Taxonomic status of *Chara tenuispina* A. Br. (Streptophyta: Charales) based on LM morphology, *matK*, *atpB* and *rbcL* of cpDNA sequences

Jacek URBANIAK*1 & Michal COMBIK2

1Department of Botany and Plant Ecology, Wrocław University of Environmental and Life Sciences, pl. Grunwaldzki 24a, 50–363 Wrocław, Poland; *Corresponding author e-mail: jacek.urbaniak@up.wroc.pl
2W. Szafer Institute of Botany, Polish Academy of Science, ul. Lubicz 46, 31–512, Kraków, Poland

**Abstract:** *Chara tenuispina* A. Br. is an extremely rare species of the genus *Chara* L., which inhabits freshwater and shaded, shallow water environments on calcareous peat bogs, as compared to *C. globularis* THULL., which is a widespread species. To re-examine the taxonomic system proposed by WOOD & IMAHORI (1965), who treated *C. tenuispina* as a variety of *C. globularis*, we studied the morphology of both species, as well as their phylogenetic relationships, based on three cpDNA gene sequences (*atpB, matK, rbcL*). In general, the species do not differ significantly in their general appearance, but certain details, such as prolonged stipulodes in upper rows below the branchlet, or the extremely long spine cells in *C. tenuispina*, may be noted as distinguishing characteristics. In addition, the results of sequence analysis demonstrated that these species are phylogenetically separated, forming distinct clades. This supports the taxonomic interpretation that *C. tenuispina* is a distinct species rather than variety of *C. globularis*.

**Key words:** *Chara tenuispina, Chara globularis*, charophytes, dimensions, Europe, morphology, molecular, phylogeny, taxonomy

**INTRODUCTION**

Representatives of the genus *Chara* L. belong to a Streptophyta group, and grow as submerged macrophytes, in standing or slow flowing waters, on all continents except of Antarctica (WOOD & IMAHORI 1965). Species level taxonomy in the genera *Chara* L. and *Nitella Agardh* is controversial, mostly due to overlapping morphological variations, apparent intermediate forms between taxa, and the unknown extent to which a phenotypic plasticity or developmental differences contribute to for morphological variation (O’REILLY et al. 2007; SAKAYAMA et al. 2002, 2009; URBANIAK 2009, 2010, 2011a, 2011b; URBANIAK & COMBIK 2013).

Many nineteenth–century researchers attempted to characterize the degree of morphological variation in the genus *Chara or Nitella* and discovered traits that could be used to circumscribe distinct species (MIGULA 1897; GROVES & BULLOCK–WEBSTER 1924; OLSEN 1944; ROMANOV et al. 2015). As a result, a narrow and monomorphic species concept has been used in the genus *Chara*, which has resulted in the description of many species (BRAUN & NORDSTEDT 1882; CORILLION 1957; URBANIAK 2007). However, due to various problems concerning phenotypic plasticity and overlapping morphological variation in many traits, WOOD & IMAHORI (1965) adopted a wider species concept; they subdivided the genus *Chara* into fewer, more polymorphic species. Indeed, they recognized only 18 species worldwide, together with a number of varieties and forms.

The existence of such different interpretations – the monomorphic and polymorphic species concept – as well as the taxonomical difficulties in delimiting various species in the family Characeae, is most likely caused by a lack of methods to determine objectively which characteristics serve to delimit the species, within the genus (MEIERS et al. 1999) such as in the case of *C. tenuispina* A. Br. Such classification problems are typical not only for the genus *Chara* (MANNSCHEICK et al. 2002; O’REILLY et al. 2007; URBANIAK & COMBIK 2013), but also for the genus *Nitella* (SAKAYAMA et al. 2002). As certain intermediate forms exist between *C. tenuispina* A. Br. and *C. globularis* THULL., authors treat *C. tenuispina* in different ways: they either consider it to be a separate species (KRAUSE 1997; URBANIAK & GARIKA 2014) or reject species concepts (WOOD 1962; WOOD & IMAHORI 1965) and consider it as a variety of *C. globularis* (Table 1).

Previous studies of oospore morphology using
scanning electron microscopy (SEM), together with oospore dimensions, demonstrated that certain infra-
specific Chara species identified by Wood (1962) and
Wood & Imaiho (1965) should be recognized as dis-
tinct species (Urbaniaik & Blazencic 2012). A detailed
analysis showed that the oospore dimensions of Chara
tenuisipina (C. globularis var. tenuisipina R. D. Wood)
differed significantly from those of C. globularis (C.
globularis var. globularis Thull. R. D. Wood), C.
connivens Salza. (C. globularis f. connivens R. D.
Wood) and C. virgata Kotz. (C. globularis var. virgata
R. D. Wood), suggesting that C. tenuisipina should be
treated as a distinct species. However, all of the above-
listed species had a similar pustular type of oospore
wall ornamentation, which indicates that they share a
rather close taxonomic relationship (Urbaniaik 2011a).
Therefore, a re-assessment of morphology is neces-
sary, using molecular analyses on mature material to
determine the taxonomic status for both species, with
particular attention to the taxonomic relationship be-
tween C. tenuisipina and C. globularis.

Recently, several specimens of C. tenuisipina
were collected from Poland. Based on their morpho-
logical analyses these could be classified as separate
species, and not as subspecies of C. globularis as sug-
gested by the classification proposed by Krause (1997)
and Urbaniaik & Gabka (2014). Additionally, we col-
lected and studied some other representatives of the
genus Chara. In order to re-examine the taxonomic
status of C. tenuisipina, the present study examined
combined light microscope (LM) morphology in de-
tail and performed molecular phylogenetic analyses
to explain their taxonomic status. These phylogenetic
analyses were based on three plastid gene sequences
(matK, atpB, rbcL) of the cpDNA from field-collec-
ted material. Consequently, we found that both species
(C. globularis and C. tenuisipina) should be treated as
separate species, rather than varieties of C. globularis.

MATERIALS AND METHODS

We collected mature specimens of C. tenuisipina and C.
globularis and re-examined their taxonomic status, using
molecular biology methods and L.M. C. baltica Bruz.,
C. contraria A. Br., C. filiformis Hertsh., C. hispida L., C. in-
termedia A. Br. and C. polyacantha A. Br. were collected
and studied as well; all specimens were collected from natu-
ral localities in Poland (Supplementary table S1). Specimens
of C. tenuisipina were collected from two lakes in the Wiel-
kopska region: Drążyniec and Crzme and on the peat bog
near Wągrowiec, and were examined separately using keys
from Krause (1997) and Urbaniaiik & Gabka (2014). Latin
names of species, as well as author names were presented
according to Algaebase website, http://www.algaebase.org
(Guiry & Guiry 2012). Fresh plant material was collected in
the field, placed in glass jars and it was rapidly transported
to the laboratory. To reduce the influence of contaminating
DNA from epiphytes, large filamentous green algae were re-
moved from young plant shoots by dissection under a ste-
reomicroscope (SMZ 800, Nikon, Tokyo, Japan) and were
cultured in laboratory conditions (at room temperature, with
light from a north-facing window) in jars filled with filtered
lake water (20–30 μm mesh). Only fresh, newly-grown tis-
ues was used for analysis. The morphological characteristics
of C. tenuisipina and C. globularis were described (Figs
1–6, Table 2). Additionally, we collected oospores, mature
oogonia and antherida from the fresh C. tenuisipina and C.
globularis samples. These were measured in a laboratory us-
ing Nikon Eclipse E200 microscope, with length and width
measured and analyzed using ANOVA parametric tests. All
statistical analyses were performed using the Statistica 12
package (StatSoft, 2014, Tulsa, OK United States). All the
investigated oospores as well as specimens were deposited in
herbarium in the Department of Botany and Plant Ecology.
In addition to the morphological observations, a molecular
technique – sequencing of the matrurate chloroplast (matK),
ATP synthase (atpB) and ribulose–1,5–bisphosphate
carboxylase/oxygenase (rbcL) was used. Chloro-
plast–encoded plastid DNA genes, such as atpB, matK, psaB, rbcL, as well as the nuclear internal transcribed se-
quence (ITS) was used previously in establishing the phylo-
geny of certain land plants (Hoot 1995; Hoot et al. 1995) and
algae, especially the Characeae (Sanders et al. 2003; Rindt
et al. 2004; Sakayama et al. 2002) and are used as barcode
markers. Before analysis, DNA variation was tested on a subset
of six samples of the genes listed above, to test genetic varia-
tibility. Three of them, plastid matK, atpB and rbcL, were used
to resolve the phylogenetic relationships of C. tenuisipina.

Total genomic DNA was isolated from fresh tissue
using freeze–dried, powdered material using a DNeasy Plant
Mini Kit (Qiagen, Hilden, Germany) according to the manu-
ufacturer’s protocol. Cells were disrupted using the Mixer Mill
MM400 (Retsch, Haan, Germany). The quality and quantity
of the DNA were determined using a fluorometer (Eppendorf,
Hamburg, Germany), but the integrity of the extracted DNA
was estimated on 1% TBE–agarose gel. The PCR amplifi-
cation and sequencing of the atpB gene were accomplished
using the primers described by Sakayama et al. (2004), while
that of the rbcL and matK made use of primers described by
Sakayama et al. (2009) and Sanders et al. (2003), respec-
tively. Analyses were performed in a Veriti gradient Thermal
Cycler (Applied Carlsbad, CA, USA Each 20 μl reaction con-
tained 10 mM each of dATP, dCTP, dGTP, and dTTP; 0.5 μM
each of primer, 4.0 μl 5’ reaction buffer, 0.2 μl Phusion Hot
Start II HF DNA Polymerase (Thermo Scientific, Waltham,
MA, USA.) and 1 μl of total genomic DNA. The PCR cycle
consisted of an initial denaturation at 98 °C for 20 sec.,
followed by 33 cycles at 98 °C for 5 sec., followed by testing
the adequate annealing temperature by means of the gradient
method for 15 sec., and elongation 72 °C for 30 sec., with a
final extension of 5 min. at 72 °C. The PCR products were
examined for correct length, yield and purity under UV light
on 1% agarose gels, stained with ethidium bromide. PCR
products were purified prior to sequencing reactions, using
the Exo–BAP Mix (Eurx, Gdańsk, Poland), and sequenced
using the amplification primers.

All molecular analyses were performed at the De-
partment of Botany and Plant Ecology, Wrocław University
of Environmental and Life Sciences, apart from the sequenc-
ing, post–reaction cleaning and reading, which were per-
fomed by Genomed sequencing service (Warsaw, Poland)
using an ABI 377XL Automated DNA Sequencer (Applied
Biosystems, Carlsbad, CA, USA).

The atpB, matK and rbcL DNA sequence data were

Urbaniak & Combik: Taxonomy of Chara tenuisipina
analyzed separately.

For the atpB gene phylogeny, additional sequences from the representatives of different genera: (Chara, Laphrothamnium, Lychnothamnus, Nitellopsis, Niella, and Tolypella), which were used to delineate an out-group, were received from GeneBank (Table 1, Figs 8–10). These genera were selected as an out-group.

The atpB DNA sequences analyzed in this study were 1,029 bp and correspond to position 241–1,269 of the Chara vulgaris atpB gene (Turmel et al. 2006). Prior to the phylogenetic analyses, we aligned the atpB DNA sequences using Clustal W (Thompson et al. 1994), and the alignment was subsequently adjusted by eye. A tree was constructed using PhyML 3.0 by the maximum likelihood (ML) method (Guindon & Gascuel 2003). Prior to the analysis, the Kakusan4 (Tanabe 2011) was used to identify the sequence evolution model that fit the dataset using Akaike’s Information Criterion (AIC). The bootstrap proportions (BP) (Felsenstein 1985) used for ML analyses and selected with the GTR+G model selected by Kakusan4 (Tanabe 2011) were calculated based on 100 replicates of heuristic searches. The BI analyses were performed using MrBayes 3.1.2. (Ronquist & Huelsenbeck 2003). The Bayesian inference (BI), maximum parsimony (MP) and neighbor–joining (NJ) trees were also constructed and compared the topologies of the obtained trees to establish and validate the phylogenetic position of the studied species. The substitution models used for each codon position of the atpB gene in the BI analyses were GTR+I (1st codon position), GTR+I+G (2nd codon position), and GTR+G (3rd codon position), which were estimated based on AIC and selected by MrModeltest 2.3 (Nylander et al. 2004) implemented in PAUP* 4.0b10 (Swoford 2002). The parameters of the substitution models for each codon position were unlinked. The Markov Chain Monte Carlo (MCMC) process was stopped at 1,000,000 generations, and first 25% of generations were discarded as burn–in, whereas the remaining trees were used to calculate a 50% majority–rule tree and to determine the posterior probabilities (PP) of individual branches. The average standard deviations of the split frequencies were below 0.01, indicating convergence of the iterations. BPs for MP analyses based on 1,000 replications of full heuristic searches with the TBR branch–swapping algorithm, and those for NJ analyses (Saito & Nei 1987) under the JC model (Jukes & Cantor 1969) based on 1,000 replications, were conducted using PAUP* 4.0b10 (Swofford 2002).

In case of matK gene, additional DNA sequences from the genus Chara, as well as other data from the genus Laphrothamnium were obtained for phylogenetic analysis from GeneBank (Fig. 11) that were selected as an outgroup based on previous study of McCourt et al. 1996; Karol et al. 2001; Sakayama et al. 2002, 2004; Sanders et al. 2003 and Sakayama et al. 2009). The matK DNA sequences analyzed in this study were 1,203 bp (excluding gaps) and correspond to position 79–1,314 of the Chara vulgaris matK gene (Turmel et al. 2006). Prior to the phylogenetic analyses, the matK gene sequences were aligned based on the amino acid translations by MUSCLE (Edgar 2004a, 2004b) using TranslatorX (Abascal et al. 2010). The phylogenetic trees based on the matK gene dataset were constructed in similar way as in the case of atpB gene trees, however, for ML analyses, a GTR model was selected. For the BI analyses, GTR+I, GTR+G and GTR+I+G, and models were selected for 1st, 2nd and 3rd codon positions, respectively.

In the case of rbcL gene, additional DNA sequences of closely related Chara species, as well as N. obtusa, L. barbatus, N. flexilis, N. acuminata and two representatives of genus Tolypella were obtained for phylogenetic analysis from GeneBank (Fig. 12). Representatives of genera Nitellopsis, Niella, Lychnothamnus and Tolypella were selected as an outgroup according to previous study (McCOURT et al. 1996, 1999; Karol et al. 2001; Sakayama et al. 2002, 2004; Sanders et al. 2003 and Sakayama et al. 2009). The rbcL DNA sequences analyzed in this study were 1,244 bp (excluding gaps) and correspond to position 5–1,249 of the Chara vulgaris rbcL gene (Turmel et al. 2006). Before the phylogenetic analyses, rbcL gene sequences were aligned based on the amino acid translations by MUSCLE (Edgar 2004a, 2004b) using TranslatorX (Abascal et al. 2010). The phylogenetic trees based on the rbcL gene dataset were constructed using the same methods as the ones used to construct the atpB and matK gene trees, however, for ML analyses, a GTR model was selected. For the BI analyses, GTR+G, GTR+I+G and GTR+I and models were selected for 1st, 2nd and 3rd codon positions, respectively.

### Results

The specimens C. globularis and C. tenuispina sensu Krause (1997) examined in the present study are described in detail in Table 2. In general, both plants were monoeocious with gametangia at lowest branchlet nodes, being from small to medium size, having a delicate and slender appearance. Specimens of C. teniuspina were up to 25 cm high, with a smaller axis diameter, while C. globularis specimens were larger, with the axis diameter of up to 2.2 mm. Both plants were noted to be from slightly to moderately encrusted, green to light green in color, and had triplostichous thylacanthous cortex, and were partly isostichous on older internodes (Table 2). A number of other morphological differences, allowing both species to be easily distinguished, were also noted, they are as follows: C. teniuspina possessed spine cells, in most cases only on the upper parts of the axis internodes, which were longer than the axis diameter. C. tenuispina also had two rows of stipulodes, which were elongated, acute, and longer than the axis diameter (Figs 7–9), whereas, in C. globularis, spine cells and stipulodes were absent (Figs 2, 4) or rudimentary (Figs 3, 5). The oogonia and antheridia on both species were formed on lower branches. The oogonia were 640–755 µm long and 430–560 µm wide for C. tenuispina, and 710–820 µm and 430–565 µm wide for C. globularis. Oospores from all investigated populations differed in their length (KW = 112.7, P < 0.05) and width (KW = 161.1, P < 0.05) (Table 2). The results also show that C. globularis oospores differed significantly in their length and width from the C. tenuispina.

Furthermore, both species grow in Poland in different ecological conditions. While C. globularis is a cosmopolitan species, found in different aquatic habitats such as lakes, ponds, pools and peatland exploitation pools, with a wide ecological range, growing
in both mesotrophic and eutrophic water, *C. tenuispina* has a narrow ecological amplitude and can be found in fresh water peatlands or in the lake littoral zone, in shallow water, between vascular plants.

Analyzed gene (*atpB, matK* and *rbcL*) trees showed a different resolution. The *rbcL* tree presented the best resolution and the phylogenetic relationships were consistent with the previous *rbcL* phylogeny (Sakayama et al. 2009). Out of the 1,133 characters included in the *atpB* sequence analyses, 162 were informative with respect to parsimony. All types of analyses produced trees with similar topology (Fig. 10). The two isolates of *C. tenuispina* had identical sequences and formed separate branches on the tree, and were
supported by high bootstrap values in the ML, BI, MP and NJ analyses. The C. globularis sequences, collected from NCBI, formed a robust clade with C. virgata and two specimens of C. connivens, which could indicate a close relationship between them. These sequences were located in different places on the tree than C. tenuispina.

In the case of the matK DNA sequences, out of the 1,203 analyzed base pairs, 143 had potentially parsimony–informative characteristics. All types of analyses produced trees with a very similar topology (Fig. 11). The two specimens of C. tenuispina from both localities had almost identical sequences, supported by moderately high bootstrap values in the ML, BI, MP and NJ analyses, and were located on a separate branch to C. globularis. The C. globularis sequence data, collected from the NCBI, were located in two different places on the tree, which could be the result of misidentifcation.

The rbcL analysis appears to have provided the best results: out of the 1,072 analyzed base pairs, 143 were parsimony–informative (Fig. 12). All nine of the analyzed strains of C. tenuispina, from three localities, had identical sequences and formed one robust clade supported by 100% bootstrap values and 1.00 PP in the MP, ML, NJ and BI analyses. Sequences representing C. globularis were divided between two cladises, which were placed close together but intermixed with a C. connivens species with a similar morphology, and were supported by moderately high bootstrap values in the ML, BI, MP and NJ analyses.

**DISCUSSION**

Differences in the morphological characteristics, oospore dimensions and the results of wall analysis using SEM, for various species from the genus Chara, demonstrated that the taxonomy within this genus is problematic (UBRANIAK 2011a, 2011b; UBRANIAK et al. 2012). The analyzed vegetative morphology of C. globularis and C. tenuispina do not significantly differ from each other, whereas oospore dimensions showed significant differences. Both species have a delicate and slender appearance; they are from a small to medium size, with a similar number of occasionally–incurved branches. C. tenuispina (Figs 7–9), exhibited much longer stipulodes below the branches, as well as longer spine cells than C. globularis. These characters may be used for distinguishing C. tenuispina from C. globularis, which has short or almost invisible stipulodes, and rudimentary or absent spine cells (Figs 3–5). C. tenuispina is a rare species in Europe, but the comparison of different specimens collected from herbaria: B, C, GTB, H, KRA, L, LU, POZ, S, W, WA, WRSL, (BRSL) – shortcuts after NYBG Index Herbariorum (Thiers 2013), http://sweetgum.nybg.org/science/ih/, confirm that the morphological characteristics of both the species listed above, as well as those in Table 2, are consistent. This LM observation contradicted the description provided by KRAUSE (1997) and UBRANIAK & GAŁKA (2014).

The results based on the three cpDNA sequences show that C. tenuispina and C. globularis form separate clades on the phylogenetic trees, which is congruent with the LM morphological analyses, as described above. Previously analyzed oospores of C. tenuispina, differed from C. globularis, in that the former displayed pustular ornamentation on the fossa wall, whereas C. globularis had a smooth fossa wall. Additionally, significant differences between the oospore dimensions of both species have been found (UBRANIAK 2011a).

In the case of the phylogenetic trees constructed on atpB and rbcL genes, C. globularis formed cladises with C. connivens, and neither species was separated (Fig. 10, 12). The separation of C. globularis and C. connivens was observed on the matK phylogenetic gene (Fig. 11). According to WOOD & IMAIHORI (1965) and SAKAYAMA et al. (2009), C. connivens (C. globularis f. connivens) can be clearly distinguished from C. globularis by having incurved, whorled branchlets, and in general by having a different habitus. Moreover, SAKAYAMA et al. (2009) indicate that SEM oospore wall ornamentation of C. connivens is different than oospores of C. globularis, this observation that has also been confirmed by UBRANIAK (2011a). The oospores of C. connivens and C. globularis may bear a similar type of ornamentation, but the elongated projections and papillate ornamentation in C. connivens seem to be a stable feature in inter–population variation, and it is therefore highly distinctive for this species. Similarly, UBRANIAK (2011a) found significant differences between the oospore dimensions of both species.

The close taxonomic relationship between charophyte species is well known, possibly reflecting the high degree of morphological similarity between phylogenetically, closely–related species (MEERS et al. 1999; UBRANIAK & COMBIK 2013). The results of the study do not support the taxonomic interpretation proposed by WOOD & IMAIHORI (1965) to combine separate species into a macrospecies. Although the taxonomic criteria for distinguishing separate species are not clear, the combined data, based on LM or SEM analyses and molecular data, are helpful in understanding the various taxonomic ideas of dividing organisms into species and macrospecies and the so–called taxonomic continuum between closely related species in the genus Chara.

The genetic differences between species are mirrored by differences in morphological characteristics and suggesting existence of distinct species.

This is because various species might represent distinct taxa, but are masked by phenotypic or genotypic adaptation to different environmental conditions.

**Science/ih**, confirm that the morphological characteristics of both the species listed above, as well as those in Table 2, are consistent. This LM observation contradicted the description provided by KRAUSE (1997) and UBRANIAK & GAŁKA (2014).

The results based on the three cpDNA sequences show that C. tenuispina and C. globularis form separate clades on the phylogenetic trees, which is congruent with the LM morphological analyses, as described above. Previously analyzed oospores of C. tenuispina, differed from C. globularis, in that the former displayed pustular ornamentation on the fossa wall, whereas C. globularis had a smooth fossa wall. Additionally, significant differences between the oospore dimensions of both species have been found (UBRANIAK 2011a).

In the case of the phylogenetic trees constructed on atpB and rbcL genes, C. globularis formed cladises with C. connivens, and neither species was separated (Fig. 10, 12). The separation of C. globularis and C. connivens was observed on the matK phylogenetic gene (Fig. 11). According to WOOD & IMAIHORI (1965) and SAKAYAMA et al. (2009), C. connivens (C. globularis f. connivens) can be clearly distinguished from C. globularis by having incurved, whorled branchlets, and in general by having a different habitus. Moreover, SAKAYAMA et al. (2009) indicate that SEM oospore wall ornamentation of C. connivens is different than oospores of C. globularis, this observation that has also been confirmed by UBRANIAK (2011a). The oospores of C. connivens and C. globularis may bear a similar type of ornamentation, but the elongated projections and papillate ornamentation in C. connivens seem to be a stable feature in inter–population variation, and it is therefore highly distinctive for this species. Similarly, UBRANIAK (2011a) found significant differences between the oospore dimensions of both species.

The close taxonomic relationship between charophyte species is well known, possibly reflecting the high degree of morphological similarity between phylogenetically, closely–related species (MEERS et al. 1999; UBRANIAK & COMBIK 2013). The results of the study do not support the taxonomic interpretation proposed by WOOD & IMAIHORI (1965) to combine separate species into a macrospecies. Although the taxonomic criteria for distinguishing separate species are not clear, the combined data, based on LM or SEM analyses and molecular data, are helpful in understanding the various taxonomic ideas of dividing organisms into species and macrospecies and the so–called taxonomic continuum between closely related species in the genus Chara.

The genetic differences between species are mirrored by differences in morphological characteristics and suggesting existence of distinct species.

This is because various species might represent distinct taxa, but are masked by phenotypic or genotypic adaptation to different environmental conditions.
Fig. 10. Phylogenetic tree inferred from maximum-likelihood (ML) analysis of atpB gene sequence data for the Charophyceae (Characeae) and outgroup taxa, with bootstrap support (BP) indicated at the nodes. Upper left number from maximum likelihood, right from bayesian interference, bottom left from maximum parsimony and right from neighbour joining. The BP values lower than 60% are not shown. Branch lengths are proportional to the amount of sequence change.
Fig. 11. Phylogenetic tree inferred from maximum-likelihood (ML) analysis of matK gene sequence data for the Charophyceae (Characeae) and outgroup taxa, with bootstrap support (BP) indicated at the nodes. Upper left number from maximum likelihood, right from bayesian interference, bottom left from maximum parsimony and right from neighbour joining. The BP values lower than 60% are not shown. Branch lengths are proportional to the amount of sequence change.
Fig. 12. Phylogenetic tree interfered from maximum-likelihood (ML) analysis of \textit{rbcL} gene sequence data for the Charophyceae (Characeae) and outgroup taxa, with bootstrap support (BP) indicated at the nodes. Upper left number from maximum likelihood, right from bayesian interference, bottom left from maximum parsimony and right from neighbour joining. The BP values lower than 60 % are not shown. Branch lengths are proportional to the amount of sequence change.
| Species               | GenBank number / collection information                                                                 |
|----------------------|----------------------------------------------------------------------------------------------------------|
| **atpB**             |                                                                                                          |
| C. australis Brown   | -                                                                                                        |
|                      | -                                                                                                        |
|                      | HF913625/ Sweden                                                                                         |
|                      | HF913630/ Sweden                                                                                         |
| C. aspera Willd.     | -                                                                                                        |
|                      | -                                                                                                        |
|                      | HF91363/ Sweden                                                                                         |
| C. baltica Bruz.     | **KP791876**/ Chalupy, Poland                                                                        |
|                      | **KP791874**/ Jastarnia, Poland                                                                       |
|                      | HF91363/ Sweden                                                                                         |
|                      | -                                                                                                        |
|                      | -                                                                                                        |
| C. braunii C. Gmell. | -                                                                                                        |
|                      | -                                                                                                        |
|                      | AB60667/ New Zealand                                                                                     |
|                      | AB440258/ Lake Haryunuma, Japan                                                                         |
| C. connivens Salzm. | AF408782                                                                                                 |
|                      | HF913628/ Sweden                                                                                         |
|                      | -                                                                                                        |
| C. contraria A. Br. | **KR349169**/ Lake Wigry, Poland                                                                       |
|                      | HF913639/ Sweden                                                                                         |
|                      | -                                                                                                        |
|                      | -                                                                                                        |
|                      | KP876010/ Lake Biale, Poland                                                                           |
|                      | KP791860/ Lake Brozane, Poland                                                                          |
|                      | KP791864/ Lake Kownackie, Poland                                                                        |
|                      | KP791865/ Lake Budzislawskie, Poland                                                                    |
| C. coralina Klein.   | -                                                                                                        |
|                      | -                                                                                                        |
|                      | AB359167/ Pond Okumaohike, Japan                                                                        |
| C. fibrosa C. Agardh | -                                                                                                        |
|                      | -                                                                                                        |
|                      | AB359168/ Hiroshima, Japan                                                                              |
| C. filiformis Hertsch| -                                                                                                        |
|                      | -                                                                                                        |
|                      | KP791867.1/ Lake Staw Studzieniczcy, Poland                                                            |
|                      | KP791867/ Lake Staw, Poland                                                                            |
| C. globularis Thull. | HF913627/ Sweden                                                                                         |
|                      | -                                                                                                        |
|                      | -                                                                                                        |
|                      | KP876012/ Lake Czarek, Poland                                                                           |
|                      | KP791866/ Pond Gnrownica, Poland                                                                        |
|                      | -                                                                                                        |
| C. hispida L.        | -                                                                                                        |
|                      | -                                                                                                        |
|                      | KP791844/ Lake Studzieniczys, Poland                                                                   |
|                      | KP791846/ Lake Paniewo, Poland                                                                         |
|                      | KP791854/ Lake Wigry, Poland                                                                            |
| Spezies | Reference | Accession Number | Location 1 | Location 2 | Location 3 |
|---------|-----------|------------------|-----------|-----------|-----------|
| C. hydropitys H. Reichl. | - | - | - | HQ380461 | - |
| C. haitensis Turpin | - | - | - | HQ380455 | - |
| C. intermedia A. Br. | HF913636/ Sweden | KP791848/ Lake near Staszów, Poland | KP791849/ Wólka Zabna, Poland | KR349171/ Żabno, Poland | KR349172/ Lake Wielkie, Poland |
| C. longisfolia Robinson | - | - | - | AY170452/ Saskatchewan, Canada | - |
| C. polyantha A. Br. | - | KP791854/ Lake Jasne, Poland | KP791855/ Lake Budzisławskie, Poland | KP791857/ Lake Kaminsko, Poland | - |
| C. rudis Leonhl. | - | KP791858/ Lake Hańcza, Poland | KP791859/ Lake Budzisławskie, Poland | KP791860/ Lake Jegłóweczek, Poland | KP791861/ Lake Staw, Poland |
| C. rusbyana Howe | - | - | - | AF097168 | - |
| C. tomentosa L. | HF913626/ Sweden | KJ395813 | - | - | - |
| C. tenuispira A. Br. | KR349167/ Lake Dążynek, Poland | KP876013/ Lake Dążynek, Poland | (KU128733, KU128734, KU128735)/ Lake Dążynek, Poland | (KU128736, KU128737, KU128738)/ Lake Czarne, Poland | (KU128739, KU128740, KU128741)/ peat bog near Wągrowiec, Poland |
| C. virgata Kütz. | HF913629/ Sweden | - | - | - | - |
| C. vulgaris L. | KR349170/ Lake Wigry, Poland | HF913631/ Sweden | DQ076305/ Pingtung, Taiwan | DQ076306/ Changhua, Taiwan | - |
| C. zeyleniaca Willd. | - | - | - | HQ380468 | - |
| L. heraldii García et Casa-nova | AY823682 | - | - | - | - |
(Urbaniak & Combik 2013). Schneider et al. (2006) showed that changes in branching occur in C. hispida and C. intermedia, in response to different light conditions, thus phenotypic plasticity and genetic adaptation to different environmental conditions underlie the morphological variability observed in many charophyte species. This in turn provides the basis for natural selection to drive macroevolution (Urbaniak & Combik 2013). The presented cpDNA analysis, in addition to the other morphological features of the habitus, showed that Chara tenuispina (C. globularis var. tenuispina R.D. Wood), and C. globularis (Chara globularis var. globularis Thuill. R. D. Wood) should be recognized as distinct species (Urbaniak & Blaženič 2012), rather than being considered as polymorphic variations of C. globularis, as proposed by Wood & Imahori (1965). Previous SEM images and oospore dimensions confirmed this conclusion (Urbaniak 2010 a). The DNA data analyses are powerful tools for taxonomists, allowing small genetic differences to be detected in order to distinguish between populations of various plant species (Groff et al., 2015). However, sometimes it is not possible to distinguish between several previously recognized taxa. This was observed by Urbaniak & Combik (2013) for several species of the genus Chara from the sect. Hartmania: C. hispida, C. intermedia, C. polyacantha and C. rudis, with data based on the AFLP fingerprinting method showing overlapping ranges, and no detection of distinct species groups. This seems to confirm that a wide range of phenotypic variability and developmental variation exists within this section, and possibly also within other charophyte species, where the ranges of particular morphological features are wide and overlapping. Further examination of SEM oospore morphology, combined with molecular phylogenetic analyses using a larger data set (including the other variations and forms of Chara globularis sensu Wood & Imahori 1965) are necessary to provide a detailed understanding, in order to reconstruct their natural taxonomic system.

Acknowledgements
Authors would like to thank to Maciej Gąbka (Adam Mickiewicz University, Poznań, Poland) for help in collecting C. tenuispina specimens. Additionally, we thank to Hidetoshi Sakayama for useful and detailed advices on performing the phylogenetic analyzes and to an anonymous reviewer for useful comments.

References
Abascal, F.; Zardoya, R. & Telford, M.J. (2010): TranslatorX, multiple alignment of nucleotide sequences guided by amino acid translations. – Nucleic Acids Research 38: W7 – 13.
Braun, A. & Nordstedt, C.F.O. (1882): Fragment einer Monographie der Characeen. Nach den hinterlassenden Manuscripten A. Braun’s. – Abhandlungen der Königlichen Akademie der Wissenschaften zu
Table 2. Classification of *Chara globularis* and *C. tenuispina* according to different authors.

| Author/Sources | *Chara globularis* Varieties | *Chara tenuispina* Varieties |
|---------------|-----------------------------|----------------------------|
| **Wood & Imahori (1965)** | *Chara globularis* var. *globularis* f. *globularis* | *C. tenuispina* A. Br. |
| **Krause (1997)** | *Chara fragilis Desv. in Loisel.* |
| **Cirujano *et al.* (2008)** | *Chara globularis* | *Chara tenuispina* (Kütz.) R. D. Wood |

Table 3. Comparisons of morphological features *C. tenuispina* and *C. globularis*.

| Character / Feature | *C. globularis* Thuill. | *C. tenuispina* A. Br. |
|---------------------|------------------------|------------------------|
| **Habit**           | slender                | delicate and slender    |
| **Plant size**      | medium size species (40 – 60 cm high), diameter of the plant axis (0.45 – 2.2 mm) | small to medium size (5 – 25 cm high), diameter of the plant axis (0.2 – 0.9 mm) |
| **Color**           | fresh to dark green    | green to light green    |
| **incrustation**    | slightly to moderately incrusted | slightly incrusted |
| **Internodes**      | as long as branches or much longer | as long as branches or 1 – 5 times longer |
| **Branchlet**       | up to 8 branches in a whorl, long and slender, occasionally incurved, consisted of 5 – 10 segments of which last 2 – 3 were 1 – 2 celled, without cortex | 7 – 10 in a whorl, straight, divided in to 7 – 8 segments of which 5 – 6 corticated; end segment naked, elongate or acute; some plants with only with 1 - 2 corticated segments of branchlets |
| **Cortification**   | triplostichous or partially isostichous in lower parts of the plant and thylacanthous | triplostichous, sometimes irregularly, thylacanthous and isostichous on older parts of plant |
| **Spine cells**     | absent or rarely rudimentary, sometimes visible only on young internodes (Figs 2-5) | mostly only in upper parts of axis internodes; longer than axis diameter (up to 2 – 4 times), solitary and slender (Fig. 8) on lower parts of plants shorter |
| **Stipulodes**      | very small (rudimentary and papiliform) or absent (Figs 2-3) | in two rows, elongate, acute, longer than axis diameter; upper longer than lower (Figs 8-9) |
| **Monoecious/ Dioecious** | monooecious with gametangia at lowest 3 – 5 branchlet nodes | monooecious with gametangia at lowest branchlet nodes |
| **Bract cells**     | up to 5; anteriors longer or shorter than oogonium, acuminate; posteriors small, globular | slender and acute, well developed; anteriors up to 3 – 4 times longer as oogonia and usually longer than posteriors |
| **Bracteoles**      | longer or as long as mature oogonium in similar length as bract cells | |
| **Oogonia**         | 710 – 820 μm long, 430 – 565 μm wide | 640 – 755 μm long and 430 – 560 μm wide, sometimes 2 - 3 oogonia in one whorl of branchlets, richly fertile |
| **Antheridia**      | about 355 – 460 μm in diameter | about 250 – 310 μm in diameter |
| **Oospores**        | dark brown or black 540 – 740 μm long, 255 – 425 μm wide | dark brown or almost black, 440 – 520 μm long, 315 – 350 μm wide |

---

Berlin, Physikalisch–Mathematische Klasse 1882: 1–221.

Cirujano, S.; Cambra, J.; Sánchez–Castillo, P.M.; Meco, A. & Flor–Arnau, N. (2008): Carofitos (Characeae). – In: Cirujano, S. (ed.): Flora ibérica. Algas continentales. – 132 pp., Real Jardín Botánico, Madrid.

Corillion, R. (1957): Les Charophycées de France et d’Europe Occidentale. – 499 pp., Imprimerie Bretonne, Rennes.

Edgar, R.C. (2004a): MUSCLE, a multiple sequence alignment method with reduced time and space complexity. – BMC Bioinformatics 5: 113.

Edgar, R.C. (2004b): MUSCLE, multiple sequence alignment with high accuracy and high throughput. – Nucleic Acids Research 32: 1792 – 1797.

Felsenstein, J. (1985): Confidence limits on phylogenies, an approach using bootstrap. – Evolution 38: 16 – 24.

Gwoff, P.A.; Hale, A.M. & Whitlock, B.A. (2015): Chloroplast Lineages in Disjunct Western North American Populations of *Swaitia perennis* (Gentianaceae). –
Nylander, J.A.A.; Ronquist, F.; Huelsenbeck, J.P. & Nieves-Aldrey, J.L. (2004): Bayesian phylogenetic analysis of combined data. – Systematic biology 53: 47 – 67.

Olsen, S. (1944): Danish Charophyta. Chorological, ecological and biological investigations. Kongelige Danske Videnskabernes Selskabs. – Biologiske Skrifter 3: 1–240.

O’Reilly, C.L.; Cowan, R.S. & Hawkins, A.A. (2007): Amplified fragment length polymorphism genetic finger-printing challenges the taxonomic status of the near- endemic species Chara curta Nolte ex Kütz. (Characeae). – Botanical Journal of the Linnean Society. 155: 467–476.

Rind, F.; McIvor, L. & Guiry, M.D. (2004): The prasiolaes (Chlorophyta) of atlantic Europe: An assessment based on morphological, molecular, and ecological data, including the characterization of Lavesningiella radicans (Kützing) comb. nov. – Journal of Phycology 40: 977–997.

Ronquist, F. & Huelsenbeck, J.P. (2003): MrBayes 3. Bayesian phylogenetic inference under mixed models. – Bioinformatics 19: 1572–1574.

Saito, N. & Nei, M. (1987): The neighbor – joining method, a new method for reconstruction phylogenetic trees. – Molecular Biology and Evolution 4: 406–425.

Sakayama, H.; Hara, Y. & Nozaki, H. (2004): Taxonomic re-examination of six species of Nitella (Charales, Charophyceae) from Asia, and phylogenetical relationships within the genus based on rbcL and atpB gene sequences. – Phycologia 43: 91–104.

Sakayama, H.; Kasai, F.; Nozaki, H.; Watanabe, M.M.; Kawachi, M.; Shiroyo, M.; Nishihira, J.; Washitani, I. & Kreutz, L. (2009): Taxonomic reexamination of Chara globularis Thüll. (Charales, Charophyceae) from Japan based on oospore morphology and rbcL gene sequences, and the description of C. leptospora sp. nov. – Journal of Phycology 45: 917–927.

Sakayama, H.; Nozaki, H.; Kasai, F. & Hara, Y. (2002): Taxonomic re-examination of Nitella (Charales, Charophyceae) from Japan, based on microscopical studies of oospore wall ornamentation and rbcl gene sequences. – Phycologia 41: 397–408.

Sanders, E.R.; Karol, K.G. & McCourt, R.M. (2003): Occurrence of matK in and trnK group II intron in charophyte green algae and phylogeny of the Characeae. – American Journal of Botany 90: 628–633.

Schneider, S.; Ziegler, C. & Melzer, A. (2006): Growth towards light as an adaptation to high light conditions in Chara branches. – New Phytologist 172: 83–91.

StatSoft, Inc. (2014). STATISTICA (data analysis software system), version 12. Tulsa.

Swofford, D.L. (2002): PAUP* – Phylogenetic Analysis Using Parsimony (* and other methods), version 4.0b10. – Sinauer Associates, Sunderland, MA, USA.

Tanabe, A.S. (2011): Kakusan4 and Aminosan, two programs for comparing nonpartitioned, proportional and separate models for combined molecular phylogenetic analyses of multilocus sequence data. – Molecular Ecology Resources 11: 914–921.

Thompson, J.D.; Higgins, D.G. & Gibson, T.J. (1994): CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position–specific gap penalties and weight matrix choice. – Nucleic Acids Research 22:
4673–4680.
Thiers, B. (2013): Index Herbariorum: A global directory of public herbaria and associated staff. – New York Botanical Garden’s Virtual Herbarium. http://sweetgum.nybg.org/science/ih/; searched on 23 November 2015.

Turmel, M.; Otis, C. & Lemieux, C. (2006): The chloroplast genome sequence of Chara vulgaris sheds new light into the closest green algal relatives of land plants. – Molecular Biology and Evolution 23: 1324–1338.

Urbaniak, J. (2007): Distribution of Chara braunii Gmelin 1826 (Charophyta) in Poland. – Acta Societatis Botanicorum Poloniae 76: 313 – 320.

Urbaniak, J. (2009): Oospore variation in Nitella gracilis and Nitella mucronata (Charales, Charophyceae) from Poland. – Biologia 64: 252–260.

Urbaniak, J. (2010): Analysis of morphological characters of Chara baltica Bruz., C. hispida L., C. horrida Wahlst. and C. rudis A.Br. from Europe. – Plant Systematics and Evolution 286: 209–221.

Urbaniak, J. (2011a): An SEM and light microscopy study of the oospore wall ornamentation in Polish charophytes (Charales, Charophyceae) – genus Chara. – Nova Hedvigia 93: 1–28.

Urbaniak, J. (2011b): An SEM study of the oospore wall ornamentation in Polish charophytes (Charales, Charophyceae) – genus Lychnothamnus, Nitella and Nitellopsis. – Nova Hedvigia 93: 537–549.

Urbaniak, J. & Blazencic, J. (2012): SEM study of oospore characteristics in endemic and endangered Balkan Charophytes. – Cryptogamie, Algologie 33: 277–288.

Urbaniak, J.; Langangen, A. & Van Raam, J. (2012): Oospore Wall Ornamentation in the Genus Tolypella (Charales, Charophyceae). – Journal of Phycology 48: 1538–1545.

Urbaniak, J. & Combik, M. (2013): Genetic and morphological data fail to differentiate Chara intermedia A.Br. from C. baltica Bruz., or C. polyacantha A.Br. and C. rudis A.Br. from C. hispida L. – European Journal of Phycology 48: 253–259.

Urbaniak, J. & Gąbka, M. (2014): Polish Charophytes. An illustrated guide to identification. – 120 pp., Wydawnictwo Uniwersytetu Przyrodniczego we Wrocławiu, Wrocław.

Wood, R.D. (1962): New combinations and taxa in the revision of Characeae. – Taxon 11: 7–25.

Wood, R.D. & Imahori, K.A. (1965): Monograph of the Characeae. – In: Wood, R.D & Imahori, K. (eds): A revision of the Characeae . – 904 pp., J. Cramer Verlag, Weinheim.

© Czech Phycological Society (2017)
Received January 4, 2016
Accepted April 6, 2016