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Amplified fragment length polymorphism (AFLP)-based differentiation of selected *Chara* species

by

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Abstract

Charophytes are a group of green algae that grow in various types of water ecosystems and are characterized by a high degree of plasticity and morphological variation. To analyze the genetic diversity and taxonomic rank of several species from the genus *Chara*, the fingerprinting technique of Amplified Fragment Length Polymorphism (AFLP) was applied. We studied species that belong to sect. Grovesia (*C. tenuispina*, *C. globularis*, *C. virgata*, *C. aspera* and *C. strigosa*) and two species from the sect. Hartmania (*C. intermedia* and *C. hispida*). The individuals were collected in the field in north-eastern, central and eastern Poland. The species were identified based on morphological features and then analyzed using the AFLP fingerprinting method. UPGMA clustering and PCA analysis as well as morphological analysis revealed a clear separation of *C. tenuispina* and *C. globularis*, which formed separate clusters supported by high bootstrap values. Therefore, these species were distinguish as separate taxa, rather than varieties of *C. globularis*. Similarly, *C. virgata* also formed a separate cluster, thereby confirming that this taxon is a separate species, rather than a variety of *C. globularis*. The AFLP analysis did not show any differentiation between *C. aspera* and *C. strigosa*. The presented results do not fully support the taxonomic interpretation for the existence of several polymorphic species with numerous variations and forms, however, in some examples, the distinctive nature of the reproduction system may be used as a distinguishing feature of the taxa.

Key words: *Chara*, AFLP, Poland, morphology, molecular taxonomy

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Introduction

Charophytes (order Charales, family Characeae) are a group of highly developed green algae that grow as submerged macrophytes in standing, slow-flowing freshwater and brackish waters, on all continents except Antarctica (Wood & Imahori 1965). These green algae (known as stoneworts) have been suggested to be a sister group of embryophytes (McCourt et al. 2004). Specimens from the genus *Chara* L. are sometimes not easy to identify, mostly due to overlapping morphological variations, apparent intermediate forms between taxa, and the unknown extent to which phenotypic plasticity or developmental differences account for morphological variation. Identification problems such as these were addressed during the initial phase of taxonomic studies of charophyte morphology. Several researchers have attempted to characterize the degree of morphological variation in charophytes and have discovered certain traits that may be used to distinguish distinct species (Migula 1897; Groves, Bullock-Webster 1924; Olsen 1944). As a result, a narrow and monomorphic species concept has been developed for the genus *Chara*, which has resulted in the identification of many species (Braun, Nordstedt 1882; Corillion 1957; Urbaniak 2007; Urbaniak et al. 2008; Urbaniak 2010; Urbaniak et al. 2012). However, due to many problems concerning the phenotypic plasticity and overlapping morphological variation in many traits, Wood (1962) and Wood & Imahori (1965) adopted a wider species concept, dividing the family Characeae into two tribes: Charae (including the genera *Chara*, *Nitellopsis*, *Lamprothamium*, *Lychnothamnus*) and Nitellae (including the genera *Nitella* and *Tolypella*). This reduced the number of species and only 19 *Chara* species were recognized worldwide, instead of many microspecies described at that time. The existence of such different interpretations as well as taxonomic difficulties in identifying various charophytes most likely result from the lack of suitable methods to objectively determine which characteristics can be used to distinguish a species within the genus (Meiers et al. 1999).

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; Sakayama et al. 2006; Urbaniak 2011b). Recent studies of oospore dimensions and wall ornamentation morphology of the genus *Chara,* with the use of scanning electron microscopy (SEM), did not give clear and satisfactory results. Although oospore dimension analysis could distinguish some of the taxa classified by Wood (1962) and Wood & Imahori (1965) as varieties or forms (which should in fact be recognized as distinct species), it did not work for all the taxa. Such analysis was performed for *C. globularis*, *C. tenuispina* and *C. virgata* (Urbaniak 2011a; Urbaniak & Blazencic 2012). On the other hand, oospore analysis performed on sect. Hartmania (*C. hispida*, *C. intermedia*, *C. polyacantha*, *C. rudis*) indicated a very close relationship between the taxa due to a similarity in oospore wall ornamentation. Since the results based on oospore morphology do not provide a definitive answer, the presented study examines Wood & Imahori's (1965) hypothesis concerning the taxonomic relationships within the genus *Chara,* using a molecular fingerprinting technique (AFLP) and morphological analysis. In particular, we investigate whether the taxa belonging to the genus sect. Grovesia (*C. aspera* Willd., *C. globularis* Thuill., *C. strigosa* A.Br., *C. virgata* Kütz. and *C. tenuispina* A.Br.) should be regarded as conspecific, according to Krause (1997), or they should be reduced to forms and varieties of *C. globularis*.

Materials and methods

Plant sampling

The studied specimens of *C. aspera, C. globularis, C. hispida, C. intermedia*, *C. tenuispina* and *C. virgata* were collected from natural localities in north-eastern, central and eastern Poland. Each species was collected from several localities, except for *C. strigosa*, which was found only in Lake Hańcza in northern Poland. Details on the collection sites can be found in Table 1.

Plants were collected by hand in shallow places, up to 0.5 m deep, or using a hook at deeper localities. The collected plants (Table 2) were examined using Krause (1997) and Urbaniak & Gąbka (2014) identification keys.

Fresh plant material (5-10 individuals per species) was collected in the field, placed in glass jars (each species separately, in 2-3 jars) and quickly transported to the laboratory. To reduce the influence of alien DNA from epiphytes, large filamentous algae were removed from a cultured plant by dissection with needles under a stereomicroscope (SMZ 800, Nikon, Tokyo, Japan). Charophytes were cultured in laboratory conditions (at room temperature with light from a north-facing window) for one month. After that, all plants were placed in new jars filled with tap water. Only fresh, newly grown tissues (young shoots) were used for analysis.

Morphological characteristics of the investigated species were described in detail and the descriptions were completed with photographs of the

Table 1

Classification of the analyzed *Chara* species according to different authors

Krause (1997)	Wood & Imahori (1965)	Cirujano et al. (2008)
C. aspera Deth. ex Willd. 1809	*Chara globularis* var. *aspera* (Deth. ex Willd. 1809) R.D. Wood 1962	*Chara aspera* var. *aspera* Deth. ex Willd. 1809
C. globularis Thuill. 1799	*Chara globularis* var. *globularis* f. *globularis* R.D. Wood 1962	*Chara fragilis* Desv. in Loisel. 1810
C. hispida L.	*Chara hispida* var. *major* f. *major* (Hartm.) R.D.Wood 1962	*Chara hispida* var. *major* (Hartm.) R.D. Wood 1962
C. intermedia A.Br. 1859	*Chara hispida* var. *major* f. *intermedia* (A.Br.) R.D.Wood 1962	*Chara hispida* var. *hispida* (Hartm.) R.D. Wood 1962
C. strigosa A.Br 1847	*Chara globularis* f. *strigosa* (A. Br.) R.D. Wood 1962	-
C. tenuispina A.Br. 1835	*Chara globularis* var. *tenuispina* (Kütz.) R.D. Wood 1962	-
C. virgata (C. delicatula) Kütz. 1834	*Chara globularis* var. *virgata* (A. Br.) R.D. Wood 1962	-

Table 2

The studied species and populations

Species	Locality with the number of individuals	Date of collection
C. aspera	Lake Mauda, Suwałki Lakeland, (31-34) Lake Pobłędzie, Suwałki Lakeland (35-38) Lake Wigry, Augustów Lakeland, (51-55) Lake Hańcza, Suwałki Lakeland (81-85)	Jul. 2014 Aug. 2013 Aug. 2013 Jul. 2014
C. globularis	fish pond near Szczodre, Silesian Lowland, (201-205) Lake Czarne, Łęczna-Włodawa Lakeland, (206-210) pond near Niemodlin, Opolska Upland, (211-216)	Jul. 2014 Jul. 2014 Jul. 2014
C. hispida	Lake Okmin, Suwałki Lakeland, (21-24) Lake Szelment, Suwałki Lakeland, (25-28)	Jul. 2014 Jul. 2014
C. intermedia	Lake Czarne, Łęczna-Włodawa Lakeland, (91-94) pond near Staszów, Staszów Upland, (95-97)	Jul. 2014 Aug. 2013
C. strigosa	S part of Lake Hańcza, Suwałki Lakeland, (71-76) SE part of Lake Hańcza, Suwałki Lakeland, (131-134)	Jul. 2014 Jul. 2014
C. tenuispina	Lake Drążynek, Wielkopolskie Lakeland, (101-104) Lake near Wągrowiec, Wielkopolskie Lakeland, (105-108)	Sept. 2013 Sept. 2013
C. virgata	Lake Wigry, Augustów Lakeland, (1a-4a) Lake Serwy, Augustów Lakeland, (301-305)	Aug. 2013 Aug. 2013

discriminatory characteristics (Fig. 1a-l). We have paid special attention to the following morphological characters that allow differentiation between the species studied: species monoecious/dioecious, types of cortification, plant aulacanthous/thylacanthous, presence/absence of spine cells, types of spines (rudimentary/as long as axis diameter/longer than axis diameter), spine cells solitary/in pairs/in bunches, presence of stipulodes, both rows of stipulodes well developed/stipulodes rudimentary/upper row prolonged, presence/absence of antheridia/oogonia (Urbaniak & Gąbka 2014).

DNA isolation and AFLP fingerprinting

In addition to the morphological observations, genetic fingerprinting was carried out using amplified fragment length polymorphisms (AFLPs). The advantages of the AFLP procedure are as follows: *i*) no *a priori* knowledge about the target

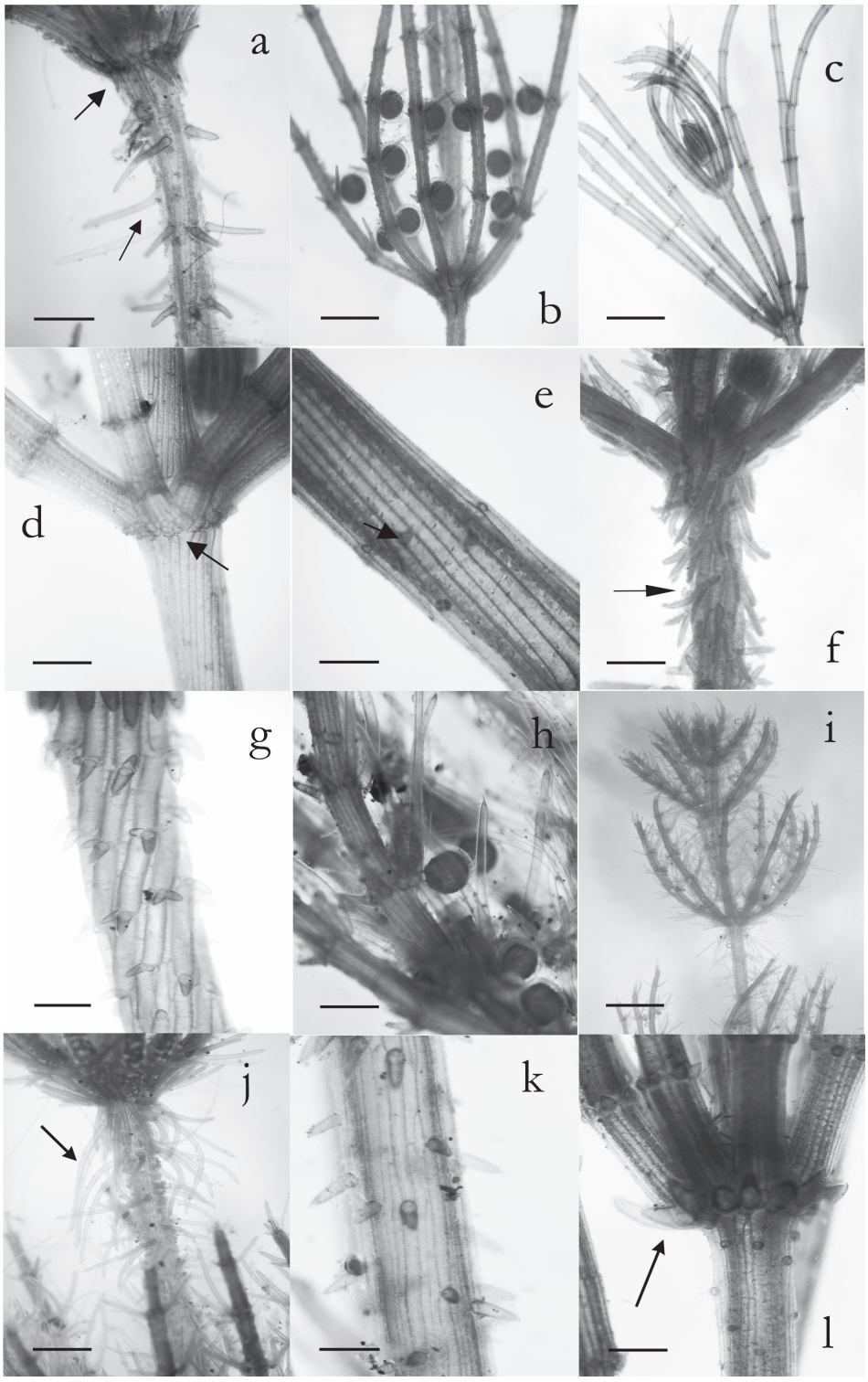

Figure 1

LM images of the studied species: **a.** Stipulodes (upper arrow) and long spine cells (arrow in the middle of the axis) on *C. aspera* axis; **b.** Antheridia on male *C. aspera*; **c.** Upper branchlet of *C. globularis* axis; **d.** Rudimentary stipulodes below *C. globularis* branchlet; **e.** Short and reduced spine cells on *C. globularis* axis; **f.** Long spine cells on axis *C. hispida*; **g.** Spine cells in bunches on *C. intermedia* main axis; **h.** Antheridia and oogonia on dioecious *C. strigosa*; **i.** General habit of *C. tenuispina*; **j.** Long spine cells of *C. tenuispina* on the upper part of the main axis; **k.** Spine cells of *C. tenuispina*; **l.** Long stipulodes below the branchlet on *C. virgata*

Scale bars: Figs 1, 4, 12, 100 µm; Figs 2, 3, 6, 8, 9, 10, 300 µm; Figs 5, 50 µm; Figs 7, 11, 150 µm

genome is necessary, *ii*) highly efficient detection of polymorphisms, and *iii*) universal applicability combined with high capacity for discrimination and reproducibility (O'Reilly et al. 2007; Janssen et al. 1996). This method is informative about genetic diversity between and within populations and was used to estimate genetic distances between the species. Total genomic DNA was isolated from fresh tissue using freeze dried, powdered material and the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Cells were disrupted using a mixer mill MM400 (Retsch, Haan, Germany). The quality and quantity of the DNA was determined using a fluorometer (Eppendorf, Hamburg, Germany), but the integrity of the extracted DNA was estimated on 1% TBE-agarose gels (Weising et al. 1995).

The AFLP procedure followed that described by Vos et al. (1995) with slight modifications. Digestion, ligation, preamplification and amplification procedures were performed in a Verity Thermocycler (Applied Biosystems, Carlsbad, USA). The detailed procedure was previously described by Urbaniak & Combik (2013). For selective amplification, we used four primer pairs selected from more than twenty combinations as most useful (Table 3). After selective amplification, the fluorescently labelled products were diluted 10× in Sample Loading Solution (SLS) with the addition of DNA size standards (DNA Size Standard Kit 400), and separated on a separation gel (GenomeLab™ Linear Polyacrylamide) in an automated sequencer (GenomeLab™ GeXP Genetic Analysis System, Beckman Coulter, Brea, USA). Analysis of some individuals was repeated in order to assess the reproducibility of the AFLP technique. Raw data were collected and aligned with the internal size standard using Beckman Coulter Fragment Analysis Software.

All molecular analyses were carried out at the Department of Botany and Plant Ecology, Wrocław University of Environmental and Life Sciences and Institute of Botany, Polish Academy of Sciences, Kraków.

Table 3

Percentage of polymorphic and non-polymorphic fragments per primer combination for the analyzed *Chara* species

Primer combination	All bands	Monomorphic	Polymorphic	
	(n)	(n)	(n)	(%)
ACA/CGA	185	6	179	97
ACA/CGC	177	16	161	91
ACT/CGA	184	15	169	92
ACG/CAT	201	11	190	93

Data analysis

The AFLP fragments were analyzed for the number of monomorphic and unique loci and the percentage of polymorphism. Analysis of some individuals was repeated in order to test the reproducibility of the AFLP technique. A locus was considered to be monomorphic if the corresponding band was present in all samples. The overall percentage of polymorphic loci was calculated by dividing the number of observed polymorphic bands by the total number of detected bands using FAMD v. 1.25 (Schlüter, Harris 2006). The similarity of AFLP profiles between pairs of samples was calculated using Jaccard's similarity coefficient (Sneath, Sokal 1973), according to the formula:

$$J = S_{ij}/(S_{ij} + S_i + S_j)$$

where: S_{ij} = the number of bands shared by both species, S_i = the number of bands present only in species *i*, and S_j = the number of bands present only in species *j* (Urbaniak, Combik 2013). A UPGMA (Unweighted Pair-Group Method with Arithmetic Mean) tree based on genetic distance of Nei & Li (1979) was generated and bootstrapped using 1000 replicates with TREECON 1.3b (Van de Peer, De Watcher 1994). Principal components analysis (PCA) of the molecular data was performed with MVSP 3.21 (Kovach 2007).

Results

Morphological differentiation of the species studied is provided in Fig. 1a-l. *C. aspera* individuals had long stipulodes below the branchlet and long spine cells exceeding the axis diameter, however, these were sparse and solitary (Fig. 1a). Solitary antheridia present on the branchlet contradict that *C. aspera* is a dioecious species (Fig. 1b). While similar to *C. aspera* in the habit, *C. strigosa* had much longer stipulodes and dense spine cells, arranged in bunches, while oogonia were conjoined, with antheridia below (Fig. 1h). These morphological differences allow for these species to be distinguished from one another. *Chara globularis* and *C. tenuispina* were both light green with similar habit, which may account for Wood's (1962) suggestion that *C. tenuispina* should be considered a variety of *C. globularis*. The two species can be distinguished based on the following morphological differences: *C. tenuispina* had spine cells mostly in the upper parts of the axis internodes, which were longer than the axis diameter, as well as two rows of stipulodes, which were elongated, acute, and longer than the axis diameter

(Fig. 1i-k). *Chara globularis*, however, had spine cells, while stipulodes were either absent or rudimentary (Fig. 1d-e). Similarly, Urbaniak & Gąbka (2014) stated that the same features allow *C. tenuispina* and *C. globularis* to be distinguished. Additionally, both species differ in ecological preferences and grow in different conditions. *Chara globularis* is a cosmopolitan species that can be found in different aquatic habitats such as lakes, ponds, pools and peatland exploitation pools, and has a wide ecological range, growing in mesotrophic and eutrophic water. In contrast, *C. tenuispina* has a narrow ecological amplitude and can be found in freshwater peatlands or in the littoral zone of lakes, in shaded and shallow water between vascular plants.

Compared to *C. intermedia* (Fig. 1g), *C. hispida* (Fig. 1f) had spine cells longer than the axis diameter and they occurred in bunches or in the group of three. Whereas spine cells of *C. intermedia* were shorter and occurred in pairs. *C. virgata* was characterized by longer upper whorls of stipulodes below the branchlet, and short and rudimentary spine cells (Fig. 1l).

The analyzed data showed high reliability of the AFLP bands (more than 96%). Prior to analysis, we tested approximately 20 different primer combinations *Eco*RIxxx–*Mse*Ixxx, ranging in size from 80 to 450 bp (more bands were tested but could not be scored with confidence across gels). Out of these 20 primer combinations, four were used for further analysis (Table 3). The highest intraspecific polymorphism rate was determined for *C. globularis* (76%) and *C. tenuispina* – 67-51%. The polymorphism rate for *C. aspera* was 76% and for *C. strigosa* – 69%. The lowest polymorphism rates were determined for *C. intermedia* (60-58%), *C. hispida* (66%) and *C. virgata* (68%). Similarities in the AFLP profiles for pairs of samples, calculated according to Jaccard's genetic similarity index, were 0.08-0.71. This score indicates that individuals of *C. tenuispina* and *C. globularis* showed a high degree of Jaccard's genetic similarity (Table 4).

Cluster analysis of the AFLP data showed the separation of several groups of species (Fig. 2). However, not all species were clearly separated and several specimens were intermixed with other species. Specimens belonging to both *C. tenuispina* and *C. globularis* formed separate clusters with high bootstrap support. *C. virgata* also formed separate cluster. The PCA analysis showed a separation into two distinct groups: one of them comprised mostly individuals of *C. globularis* (Group 1) and the second one – *C. tenuispina* (Group 2) (Fig. 3). A few individuals of *C. virgata* and *C. tenuispina* were intermixed with the *C. globularis* group. Representatives of *C. virgata* populations formed two subclades in one cluster, intermingled with individuals of *C. globularis* with high bootstrap values (Fig. 2).

Other specimens belonging to *C. hispida* and *C. intermedia* formed mixed clades; single individuals were intermixed or clustered together with other species. Data points representing *C. aspera*, *C. strigosa*, *C. virgata*, *C. hispida* and *C. intermedia* could not be distinguished using PCA analysis and formed an indistinguishable group of species (Group 3) (Fig. 3). The third group contains species with different morphological characters, which normally can be used to distinguish one species from the other. Similarly, the UPGMA method was not able to differentiate between *C. aspera* and *C. strigosa*. Within the *C. aspera* and *C. strigosa* group/clusters in the UPGMA analysis, individuals that belonged to different populations were basically grouped together, forming separate subclusters supported by medium to high bootstrap values (Fig. 2). Unfortunately, both species subgroups were intermingled and did not form separate clades

Table 4

Jaccard's genetic similarity index between pairs of plants belonging to different species

	C. globularis	*C. hispida*	*C. intermedia*	*C. strigosa*	*C. tenuissima*	*C. virgata*
C. aspera	0.16	0.18	0.14	0.68	0.37	0.21
C. globularis		0.27	0.30	0.47	0.69	0.49
C. hispida			0.71	0.29	0.08	0.1
C. intermedia				0.20	0.12	0.25
C. strigosa					0.31	0.38
C. tenuissima						0.46

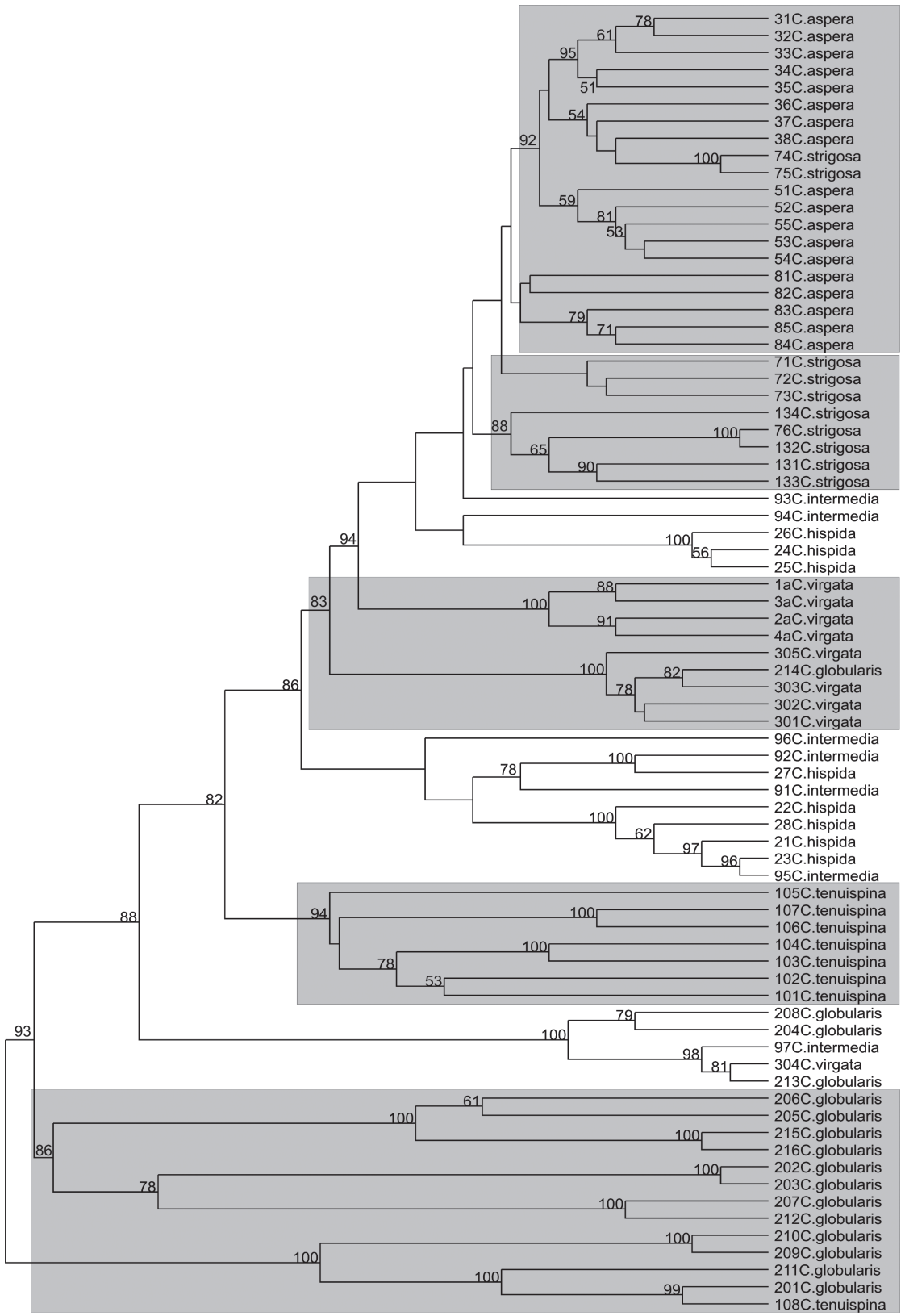

Figure 2

Neighbor-joining dendrogram based on AFLP markers

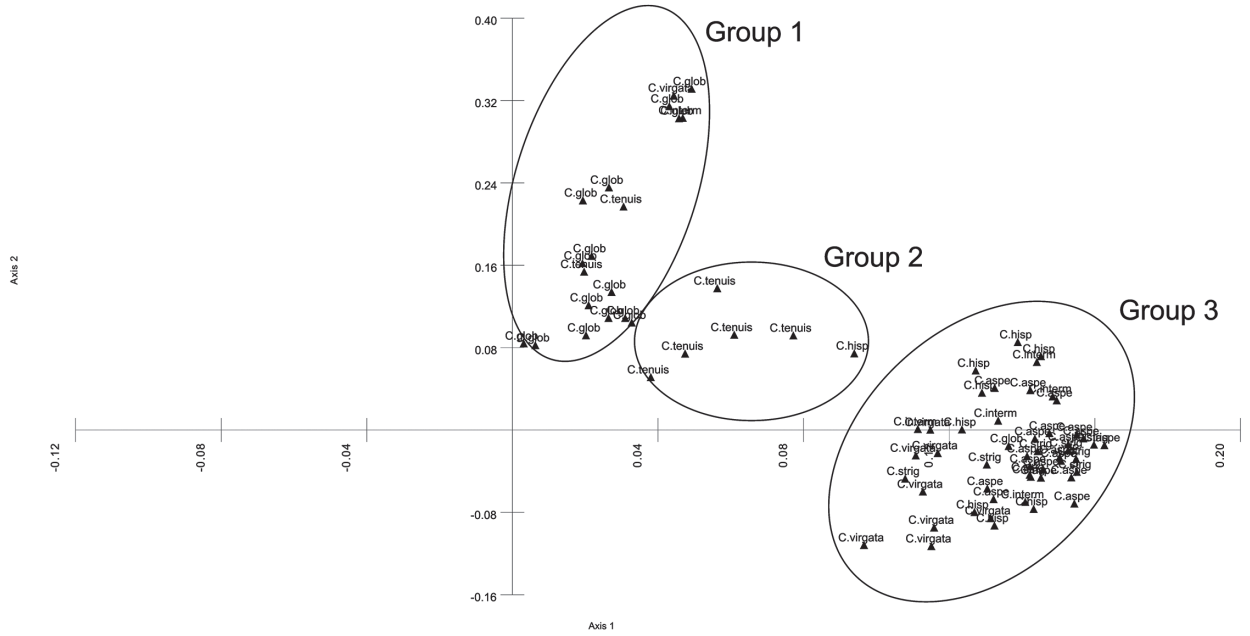

Figure 3

Principal Component Analysis of AFLP of the analyzed species

for each species, which would allow for the distinction between these two taxa.

Discussion

The primer combinations used for AFLP analysis produced a mean percentage of 96% for polymorphic bands. Similar values were obtained by Mannschreck et al. (2002), Bögle et al. (2007) and Urbaniak & Combik (2013) for other charophyte species.

Previously, oospore dimensions showed that *Chara tenuispina* A. Braun 1835 (*C. globularis* var. *tenuispina* R.D.Wood 1962) and *C. globularis* Thuill. 1799 (*Chara globularis* var. *globularis* f. *globularis* R. D. Wood 1962) should be recognized as distinct species (Urbaniak 2011a) rather than polymorphic variations of *C. globularis,* as proposed by Wood & Imahori (1965). This was not confirmed by SEM images of oospore wall ornamentation (Urbaniak 2011a). Results from the presented UPGMA and PCA analyses suggest a separation of both taxa, which was further supported by high bootstrap values (Figs 2-3). Therefore, we suggest that *C. tenuispina* should be classified as a separate species rather than a form of the polymorphic species group *C. globularis* as suggested by Wood & Imahori (1965). Our study based on the AFLP fingerprinting method also confirms recent results obtained by Schneider et al. (2016), who successfully distinguished *C. globularis* from *C. tenuispina* using barcode genes. In this case, molecular analysis performed on specimens collected from various locations is consistent with morphological characteristics. Both species have a similar appearance (slender habit, no spine cells on the triplostichous cortex), but *C. tenuispina* has much longer stipulodes with spine cells longer than the axis diameter, especially directly below the branchlet (Fig. 1j). This shows that both characters are valid in morphological differentiation, and we agree with Krause (1997) and Urbaniak & Gabka (2013) that this feature is an important character to distinguish between the two species.

Previously, Urbaniak (2011a) found that oospores of *C. globularis* and *C. virgata* formed one aggregation and did not differ in their ornamentation, thereby suggesting a close taxonomic relationship between the taxa. Such statement can be true as long as our results do not fully separate both species. Individuals of *C. globularis* and *C. virgata* were mixed in PCA analysis with *C. tenuispina* (Group 1) or formed one undifferentiated group of species together with *C. aspera, C. strigosa, C. virgata, C. hispida* and *C. intermedia* (Group 3) (Fig. 3). In the UPGMA figure, *C. globularis* and *C. virgata* did not form one and clearly separated cluster supported by high bootstrap values, but almost all *C. globularis* individuals are located next to each other in two groups supported by high bootstrap values. Some individuals of *C. virgata* were also mixed with other species, however, the collected specimens of *C. virgata* were very similar to those described by Krause (1997), i.e. with prolonged upper rows of stipulodes below

the branchlet, clearly triplostichous, with thylacanthous cortex, rudimentary spine cells on the axis and white, not spherical bulbils on the rhizoids. They differed from *C. globularis* specimens which had rudimentary stipulodes and lack spine cells on the cortex (Fig. 1l). This shows that AFLP results are in partial disagreement with the morphological traits that differentiate *C. globularis* from *C. virgata* and also from other species, e.g. those belonging to the sect. Hartmania that are significantly different. Based on *mat*K and *rbc*L genes, Schneider et al. (2016) clearly separates *C. globularis* from *C. virgata* but accepts specimens without spines and with (usually only slightly) elongated stipulodes as *C. globularis*. We agree with the common meaning that *C. globularis* does not have elongated stipulodes and the specimens equipped with such stipulodes should be treated as transitional forms. On the other hand, stipulodes are not the only characters that may be taken into account. The observed morphological differentiation, particularly in *C. globularis* is huge (Migula 1897), so is the number of disputed morphological characters.

Group 3 includes individuals not only from *C. virgata*, but also from *C. globularis* and *C. tenuispina*. This again confirms close taxonomic/phylogenetic affinity between these species (monoecious with triplostichous cortex and delicate slender habitus). In other words, it is possible that despite the accurate identification before laboratory culturing and analysis, some specimens were wrongly identified, even though we were very careful in this particular case. Therefore, one of the likely explanations is that our molecular methods do not fully reflect the morphological differentiation.

Some individuals of *C. globularis* and *C. virgata* formed an unresolved group with triplostichous: *C. strigosa*, *C. aspera* and diplostichous *C. hispida* and *C. intermedia* – group 3 (Fig. 3). All these species are monoecious, except for *C. aspera*, which is a dioecious species, but this difference was again not consistent with AFLP analysis. We were not able to differentiate species in this group by PCA analysis, especially monoecious and dioecious species. In the UPGMA tree, *C. aspera* and *C. strigosa* formed a compact group of individuals, and only a few *C. strigosa* individuals were found among *C. aspera*. Unfortunately, none of the analyses show a clear acceptable differentiation between *C. aspera* and *C. strigosa*. Specimens of both species formed several subclades, instead of distinct branches (Fig. 2). The other examined specimens, for which there are doubts regarding their taxonomic differentiation, belong to the taxa: *C. hispida* and *C. intermedia* (sect. Hartmania). They differ from other individuals included in the same Group 3 in size, cortification, presence of spine cells and general appearance (Fig. 3).

Similarly, significant differences in morphology, such as the habitus or the type of cortification that allow for routine identification of charophytes, also did not confirm our results as useful for distinguishing between the studied species. Urbaniak & Combik (2013), who used AFLP, and later Schneider et al. (2015), who used barcode genes, did not find differences between *C. hispida* and *C. intermedia* in a much larger data set. With the current state of knowledge, the statement of Wood & Imahori (1965) about very close taxonomic relationships between the species from sect. Hartmania seems to be acceptable.

The AFLP fingerprinting method is a useful tool for taxonomists, which can detect small genetic differences between populations of various plant species. This was successfully done in the case of *C. globularis* and *C. tenuispina* identification. Additionally in this case, morphological characters clearly correlate with the identification based on molecular methods: AFLP and *rbc*L+*mat*K (Schneider et al. 2016). Sometimes, however, it is not possible to distinguish between the closely related taxa. This is the case of *C. aspera* and *C. strigosa*, *C. hispida* and *C. intermedia* or other species from the sect. Hartmania. The genetic differences between them can be reflected in morphological differences and the phylogenetic relationships can be inferred from this knowledge. Numerous examples show that this is not a rule. Another good example are *C. aspera* and *C. strigosa*, which have similar habits, but significantly differ in their reproduction system. The method of reproduction is the most important feature used to distinguish between the species. Therefore, based on Mayr's classical definition of biological species, *C. aspera* and *C. strigosa* should be treated as separate species. Our results are only partly contradictory, however, much more data are needed to clearly define their taxonomic relationships. It is also possible that application of other methods would help. Barcode markers, *mat*K and *rbc*L used by Schneider et al. (2016), were also unable to relate the morphology and reproduction system to genetic data. They successfully distinguish both species, but surprisingly *C. strigosa* formed one clade with *C. virgata*.

According to Bögle et al. (2010), who analyzed differences between populations of *C. baltica* and *C. intermedia* collected along the north-south gradient across Europe (from Sweden to Germany) using AFLPs, populations from the central part of the distribution demonstrated intermediate features, suggesting that individuals in this area might represent features of a common ancestor.

One of the possible explanations is a taxonomic continuum between closely related species, especially between species from sect. Hartmania (*C. baltica, C. hispida*, *C. horrida*, *C. intermedia*, *C. polyacantha* and *C. rudis*). This was observed previously when using the fingerprinting method (Urbaniak & Combik 2013). The species continuum shows that only some species are completely different from the others. At the same time, species that appear to be similar, differ greatly from each other when found in nature, both in morphological and molecular terms (Sepp & Paal 1998). This is observed based on the presented results in the case of *C. tenuispina* but also other *Chara* species that can be treated as separate species: *C. baueri, C. canescens, C. globularis*. Most of the other species, e.g. *C. baltica, C. hispida, C. intermedia, C. polyacantha* and *C. rudis*, can be completely indistinguishable using morphological and molecular markers and convincing evidence for their distinction are still lacking. They still can be treated as variations or forms of other species (Urbaniak & Combik 2013; Schneider et al. 2016). It is possible that the continuum of species indicates that hybridization between species occurred in the past and probably occurs today.

Other authors (Schneider et al. 2015) were also unable to distinguish the species using barcode markers. Various species might represent distinct taxa but are masked by phenotypic or genotypic adaptations to different environmental conditions. Furthermore, Schneider et al. (2006) showed that changes in branching occur in *C. hispida* and *C. intermedia* as a response to different light conditions. Blindow & Schütte (2007) concluded that morphological differences between freshwater and brackish water populations of *C. aspera* were at least partly explained by plastic responses to salinity, rather than by genetic differences. Thus, phenotypic plasticity and genetic adaptation to different environmental conditions underlie the morphological variability observed in many charophyte species, which in turn provides the basis for natural selection to drive macroevolution (Urbaniak & Combik 2013). The close taxonomic relationship between charophyte species (and within various sections of charophytes) is commonly known and is reflected in a high degree of morphological similarity between phylogenetically closely related species (Meiers et al. 1999; Urbaniak& Combik 2013). While the results of this study do not fully support the taxonomic interpretation proposed by Wood & Imahori (1965), they do not exclude the possibility of combining separate species into macrospecies. Although taxonomic criteria for distinguishing between separate species are not clear, the idea of dividing organisms into species and macrospecies is generally acceptable, more attention should be paid to research on the observed phenotypic plasticity of charophytes in laboratory conditions and their correlation with molecular markers.

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