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Taxonomic status of *Chara tenuspina* A. Br. (Streptophyta: Charales) based on LM morphology, *mat*K, *atp*B and *rbc*L of cpDNA sequences

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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* Thuill., 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 (*atp*B, *mat*K, *rbc*L). 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* (Mannschreck 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* Thuill., authors treat *C. tenuispina* in different ways: they either consider it to be a separate species (Krause 1997; Urbaniak & Gąbka 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 infraspecific *Chara* species identified by Wood (1962) and Wood & Imahori (1965) should be recognized as distinct species (Urbaniak & Blaženič 2012). A detailed analysis showed that the oospore dimensions of *Chara tenuispina* (*C. globularis* var. *tenuispina* R. D. Wood) differed significantly from those of *C. globularis* (*C. globularis* var. *globularis* Thuill. R. D. Wood), *C. connivens* Salzm. (*C. globularis* f. *connivens* R. D. Wood) and *C. virgata* Kütz. (*C. globularis* var. *virgata* R. D. Wood), suggesting that *C. tenuispina* 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 (Urbaniak 2011a). Therefore, a re–assessment of morphology is necessary, using molecular analyses on mature material to determine the taxonomic status for both species, with particular attention to the taxonomic relationship between *C. tenuispina* and *C. globularis*.

Recently, several specimens of *C. tenuispina* were collected from Poland. Based on their morphological analyses these could be classified as separate species, and not as subspecies of *C. globularis* as suggested by the classification proposed by Krause (1997) and Urbaniak & Gąbka (2014). Additionally, we collected and studied some other representatives of the genus *Chara*. In order to re–examine the taxonomic status of *C. tenuispina*, the present study examined combined light microscope (LM) morphology in detail and performed molecular phylogenetic analyses to explain their taxonomic status. These phylogenetic analyses were based on three plastid gene sequences (*mat*K, *atp*B , *rbc*L) of the cpDNA from field–collected material. Consequently, we found that both species (*C. globularis* and *C. tenuispina)* should be treated as separate species, rather than varieties of *C. globularis*.

Materials and Methods

We collected mature specimens of *C. tenuispina* and *C. globularis* and re–examined their taxonomic status, using molecular biology methods and LM. *C. baltica* Bruz., *C. contraria* A. Br., *C. filiformis* Hertsch, *C. hispida* L., *C. intermedia* A. Br. and C. *polyacantha* A. Br. were collected and studied as well; all specimens were collected from natural localities in Poland (Supplementary table S1). Specimens of *C. tenuispina* were collected from two lakes in the Wielkopolska region: Drążynek and Czarne and on the peat bog near Wągrowiec, and were examined separately using keys from Krause (1997) and Urbaniak & Gąbka (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 removed from young plant shoots by dissection under a stereomicroscope (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 tissue was used for analysis. The morphological characteristics of *C. tenuispina* and *C. globularis* were described (Figs 1–6, Table 2). Additionally, we collected oospores, mature oogonia and antherida from the fresh *C. tenuispina* and *C. globularis* samples. These were measured in a laboratory using 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 maturase chloroplast (*mat*K), ATP synthase (*atp*B) and ribulose–1,5–bisphosphate

carboxylase/oxygenase (*rbc*L) was used. Chloroplast–encoded plastid DNA genes, such as *atp*B, *mat*K, *psa*B, *rbc*L, as well as the nuclear internal transcribed sequence (ITS) was used previously in establishing the phylogeny of certain land plants (Hoot 1995; Hoot et al. 1995) and algae, especially the Characeae (Sanders et al. 2003; Rindi 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 variability. Three of them, plastid *mat*K, *atp*B and *rbc*L, were used to resolve the phylogenetic relationships of *C. tenuispina*.

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 manufacturer'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 amplification and sequencing of the *atp*B gene were accomplished using the primers described by Sakayama et al. (2004), while that of the *rbc*L and *mat*K made use of primers described by Sakayama et al. (2009) and Sanders et al. (2003), respectively. Analyses were performed in a Veriti gradient Thermal Cycler (Applied Carlsbad, CA, USA Each 20 μl reaction contained 10 mM each of dATP, dCTP, dGTP, and dTTP; 0.5 μM of each primer, 4.0 μl 5′ reaction buffer, 0.2 μl Phusion Hot Start II HF DNA Polymerase (Thermo Scientific, Waltham, MA, USA.) and 1.0 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 Department of Botany and Plant Ecology, Wrocław University of Environmental and Life Sciences, apart from the sequencing, post–reaction cleaning and reading, which were performed by Genomed sequencing service (Warsaw, Poland) using an ABI 377XL Automated DNA Sequencer (Applied Biosystems, Carlsbad, CA, USA).

The *atp*B, *mat*K and *rbc*L DNA sequence data were

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analyzed separately.

For the *atp*B gene phylogeny, additional sequences from the representatives of different genera: (*Chara, Lamprothamnium, Lychnothamnus , Nitellopsis, Nitella* 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 *atp*B DNA sequences analysed in this study were 1,029 bp and correspond to position 241–1,269 of the *Chara vulgaris atp*B gene (Turmel et al. 2006). Prior to the phylogenetic analyses, we aligned the *atp*B 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 *atp*B 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 (Swofford 2002). The parameters of the substitution models for each codon position were unlinked. The Markov chain Monte Carlo iteration 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).

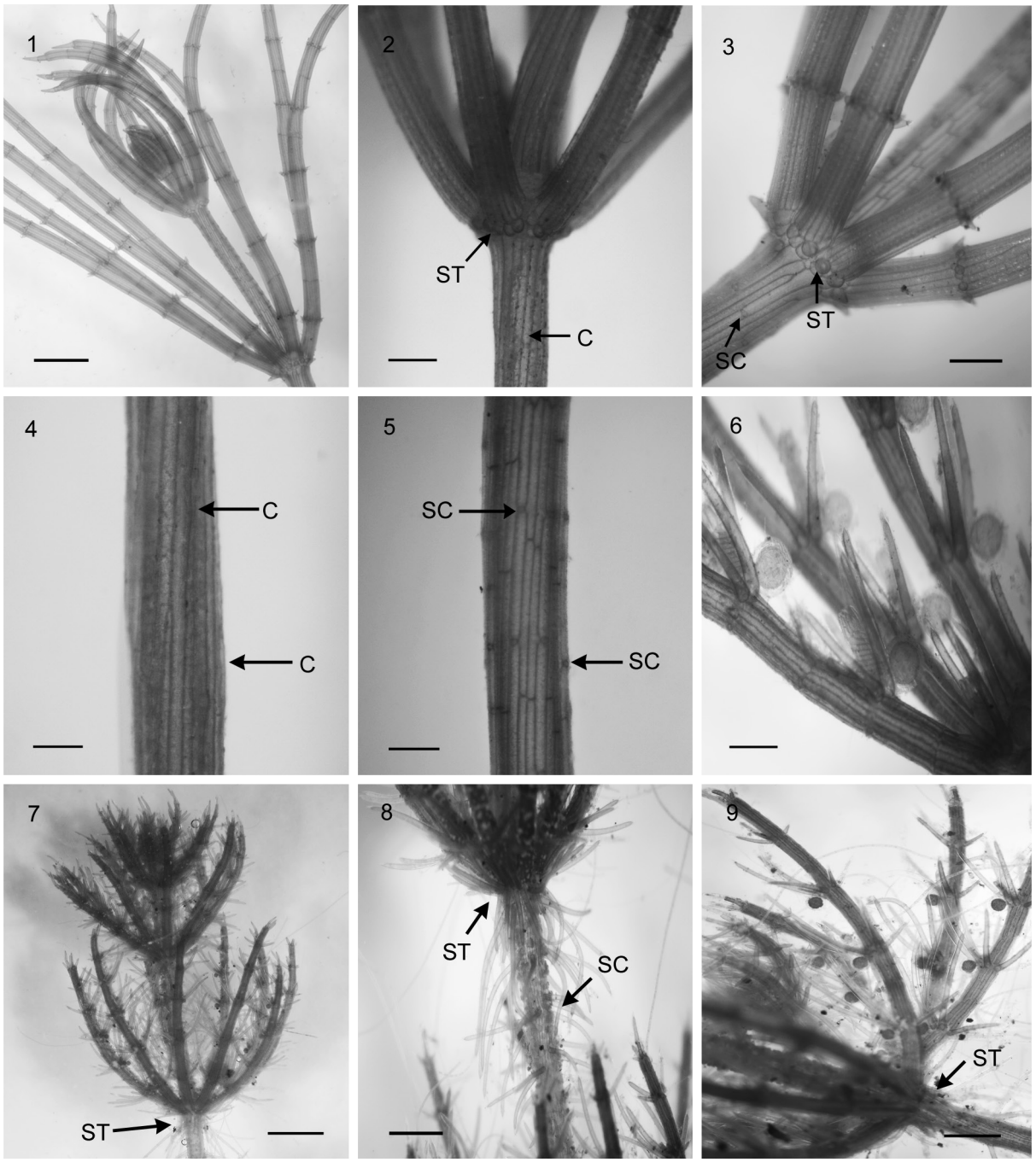
In case of *mat*K gene, additional DNA sequences from the genus *Chara*, as well as other data from the genus *Laprothamnium* were obtained for phylogenetic analysis from GeneBank (Fig. 11) that were selected as an outgroup based on previous study of McCourt et al. 1996; Karol et al. 2001; Sakayama et al. 2002, 2004; Sanders et al. 2003 and Sakayama et al. 2009). The *mat*K DNA sequences analysed in this study were 1,203 bp (excluding gaps) and correspond to position 79–1,314 of the *Chara vulgaris mat*K gene (Turmel et al. 2006). Prior to the phylogenetic analyses, the *mat*K 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 *mat*K gene dataset were constructed in similar way as in the case of *atp*B 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 *rbc*L 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, Nitella, Lychothamnus* 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 analysed in this study were 1,244 bp (excluding gaps) and correspond to position 5 – 1,249 of the *Chara vulgaris rbc*L gene (Turmel et al. 2006). Before the phylogenetic analyses, *rbc*L 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 *rbc*L gene dataset were constructed using the same methods as the ones used to construct the *atp*B and *mat*K 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 monoecious with gametangia at lowest branchlet nodes, being from small to medium size, having a delicate and slender appearance. Specimens of *C. tenuispina* 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. teniuspina* 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



Figs 1–9. LM images of *C. tenuispina* and *C. globularis*: (1) part of main axis of *C. globularis*; upper branchlet with branches; (2) main axis of *C. globularis* with rudimentary stipulodes (ST) below whorl of branches and isostichous cortex (C) without visible spine cells; (3) rudimentary stipulodes (ST) below whorl of branches, and rudimentary, but visible spine cells (S) on *C. globularis* axis; (4) part of main axis of *C. globularis* with isostichous cortex (C) without visible spine cells; (5) rudimentary spine cells (S) on *C. globularis* axis; (6) branches *C. globularis* with oogonia and antheridia; (7) part of main axis of *C. tenuispina* with very long stipulodes (S) and spine cells (SC); (8) part of main axis of *C. tenuispina* with long stipulode (S) below branchlet and spine cells (SC) on the main axis; (9) upper branchlet of *C. tenuispina* with long spine cells (S) below. Scale bar 200 µm (1, 8–9), 150 µm (2), 50 µm (3), 100 µm (4–6), 300 µm (7).

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 (*atp*B, *mat*K and *rbc*L) trees showed a different resolution. The *rbc*L tree presented the best resolution and the phylogenetic relationships were consistent with the previous *rbc*L phylogeny (Sakayama et al. 2009). Out of the 1,133 characters included in the *atp*B 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

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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 *mat*K 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 misidentification.

The *rbc*L 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 clades, 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 (Urbaniak 2011a, 2011b; Urbaniak 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 characteristics may be used for distinguishing *C. tenusipina* 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 Urbaniak & Gąbka (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. teuispina,* 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 (Urbaniak 2011a).

In the case of the phylogenetic trees constructed on *atp*B and *rbc*L genes, *C. globularis* formed clades with *C. connivens,* and neither species was separated (Fig. 10, 12). The separation of *C. globularis* and *C. connivens* was observed on the *mat*K phylogenetic gene (Fig. 11). According to Wood & Imahori (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 Urbaniak (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, Urbaniak (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 (Meiers et al. 1999; Urbaniak & Combik 2013). The results of the study do not support the taxonomic interpretation proposed by Wood & Imahori (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

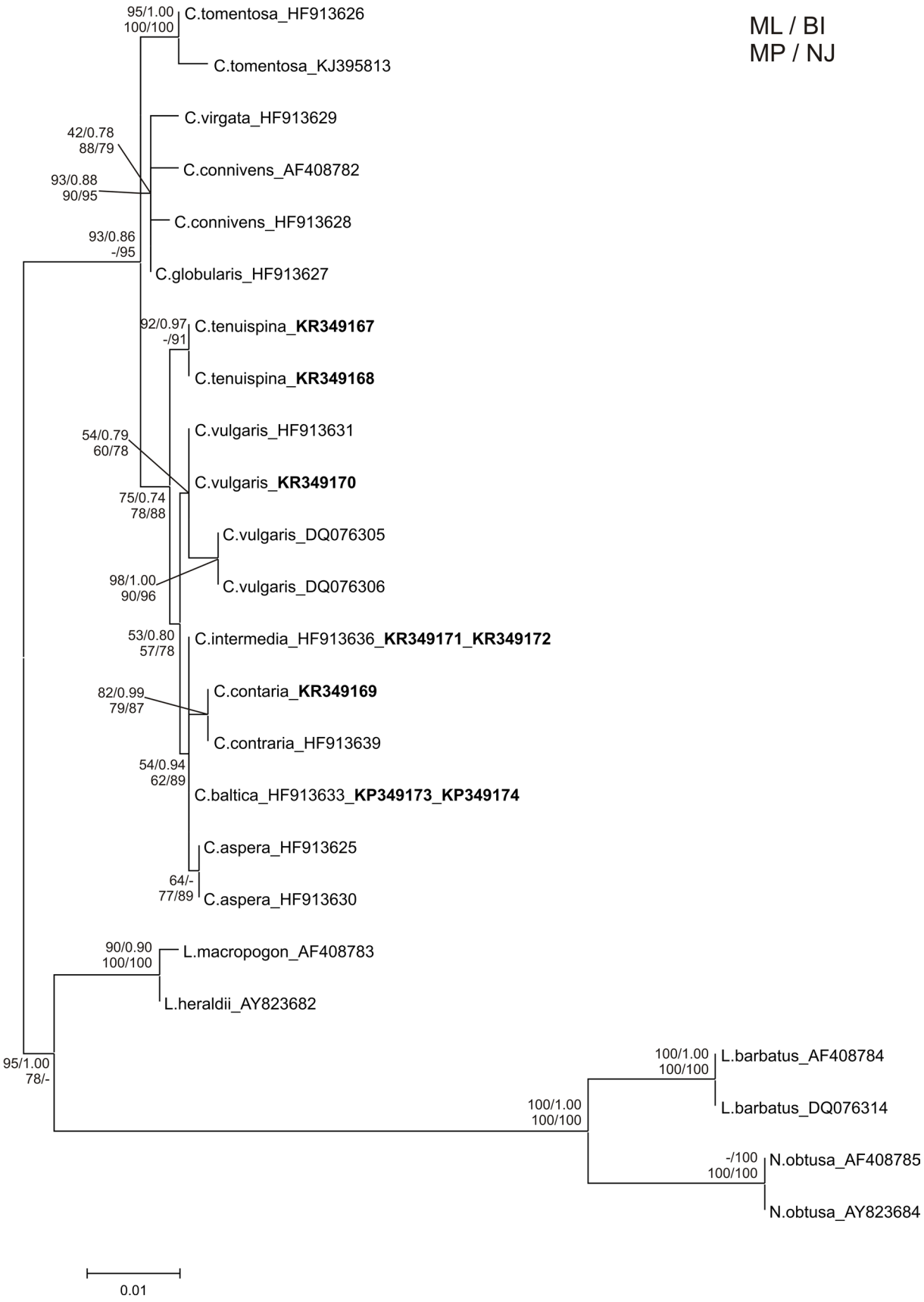

Fig. 10. Phylogenetic tree interfered from maximum–likelihood (ML) analysis of *atp*B gene sequence data for the Charophyceae (Characeae) and outgroup taxa, with bootstrap support (BP) indicated at the nodes. Upper left number from maximum likelihood, right from bayesian interferrence, bottom left from 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.

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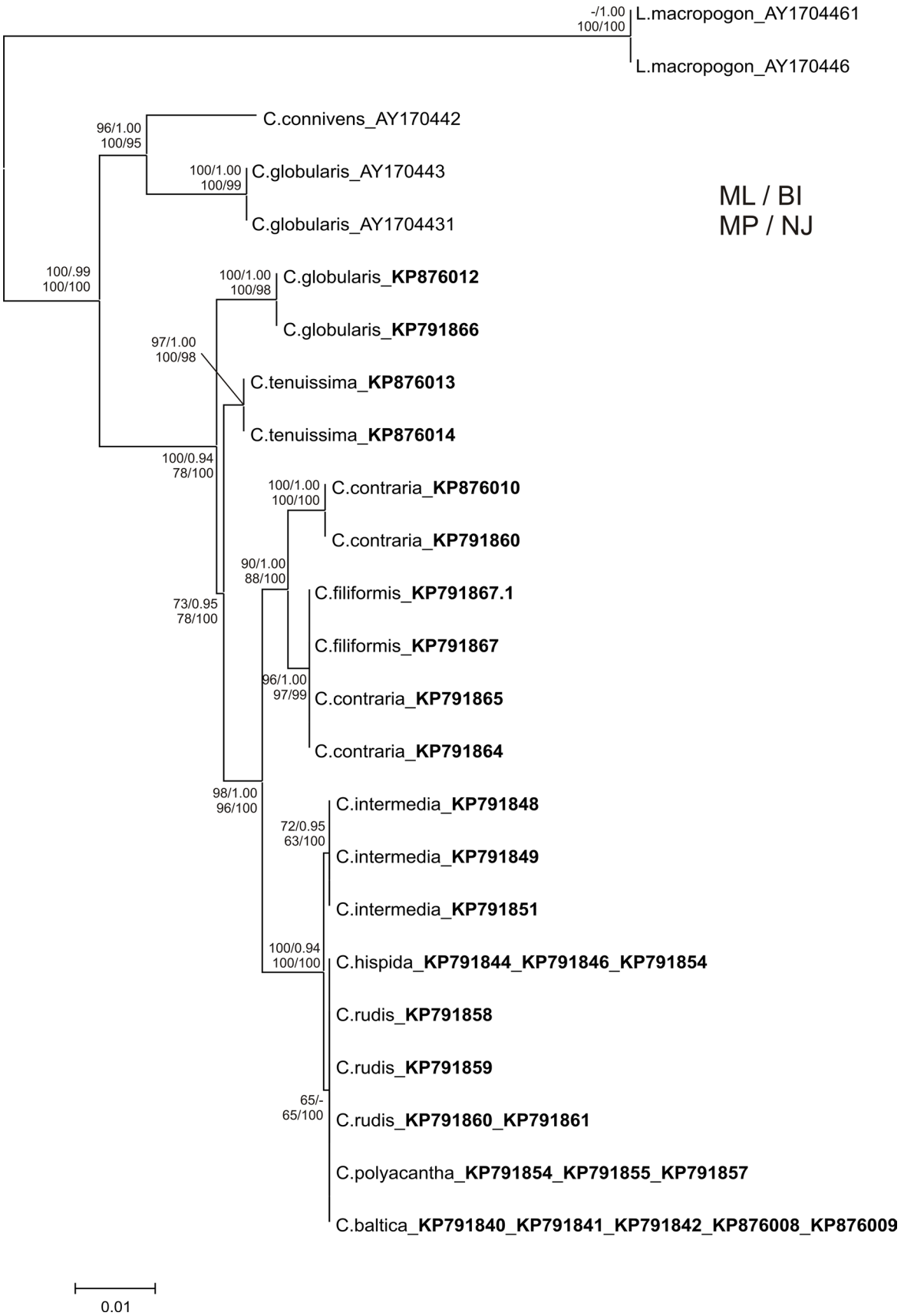

Fig. 11. Phylogenetic tree interfered from maximum–likelihood (ML) analysis of *mat*K gene sequence data for the Charophyceae (Characeae) and outgroup taxa, with bootstrap support (BP) indicated at the nodes. Upper left number from maximum likelihood, right from bayesian interferrence, bottom left from 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.

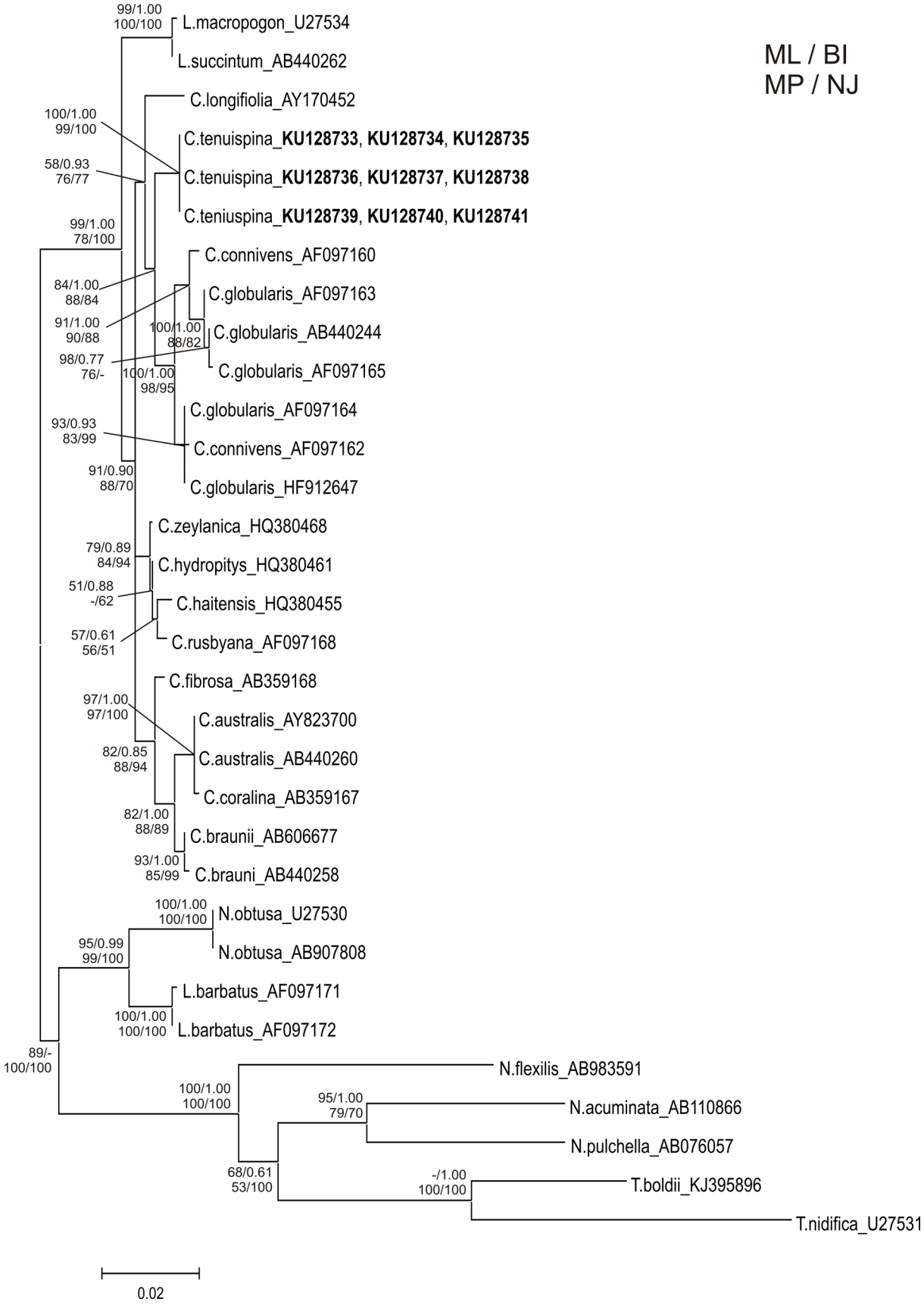

Fig. 12. Phylogenetic tree interfered from maximum–likelihood (ML) analysis of *rbc*L gene sequence data for the Charophyceae (Characeae) and outgroup taxa, with bootstrap support (BP) indicated at the nodes. Upper left number from maximum likelihood, right from bayesian interferrence, bottom left from 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.

Table 1. Species studied including location and GenBank accession numbers of used sequences in phylogenetic study.

Species	GenBank number / collection information		
	***atp*B**	***mat*K**	***rbcL***
C. australis Brown	-	-	AY823700
	-	-	AB440260
C. aspera Willd.	HF913625/ Sweden	-	-
	HF913630/ Sweden	-	-
C. baltica Bruz.	**KP791873**/ Chałupy, Poland	**KP791840**/ Władysławowo, Poland	-
	KP791874 / Jastarnia, Poland	**KP791841**/ Chałupy, Poland	-
	HF913633/ Sweden	**KP791842**/ Jastarnia, Poland	-
	-	**KP876008**/ Baltic Sea, Poland	-
	-	**KP876009**/ Baltic Sea, Poland	-
C. braunii C. Gmell.	-	-	AB606677/ New Zeland
	-	-	AB440258/ Lake Haryunuma, Japan
C. connivens Salzm.	AF408782	AY170442	AF097160
	HF913628/ Sweden	-	-
C. contraria A. Br.	**KR349169**/ Lake Wigry, Poland	**KP876010**/ Lake Białe, Poland	-
	HF913639/ Sweden	**KP791860**/ Lake Brozane, Poland	-
	-	**KP791864**/ Lake Kownackie, Poland	-
	-	**KP791865**/ Lake Budzisławskie, Poland	-
C. coralina Klein.	-	-	AB359167/ Pond Okumaohike, Japan
C. fibrosa C. Agardh	-	-	AB359168/ Hiroshima, Japan
C. filiformis Hertsch	-	**KP791867.1**/ Lake Staw Studzieniczny, Poland	-
		KP791867/ Lake Staw, Poland	
	-		-
C. globularis Thuill.	HF913627/ Sweden	AY170443	AF097163
	-	AY170443.1	AB440244/ Lake Tanne-To, Japan
	-	**KP876012**/ Lake Czarne, Poland	AF097165
	-	**KP791866**/ Pond Grabownica, Poland	AF097164
	-	-	AF097162
	-	-	HF912647/ Sweden
C. hispida L.	-	**KP791844**/ Lake Studzieniczne, Poland	-
	-	**KP791846**/ Lake Paniewo, Poland	-
	-	**KP791854**/ Lake Wigry, Poland	-

Table 1 Cont.

C. hydropitys H. REICH.	-	-	HQ380461
C. haitensis TURPIN	-	-	HQ380455
C. intermedia A. BR.	HF913636/ Sweden **KR349171**/ Żabno, Poland **KR349172**/ Lake Wielkie, Poland	**KP791848**/ Lake near Staszów, Poland **KP791849**/ Wólka Żabna, Poland **KP791851**/ Okopy, Poland	- - -
C. longifolia ROBINSON	-	-	AY170452/ Saskatchewan, Canada
C. polyacantha A. BR.	- - -	**KP791854**/ Lake Jasne, Poland **KP791855**/ Lake Budzisławskie, Poland **KP791857**/ Lake Kaminsko, Poland	- - -
C. rudis LEONH.	- - - -	**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. tenuispina A. BR.	**KR349167**/ Lake Drążynek, Poland **KR349168**/ Lake Czarne, Poland -	**KP876013**/ Lake Drążynek, Poland **KP876014**/ Lake Czarne, Poland -	(**KU128733, KU128734, KU128735**)/ Lake Drążynek, 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 CASANOVA	AY823682	-	-

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Table 1 Cont.

L. macropogon I. L. Ophel	AF408783 -	AY170446 AY170446	U27534 -
L. succintum R. D. Wood	-	-	AB440262/ Oike, Tebajima, Japan
L. barbatus Leonh.	DQ076314/ Elizabeth Creek, Australia AF408784	-	AF097171/ Queensland, Australia
		-	AF097172/ Poland
N. obtusa J. Groves	AF408785 AY823684	- -	U27530/ Stadtwald, Germany AB907808/ Lake Biwa, Japan
N. flexilis C. Agardh	-	-	AB983591/ Germany
N. acuminata A. Br.	-	-	AB110866/ Malaysia
N. pulchella Allen	-	-	AB076057
T. boldii Sawa	-	-	KJ395896
T. nidifica Leonh.	-	-	U27531/ Ombre Pond, France

(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.

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Table 2. Classification of *Chara globularis* and *C. tenuispina* according to different authors.

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

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 green 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	monoecious with gametangia at lowest 3 – 5 branchlet nodes	monoecious 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

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