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Genetic and morphological variation in *Chara contraria* and a taxon morphologically resembling *Chara connivens*

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Genetic and morphological variation in *Chara contraria* and a taxon morphologically resembling *Chara connivens*

Charophyte species delineation is regularly based on a set of thallus morphological characteristics, but considering pronounced phenotypic plasticity, difficulties and doubts commonly occur in *Chara* species determination. DNA barcoding may contribute to solving these challenges. Here we characterize *Chara contraria* with an unusual set of morphological characteristics, and specimens morphologically resembling *Chara connivens* collected in Serbia, by describing their morphological traits and analysing *matK* barcoding results. Our results indicated that dioecious *Chara* specimens, tentatively determined as *Chara "connivens"* based on morphological traits, were genetically more closely related to *C. globularis*. These *Chara "connivens"* specimens formed a sister group to a monophyletic *C. globularis* cluster, suggesting that it may be neither *C. connivens* nor *C. globularis*. We strongly encourage further barcoding of *C. "connivens"* samples from freshwater, in order to find out if there are consistent genetic differences between the dioecious freshwater *C. "connivens"* and monoecious *C. globularis*. Barcoding of *matK* placed the monoecious *Chara* specimens, which based on morphological characteristics initially were determined as *C. virgata*, into the *C. contraria* group. This indicates that the microscopic traits which commonly are used for *Chara* species determination sometimes are misleading. In general, our study challenges the commonly used phenetic species concept in Charophyte taxonomy and illustrates the importance of molecular approaches to evaluate the validity of morphological characteristics of the plant thallus in species delineation.

Keywords: algae; barcode; Charales; charophyte; *matK*

Introduction

Charophytes are macroscopic benthic algae with a complex morphology, vaguely resembling Equisetum (Pukacz et al. 2014.; Schneider et al. 2016). They are also called stoneworts, due to the calcium carbonate encrustations which are firmly attached to the surface of their thalli (Pełechaty et al. 2013). Charophytes are well known providers of ecosystem services. By

providing habitat, shelter, and food to various organisms, charophytes contribute to maintaining biodiversity in freshwater ecosystems (Schneider et al. 2015). Charophyte meadows effectively act as nutrient sinks, by incorporating nutrients in biomass, co-precipitation of phosphorus with calcium carbonate and restriction of sediment resuspension, thereby enhancing water clarity and quality (Kufel and Kufel 2002). At the same time, charophytes are sensitive to environmental impacts, e.g. eutrophication and climate change (Blindow, 1992; Auderset Joye and Rey-Boissezon, 2015). Because of their species-specific sensitivity to eutrophication, charophytes are often used as bioindicators to indicate eutrophication or ecological status of water bodies according to the Water Framework Directive (e.g. Melzer, 1999; Schneider et al. submitted). Consequently, accurate identification of charophytes is important, in order to assess the ecological status of water bodies as correctly as possible.

Morphological characteristics of the plant thallus are commonly used for charophyte species delineation (Schneider et al. 2015). However, this often is difficult, because the morphology of a species may vary, and morphological traits used for species delineation may overlap between species or differ among determination keys (Boegle et al. 2007). Both, environmental conditions and genetic differences may contribute to phenotypic plasticity of charophytes (Boegle et al. 2007; Schneider et al. 2016). It has recently been shown that characters which commonly are used for *Chara* species delineation – such as the number and length of spines and stipulodes, do not coincide with genetic differences (Schneider et al. 2016).

DNA barcoding is a reliable tool for detecting genetic variability and phenotypic plasticity within genetically similar units. It has successfully been applied also in charophytes (e.g. Schneider et al. 2015; Karol et al., 2018). In DNA barcoding, short regions of DNA (barcodes) are sequenced and matched to a reference library. *MatK*, a rapidly evolving coding

section of the plastid genome, is, along with *rbcL,* recommended as standard DNA barcode for plants (CBOL Plant Working Group 2009), and has previously successfully been used for barcoding of *Chara* species (Schneider et al. 2016; Karol et al., 2018; Langangen et al., in press).

Chara contraria is described as an extratropical cosmopolite mainly occurring in the Northern hemisphere (Krause 1997). The species is relatively common in Europe (Doege and van de Weyer 2016). *Chara contraria* inhabits various types of water bodies, but it seems to prefer larger lakes, either natural or artificial, which can be colonized to greater depths (Doege and van de Weyer 2016). *Chara contraria* has frequently been found in Serbia and is currently regarded as being at Low Risk (nearly threatened) in the Red List of species in Serbia (Blaženčić 2014).

Chara connivens is considered to be a brackish water species, but it also occurs in freshwater habitats such as lakes, ditches and temporary ponds (Torn and Martin 2004). *Chara connivens* occurs in Europe, Africa and Northern Asia (Torn and Martin 2004). In Europe, it is reported mainly from coastal habitats, from Scandinavia to the Mediterranean (Torn and Martin 2004; Becker 2016), but also from inland waters in central and southern Europe, such as lake Balaton in Hungary and the Danubian floodplain in Romania and the Balkans (Krause 1997; Blaženčić et al. 2006). Still, this species is not common outside coastal habitats, and by now in Serbia it was found only in one locality, thus it is considered critically endangered (Blaženčić 2014).

Difficulties and doubts commonly occur in *Chara* species determination, and we also experienced doubts in the determination of samples which showed “untypical” morphological characteristics. Barcoding of genetic markers may contribute to solving these challenges. We here use barcoding of *matK*, together with the characterization of morphological traits, to characterize the morphological and genetic variability of samples collected in Serbia. One of

the samples was preliminarily identified as *C. connivens,* while the morphology of the other sample had characters from *C. contraria* and *C. virgata*. Since the barcoding results clearly identified this sample as *C. contraria*, we refer to it as *C. contraria* in the entire manuscript.

Methods

Localities and charophyte sampling

Samples of *C. contraria* and *C. connivens* were collected in Dulin pond and Sava lake.

Dulin Pond is a shallow (maximum depth 1.5 m; surface area 11.8 ha; 68 m a.s.l.) permanent water body situated in the Nature Reserve Deliblato Sands at the southern margin of the Pannonian Plain, along the river Danube (44°51'11.7"N, 21°17'52.5"E). It is separated from the Danube-Tisa-Danube Canal by a narrow bank and situated nearby a regional highway (Fig. 1). The total phosphorus concentration is high (400 µg TP/l; unpublished data from a single measurement in June 2017). Other available data on water chemistry indicate that Dulin Pond contains freshwater (not brackish water; Ca 17 mg/l, Mg 14 mg/l, conductivity 226 µS/cm, total hardness 5.5 dH; average values from 3 measurements in 2018).

Sava lake is a permanent water body situated in the city of Belgrade (44° 47’ 02.28” N, 20° 23’ 25.64” E; 73 m a.s.l.; surface area 81.7 ha, maximum depth 12 m, average depth 4.5 m). It is a former arm of the river Sava. The lake is intensively used for recreation. Total phosphorus concentrations are around 10 µg/l (average of measurements taken at 6 sites around the lake taken in October 2017 and April 2018). The macrophyte vegetation is dominated by *Myriophyllum spicatum* L., and the macrophytes are regularly removed in the littoral during the summer season, to enable recreational use of the lake.

[Figure 1 near here]

Macrophytes in Dulin pond were collected in June 2017 along two transects, and fresh *Chara* samples were collected in August 2017 at one sampling point for genetic analyses (Fig. 1), by using a grapnel and a rake. Macrophytes in Sava lake were collected in July 2017 at the easternmost shore of the lake, by using a grapnel, and by snorkelling. Charophyte specimens which were difficult to determine due to unusual morphology were analysed by barcoding. Light microscope (LM) images of taxonomically relevant morphological characteristics of *Chara* specimens were obtained by using Carl Zeiss AxioImager M1 microscope and a digital camera AxioCam MRc5 with AxioVision 4.8 software.

DNA barcoding

Two different methods were used to isolate genomic DNA from *Chara* samples investigated in this study. An overview over which samples were analyzed with which method is given in Table 1. The sequence data were deposited in the European Nucleotide Archive (ENA) under the accession numbers given in Table 1.

Method A: Genomic DNA from *Chara* material was isolated after Schneider et al. (2016). PCR for the *matK* gene was performed on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Oslo, Norway) using the iProof High-Fidelity PCR Kit (Bio-Rad Laboratories, Oslo, Norway). Amplification of the *matK* gene region was conducted using the primers F-Chara (agaatgagcttaaacaaggat) and R-Chara (acgatttgaacatccactataata). The following cycling protocol was used: one cycle of 5 min at 94 °C, and then 35 cycles each consisting of 10 s at 94 °C, 20 s at 62 °C, and 20 s at 72 °C, followed by a final elongation step of 72 °C for 5 min. PCR products were visualized by 1.5% agarose gel electrophoresis with GelRed staining (GelRed® Nucleic Acid Gel Stain (Biotium, Fremont, USA)) and UV illumination. For sequencing the same primers and the intermediate primers

charaintF (gatggctattcaagcagga), charaintR (ctaccgataagttcgtcct), charaBt2F (datatggcaacaycaaaagac) and charaBT2R (atacagaccatgcagcytt) were used. For each PCR product, both strands were sequenced on an ABI 3730 Avant genetic analyser using the BigDye terminator V.3.1 cycle sequencing kit (Applied Biosystems, (Applied Biosystems, Thermo Fisher Scientific Oslo, Norway) according to the manufacturer's instructions.

Method B: Preparation of total DNA was performed using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. Amplification of the *matK* gene region was performed with a Taq PCR Master Mix (Qiagen, Hilden, Germany), using the primers *matK*-F2 (aatgagcttaaacaaggattc) and *matK*-R1b (gcagccttatgaattggatagc). The following PCR protocol was used: 10 cycles of one minute each at 94° C, 55° C, and 72° C, followed by one minute each at 94° C, 52° C, and 72° C for 25 cycles. The amplified DNA was purified with the Biometra-innuPrep Gel ExtractionKit (Analytik Jena, Jena, Germany) according to the manufacturer's instructions and was sequenced directly on a 3130×L GeneticAnalyzer (Applied Biosystems, NY, USA) using the BigDye terminator V.1.1 cycle sequencing kit (Applied Biosystems, Thermo Fisher Scientific, Darmstadt, Germany). Sequencing primers were identical to the primers that were used for the PCR reactions. Achieved sequences were proofed and manually edited using the BioEdit programme (Hall 1999).

[Table 1 near here]

Phylogenetic analyses

Sequences were analysed and aligned using Seqassem (version 04/2008) and Align (version 03/2007) MS Windows-based manual sequence alignment editor (SequentiX - DigitalDNA Processing, Klein Raden Germany) to obtain DNA sequence alignments, which were then corrected manually. In addition to two samples collected in Dulin pond and one sample from Sava lake, a *matK* set containing 50 other *Chara* sequences (Table 1), and 1067 nucleotide

positions were used for phylogenetic analysis. *Nitellopsis obtusa* (AY170447) was used as an outgroup taxon in the *matK* tree. The dataset was analyzed using maximum likelihood (ML), maximum parsimony (MP) and distance (neighbor-joining (NJ)) in MEGA version 7 (Kumar, Stecher, and Tamura 2016) and using Bayesian inference (BI) in MrBayes (No. of generations: 2,000,000; burn-in fraction: 0.25) (Ronquist et al.2012). GTR+G was selected as the best-fitting evolutionary model for the *matK* gene region. ML, MP, and distance analyses were performed with 1000 bootstrap replicates in MEGA version 7 (Kumar, Stecher, and Tamura 2016).

Results

Macrophyte species composition

The following floating and submerged macrophyte species were recorded in Dulin pond: *Nymphaea alba* L., *Nuphar lutea* (L.) Sm., *Ceratophyllum demersum* L., *Elodea nuttallii* (Planch.) St. John, *Myriophyllum spicatum*, *Najas marina* L., *Najas minor* All., *Polygonum amphibium* L., *Potamogeton lucens* L., *Stuckenia pectinata* (L.) Börner, *Trapa natans* L., *Chara globularis* Thuill., *Chara contraria* A. Braun ex Kütz. and *Nitellopsis obtusa* (Desv.) J. Groves. In addition, we found dioecious *Chara* specimens more closely described below.

The following macrophyte taxa were recorded at the sampling site in Sava lake: *Myriophyllum spicatum* L., *Najas minor* All., *Najas marina* L., *Potamogeton nodosus* Poir., *Potamogeton pusillus* L., *Stuckenia pectinata* (L.) Börner, *Zannichellia palustris* L., *Tolypella intricata* (Trentep. ex Roth) Leonh., and *Nitella* C. Agardh sp. In addition, we found *Chara* specimens more closely described below.

Description of Chara specimen with "unusual" morphology

Chara "connivens" from Dulin pond

We found dioecious *Chara* specimens (Fig. 2) which tentatively were determined as *Chara "connivens"*, although the branchlets were longer than what is given in the determination keys (Table 2). *Chara "connivens"* was most abundant and partly dominant in the shallow littoral, up to 0.8-1 m depth, where it occurred mostly together with *Chara globularis*. Gametangia were well developed. In male plants, branchlets were markedly connivent and shorter than in female plants, where branchlets were straight and usually longer (Fig. 2). Species traits of *C. connivens*, as given in the most commonly used charophyte determination literature in Europe, together with the respective traits we found in the samples from Dulin pond, are given in Table 2.

[Figure 2 near here]

[Table 2 near here]

Chara contraria from Sava lake

We found monoecious *Chara* specimens which initially were determined as *C. virgata*. These specimens were found relatively sparsely at around 4-6 m water depth, together with *Myriophyllum spicatum*. The plants were roughly 20 cm long, and the plant habitus generally resembled *C. contraria*. However, the microscopic traits did not match the description of *C. contraria* given in the literature. *C. contraria* is generally described as diplostichous, with (slightly) elongated stipulodes in two tiers and short but generally (slightly) elongated spines (Krause 1997). In contrast, our samples were mainly triplostichous, had very short rudimentary spine cells, and globular stipulodes (sometimes the upper row of stipulodes was slightly elongated) (Fig. 3).

[Figure 3 near here]

Barcoding results

Both, *C. contraria* and *C. virgata* formed monophyletic clusters supported by bootstrap values ≥ 99%, and the species were well separated from each other and from other species (Fig. 4). Despite its untypical morphology, which microscopically resembled *C. virgata*, barcoding of *matK* clearly placed the sample from Sava lake (S110) into a group with 20 other *C. contraria* samples collected in seven different countries within Europe (Fig. 4; Table 1). There was some genetic variability within *C. contraria*, but sample S110 was identical to three samples from Germany, and one sample from Greece (Fig. 4; Table 1).

Our samples of *C. connivens* also formed a well-defined cluster supported by a bootstrap value of 100%. However, *C. connivens* AY170422 obtained from Genbank was located in a separate cluster (Fig. 4). Samples S111 and S112, however, which were collected in Dulin pond, were closely related to *C. globularis,* and formed a sister group to a monophyletic *C. globularis* cluster. The clearest morphological difference between *C. globularis* and *C. connivens* is that *C. globularis* is monoecious, while *C. connivens* is dioecious (Krause, 1997). Our samples clearly were dioecious (Table 2), which suggests that it is *C. connivens*. However, barcoding results so far indicate that it may be neither *C. connivens* nor *C. globularis* (Fig. 4).

[Figure 4 near here]

Discussion

The phenetic species concept, which Charophyte taxonomy generally relies on, was in the last decades often challenged by molecular approaches, aiming to evaluate the validity of morphological characteristics of the plant thallus in species delineation (Boegle et al. 2007; Kato et al. 2010, Boegle et al. 2010a, 2010b, Urbaniak and Combik 2013; Schneider et al. 2016; Nowak, Schubert, and Schaible 2016;). Schneider et al. (2016) challenged the

usefulness of a number of morphological traits, such as partial or total loss of cortication, sex differentiation, or the number and length of spine cells, bract cells and stipulodes for *Chara* species differentiation, and highlighted the importance of genetic support for species delineation. In Schneider et al. (2016), 47 individuals from nine different countries in Europe formed the "*C. contraria*-cluster". Among these 47 individuals were monoecious and dioecious specimens, individuals with short and elongated branchlets, as well as ecorticated, partly ecorticated and normally corticated specimens. Interestingly, however, all individuals were diplostichous (Schneider et al. 2016). In contrast, our *C. contraria* sample from Sava lake (S110) was mainly triplostichous (Fig. 3). This indicates that also the number of cortex cell rows may be variable within a genetically homogeneous *Chara* group. Occasional occurrence of triplostichous cortex in *C. contraria* was mentioned before, but exclusively as an irregularity and anomaly in cortex development (Wood and Imahori 1965; Mouronval et al. 2015; Doege and van de Weyer 2016).

Commonly used determination keys (Wood and Imahori 1965, Gollerbah and Krasavina 1983; Krause 1997; Schubert and Blindow 2004; Urbaniak and Gąbka 2014; Mouronval et al. 2015; etc.) use a set of morphological traits such as cortication, length and number of spine cells, length of stipulodes and bract cells, as well as sex differentiation, to differentiate *Chara* species. Our results, together with earlier studies, have shown that all these traits vary within genetically homogeneous groups. Because of the microscopic traits (triplostichous, short stipulodes with the upper row sometimes a little elongated, rudimentary spine cells) we initially wrongly determined the *C. contraria* sample (S110) from Sava lake as *C. virgata*. Only the overall plant habitus resembled *C. contraria*, mainly because the specimen were more «greyish green» than *C. virgata* usually is, and because the branches were curved, rather than straight as is commonly observed in *C. virgata*. This made us doubt our determination, and double-check it using DNA-barcoding. Species determination keys

usually contain a short description of plant habitus, but this is not based on a uniform set of traits and relies on subjective impression. Habitus thus is usually only marginally (if at all) considered in species delineation. On the other hand, the experienced eye of the professional can recognize the habitus of some *Chara* species at the first sight. Unfortunately, habitus as morphological trait can hardly be uniformly and unambiguously described in practice. Nevertheless, we suggest that overall plant habitus may be "trusted" more in *Chara* species determination, particularly when overall habitus and microscopic morphological traits do not match, as was the case in our *C. contraria* sample from Sava lake.

In our barcoding results, the dioecious *C. "connivens*" from Dulin pond did not cluster with other *C. connivens* samples, but instead formed a separate cluster within *C. globularis* where it nevertheless was separated from the monoecious *C. globularis* samples (Fig. 4). This could mean that our *C. "connivens*" from Dulin pond may belong to a new, hitherto undescribed, species. Following the traditional determination keys, morphological traits (triplostichous, reduced spines and stipulodes, dioecious) clearly lead to *C. connivens*. However, typical *C. connivens* is described from brackish water (Torn and Martin 2004; Urbaniak and Gąbka 2014; Mouronval et al. 2015), and only occasionally from eutrophic calcareous freshwaters characterized by high conductivity (Brzeska et al. 2015; Becker 2016). Dulin pond, however, is a typical freshwater environment, characterized by high phosphorus concentrations but relatively low conductivity and water hardness, compared to the general ecological preferences of *C. connivens* (Becker 2016). According to the same author, such conditions could be comparable with a habitat of *C. connivens* in Lower Saxony in Germany (Becker, own data, cited in Becker 2016). We hypothesize that the typical brackish water "connivens" may be a different species than specimens with a similar morphology from freshwater. However, more data are needed before conclusions can be drawn.

Specimens of *C. connivens* were once determined in Serbia before, in 1983 in a channel near Silver lake, about 15 km from Dulin pond (Blaženčić 2014). There was always a hint of doubt attached to this record, because also this location is a typical freshwater habitat. Hitherto unpublished drawings of this material (Fig. 5 a) show that they were morphologically similar to the samples we found in Dulin pond. Cytological analyses done at the time showed that the chromosome number was 21 (Fig. 5b), which is more than what Proctor (1971) found in *C. connivens* (n=14), but less than in *C. globularis* (n=28/42/ca.77). We therefore hypothesize that this old record of *C. "connivens"* found in the channel near Silver lake was the same species as the sample we now found in Dulin pond. Unfortunately, no herbarized material of any of these specimens exists, and no new findings at this locality have been made since 1983.

[Figure 5 near here]

The question whether dioecious and monoecious forms of otherwise morphologically similar *Chara* individuals automatically are to be considered as distinct species has been debated for a long time. Wood and Imahori (1965) did not recognize monoecious and dioecious forms as separate species. This was criticized by Sarma and Khan (1967) who, based on cytological findings, argued that dioecism and monoecism is a reliable species delineation character. Proctor (1975) found that some strains (very few, not all) of dioecious *C. connivens* and monoecious *C. globularis* can cross with one another, although their hybrid offsprings (recovered at low frequences) were invariably monoecious and self-sterile. He suggested that at some point during evolution *C. connivens* (or a closely related dioecious form) gave rise to the monoecious *C. globularis*, but in present, he suggested that these two fully deserve recognition as distinct species. Also, Krause (1997) considered dioecism and monoecism as fully reliable character for species delineation. Schneider et. al. (2016) showed that sex differentiation (monoecious-dioecious) was not always reliable to separate species,

because dioecious *C. arcadiensis* and *C. imperfecta* were not genetically different from monoecious individuals within the *C. contraria* cluster, while monoecious *C. tenuispina* indeed was different from dioecious *C. aspera*. Kato et al. (2010) showed that *rbc*L sequence data of monoecious *C. altaica* and dioecious *C. canescens* were identical, and we also have an unpublished sequence of a monoecious *Chara* sample which genetically clusters with dioecious *C. canescens*. We therefore suggest that monoecism-dioecism in some but not all cases indicates genetic separation of otherwise similar species. In some cases, the separation has gone a long way, and this is reflected in different barcodes. In other instances, separation is relatively new, such that barcoding differences have not yet become apparent.

In this context, we suggest that the dioecious *C. "connivens*" samples from Dulin pond are unusual - different from monoecious *C. globularis*, but also from the other dioecious *C. connivens* samples included in this study. We encourage further barcoding of *C. "connivens*" samples from freshwater, in order to find out if there are consistent genetic differences between the dioecious freshwater *C. "connivens*" and monoecious *C. globularis*. Overall, our study illustrates that using morphological traits for *Chara* species determination is challenging and stresses the importance of general plant habitus as a taxonomic trait.

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Identification	Field ID	GenBank accession number	Method	Coll. year	Country	Author
C. aspera	MB76	LR590598	A	2001	France	Willd. 1809
C. connivens		AY170442				Salzm. ex A. Braun 1835
C. connivens	GJ31	MK914581	B	2009	Sweden	
C. connivens	GJ37	MK914581	B	2009	Sweden	
C. connivens	GJ38	MK914582	B	2009	Sweden	
C. connivens	EST-HG8	MK914584	B	2018	Estonia	
C. connivens	001NS42	MK914583	B	2012	Poland	
C. "connivens"	**S111**	**LR590596**	**A**	**2017**	**Serbia**	
C. "connivens"	**S112**	**LR590597**	**A**	**2017**	**Serbia**	
C. contraria	10AD10c	MK914585	B	2010	Germany	A. Br. ex Kütz. 1845 s. str.
C. contraria	10AD10e	MK914586	B	2010	Germany	
C. contraria	10AD22b	MK914594	B	2010	Germany	
C. contraria	12AD18e	MK914587	B	2012	Germany	
C. contraria	12AD21a	MK914588	B	2012	Germany	
C. contraria	12AD21b_f	MK914589	B	2012	Germany	
C. contraria	12AD2a	MK914595	B	2012	Germany	
C. contraria	12AD2c	MK914596	B	2012	Germany	
C. contraria	D-DS01-1	MK914590	B	2018	Germany	
C. contraria	CS29	MK914591	B	2012	France	
C. contraria	CS34	MK914592	B	2012	France	
C. contraria	DH5d	MK914593	B	2011	UK	
C. contraria	M17	LR590599	A	2008	Norway	
C. contraria	M25	LR590600	A	2008	Norway	
C. contraria	MB22	LR590601	A	2005	Greece	
C. contraria	MB70	LR590602	A	2000	Austria	
C. contraria	MB82	LR590603	A	2000	Germany	
C. contraria	TK82	MK914606	B	2009	Sweden	
C. contraria	TK86	MK914607	B	2009	Sweden	
C. contraria	TK88	MK914608	B	2009	Sweden	
C. contraria	**S110**	**LR590604**	**A**	**2017**	**Serbia**	
C. globularis	16	LR590605	A	2009	Macedonia	Thuillier 1799
C. globularis	17	LR590606	A	2009	Macedonia	
C. globularis	D-BW-CG2	MK914602	B	2018	Germany	
C. globularis	DH6a	MK914597	B	2011	UK	
C. globularis	DH7c	MK914598	B	2011	UK	
C. globularis	GJ29	MK914599	B	2009	Sweden	
C. globularis	GJ30	MK914600	B	2009	Sweden	
C. globularis	IW5a	MK914603	B	2012	Germany	
C. globularis	IW5b	MK914601	B	2012	Germany	
C. globularis	MB29	LR590607	A	2005	Sweden	
C. globularis	MB60	LR590608	A	2001	France	
C. globularis	T83	LR590609	A	2011	Norway	
C. hispida	49	LR590610	A	2012	Germany	(L.) Hartm. 1820

C. tomentosa	S18	LR590611	A	2009	Macedonia	L. 1753
C. virgata	10	LR590612	A	2009	UK	Kütz. 1834
C. virgata	39	LR590613	A	2012	Finland	
C. virgata	50	LR590614	A	2012	Germany	
C. virgata	GJ41	MK914604	B	2009	Sweden	
C. virgata	GJ43	MK914605	B	2009	Sweden	
C. virgata	S12	LR590615	A	2009	Norway	
C. virgata	T79	LR590616	A	1992	Norway	
C. virgata	T86	LR590617	A	1929	Norway	
C. vulgaris	MB53	LR590618	A	2001	France	L. 1753
Nitellopsis obtusa		AY170447				(Desvaux in Loisel.) J. Groves 1919

Table 1. List of 53 *Chara* individuals (and one *Nitellopsis obtusa*) used in the present study. "Method" refers to the method used for DNA-sequencing described in DNA barcoding subsection in Materials and Methods. Specimen collected in Dulin pond (S111 and S112) and Sava lake (S110) are marked in bold.

Literature source	Wood and Imahori 1965.	Gollerbah and Krasavina 1983	Krause 1997.	Schubert and Blindow 2004.	Urbaniak and Gąbka 2014.	Mouronval *et al*. 2015.	Dulin Pond sample description
Species name	*Chara globularis* f. *connivens* (Salzm. ex A.Br.) R. D. W. (*C. connivens* Salzm. ex A.Br.)	*Chara connivens* Salzm. ex A.Br.	*Chara connivens* Salzmann ex A. Braun 1835	*Chara connivens* Salzm. ex A.Braun 1835	*Chara connivens* Salzm. ex A.Braun 1835	*Chara connivens* Salzmann ex Braun	*Chara* "*connivens"*
Height	Up to 40 cm	(10)15-25 (40) cm	small, rarely medium size, 15 cm, rarely up to 40 cm high	mostly of small size, 15 cm, rarely up to 40 cm	small and slender plant (5-15 cm), up to 25 cm	(10) 15-30 (40) cm	(8.7) 13-15 (20)
Habitus description	dioecious, slender, delicate green, more or less incrusted	dioecious, light green, slender, weakly branched, yet many shoots from the base, weakly incrusted, still fragile	dioecious, light green, not incrustated, fragile. When removed from water keeping shape.	dioecious,fresh green, not or lightly incrusted	dioecious, yellowish to light green, not or only slightly incrusted	dioecious,the upper parts have whorls of fertile branches, lower parts of talli usually have 5-6 sterile whorls, separated by long internodia. weakly branched, weakly or not incrusted	dioecious, light green, slightly encrusted moderately branched, slender, many shots from the base when fresh, not fragile, moderately tough
Axes							

diameter	up to 0.4 mm	0.5 mm	0.4-0.6 mm		0.3-1.4 mm	0.4-0.6 (0.8) mm	0.35-0.7 mm
Internodes							
1. x branchlet lentht	**1.** 2-4 x branchlet legnth	**1.** up to 6 x branchlet length	**1.**	**1.** up to 6 x branchlet length	**1.** shorter or as long as branches	**1.** 0.5 – 4 branchlet length	**1.** up to 6 x branchlet legth
2. lenght	**2.** up to 5 cm	**2.**	**2.**	**2.**	**2.**	**2.**	**2.** (1) 2 – 6 (8) cm
Cortex	triplostichous, isostichous	triplostichous, isostichous	triplostichous, isostichous	triplostichous, isostichous	triplostichous, isostichous	triplostichous, isostichous	triplostichous, isostichous
Spine cells							
1. description	**1.** rudimentary	**1.** rudimentary, globular, wart like	**1.** inconspicuous, papilliform	**1.** almost lacking, papilliform	**1.** lacking or rudimentary, papilliform	**1.** rudimented or absent	**1.** globular - short conical
2. dimension	**2.**	**2.**	**2.**	**2.**	**2.**	**2.**	2.
Stipulodes							
1. description	**1.** 2 tiers, globular cells, in upper row slightly larger than lowers	**1.** 2 tiers, 2 sets per branchlet, slightly larger cells in upper row	**1.** rudimentary	**1.** papilliform	**1.** 2 tiers, rudimentary almost globular	**1.** 2 tiers, 2 sets per branchlet, cells are subspheric, very weakly developed or indistinct, especially at the lower nodes	**1.** 2 tiers, 2 sets per branchlet. In lower row rudimentary or indistinct, globular. In the upper row elongated and conical shape
2. cell dimensions	**2.**	**2.**	**2.**	**2.**	**2.**	**2.**	**2.**
a) upper row	**a)** 75-150 µm long, 75-105	**a)**	**a)**		**a)**	**a)**	**a)** 50 – 160

	µm wide in the base						(223) µm long, 40 – 100 (120) µm wide in the base
b) lower row	**b)** 60-100 µm, sometimes absent	**b)**	**b)**		**b)**	**b)**	**b)** 40 – 150 µm
Branchlets							
1. number in a whorl and description	**1.** usually 8 in a whorl, strongly incurved	**1.** in male plants brnchlets connivent and shorter in comparison to females 6-10	**1.** 6-10 in a whorl, connivent in male plants, longer in female plants	**1.** in a whorls, 6-8 branchlets, curved inwards in male plants	**1.** 6-9, in male plants branchlets curved inward	**1.** 6-10, branches stiff and in upper parts of talli strongly curved in in male plants, while straight or slightly curved in female plants. In the lower parts of talli, branches are rather divergent in both sexes	**1.** 7-9 (11), in male plants branchlets connivent and shorter in comparison to females where branchlets are straight and longer
2. lenght	**2.** up to 1.4 cm	**2.** 0.5-1.6 cm	**2.**	**2.**	**2.**	**2.**	**2.** (0.6) 1 -3.3 cm
3. number of segments	**3.** 8-10	**3.**	**3.** 6-11	**3.** 8-10	**3.** 6-10	**3** In male plants branches	3. (6) 7 – 10 (13)

						relatively short, strongly curved 6-9 segments, in females long and thin 8-13 segments	
4. cortication of segments	**4.** 7-9 are corticated, end segment ecorticated	**4.** 6-10 corticated and 1-ecorticated	**4.** upper 1-2 ecorticated, short	**4.** upper 1-2 ecorticated	**4.** 6-8 corticated, the last one ecorticated	**4.** 1-3 terminal segments acorticated	**4.** Terminal segment ecorticated
5. terminal segment	**5.**	**5.**	**5.**	**5.**	**5.**	**5.**	**5.**
a) number of cells in terminal segment	**a)** 1-2 cell	**a)** 1-2 cell	**a)** 1-2	**a)** 1-2	**a)** 1-2	**a)**	**a)** 1 -2 cell
b) terminal cell	**b)**	**b)** short, conical, obtuse	**b)** short, ecorticated	**b)** short, ecorticated	**b)**	**b)**	**b)** ecorticated, acuminate
Bract cell	7-8, rudimentary, short conical	rudimentary, 6-8	rudimentary, 5-7		7-8 very short, rudimentary		5 – 6, conical, very short, rudimentary
Bracteole	2-4 in females, 2 in males, well developed, as long as or slightly shorter than oogonia,	2-4 in females, 2 in male plants	2-3, as long as gametangia		papilliform or shorter than oogonia		4 in females, 2 in males; well developed in females, about half as long as or slightly

	acuminate.						shorter than oogonia
Gametangia							
1. single conjoined	**1.** on separate plants, solitary	**1.** solitary	**1.**	**1.**	**1.**	**1.** solitary	**1.** on separate plants, sollitary
2. position at the branchlet	**2.** 3-4 lowest branchlet nodes	**2.** 1-3 lowest branchlet nodes	**2.** 3-4 lowest branchlet nodes	**2.**	**2.** lowest branchlet nodes	**2.** 3-4 upper nodes	**2.** 3-4 lowest branchlet nodes
Oogonia							
1. number in node	**1.** 1, long, elipsoid	**1.** 1, long, elipsoid	**1.**	**1.**	**1.**	**1.**	**1.** 1, usually long, ellipsoid
2. dimensions (length x height)	**2.** 695 µm-690 µm (excl. coronula) x 330 µm -375 µm	**2.** (675) 750 - 1150 µm (excl. coronula) x 320-550 µm	**2.** 650-750 µm (excl. coronula) x 330-400 µm	**2.** 650-1100 µm x 320-550 µm	**2.**605-775 µm x 330-410 µm	**2.** ellipsoid	**2.** 420 – 810 µm (excl. coronula) x 250 – 460 µm
3. number of convolutions	**3.** 14-15	**3.** 13-14	**3.** 14-15	**3.** 13-15	**3.**	**3.**	**3.** 12– 15
4. coronula	**4.** elongated, conical, 225-240 µm x 150-180 µm	**4.** tight, 200-240 µm x 150-185 µm, cells conical, wide in base, narrow on top	**4.** elongated **,** 200-240 µm x 160 µm	**4.**	**4.**	**4.** 200-240 µm, cells converge to form conical tip	**4.** 110 -160 µm x 100-150 µm, cells conical, wide in base, narrow on top
Oospore							
1. color	**1.** dark brown to black, long ellipsoid to cylindrical,	**1.** dark brown to black, long	**1.** dark brown to black	**1.** dark brown to black	**1.** dark brown or black, long, elipsoid to cylindrical	**1.**	**1.** dark brown to black

	terminating in basal claws	cylindrical					
2. dimensions (lenght x height)	**2.** 555-580 µm x 300-320 µm	**2.** 500-770 µm x 240-350 (440) µm	**2.** 500-700 µm x 250-350 µm	**2.** 500-700 µm x 240-350 µm	**2.**485-595 µm x 205-325 µm	**2.**	**2.** 480-550 µm x 270-340 µm
3. ridges description	**3.** faint ridges	**3.** thin	**3.**strong	**3.** weakly distinguishable ridges	**3.**	**3.**	**3.** medium prominent, distinguishable
4. ridges number	**4.** 12-13	**4.** 11-13	**4.12-14**	**4.** 11-14	**4.**	**4.**	**4.** 9-12
5. fossa	**5.** 49 µm	**5.**	**5.**	**5.**	**5.**	**5.**	**5.** 40-60 µm
6. membrane coloration	**6.** dark reddish brown, opaque	**6.** thick, stout, opaque,dark reddish brown	**6.**	**6.**	**6.**	**6.**	**6.** light brown
7. membrane structure	**7.** smooth or minutely granulate	**7.** granulated	**7.**	**7.**	**7.**	**7.**	**7.** smooth
Antheridia							
diameter and description	up to 540-600 µm	(500) 600-700 (1100) µm	600-1000 µm, orange-red		650-1000 µm	0.6-1.1 mm, intense red, 1, rarely in pair	(450) 600-650 (710), intense red / orange

Table 2. Species traits of *Chara connivens*, as described in the most commonly used charophyte determination literature in Europe, and traits measured in 119 (66 male and 53 female) samples from Dulin pond.

Figure captions

Figure 1. Map of the sampling localities.

Figure 2. *Chara "connivens"* from Dulin Pond. a) habitus of male (left) and female plant (right); b) branchlet of the male plant with antheridia; c) branchlet of the female plant with oogonia; d) antheridium; e) oogonium; f) stipulodes; g) triplostichous, isostichous cortex with rudimentary spines. Scale 200 μm.

Figure 3. *Chara contraria* from Sava lake. a) habitus; b) short papillary stipulodes; c) triplostichous cortex, rudimentary spines. Scale 200 μm.

Figure 4. Maximum Likelihood tree of the matK gene of Chara spp. The scale bar indicates 2% sequence divergence. Bootstrap values above 50 are included. Bootstrap values are depicted in the following order: ML/BI/MP/NJ. Sample S110 (in bold) is from Sava lake, and samples S111 and S112 (in bold) are from Dulin pond.

Figure 5. a) Drawings of the unusual *C. connivens* found in the channel near the Silver Lake, Serbia in 1983; b) chromosomes in actively growing cells of antheridial filaments of the same specimens (drawings & cytological analysis done by P.Firbas).