run as burn-in using logCombiner v1.82, a maximum clade credibility (MCC) tree was calculated in TreeAnnotator v1.82 and visualized in FigTree 1.4.0 (Rambaut, 2012). We also analyzed our data using the maximum likelihood (ML) and neighbor joining algorithms in MEGA version 6 (Tamura *et al.*, 2013), and the results are given in Figs. S1 and S2. In the trees, we defined clusters as "monophyletic morphospecies or group of morphospecies".

Results and discussion

Consistency between barcode haplotypes and morphological species boundaries

BI analysis of the *matK* locus separated the 324 *Chara* individuals into 11 well-defined groups which were supported by posterior probabilities >= 0.9% (Fig. 2). A 12th group was formed by our samples of *C. connivens*, which was, however, not monophyletic to a sample of the same species obtained from GenBank. With the exception of *C. connivens*, gene sequence similarities of the *Chara* individuals within each of the 12 groups generally were above 99% (Table S2). The same 12 groups as in the BI tree were recovered using ML and NJ (Figs. S1 and S2), and only few differences with respect to support of groups occurred. Also, two individuals of the charophyte genus *Lamprothamnium* formed a separate group. For better overview, the results are presented as summarized tree (Fig. 2). Complete trees are given in Figs. S1 and S2. The names we use for labelling the groups refer to the oldest described species in each group.

C. hispida-cluster

The first group (labelled *C. hispida*-cluster; Fig. 2) is a large cluster containing 142 individuals which have traditionally been assigned to 10 different taxa (*C. aculeolata, C. baltica, C. calveraensis, C. corfuensis, C. hispida, C. horrida, C. intermedia, C. liljebladii, C. polyacantha, C. rudis*). They originate from 11 different countries in Europe (from Norway in the North to Greece in the South, and from Ukraine in the East to Spain in the West), in addition to Argentina. There was little genetic variation within the 142 individuals in the *C.*

hispida-cluster (Fig. 2, Table S2). BI divided the *C. hispida*-cluster into two subgroups (Fig. 2). These were, however, not consistent with morphological species boundaries or with geographic origin (Fig. S1, Table S1), and we are not aware of ecological differences (e.g. different habitat types) between the two sub-groups either. Morphological variation among the individuals that formed the *C. hispida*-cluster was considerable, ranging from short to elongated spines, stipulodes and bract cells, from single to fasciculate spines, as well as from aulacanthous to isostichous and tylacanthous cortication. Also, *C. baltica*, *C. liljebladii* and *C. horrida* are brackish water species, while the other taxa in this cluster occur in freshwater (Krause, 1997). However, the individuals that formed the *C. hispida*-cluster all were monoecious and diplostichous with corticated stem and branchlets, spines were present on the stem cortex, they had two well-developed rows of stipulodes, and the stem was moderately stout to stout (internode diameter > ~0.9 mm).

Our results for the *C. hispida*-cluster are consistent with those of Schneider *et al.* (2015*b*), and enhance their reliability by including almost three times as many samples that originated from a wider geographical range. They are also consistent with AFLP studies (Boegle *et al.*, 2010*a*; Urbaniak & Combik, 2013) that indicated a continuum may exist within taxa included in this cluster rather than discreet entities. Our results support Wood & Imahori (1965), who assumed a close phylogenetic relationship among the taxa included in the *C. hispida*-cluster. In contrast to Wood & Imahori (1965), our results indicate that *C. calveraensis* also is part of the *C. hispida*-cluster (Fig. 2).

C. contraria-cluster

A second cluster (labelled *C. contraria*-cluster; Fig. 2) contained 47 individuals which have traditionally been assigned to seven different taxa (*C. arcadiensis*, *C. contraria*, *C. denudata*, *C. filiformis*, *C. gymnophylla*, *C. imperfecta*, *C. ohridana*). They originate from 9 different countries in Europe (from Norway in the North to Greece in the Southeast and Ireland in the West), in addition to Nepal and Canada. Most individuals had identical sequences on the 518 positions of the *matK* gene. However, a sample of *C. gymnophylla* from Nepal, and a group containing three individuals of *C. contraria* from Germany and one from Greece differed by one basepair from the other samples in this group, respectively (Figs. S1 and S2). Although the three individuals from Germany were partly ecorticated, the individual from Greece had normal cortication, such that the subgroup did not reflect consistent morphological differences

to the other *C. contraria* samples. In general, there was considerable morphological variation among the individuals that formed the *C. contraria*-cluster. The samples that were identified as *C. arcadiensis* and *C. imperfecta* were dioecious, while all other individuals were monoecious. Individuals that were identified as *C. filiformis* had extremely short branchlets, while branchlets of the other specimens were of normal length. *C. gymnophylla* had ecorticated branchlets, *C. ohridana* and *C. imperfecta* were entirely ecorticated, while the other specimens had a normally developed cortex. Among the samples that were identified as *C. contraria* were some individuals with a poorly developed cortex, some with elongated spines (determined as *C. contraria* var. *hispidula*), but most resembled the typical *C. contraria* (Krause, 1997). The only morphological traits that were shared by all individuals of the *C. contraria*-cluster were the two well-developed rows of stipulodes, and the slender to moderately stout stem (internode diameter roughly < 0.9 mm). The individuals that had a corticated stem were all diplostichous and tylacanthous (but the spines were of varying length). To our knowledge, no other published information exists with respect to genetic differentiation of taxa within the *C. contraria*-cluster, but Corillion (1957) suggested a close relationship among *C. contraria*, *C. denudata* and *C. filiformis*, based on culturing experiments. Wood & Imahori (1965) grouped all taxa that were included in the *C. contraria*-cluster into *C. vulgaris*. Our data thus only partly support their view, since the taxa that formed the *C. contraria*-cluster indeed were closely related with each other, but not with *C. vulgaris* which formed a separate cluster. Instead, the species that formed the *C. contraria*-cluster were most closely related to the *C. hispida*-cluster (Figs. 2, S1, S2).

C. aspera-cluster

A third cluster (labelled *C. aspera*-cluster) consisted of 39 individuals which have traditionally been assigned to two different species (*C. aspera*, *C. galioides*). They originate from seven countries in Europe (from Norway in the North to Greece in the Southeast and Spain in the Southwest). While all individuals of *C. aspera* and two individuals of *C. galioides* from Greece had identical sequences on the 518 positions of the *matK* gene, the samples of *C. galioides* from France and from Spain each formed their own subgroup (Fig. 2). *C. aspera* and *C. galioides* are known to be morphologically similar to each other. The only consistent difference is the larger diameter of the antheridium of *C. galioides* (Wood & Imahori, 1965; Krause, 1997), although *C. galioides* often also has a wider stem diameter than *C. aspera* (Flor-Arnau *et al.*, 2006). Both taxa are dioecious, slender to moderately stout (axis

diameter < ~0.9 mm), triplostichous, have two well-developed rows of stipulodes, and spines and stipulodes are acute.

Mannschreck (2003) was able to separate *C. aspera* from *C. galioides* by AFLP. However, her samples of *C. galioides* all were from France. In fact, the five individuals of *C. galioides* from France we have in our dataset were taken from the same herbarium sheets which also were used by Mannschreck (2003). Our results agree with Mannschreck (2003) in that the French specimens of *C. galioides* indeed are genetically different from *C. aspera*. However, the samples from Spain and Greece show that polyphyletic clades of *C. galioides* exist, and that *C. galioides* is not consistently separated from *C. aspera* (Fig. 2). Our results partly support Wood and Imahori (1965) who assumed a close phylogenetic relationship between *C. aspera* and *C. galioides*. However, they regarded them as forms of *C. globularis*, which according to our results is not the case.

C. canescens - *C. tenuispina*

A fourth cluster consisted of 14 individuals of *C. canescens* originating from Sweden, Spain, and Greece. Only the sample from Greece differed in one basepair from the other *C. canescens* samples. *C. canescens* is generally differentiated by its haplostichous cortex from all other *Chara* species, and has species rank both in Wood and Imahori (1965) and Krause (1997). Our data support the status of *C. canescens* as a well-defined species, both genetically and morphologically.

Another cluster consisted of two individuals of *C. tenuispina* from Germany. *C. tenuispina* is triplostichous, monoecious, and has long and slender spine cells. Our results support Krause (1997) who gave this taxon species rank, but not Wood & Imahori (1965) who regarded *C. tenuispina* as variety of *C. globularis*.

C. vulgaris-cluster

A sixth cluster (labelled *C. vulgaris*-cluster) contained 23 individuals which have traditionally been assigned to two species (*C. gymnophylla*, *C. vulgaris*). They originate from six countries in Europe (from Sweden in the North to Greece in the Southeast and the UK in the West), in addition to Egypt. There was little genetic variation among the individuals that formed the *C. vulgaris*-cluster, but the two samples of *C. gymnophylla* (both collected in Greece) formed a

subgroup. *C. gymnophylla* morphologically differed from the *C. vulgaris* samples by their entirely ecorticated branchlets (note: upper parts of *C. vulgaris* branchlets may also be ecorticated). Because the differences in *matK* sequence between *C. vulgaris* and *C. gymnophylla* were small (Figs. S1 and S2), and our samples of aulacanthous *C. gymnophylla* originated from one country only, we advocate analyzing more samples to determine if *C. gymnophylla* is generally separated from *C. vulgaris*.

Samples MB8 and MB56 were originally determined as *C. contraria*. Their morphology was intermediate between *C. vulgaris* and *C. contraria*, since the samples were tylacanthous (which is indicative of *C. contraria;* MB8: isostichous to slightly tylacanthous), and had elongated bract cells (which is typical for *C. vulgaris*). However, the same combination of morphological traits was found in some individuals that genetically clustered to *C. contraria*. Problems of differentiation between *C. vulgaris* and *C. contraria* have been reported before (Mannschreck, 2003), but have traditionally been solved by assigning tylacanthous forms to *C. contraria* (Wood & Imahori, 1965; Krause, 1997). Our results carefully indicate that tylacanthous forms that genetically cluster to *C. vulgaris* may exist.

Our results also indicate that *C. gymnophylla* consists of two genetically separate groups (Fig. 2). Neither Wood & Imahori (1965) nor Krause (1997) differentiated between tyla- and aulacanthous forms of *C. gymnophylla*. In our samples, the two aulacanthous individuals of *C. gymnophylla* from Greece clustered to *C. vulgaris*, while the tylacanthous individual from Nepal clustered to *C. contraria* (Fig. 2). Taken together, our understanding of *C. contraria*, *C. vulgaris* and *C. gymnophylla* is that *C. contraria* generally is tylacanthous, while *C. vulgaris* mainly is aulacanthous. In addition, *C. vulgaris* generally has elongated bract cells. A typical *C. vulgaris* is slender to moderately stout (axis diameter < ~0.9 mm), but exceptions occurred among our samples. Tylacanthous individuals with ecorticated branchlets belong to *C. contraria (C. contraria* var. *gymnophylla)*, while aulacanthous individuals with ecorticated branchlets are closely related to *C. vulgaris (C. vulgaris* var. *gymnophylla)*. Regrettably, tylacanthous individuals of *C. vulgaris* may also exist. These individuals can to our knowledge not morphologically be differentiated from *C. contraria* (but we have not analyzed oospore morphology, which may be useful for *Chara* species determination; Urbaniak and Blazencic, 2012).

C. tomentosa – C. globularis – C. connivens

A well-defined cluster consisted of 9 individuals of *C. tomentosa* from five countries in Europe. They shared identical sequences on the *matK* gene. *C. tomentosa* is a large, robust plant with inflated bract cells. It is the type species of the genus *Chara* (Wood and Imahori, 1965) and has species rank both in Wood & Imahori (1965) and Krause (1997). Our data support the status of *C. tomentosa* as one of the few species where little doubt exists with respect to species delineation.

The next cluster was formed by 17 individuals of *C. globularis* collected from six countries in Europe. There was little genetic variation among the *C. globularis* individuals, and no consistent morphological differences were apparent among them. *C. globularis* is often difficult to separate from *C. virgata*, and intermediate forms exist that have morphological traits of *C. globularis* (no spines or papillae on the cortex) and *C. virgata* (elongated upper row of stipulodes). Our results clearly separate *C. globularis* from *C. virgata*, and assign specimens without spines but with (usually only slightly) elongated upper stipulodes to *C. globularis* (samples IW 5a, IW 13, MB 62, MB 69). Our results do not support Wood & Imahori (1965), who assigned strains with elongated upper stipulodes to *C. virgata* but instead included strains with papillar spines in *C. globularis*. We agree with Krause (1997) that *C. globularis* does not have spines, but extend Krause's description of this species to also include specimens with (slightly) elongated upper stipulodes.

Our samples of *C. connivens* consisted of three genetically homogenous individuals collected from Sweden, but their monophyly with sample AY170442 of the same species obtained from GenBank (originating from Northeastern Spain; Sanders *et al.*, 2003) could not be established (Fig. 2). More samples are necessary to determine if this is due to a misidentification, a sequencing error, or if *C. connivens* consists of two closely related taxa. *C. connivens* is morphologically similar to *C. globularis*, and the former differs from the later by its incurved branchlets (*C. globularis*: straight or only slightly incurved), and its dioecious sex (*C. globularis*: monoecious; Krause, 1997). Wood & Imahori (1965) even recognize mon- and dioecious forms of both *C. globularis* and *C. connivens*, and use the incurved branchlets as the only differentiation between these two species. That incurved branchlets generally reflect genetic differences between *Chara* species seems highly unlikely, because they can be induced by high light conditions (Schneider *et al.*, 2006; 2015*c*). Nevertheless, this trait seems useful for differentiating *C. connivens* from *C. globularis*.

C. strigosa-virgata-cluster

Another cluster (labelled *C. strigosa-virgata*-cluster) consisted of 23 specimens belonging to *C. virgata* (17; from five countries in Europe) and *C. strigosa* (5; from three countries in Europe). Sample AY170443 obtained from Genbank (originating from Eastern Germany; Sanders *et al.*, 2003) clearly has been misidentified (registered as *C. globularis*). All samples shared identical sequences on the *matK* gene in spite of conspicuous morphological differences in spine cells and stipulodes (*C. virgata*: only the upper row of stipulodes is well developed, spine cells are rudimentary; *C. strigosa*: two well-developed rows of stipulodes, spine cells are elongate and fasciculate). Our results are consistent with Schneider *et al.* (2015*b*) and Mannschreck (2003), and enhance their reliability by including more samples from a larger geographic area. To our knowledge, our results are at odds with all existing *Chara* determination literature, which either treats them as different species, or, in case of Wood & Imahori (1965), relates *C. strigosa* to *C. aspera*, which is clearly not the case (Fig. 2).

C. longifolia – C. baueri – Lamprothamnium sp.

C. longifolia (both our sample and AY170444 obtained from GenBank (Sanders *et al.*, 2003) originate from Canada) and *C. baueri* (two samples from Germany), each formed their own cluster. Wood and Imahori (1965) list *C. longifolia* as *C. hornemannii* f. *longifolia*. Our sample did not completely match the description of Wood & Imahori (1965), but was intermediate between *C. hornemannii* f. *hornemannii*, f. *nordhoffiae* and f. *longifolia* (axis stout, branchlets ecorticate and shorter than internode length, spines absent, one row of elongated stipulodes). *C. baueri* also had ecorticated branchlets and only one row of stipulodes. This species has long been regarded as extinct, and has only recently been rediscovered in few localities in Germany and Poland (Pukacz *et al.*, 2012). Krause (1997) and Wood & Imahori (1965) agree on the status of *C. baueri* as a separate species, and our data support their assumption. However, we have no data on *C. braunii*, which, according to Krause (1997) may be closely related to *C. baueri*. Our results support the assumption of Wood and Imahori (1965) that *C. longifolia* and *C. baueri* would be phylogenetically quite distinct from other *Chara* species. *C. baueri* may even be closer related to the genus *Lamprothamnium* than to *Chara* (Fig. 2). Earlier studies based on 18S rDNA sequences (Meiers *et al.*, 1999), AFLP (Mannschreck, 2003) and multi-gene sequences (Pérez *et al.*,

2014) placed *Lamprothamnium* within the genus *Chara*. Our results support these findings (Fig. 2).

What may explain morphological variation within homogenous genetic groups?

Many algal species are known to exhibit substantial intraspecific morphological variation, either as a result of genetically controlled polymorphism or environmentally induced plasticity (see Leliaert *et al.*, 2014 for a review). Also in *Chara*, phenotypic plasticity may be environmentally induced, e.g. by light intensity, water temperature, nutrient concentrations, salinity and wave exposure (Blindow and Schuette, 2007; Bociag *et al.*, 2013; Sato *et al.*, 2014; Schneider *et al.* 2015*c*). These environmental factors affect morphological traits like shoot and branchlet length, branchlet curvature, formation of sex organs, and plant branching pattern. Corillion (1957) has shown that culturing conditions may impact shoot and branchlet cortication and the length of stipulodes in *Chara*. However, an impact of environmental factors on the number and length of spines and the number of stipulodes has to our knowledge not yet been demonstrated. Growth and morphology of the marine green alga *Ulva* is influenced by epiphytic bacteria, which may result in anything from "pincushion" morphology via tubes to foliaceous growth (Provasoli & Pintner, 1980; Marshall *et al.*, 2006). Although, to our knowledge, a possible impact of bacteria on *Chara* has not yet been tested, we suspect that the environment may influence *Chara* morphology to a greater extent than hitherto demonstrated.

Heritable phenotypic modifications in the absence of differences in plant barcodes may also be caused by epigenetic variation, such as DNA methylation (Cubas *et al.*, 1999; Zhang *et al.*, 2013), or polyploidy (Schranz & Osborn, 2004). Indeed, variability in chromosome numbers has been reported not only within *Chara* species (Prasad & Verma, 1985), but even in different cells of a single *Chara* individual (Chaudhary & Dash, 1991). Likewise, DNA methylation may differ among individuals of the same *Chara* species (Kunachowicz *et al.*, 2001).

Lastly, in clades where speciation has been very recent, barcode sequences may be shared among related taxa (Hollingsworth *et al.*, 2011). Thus, the *matK* marker we used in our study may have been too conservative, and other loci or genetic fingerprinting techniques may have provided a better resolution. However, *matK* has recently been shown to agree well with results based on *rbc*L and ITS2 in *Chara* (Schneider *et al.*, 2015*b*). Likewise, Schaible *et al.*

(2009) found a good agreement among AFLP, *rbc*L and SNP markers for different populations of *C. canescens*. Also in our study, barcoding results were consistent with previous studies using AFLP. Although additional markers, e.g. the ribosomal marker 18S rRNA, may have improved resolution, it seems unlikely that major differences between *matK* and other commonly used markers would have occurred in our dataset.

Which morphological traits reflect genetic variation?

Generally, there was little genetic but substantial morphological variation within most of the clusters in our study. The morphological variation included traits which are commonly used for *Chara* species determination, like the length and number of spine cells (*C. hispida*-cluster, *C. strigosa-virgata*), the length of stipulodes and bract cells (*C. hispida*-cluster, *C. strigosa-virgata*), cortication (tylacanthous, isostichous, aulacanthous, and even absent cortication; *C. hispida*-cluster, *C. contraria*-cluster), as well as sex differentiation (monoecious – dioecious; *C. contraria*-cluster). In addition, the usefulness of traits to morphologically differentiate among the clusters was not consistent across all clusters. For example, the *C. hispida*-cluster included tyla- and aulacanthous individuals, while *C. contraria* and *C. vulgaris* could, with few exceptions, be differentiated by using this morphological trait. Likewise, the *C. contraria*-cluster contained monoecious and dioecious individuals, while this trait indeed seemed useful for differentiating *C. connivens* from *C. globularis*, and also for differentiating *C. tenuispina* from *C. aspera*. Identical barcoding sequences for monoecious and dioecious individuals have previously been shown for *C. canescens* and *C. altaica* (Kato *et al.*, 2010; *C. altaica* is a taxon described from Japan which Wood & Imahori (1965) would consider to be a monoecious strain of *C. canescens*). Even traits which have been shown to be influenced by the environment, may in some cases be useful for species differentiation (*C. connivens* may be differentiated by its incurved branchlets from *C. globularis*, even though incurved branchlets in *Chara* may be caused by high light conditions; Schneider *et al.*, 2015*c*).

These examples reflect that morphological traits for specific taxa indeed may serve as diagnostic tools for species delimitation, but that they are not generally suitable for inferring genetic differentiation. When two lineages separate, they may eventually become morphologically distinct. These morphological differences may then serve as diagnostic tool for species delimitation. In other instances, however, the same morphological variation may occur as polymorphism or environmentally induced plasticity within one species. For this

reason, we discourage the description of new *Chara* species based exclusively on morphological differences like partial or total loss of cortication, sex differentiation, or the number and length of spine cells, bract cells and stipulodes.

With the obvious exception of ecorticated forms, stem cortication (haplo-diplo-triplostichous) was consistent within each cluster and may therefore be useful for species delineation. Here, the determining factor was the main type of cortication. Individual *Chara* plants may well be irregularly corticated, i.e. some parts may appear diplo-, and others triplostichous. According to our results, however, cortication is not phylogenetically informative, because the triplostichous species within the *C. aspera*-cluster were polyphyletic to the triplostichous *C. globularis*, *C. connivens*, and *C. strigosa*-*virgata*-cluster (Fig. 2).

Consequences for species delineation in Chara

Because speciation is a process and not a single event in time, uncertainty about species boundaries is inevitable in recently diverged lineages (Leliaert *et al.*, 2014). This explains why some species boundaries remain obscure, in spite of the increasing amount of available genetic information. Algal species are generally viewed as separately evolving metapopulation lines (Leliaert *et al.*, 2014). When two lineages separate, they will eventually acquire genetic differences. These differences often can first be detected with high-resolution methods like e.g. AFLP (Roy *et al.*, 2010; Bog *et al*., 2015), while more conservative markers like *rbc*L in the earlier phase of speciation are likely to be similar between the lineages. For the taxa in our dataset, neither AFLP, nor *matK*, *rbc*L or ITS2 sequences were able to discriminate between *C. virgata* and *C. strigosa* (Fig. 2; Mannschreck, 2003; Schneider *et al.*, 2015*b*). This indicates that, if *C. strigosa* and *C. virgata* indeed should "evolve separately", then the separation must have occurred relatively recent. It therefore indicates that *C. virgata* and *C. strigosa* may be regarded as varieties within one species, which may or may not eventually evolve into separate species.

The situation is more complicated for the *C. hispida*-cluster. Here, AFLP studies partly differed in which species they were able to separate from each other (Boegle *et al.*, 2007; 2010*a*, 2010*b*; Urbaniak & Combik, 2013). Data from some of these studies indicated a continuum may exist among the taxa included in the *C. hispida*-cluster (Boegle *et al.*, 2010*a*; Urbaniak & Combik, 2013). Together with almost identical *matK*, *rbc*L and ITS2 sequences (this study; Schneider *et al.*, 2015*b*), this suggests a recent and ongoing speciation among the

taxa included in the *C. hispida*-cluster. Specifically, not even AFLP could differentiate among *C. liljebladii*, *C. horrida* and *C. baltica* (Boegle *et al.*, 2010*b*), and not among *C. hispida*, *C. rudis* and *C. polyacantha* either (Urbaniak & Combik, 2013). In contrast, several studies were indeed able to separate different taxa between these sub-clusters from each other (Mannschreck, 2003; Boegle *et al.*, 2007, 2010*a*, 2010*b*; Urbaniak & Combik, 2013). These same studies disagreed about the differentiation between *C. intermedia* and *C. baltica*. In summary, we conclude that i) all taxa within the *C. hispida*-cluster are closely related with each other, ii) *C. liljebladii*, *C. horrida* and *C. baltica* on the one hand (= *C. baltica* s.l.), as well as *C. hispida*, *C. rudis* and *C. polyacantha* on the other hand (= *C. hispida* s.l.) likely are conspecific, iii) *C. baltica* s.l. and *C. hispida* s.l. likely are products of relatively recent speciation, but iv) for the other taxa in the *C. hispida*-cluster more high resolution genetic analyses are needed before conclusions with respect to species status can be drawn. The same is true for the other unresolved taxa in our study. However, our results indicate that all taxa within a cluster are phylogenetically closely related with each other.

Our data also indicate that *C. gymnophylla* should be divided into tylacanthous forms (which are closely related to *C. contraria*), and aulacanthous forms (which are related to *C. vulgaris*; Fig. 2). We propose to tentatively name them *C. contraria* var. *gymnophylla*, and *C. vulgaris* var. *gymnophylla*, respectively. However, more samples than the three we had in our dataset are necessary before conclusions with respect to species status should be drawn.

Conclusions

Our results show considerable morphological variation within genetically homogeneous groups (e.g. *C. hispida*-cluster, *C. contraria*-cluster, *C. strigosa*-*virgata*). In addition, species within genetically homogeneous groups partly prefer different habitat types (*C. baltica*, *C. horrida* and *C. liljebladii* are brackish water species, while the other species in the *C. hispida*-cluster typically occur in freshwater; *C. virgata* typically occurs in calcium-poor habitats, while *C. strigosa* typically occurs in calcium-rich habitats (Rey-Boissezon & Auderset Joye, 2015); note, however, that Torn *et al.* (2015) also found *C. strigosa* in low-alkalinity habitats). On the other hand, our results also show substantial genetic differences among morphologically similar species (e.g. *C. virgata* - *C. globularis* - *C. connivens*). No morphological trait consistently reflected genetic differences or differences in habitat.

This seems to indicate that different OTUs (operational taxonomic units) may be useful, depending on the aim of a study: a) for conservation of genetic diversity, taxa within genetically homogeneous groups may be combined; b) for bioindication purposes, e.g. ecological status assessment according to the Water Framework Directive, taxa which prefer different habitats should be separated from each other, because they may have bioindicative value irrespective whether or not they are phylogenetically closely related; and c) for protection of habitat types, e.g. according to the Habitats Directive, taxa which have similar ecosystem functions may be lumped. Little information is available with respect to ecosystem function of different *Chara* species, and we encourage studies that aim to quantify e.g. the influence of different charophyte species on ecosystem carbon and phosphorus balances (Kufel *et al.*, 2013), as food or habitat for other organisms (Schmieder *et al.*, 2006), as well as for bioremediation of pollutants (Schneider & Nizzetto, 2013). However, while different OTUs may be useful for different purposes, the decision whether or not a taxon should have species rank should be based on phylogenetic criteria. Our results indicate that all taxa within a cluster are phylogenetically closely related with each other and may be viewed as belonging to a macro-species sensu Wood & Imahori (1965). Regrettably, our results also indicate that morphology of *Chara* species may not be used for inferring phylogenetic distance.

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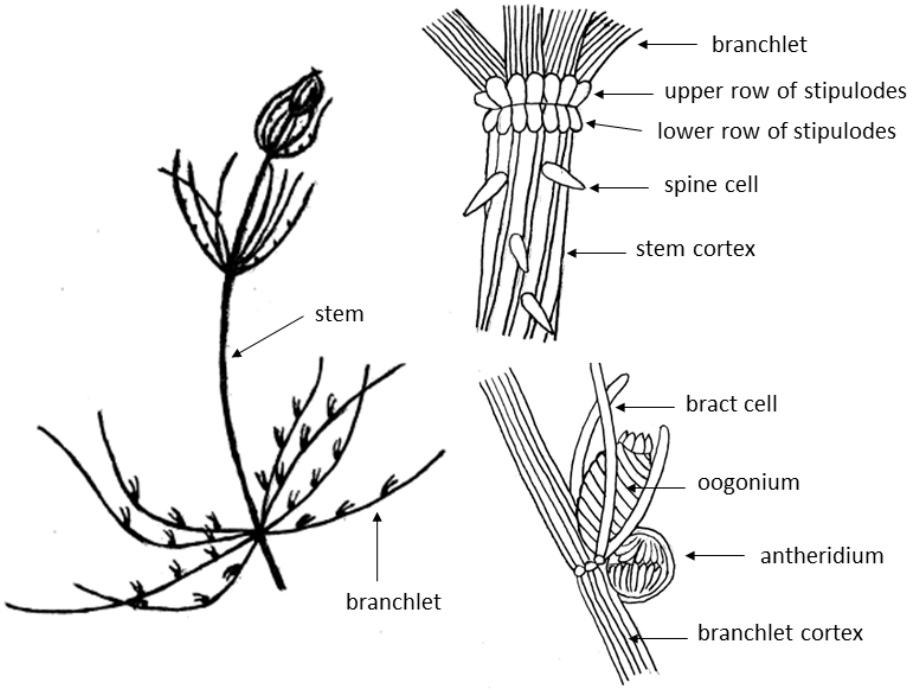
Figure captions



745 **Fig. 1.** Schematic drawing of a monoecious diplostichous tylacanthous *Chara* specimen with single spines (e.g. *C. contraria*). For the sake of clarity, we use "bract cells" as a collective term for "bract cells, bractlets and bracteoles" throughout the manuscript (see e.g. Wood & Imahori (1965) for a detailed description).

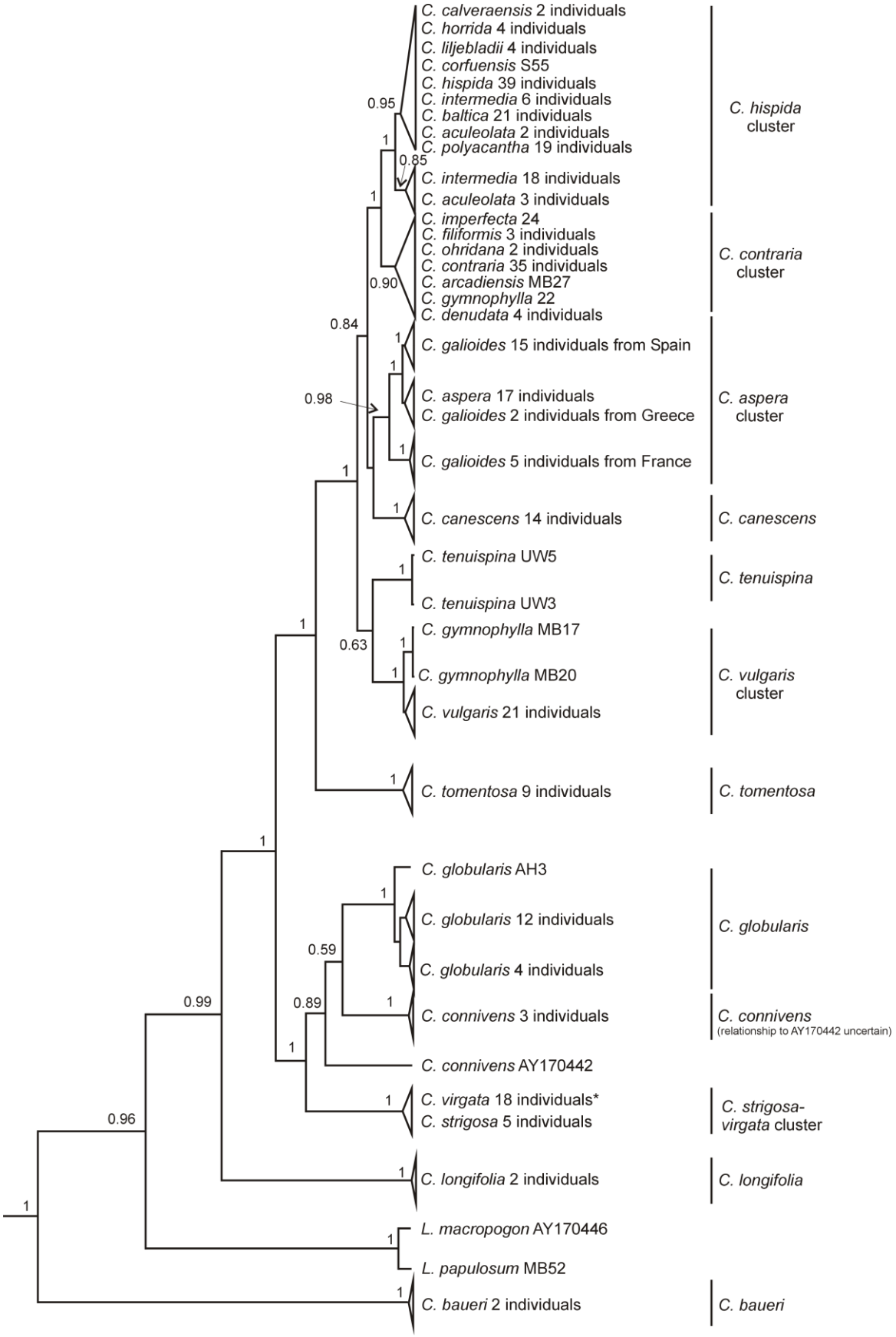

C. calveraensis 2 individuals
C. horrida 4 individuals
C. liljebladii 4 individuals
C. corfuensis S55
C. hispida 39 individuals
C. intermedia 6 individuals
C. baltica 21 individuals
C. aculeolata 2 individuals
C. polyacantha 19 individuals
C. intermedia 18 individuals
C. aculeolata 3 individuals
C. hispida cluster
C. imperfecta 24
C. filiformis 3 individuals
C. ohridana 2 individuals
C. contraria 35 individuals
C. arcadiensis MB27
C. gymnophylla 22
C. denudata 4 individuals
C. contraria cluster
C. galioides 15 individuals from Spain
C. aspera 17 individuals
C. galioides 2 individuals from Greece
C. galioides 5 individuals from France
C. aspera cluster
C. canescens 14 individuals
C. canescens
C. tenuispina UW5
C. tenuispina UW3
C. tenuispina
C. gymnophylla MB17
C. gymnophylla MB20
C. vulgaris 21 individuals
C. vulgaris cluster
C. tomentosa 9 individuals
C. tomentosa
C. globularis AH3
C. globularis 12 individuals
C. globularis 4 individuals
C. globularis
C. connivens 3 individuals
C. connivens
(relationship to AY170442 uncertain)
C. connivens AY170442
C. virgata 18 individuals*
C. strigosa 5 individuals
C. strigosa-virgata cluster
C. longifolia 2 individuals
C. longifolia
L. macropogon AY170446
L. papulosum MB52
C. baueri 2 individuals
C. baueri
0.95
1
0.85
1
0.90
0.84
1
1
0.98
1
1
1
0.63
1
1
1
1
1
0.59
0.89
1
1
0.99
0.96
1
1
1
0.02

Fig. 2. Phylogenetic relationships of 327 charophyte samples inferred through Bayesian analyses of 518 bp of the *matK* gene. Bayesian inference posterior probability values above 0.5 are shown at the nodes in the tree. Outgroup *Nitella obtusa* AY170447 is not shown in the tree. For better overview, sample IDs are indicated only for those samples where a species is represented by only one sample in a branch; in all other cases the number of individuals is given. The bar indicates 2% sequence divergence. * *C. virgata* includes sample AY170443 obtained from GenBank, which clearly was misidentified.

Table 1. Primers used in this study; numbers refer to different working groups: a) Norwegian Institute for Water Research, b) University of Rostock, c) Canadian Centre for DNA Barcoding (CCDB)

a	F-matk-Chara	AGAATGAGCTTAAACAAGGAT
	R-matk-Chara	ACGATTTGAACATCCACTATAATA
	Chara-matK-BT2F	DATATGGCAACAYCAAAAGAC
	Chara-matk-BT2R	ATACAGACCATGCAGCYTT
b	matKF2	AATGAGCTTAAACAAGGATTC
	matKR1a	CGTCCATGTAGATCTAATACTAG
c	Chara_matKF2	GAACGAATCCGTGATAAAAGC
	Chara_matKR2	CTTCGGCCTTTCAAAAAGAA

Table 1. Primers used in this study; numbers refer to different working groups: a) Norwegian Institute for Water Research, b) University of Rostock, c) Canadian Centre for DNA Barcoding (CCDB).

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Supplementary material

Table S1. List of 327 individuals (324 from the genus *Chara*, two *Lamprothamnium* and one *Nitellopsis*) used in the present study. NHM = Natural History Museum, University of Oslo, Norway; NIVA = Norwegian Institute for Water Research; Uni Rostock = University of Rostock, Germany.

Table S2. Gene sequence similarities [%] among and within the 12 *Chara* groups, based on 518 positions of the *matK* gene.

	C. hispida cluster	*C. contraria* cluster	*C. aspera* cluster	*C. canescens*	*C. tenuispina*	*C. vulgaris* cluster	*C. tomentosa*	*C. globularis*	*C. connivens*	*C. strigosa/virgata* cluster	*C. longifolia*	*C. baueri*
C. hispida cluster	99.03-100	98.07-99.23	97.49-98.65	96.72-97.68	97.49-98.26	97.1-97.88	96.14-96.53	94.59-95.56	94.02-94.59	94.4-94.98	94.59-95.37	90.15-90.35
C. contraria cluster		99.61-100	98.65-99.23	97.88-98.26	98.84-99.03	98.26-98.65	96.53-96.72	95.56-96.33	94.98-95.37	95.17-95.37	95.56-95.75	90.54-90.73
C. aspera cluster			99.23-100	97.68-98.26	98.65-99.03	98.07-98.65	96.53-97.3	95.37-96.33	94.79-95.37	94.98-95.37	95.37-95.75	90.73-91.12
C. canescens				99.81-100	97.88-98.07	97.3-97.68	95.75-95.95	95.17-95.95	94.21-94.59	94.59-94.79	94.98-95.17	90.54
C. tenuispina					100	98.65-98.84	96.91	95.95-96.53	95.37-95.56	95.56	95.95	90.93
C. vulgaris cluster						99.61-100	96.53-96.72	95.56-96.53	94.98-95.56	95.37-95.56	95.56-95.75	91.31-91.51
C. tomentosa							100	95.95-96.53	95.37-95.56	95.17	95.37	90.54
C. globularis								99.23-100	96.72-97.49	96.53-97.1	95.56-96.14	90.35-90.93
C. connivens									96.91-100	96.33-96.53	95.37	90.73-90.93
C. strigosa/virgata cluster										100	95.75	90.93
C. longifolia											100	92.47
C. baueri												100

Fig. S1. Bootstrapped condensed maximum likelihood tree of *matK* sequence from 327 charophyte samples. Note that branch length is not related to genetic similarity.

Fig. S2. Neighbor joining tree of *matK* sequence from 327 charophyte samples. The scale bar indicates the estimated number of nucleotide substitutions per site.

Fig. 1

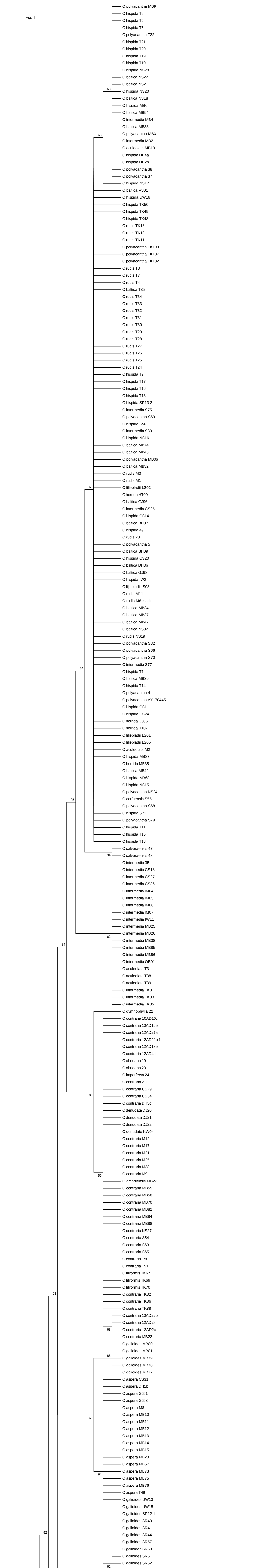

Fig. 2

Table 1

Identification	Identification	Identification	Identification	Identification	Identification	Identification
C. aculeolata	M2	2008	Norway	NIVA	Kütz. in Rchb. 1832	Hartmania
C. aculeolata	MB19	2005	Germany	NIVA		
C. aculeolata	T3	2012	Norway	NHM		
C. aculeolata	T38	2007	Norway	NHM		
C. aculeolata	T39	2010	Norway	NHM		
C. arcadiensis	MB27	2005	Greece	NIVA	tentative name for a hitherto undescribed taxon	
C. aspera	CS31	2012	France	Uni Rostock	Willd. 1809	Grovesia
C. aspera	DH1b	2011	UK	Uni Rostock		
C. aspera	GJ51	2009	Sweden	Uni Rostock		
C. aspera	GJ53	2009	Sweden	Uni Rostock		
C. aspera	M8	2008	Norway	NIVA		
C. aspera	MB10	2000	Germany	NIVA		
C. aspera	MB11	2001	Germany	NIVA		
C. aspera	MB12	2001	Germany	NIVA		
C. aspera	MB13	2001	Germany	NIVA		
C. aspera	MB14	2000	Germany	NIVA		
C. aspera	MB15	2000	Germany	NIVA		
C. aspera	MB23	2005	Sweden	NIVA		
C. aspera	MB67	2005	UK	NIVA		
C. aspera	MB73	2001	France	NIVA		
C. aspera	MB75	2001	France	NIVA		
C. aspera	MB76	2001	France	NIVA		
C. aspera	T49	2011	Norway	NHM		
C. baltica	BH07	2005	Germany	Uni Rostock	Bruzelius 1824	Hartmania
C. baltica	BH09	2005	Germany	Uni Rostock		
C. baltica	DH3b	2011	UK	Uni Rostock		
C. baltica	GJ96	2009	Sweden	Uni Rostock		
C. baltica	GJ98	2009	Sweden	Uni Rostock		
C. baltica	MB32	2004	France	NIVA		
C. baltica	MB33	2004	France	NIVA		
C. baltica	MB34	2004	France	NIVA		
C. baltica	MB37	2004	Germany	NIVA		

C. baltica	MB39	2005	Greece	NIVA		
C. baltica	MB42	2002	Sweden	NIVA		
C. baltica	MB43	2002	Sweden	NIVA		
C. baltica	MB47	2002	Sweden	NIVA		
C. baltica	MB54	2001	France	NIVA		
C. baltica	MB74	2001	France	NIVA		
C. baltica	NS02	2009	UK	Uni Rostock		
C. baltica	NS18	2010	UK	Uni Rostock		
C. baltica	NS21	2010	UK	Uni Rostock		
C. baltica	NS22	2010	UK	Uni Rostock		
C. baltica	T35	2010	Norway	NHM		
C. baltica	VS01	2013	Germany	Uni Rostock		
C. baueri	S29	2011	Germany	NIVA	A. Br. 1847	Braunia
C. baueri	S31	2008	Germany	NIVA		
C. calveraensis	47	2012	Argentinia	NIVA	R.D.W. 1965	Chara
C. calveraensis	48	2012	Argentinia	NIVA		
C. canescens	MB21	2005	Greece	NIVA	Desv. et Loisel. in Loisel. 1810	Desvauxia
C. canescens	SR47	2010	Spain	Uni Rostock		
C. canescens	SR49	2010	Spain	Uni Rostock		
C. canescens	SR51	2010	Spain	Uni Rostock		
C. canescens	SR52	2010	Spain	Uni Rostock		
C. canescens	SR53	2010	Spain	Uni Rostock		
C. canescens	SR54	2010	Spain	Uni Rostock		
C. canescens	SR55	2010	Spain	Uni Rostock		
C. canescens	SR56	2010	Spain	Uni Rostock		
C. canescens	SR70	2010	Spain	Uni Rostock		
C. canescens	SR71	2010	Spain	Uni Rostock		
C. canescens	SR72	2010	Spain	Uni Rostock		
C. canescens	SV20	2003	Sweden	Uni Rostock		
C. canescens	SV22	2003	Sweden	Uni Rostock		
C. connivens	AY170442				Salzm. ex A. Braun 1835	Grovesia
C. connivens	GJ31	2009	Sweden	Uni Rostock		
C. connivens	GJ37	2009	Sweden	Uni Rostock		
C. connivens	GJ38	2009	Sweden	Uni Rostock		

C. contraria	10AD10c	2010	Germany	Uni Rostock	A. Br. ex Kütz. 1845 s. str.	Chara
C. contraria	10AD10e	2010	Germany	Uni Rostock		
C. contraria	10AD22b	2010	Germany	Uni Rostock		
C. contraria	12AD18e	2012	Germany	Uni Rostock		
C. contraria	12AD21a	2012	Germany	Uni Rostock		
C. contraria	12AD21b_f	2012	Germany	Uni Rostock		
C. contraria	12AD2a	2012	Germany	Uni Rostock		
C. contraria	12AD2c	2012	Germany	Uni Rostock		
C. contraria	12AD4d	2012	Germany	Uni Rostock		
C. contraria	AH2	2013	Germany	Uni Rostock		
C. contraria	CS29	2012	France	Uni Rostock		
C. contraria	CS34	2012	France	Uni Rostock		
C. contraria	DH5d	2011	UK	Uni Rostock		
C. contraria	M12	2008	Norway	NIVA		
C. contraria	M17	2008	Norway	NIVA		
C. contraria	M21	2008	Norway	NIVA		
C. contraria	M25	2008	Norway	NIVA		
C. contraria	M38	1997	Norway	NIVA		
C. contraria	M9	2008	Norway	NIVA		
C. contraria	MB22	2005	Greece	NIVA		
C. contraria	MB55	2001	France	NIVA		
C. contraria	MB58	2001	France	NIVA		
C. contraria	MB70	2000	Austria	NIVA		
C. contraria	MB82	2000	Germany	NIVA		
C. contraria	MB84	2000	Germany	NIVA		
C. contraria	MB88	2000	Germany	NIVA		
C. contraria	NS27	2010	UK	Uni Rostock		
C. contraria	S54	2006	Canada	NIVA		
C. contraria	S63	2013	Norway	NIVA		
C. contraria	S65	2013	Norway	NIVA		
C. contraria	T50	2011	Norway	NHM		
C. contraria	T51	2009	Norway	NHM		
C. contraria	TK82	2009	Sweden	Uni Rostock		
C. contraria	TK86	2009	Sweden	Uni Rostock		

C. contraria	TK88	2009	Sweden	Uni Rostock		
C. corfuensis	S55	2006	Greece	NIVA	(J. Gr. Ex Fil.) R.D.W. 1965	Hartmania
C. denudata	DJ20	2012	Germany	Uni Rostock	A. Braun 1847	Chara
C. denudata	DJ21	2012	Germany	Uni Rostock		
C. denudata	DJ22	2012	Germany	Uni Rostock		
C. denudata	KW04	2010	Ireland	Uni Rostock		
C. filiformis	TK67	2009	Sweden	Uni Rostock	Hertzsch 1855	Chara
C. filiformis	TK69	2009	Sweden	Uni Rostock		
C. filiformis	TK70	2009	Sweden	Uni Rostock		
C. galioides	MB77	2001	France	NIVA	De Candolle 1813	Grovesia
C. galioides	MB78	2001	France	NIVA		
C. galioides	MB79	2001	France	NIVA		
C. galioides	MB80	2001	France	NIVA		
C. galioides	MB81	2001	France	NIVA		
C. galioides	SR12_1	2010	Spain	Uni Rostock		
C. galioides	SR40	2010	Spain	Uni Rostock		
C. galioides	SR41	2010	Spain	Uni Rostock		
C. galioides	SR44	2010	Spain	Uni Rostock		
C. galioides	SR57	2010	Spain	Uni Rostock		
C. galioides	SR59	2010	Spain	Uni Rostock		
C. galioides	SR61	2010	Spain	Uni Rostock		
C. galioides	SR62	2010	Spain	Uni Rostock		
C. galioides	SR64	2010	Spain	Uni Rostock		
C. galioides	SR65	2010	Spain	Uni Rostock		
C. galioides	SR67	2010	Spain	Uni Rostock		
C. galioides	SR76	2010	Spain	Uni Rostock		
C. galioides	SR77	2010	Spain	Uni Rostock		
C. galioides	SR78	2010	Spain	Uni Rostock		
C. galioides	SR79	2010	Spain	Uni Rostock		
C. galioides	UW13	2009	Greece	Uni Rostock		
C. galioides	UW15	2009	Greece	Uni Rostock		
C. globularis	16	2009	Macedonia	NIVA	Thuillier 1799	Grovesia
C. globularis	17	2009	Macedonia	NIVA		
C. globularis	AH1	2012	Germany	Uni Rostock		

C. globularis	AH3	2013	Germany	Uni Rostock		
C. globularis	DH6a	2011	UK	Uni Rostock		
C. globularis	DH7c	2011	UK	Uni Rostock		
C. globularis	GJ29	2009	Sweden	Uni Rostock		
C. globularis	GJ30	2009	Sweden	Uni Rostock		
C. globularis	IW13	2012	Germany	Uni Rostock		
C. globularis	IW5a	2012	Germany	Uni Rostock		
C. globularis	IW5b	2012	Germany	Uni Rostock		
C. globularis	MB28	2005	Sweden	NIVA		
C. globularis	MB29	2005	Sweden	NIVA		
C. globularis	MB60	2001	France	NIVA		
C. globularis	MB62	2001	France	NIVA		
C. globularis	MB69	2000	Germany	NIVA		
C. globularis	T83	2011	Norway	NHM		
C. "globularis"	AY170443				*misidentified in Genbank, the sample really is C. virgata*	
C. gymnophylla	22	2009	Nepal	NIVA	A. Braun 1835	Chara
C. gymnophylla	MB17	2005	Greece	NIVA		
C. gymnophylla	MB20	2005	Greece	NIVA		
C. hispida	49	2012	Germany	NIVA	(L.) Hartm. 1820	Hartmania
C. hispida	CS11	2012	Switzerland	Uni Rostock		
C. hispida	CS14	2012	Switzerland	Uni Rostock		
C. hispida	CS20	2012	Switzerland	Uni Rostock		
C. hispida	CS24	2012	Switzerland	Uni Rostock		
C. hispida	DH2b	2011	UK	Uni Rostock		
C. hispida	DH4a	2011	UK	Uni Rostock		
C. hispida	IW2	2012	Germany	Uni Rostock		
C. hispida	MB87	2001	Germany	NIVA		
C. hispida	MB6	2004	Germany	NIVA		
C. hispida	MB68	2004	Germany	NIVA		
C. hispida	NS15	2010	UK	Uni Rostock		
C. hispida	NS16	2010	UK	Uni Rostock		
C. hispida	NS17	2010	UK	Uni Rostock		
C. hispida	NS20	2010	UK	Uni Rostock		
C. hispida	NS28	2010	UK	Uni Rostock		

C. hispida	S56	2001	Germany	NIVA		
C. hispida	S71	2010	Italy	NIVA		
C. hispida	SR13_2	2010	Spain	Uni Rostock		
C. hispida	T1	2005	Norway	NHM		
C. hispida	T10	2012	Norway	NHM		
C. hispida	T11	2002	Norway	NHM		
C. hispida	T13	2011	Norway	NHM		
C. hispida	T14	2011	Norway	NHM		
C. hispida	T15	1995	Norway	NHM		
C. hispida	T16	2003	Norway	NHM		
C. hispida	T17	2010	Norway	NHM		
C. hispida	T18	2009	Norway	NHM		
C. hispida	T19	2010	Norway	NHM		
C. hispida	T2	2003	Norway	NHM		
C. hispida	T20	2010	Norway	NHM		
C. hispida	T21	2002	Norway	NHM		
C. hispida	T5	2012	Norway	NHM		
C. hispida	T6	2012	Norway	NHM		
C. hispida	T9	2012	Norway	NHM		
C. hispida	TK48	2009	Sweden	Uni Rostock		
C. hispida	TK49	2009	Sweden	Uni Rostock		
C. hispida	TK50	2009	Sweden	Uni Rostock		
C. hispida	UW16	2012	Germany	Uni Rostock		
C. horrida	GJ86	2009	Sweden	Uni Rostock	Wahlst. 1862	Hartmania
C. horrida	HT07	2005	Sweden	Uni Rostock		
C. horrida	HT09	2005	Sweden	Uni Rostock		
C. horrida	MB35	2005	Sweden	NIVA		
C. imperfecta	24	2010	Macedonia	NIVA	A. Braun in Durieu de Maisonneuve	Chara
C. intermedia	35	2012	Poland	NIVA	A. Br. in Br., Rab. and Stiz. 1859	Hartmania
C. intermedia	CS18	2012	Switzerland	Uni Rostock		
C. intermedia	CS25	2012	France	Uni Rostock		
C. intermedia	CS27	2012	France	Uni Rostock		
C. intermedia	CS36	2012	Switzerland	Uni Rostock		
C. intermedia	IM04	2005	Sweden	Uni Rostock		

C. intermedia	IM05	2005	Sweden	Uni Rostock		
C. intermedia	IM06	2005	Sweden	Uni Rostock		
C. intermedia	IM07	2005	Sweden	Uni Rostock		
C. intermedia	IW11	2012	Germany	Uni Rostock		
C. intermedia	MB86	2001	Germany	NIVA		
C. intermedia	MB2	2004	Germany	NIVA		
C. intermedia	MB25	2004	Germany	NIVA		
C. intermedia	MB26	2004	Germany	NIVA		
C. intermedia	MB38	2005	Sweden	NIVA		
C. intermedia	MB4	2004	Germany	NIVA		
C. intermedia	MB85	2003	Sweden	NIVA		
C. intermedia	OB01	2011	Ukraine	Uni Rostock		
C. intermedia	S30	2005	Greece	NIVA		
C. intermedia	S75	2013	Italy	NIVA		
C. intermedia	S77	2013	Italy	NIVA		
C. intermedia	TK31	2009	Sweden	Uni Rostock		
C. intermedia	TK33	2009	Sweden	Uni Rostock		
C. intermedia	TK35	2009	Sweden	Uni Rostock		
C. liljebladii	LS01	2013	Germany	Uni Rostock	Wallmann 1853	Hartmania
C. liljebladii	LS02	2013	Germany	Uni Rostock		
C. liljebladii	LS03	2013	Germany	Uni Rostock		
C. liljebladii	LS05	2013	Germany	Uni Rostock		
C. longifolia	AY170444				(Rob.) R.D.W. 1965	Wallmania
C. longifolia	S51	2006	Canada	NIVA		
C. ohridana	19	2009	Macedonia	NIVA	Kostic 1936	*(not listed, but must be Chara)*
C. ohridana	23	2010	Macedonia	NIVA		
C. polyacantha	37	2012	Poland	NIVA	A. Br. in Br., Rab. and Stiz. 1859	Hartmania
C. polyacantha	38	2012	Poland	NIVA		
C. polyacantha	4	2008	Spain	NIVA		
C. polyacantha	5	2008	Spain	NIVA		
C. polyacantha	AY170445					
C. polyacantha	MB3	2004	Germany	NIVA		

C. polyacantha	MB36	2005	Sweden	NIVA		
C. polyacantha	MB9	2006	Germany	NIVA		
C. polyacantha	NS24	2010	UK	Uni Rostock		
C. polyacantha	S32	2010	Germany	NIVA		
C. polyacantha	S66	2009	Italy	NIVA		
C. polyacantha	S68	2010	Italy	NIVA		
C. polyacantha	S69	2009	Italy	NIVA		
C. polyacantha	S70	2009	Italy	NIVA		
C. polyacantha	S79	2013	Italy	NIVA		
C. polyacantha	T22	2008	Norway	NHM		
C. polyacantha	TK102	2009	Sweden	Uni Rostock		
C. polyacantha	TK107	2009	Sweden	Uni Rostock		
C. polyacantha	TK108	2009	Sweden	Uni Rostock		
C. rudis	28	2010	Norway	NIVA	A. Br. in Leonhardi 1882	Hartmania
C. rudis	M1	2008	Norway	NIVA		
C. rudis	M11	2008	Norway	NIVA		
C. rudis	M3	2008	Norway	NIVA		
C. rudis	M6	2008	Norway	NIVA		
C. rudis	NS19	2009	UK	Uni Rostock		
C. rudis	T24	2010	Norway	NHM		
C. rudis	T25	2010	Norway	NHM		
C. rudis	T26	2010	Norway	NHM		
C. rudis	T27	2009	Norway	NHM		
C. rudis	T28	2008	Norway	NHM		
C. rudis	T29	2008	Norway	NHM		
C. rudis	T30	2008	Norway	NHM		
C. rudis	T31	2011	Norway	NHM		
C. rudis	T32	2011	Norway	NHM		
C. rudis	T33	2011	Norway	NHM		
C. rudis	T34	2011	Norway	NHM		
C. rudis	T4	2012	Norway	NHM		
C. rudis	T7	2012	Norway	NHM		
C. rudis	T8	2012	Norway	NHM		
C. rudis	TK11	2009	Sweden	Uni Rostock		

C. rudis	TK13	2009	Sweden	Uni Rostock		
C. rudis	TK18	2009	Sweden	Uni Rostock		
C. strigosa	KR12_11	2011	Germany	Uni Rostock	A. Braun 1847	Grovesia
C. strigosa	M7	2008	Norway	NIVA		
C. strigosa	MB49	1996	Austria	NIVA		
C. strigosa	S59	2013	Norway	NIVA		
C. strigosa	T46	2011	Norway	NHM		
C. tenuispina	UW3	2013	Germany	Uni Rostock	A. Braun 1835	Grovesia
C. tenuispina	UW5	2013	Germany	Uni Rostock		
C. tomentosa	CS21	2012	Switzerland	Uni Rostock	L. 1753	Chara
C. tomentosa	GJ01	2009	Sweden	Uni Rostock		
C. tomentosa	GJ05	2009	Sweden	Uni Rostock		
C. tomentosa	M19	2008	Norway	NIVA		
C. tomentosa	MB18	2005	Sweden	NIVA		
C. tomentosa	MB7	2004	Germany	NIVA		
C. tomentosa	S18	2009	Macedonia	NIVA		
C. tomentosa	T40	2010	Norway	NHM		
C. tomentosa	T41	2011	Norway	NHM		
C. virgata	10	2009	UK	NIVA	Kütz. 1834	Grovesia
C. virgata	39	2012	Finland	NIVA		
C. virgata	50	2012	Germany	NIVA		
C. virgata	GJ41	2009	Sweden	Uni Rostock		
C. virgata	GJ43	2009	Sweden	Uni Rostock		
C. virgata	MB40	2005	Sweden	NIVA		
C. virgata	S12	2009	Norway	NIVA		
C. virgata	S57	2012	Norway	NIVA		
C. virgata	S64	2013	Norway	NIVA		
C. virgata	T43	2011	Norway	NHM		
C. virgata	T44	2010	Norway	NHM		
C. virgata	T45	2008	Norway	NHM		
C. virgata	T78	1936	Norway	NHM		
C. virgata	T79	1992	Norway	NHM		
C. virgata	T84	1996	Norway	NHM		
C. virgata	T86	1929	Norway	NHM		

C. virgata	T88	2006	Norway	NHM		
C. vulgaris	CS28	2012	France	Uni Rostock	L. 1753	Chara
C. vulgaris	DQ229102					
C. vulgaris	GJ75	2009	Sweden	Uni Rostock		
C. vulgaris	GJ76	2009	Sweden	Uni Rostock		
C. vulgaris	GJ77	2009	Sweden	Uni Rostock		
C. vulgaris	MB16	2005	Greece	NIVA		
C. vulgaris	MB30	2006	Greece	NIVA		
C. vulgaris	MB31	2006	Greece	NIVA		
C. vulgaris	MB50	2001	France	NIVA		
C. vulgaris	MB53	2001	France	NIVA		
C. vulgaris	MB56	2001	France	NIVA		
C. vulgaris	MB59	2001	France	NIVA		
C. vulgaris	MB8	2006	Germany	NIVA		
C. vulgaris	MB83	2000	Germany	NIVA		
C. vulgaris	NC00803					
C. vulgaris	NS13	2010	UK	Uni Rostock		
C. vulgaris	NS23	2010	UK	Uni Rostock		
C. vulgaris	NS25	2010	UK	Uni Rostock		
C. vulgaris	NS26	2010	UK	Uni Rostock		
C. vulgaris	S14	2009	Poland	NIVA		
C. vulgaris	S58	2013	Egypt	NIVA		
Lamprothamnium macropogon	AY170446				(A. Br.) R.D.W. 1965	
Lamprothamnium papulosum	MB52	2001	France	NIVA	Wallroth (J. Groves) 1916	
Nitellopsis obtusa	AY170447				(Desvaux in Loisel.) J. Groves 1919	