cryptogamie
Algologie
2022 • 43 • 12

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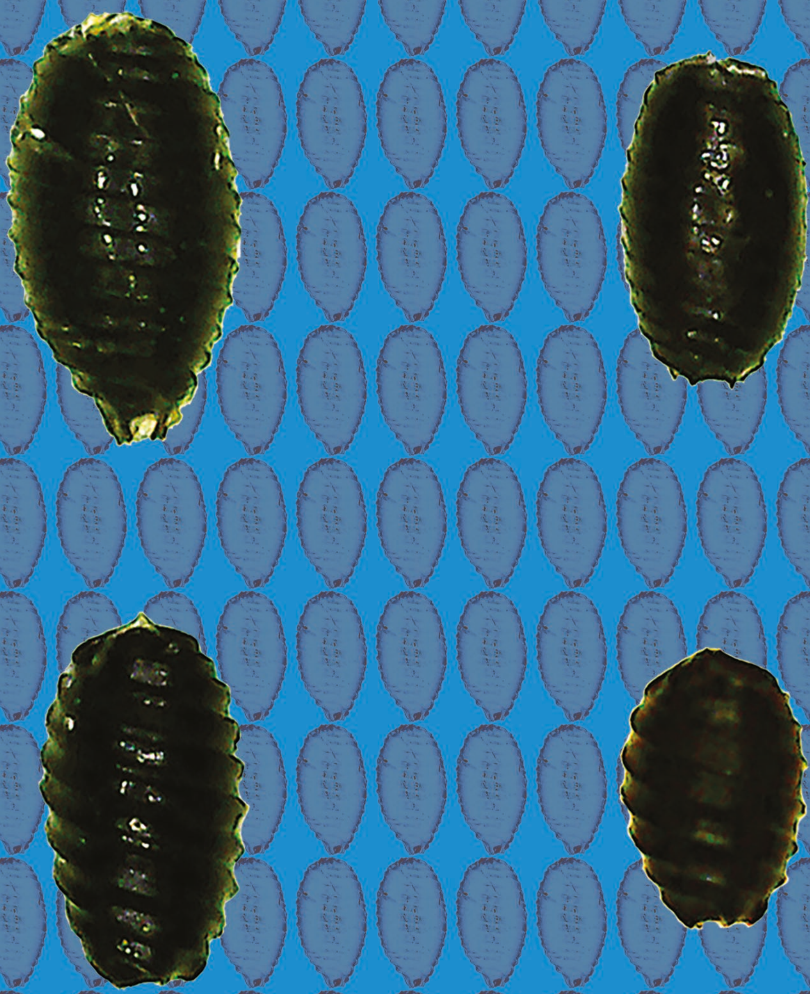

art. 43 (12) — Published on 16 November 2022
www.cryptogamie.com/algologie

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MUSÉUM NAL HIST NATURELLE

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Cryptogamie, Algologie est distribué en version électronique par / Cryptogamie, Algologie *is distributed electronically by:*

- BioOne® (http://www.bioone.org/loi/crya)

Cryptogamie, Algologie est une revue en flux continu publiée par les Publications scientifiques du Muséum, Paris
***Cryptogamie, Algologie** is a fast track journal published by the Museum Science Press, Paris*

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Tél. : 33 (0)1 40 79 48 05 / Fax : 33 (0)1 40 79 38 40
diff.pub@mnhn.fr / http://sciencepress.mnhn.fr
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ISSN (imprimé / *print*) : 0181-1568 / ISSN (électronique / *electronic*) : 1776-0984

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Submitted on 22 December 2021 | Accepted on 16 August 2022 | Published on 16 November 2022

Milovanović V., Popović S., Predojević D., Subakov Simić G., Ržaničanin A., Šinžar Sekulić J. & Trbojević I. 2022. — Oospore features among morphologically similar and closely related charophyte species: consistency and variability. *Cryptogamie, Algologie* 43 (12): 189-200. https://doi.org/10.5252/cryptogamie-algologie2022v43a12. http://cryptogamie.com/algologie/43/12

ABSTRACT
The precise determination of charophytes to the species level is important for many reasons, but the common morphological taxonomic characteristics, particularly between morphologically similar taxa can be insufficient or misleading. The aim of our study was to create a comparative analysis of the oospore's morphometric parameters in morphologically similar taxa such as *Chara globularis* Thuil., *C.* "*connivens*" P.Salzmann ex A.Braun, *C. braunii* C.C.Gmel. and *C. baueri* A.Braun (from Serbia). The potential for applying oospore features in species delineation was assessed, along with the interpopulation variability of oospore parameters in geographically remote populations of *C. globularis* and *C. braunii*. Common oospore parameters (height, width, number of ridges, width of fossa, and distance between the apical pole of the oospore and its widest part) were measured and the isopolarity index and anisopolarity index indices were calculated. Both interpopulation variability and interspecific differences were confirmed using oospore morphology for the selected charophyte species. Although interpopulation variability does in fact exist, oospore parameters clearly differ between morphologically similar taxa. These results are important from a taxonomic perspective, but also from the aspect of diaspore bank characterization and its potential application in restoration projects. The results also justify the need for the development of oospore identification keys at the local and regional level, particularly in the regions where they are completely lacking (e.g. Balkans). Collecting more data on oospore morphometry is necessary for our results to be widely used.

KEY WORDS
Charophytes,
oospores,
species delineation.

RÉSUMÉ

Caractéristiques des oospores parmi des espèces de charophytes morphologiquement similaires et étroitement apparentées : cohérence et variabilité.

La détermination précise des charophytes au niveau de l'espèce est importante sous de nombreux aspects, mais les caractéristiques taxonomiques morphologiques communes dans certains cas, en particulier entre des taxons morphologiquement similaires, peuvent être insuffisantes ou trompeuses. L'objectif de notre étude était de créer une analyse comparative des paramètres morphométriques des oospores de taxons morphologiquement similaires comme *Chara globularis* Thuil. et *C.* "*connivens*" P.Salzmann ex A.Braun, ou *C. braunii* C.C.Gmel. et *C. baueri* A.Braun (Serbie). Le potentiel d'application des caractéristiques des oospores dans la délimitation des espèces a été évalué, ainsi que la variabilité interpopulations des paramètres des oospores dans les populations géographiquement éloignées de *C. globularis* et *C. braunii*. Les paramètres courants des oospores (hauteur des oospores, largeur des oospores, nombre de crêtes, largeur du sillon et distance entre le pôle apical de l'oospore et de sa partie la plus large) ont été mesurés et les indices ISI et ANI ont été calculés. La variabilité interpopulations et les différences interspécifiques ont été confirmées en utilisant la morphologie des oospores chez les espèces de charophytes sélectionnées. Bien que la variabilité interpopulations existe effectivement, les paramètres des oospores étaient clairement différents entre les taxons morphologiquement similaires. Ces résultats sont importants du point de vue taxonomique, mais également du point de vue de la caractérisation des banques de diaspores et de son application potentielle dans les projets de restauration. Les résultats obtenus justifient également la nécessité d'élaborer des clés d'identification des oospores au niveau local et régional, en particulier dans les régions où elles font défaut (e.g. Balkans). La collecte de données supplémentaires sur la morphométrie des oospores est nécessaire pour que nos résultats soient plus largement utilisés.

MOTS CLÉS
Charophytes,
oospores,
délimitation des espèces.

INTRODUCTION

Charophytes are benthic macrophytic algae that can be found in fresh and brackish waters (Soulié-Märsche 2004), where they are recognized as providers of many ecosystem services. They are sensitive to eutrophication and climate change (Blindow 1992; Joye & Rey-Boissezon 2015), and can be used as bioindicators for the eutrophication or ecological status of water bodies (Melzer 1999; Schneider *et al.* 2020; Trbojević *et al.* 2020a). For these reasons, the precise determination of individual to species level is very important, but the common morphological taxonomic characteristics of charophytes can sometimes be insufficient or misleading. Many charophyte species are similar to each other, and their morphological identification requires a lot of expertise, time and patience (Urbaniak & Gąbka 2014), which may still result in misidentification. Trbojević *et al.* (2020a) showed that the monoecious *Chara* L. specimens from Sava lake (Serbia), were initially determined as *C. virgata* Kütz. based on morphological characteristics (cortex, stipulodes and spine cells), but were placed into the *C. contraria* A.Braun ex Kütz. group using DNA barcoding. That same study established that dioecious specimens found in Dulin pond (Serbia), initially determined as *C. connivens* P.Salzmann ex A.Braun based on their morphology, did not form a group with other specimens of *C. connivens*, but formed a separate cluster grouped with monoecious *C. globularis* Thuil. specimens (Trbojević *et al.* 2020a). In this study these particular samples from Dulin pond were used, and from now on they will be noted as *C.* "*connivens*", to avoid misinterpretation. Although DNA barcoding is a sensitive and reliable method for determining genetic differentiation and phylogenetic relationships (Nowak *et al.* 2017), morphological traits are commonly and routinely used as diagnostic tools for species delineation, and every improvement in this field would be beneficial.

The oospore, the resistant, thick-walled zygote of charophytes (Casanova 1997; Blume *et al.* 2009), has features whose description and dimensions are a part of standard determination keys of charophyte species (Migula 1897; Wood & Imahori 1965; Hollerbach & Krassavina 1983; Krause 1997; Urbaniak & Gąbka 2014). Charophytes have a unique phenology and require particular taxonomic expertise, which contributes to the generally sparse information regarding their presence and distribution in aquatic ecosystems. The occurrence of oospores in diaspore banks is constant and can be assessed at any time. Nevertheless, whether oospores could be used for species level identification remains an open question. Their dimensions in determination keys and monographs are often based on an unknown or small number of measurements (Blume *et al.* 2009) and there is often a lack of data on the methods of storing and preparing them for measurements. Moreover, whether oospores are a reliable mean of species identification tools is also challenged due to the detected differences in dimensions at the regional level (Holzhausen *et al.* 2015). However, Mandal & Ray (2004), as well as Boszke *et al.* (2008), showed that the great variability in *C. braunii* C.C.Gmel. thalli characteristics (which resulted in a considerable number of varieties and forms) did not reflect the variability of oospores of this species. This indicates that in some cases oospores may be

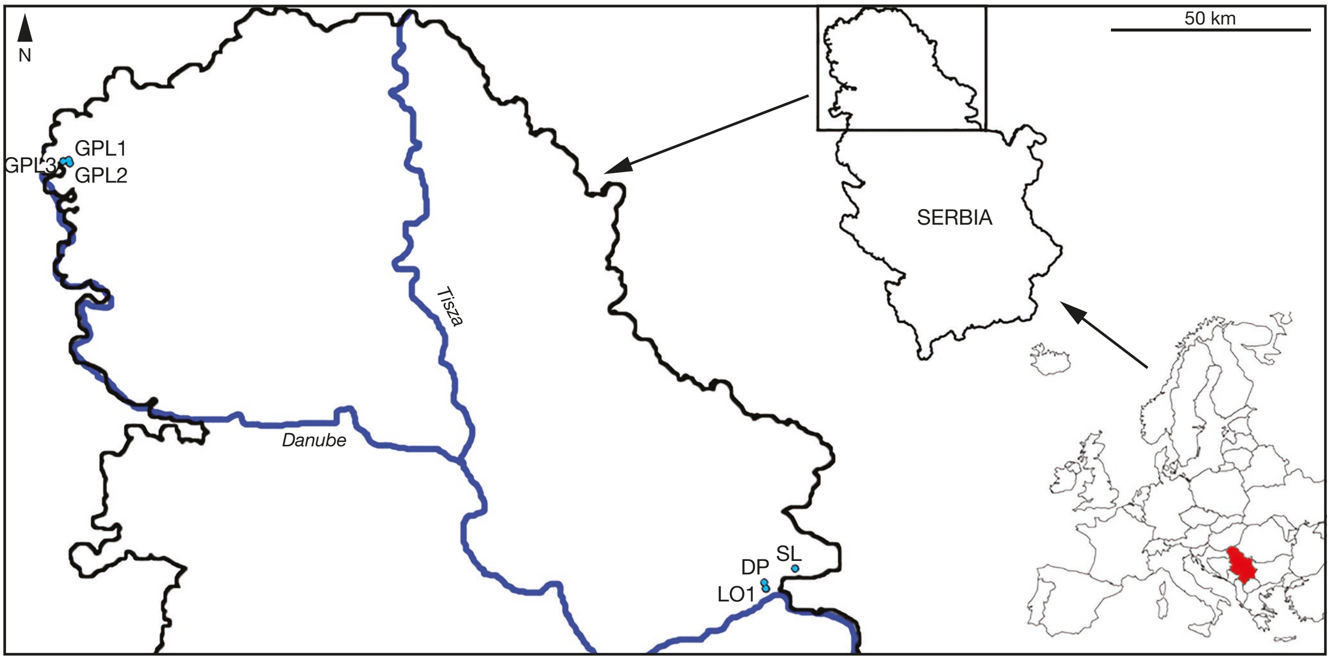

Fig. 1. — Localities investigated in this study. Abbreviations: see Material and methods.

more useful in species determination than the morphology of individuals themselves. The use of oospores for the delineation of taxa that are closely related and morphologically similar was applied on the *Chara baltica* Bruzelius "species complex" showing that the oospore characteristics differ significantly among the taxa. However, variability was high among populations belonging to the same taxon and even among individuals belonging to the same population (Blume *et al.* 2009). More studies on oospores are needed, both regionally and globally, to demonstrate their potential capacity for reliable species delineation, particularly between morphologically similar species.

Chara globularis and *C. connivens* are closely related taxa, morphologically very similar to each other, but differing in that *C. globularis* is monoecious and *C. connivens* dioecious (Urbaniak & Gąbka 2014). However, delineation of sterile specimens is problematic. *Chara baueri* A.Braun and *Chara braunii* are very similar species, differing only in the former having cortex and spine cells. Misidentification of these species is highly probable, since in some *C. baueri* specimens the cortex is poorly developed and can be found only in a few upper or even just one internode (Trbojević *et al.* 2020b and references therein). *Chara baueri* and *Chara braunii* are assumed to be closely related (Krause 1997; Urbaniak & Gąbka 2014). The aim of this study was to analyze and compare the morphometric oospore parameters between the two pairs of morphologically very similar charophyte species: *C. globularis* and *C.* "*connivens*" and *C. braunii* and *C. baueri.* We also analyzed the interpopulation variability of oospore parameters in geographically remote *C. globularis* and *C. braunii* populations. The potential of applying oospores and their features in species delineation by means of unsupervised machine learning was also assessed.

MATERIAL AND METHODS

The charophyte samples analyzed in this study were collected in the Special Nature Reserve (SNR) Gornje Podunavlje (northwest of Vojvodina, the northern province of Serbia) and in the vicinity of Bela Crkva (southeast of Vojvodina) during the vegetative season (June-August) in 2017, 2018 and 2019. The investigated sites located in the territory of SNR Gornje Podunavlje were three field ponds labeled as GPL1 (45°48'45.99"N, 18°57'38.098"E), GPL2 (45°48'45.277"N, 18°57'38.826"E) and GPL3 (45°48'52.999"N, 18°56'51"E), and Šljunkara lake in the vicinity of Bela Crkva (SL) (44°53'5.021"N, 21°23'42.309"E), Dulin pond (DP) (44°51'12.348"N, 21°17'52.296"E) and the pond in Labudovo okno (LO1) (44°50'23.316"N, 21°18'3.131"E). These last two are part of the Ramsar site Labudovo okno (Fig. 1). All three ponds in SNR Gornje Podunavlje were small and shallow field ponds (less than 0.0025 ha) and prone to drying, although complete drying was not recorded during the study period. All waterbodies in the vicinity of Bela Crkva were permanent and considerably larger (at least about 1 ha) compared to the ones in SNR Gornje Podunavlje. Each time the charophytes were sampled, the environmental parameters: temperature (T), pH, oxygen concentration (O_2 mg/L) and saturation (O_2 %), and conductivity, were measured *in situ*, using digital field instruments made by Eutech Instruments Oakton® and YSI ProODDO Optical Dissolved Oxygen Meter. Simultaneously, water samples from all localities were taken and further analyzed in the Institute for public health "Dr Milan Jovanović Batut" according to the standard analytical procedures (APHA 1995). The water chemistry parameters analyzed were: the degree of General Hardness of water (°dH), calcium ions concentration (Ca^{2+}), magnesium ions concentration

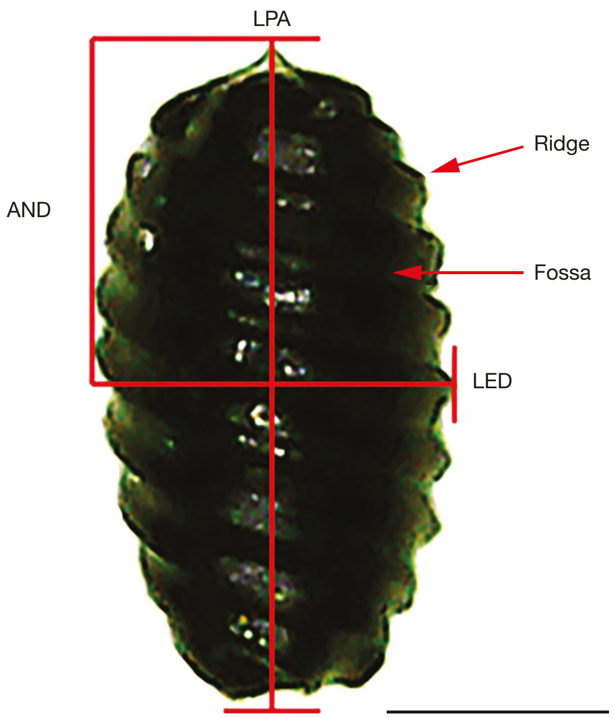

Fig. 2. — Oospore parameters. Abbreviations: see Material and methods. Scale bar: 200 µm.

(Mg^{2+}), nitrites (NO_2^-), nitrates (NO_3^-), ammonia (NH_4^+), total nitrogen (TN), orthophosphates (OP), total phosphates (TP). Measurements of environmental parameters and water collection for further analyses were always conducted between 10 am and 12 pm.

Charophyte specimens were identified using standard literature (Migula 1897; Wood & Imahori 1965; Hollerbach & Krassavina 1983; Krause 1997; Urbaniak & Gąbka 2014). Specimens without gametangia were immediately discarded. Mature oospores were mechanically collected from the previously identified individuals using forceps. Oospores were collected from 15 plants of *C. globularis*, 12 plants of *C.* "*connivens*", 22 plants of *C. baueri*, and ten plants of *C. braunii*. In order to clean the oospores, they were left in 30% H_2O_2 for 15 minutes as recommended by Holzhausen *et al.* (2015). This process made it easier to remove the spiral enveloping cells mechanically, using two pins, under a Zeiss Stereo Microscope STEMI DV4. The oospore parameters, height (LPA), width (LED), number of ridges, width of fossa and distance between the apical pole of the oospore and the LED parameter (AND), were measured on wet oospores using a Nikon YS100 light microscope (Fig. 2). The ISI (isopolarity index) and ANI (anisopolarity index) were calculated based on the measured values of LPA, LED and AND parameters, by the following formulas:

$$ISI = LPA/LED \times 100$$
$$ANI = AND/LPA \times 100$$

For each species, several micrographs of the most representative oospores were taken using a digital USB microscope camera. In order to describe membrane structure, a Carl Zeiss AxioImager M1 microscope and a digital camera AxioCam MRc5 with AxioVision 4.8 software were used.

In order to determine whether there are statistically significant differences in the measured oospore parameters between the analyzed populations of *C. globularis*, *C.* "*connivens*", *C. baueri* and *C. braunii*, a Kruskal-Wallis H test followed by Dunn's test for pairwise multiple-comparison, was performed in PMCMRplus and FSA R packages respectively (Pohlert 2021; Ogle *et al.* 2021). Unsupervised machine learning methods were used to estimate the potential of applying oospores and their features in species classification, and in some populations more than one sample of oospores was collected. Agglomerative hierarchical clustering by Ward's method, using the Euclidean distance as a measure of dissimilarity was done (Blume *et al.* 2009). The optimal number of clusters was determined using NbClust R package (Charrad *et al.* 2014). Distance matrix computation, as well as hierarchical clustering were done using R Statistical Computing Language (R Core Team 2021).

Multivariate analyses were performed using Canoco 5 for Windows (Ter Braak & Šmilauer 2012). Two RDAs were used to demonstrate oospore parameters in relation to sampling localities for *Chara globularis* and *Chara braunii* separately. The locality was used as an explanatory variable in both analyses. Three additional redundancy analyses (RDA) were performed to observe the relationship between oospore parameters (AND, ANI, ISI, LED, LPA, number of ridges, width of fossa) and nominal explanatory variables that refer to species for which these parameters were measured: 1) *Chara globularis* from all localities and *Chara* "*connivens*"; 2) *Chara globularis* from Dulin pond only and *Chara* "*connivens*"; and 3) *Chara baueri* and *Chara braunii* from all localities.

Abbreviations

Oospore parameters

- AND — distance between the apical pole of the oospore and the LED parameter;
- ANI — anisopolarity index;
- ISI — isopolarity index;
- LED — oospore width;
- LPA — oospore height.

Localities

- DP — Dulin pond;
- GPL — SNR Gornje Podunavlje;
- LO1 — pond in Labudovo okno;
- SL — Šljunkara lake.

RESULTS

Measured physical and chemical parameters of water and their variability among the investigated localities are presented in Table 1.

Generally, localities in the vicinity of Bela Crkva (SL, DP and LO1) were characterized by higher values of water hardness, Ca^{2+} and Mg^{2+} concentration, and TP in comparison to the localities in SNR Gornje Podunavlje (GPL1, GPL2 and GPL3), where nitrogen compounds (NH_4, NO_2, TN) had markedly higher concentrations.

Specimens of *Chara* "*connivens*" were found in only one of the searched localities (DP) in the vicinity of Bela Crkva, and *Chara baueri* in two different (GPL1 and GPL2) but very closely related localities in SNR Gornje Podunavlje. *Chara globularis* and *Chara braunii* specimens were collected in a few sites, including geographically remote localities (*C. globularis*: GPL1, SL and DP; *C. braunii*: GPL3 and LO1) (Fig. 1).

Representative oospores from the pool analyzed for each species are presented in Figure 3, showing the characteristic shape and color. The color of the oospores was characterized using a numerical assessment of color (RAL Deutsches Institut für Gütesicherung und Kennzeichnung e.V.).

Average values of pooled data on oospore parameters for each of the four analyzed taxa, together with the results of the Kruskal-Wallis H test and Dunn's test for multiple comparisons are given in Table 2. The Kruskal-Wallis H test has shown that the analyzed taxa were statistically different from each other in terms of the oospores' parameters.

Although the results of a non-parametric ANOVA presented in Table 2 are clear, RDA results are plotted below.

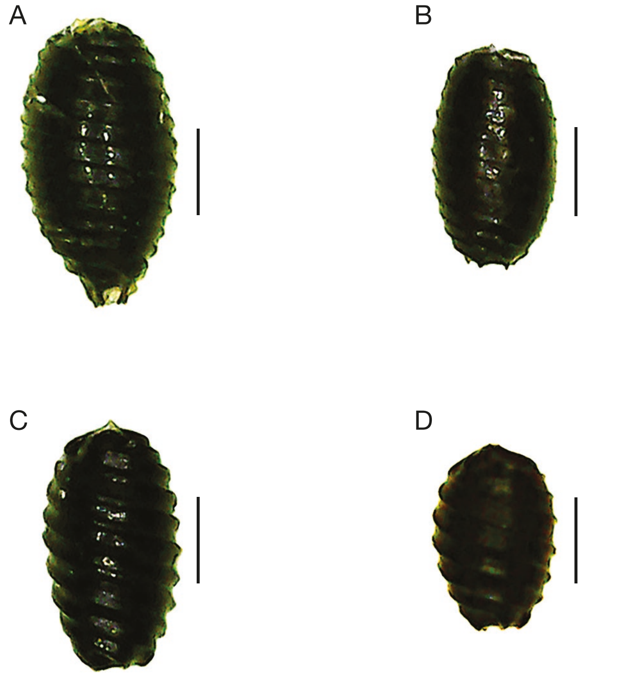

Fig. 3. — Oospores of selected charophyte species: **A**, *Chara globularis* Thuil.; **B**, *Chara* "*connivens*" P.Salzmann ex A.Braun; **C**, *Chara baueri* A.Braun; **D**, *Chara braunii* C.C.Gmel. Scale bars: 200 μm.

Chara globularis

Oospores of *Chara globularis* analyzed in this study were on average prolate (elongated) and ellipsoidal. The color varied from dark brown to black – from approximately RAL 8022 (black-brown), through RAL 9004 (signal black) to RAL 9005 (jet black) (Fig. 3A). The membrane of the oospore was brownish and slightly granular. A total number of 94 oospores was measured and included in the analysis – 54 from the vicinity of Bela Crkva (DP and SL) and 40 from the SNR Gornje Podunavlje (GPL1).

Oospore parameters of *Chara globularis* in relation to localities where this species was found are shown in Figure 4. The analysis (F = 2.1, P = 0.002) described 32% variability in our data. The sampling localities are clearly separated, as can be seen on the ordination diagram. Oospore parameters of *Chara globularis* are clearly negatively correlated with GPL1 and oriented toward the left side of the ordination diagram showing a positive correlation with DP and SL localities. More precisely, higher values of the width of the fossa, (ISI and LPA) were related to SL, while higher values of LED, ANI, number of ridges and AND were connected to the DP locality. Multivariate analysis clearly highlighted intraspecific (i.e., interpopulation) variability of *C. globularis* oospore parameters, pointing out that all parameters had higher values in specimens found in vicinity of Bela Crkva (SL and DP) in comparison to the remote locality in SNR Gornje Podunavlje (GPL1). The variability of measured parameters was clearly visible even between populations from geographically close localities (SL and DP).

Chara "*connivens*"

Oospores of *Chara* "*connivens*" in this study were on average elongated (prolate) and ellipsoidal in shape. Their color was dark brown to black – from approximately RAL 8019 (grey brown), through RAL 8022 (black brown) to graphite black (RAL 9011) (Fig. 3B). The membrane was opaque, light brown in color, and completely smooth. A total number of 62 oospores were included in the analysis, all of them from the vicinity of Bela Crkva (DP).

Comparative analysis of oospores of *Chara globularis* and *Chara* "*connivens*"

Comparing measured oospore parameters between samples of *C. globularis* from all localities and *C.* "*connivens*" (Fig. 5A), it is clear that almost all parameters correlated with *Chara globularis* – meaning that higher values of these oospore parameters are observed in this species. Only ANI showed positive correlation with *Chara* "*connivens*". This analysis described 54% variability in our data, and was significant (F = 18.4, P = 0.002).

The other analysis also compared measured oospore parameters between samples of *C. globularis* and *C.* "*connivens*", but it was conducted with data for *Chara globularis* from the DP locality only (Fig. 5B), because in this locality *C. globularis* and *C.* "*connivens*" were found together and the morphological similarity between sterile specimens was extremely high. Results of this analysis were almost the same as in the one above, only the ISI index showed no clear correlation with either of the species. This analysis described 72% variability in the data and was also significant (F = 24.6, P = 0.002). Presented analyses (Fig. 5A, B) confirm that although intraspecific (interpopulation) variability of oospore parameters exist in *C. globularis*, when compared to *C.* "*connivens*", the difference between species is clear and uniform.

Table 1. — Physical and chemical properties of water in investigated localities. When multiple measurements, values were averaged.

Locality		T (°C)	pH	EC (µS/cm)	O_2 (%)	O_2 (mg/l)	N-NH_4 (mg/l)	N-NO_2 (mg/l)	N-NO_3 (mg/l)	P-PO_4 (mg/l)	TP (mg/l)	TN (mg/l)	Hardness dH	Ca^{2+} (mg/l)	Mg^{2+} (mg/l)
SNR Gornje Podunavlje	GPL1	30.8	9.1	358	176	13.7	0.24	0.016	0.25	0.01	0.03	2.20	1.85	6.2	4.2
	GPL2	26.1	8.4	435	77	6.2	0.18	0.010	0.25	0.01	0.05	3.00	4.40	21.8	5.9
	GPL3	33.3	9.0	319	206	15.2	0.27	0.006	0.25	0.01	0.04	2.47	7.05	28.6	13.5
Bela Crkva	SL	19.0	8.7	350	142	13.2	0.06	0.010	1.10	0.01	0.07	1.3	12.30	59.0	18.0
	DP	26.2	9.6	226	128	11.2	0.16	0.004	1.13	0.10	0.18	1.76	5.85	16.0	15.5
	LO1	26.6	8.7	244	133	10.6	0.06	0.001	0.05	0.005	0.12	1.86	6.70	25.0	14.0

Table 2. — Mean values of analyzed oospore parameters with the summary of Kruskal-Wallis H test results. Group averages and their standard errors followed by the same letter are not significantly different from each other according to the Dunn's test. **n** is the number of oospores measured and included in the analysis. Abbreviations: see Material and methods.

	LPA	LED	Number of ridges	Width of fossa	AND	ISI	ANI
Kruskal-Wallis H test	X^2 = 255.26 p < 0.00001	X^2 = 192.25 p < 0.00001	X^2 = 276.73 p < 0.00001	X^2 = 142.89 p < 0.00001	X^2 = 233.55 p < 0.00001	X^2 = 102.96 p < 0.00001	X^2 = 67.354 p < 0.00001
Chara baueri (n = 127)	535.47±2.87^{b}	282.36±2.20^{a}	7.75±0.04^{b}	61.22±0.37^{b}	249.17±2.57^{b}	190.84±1.59^{c}	46.5±0.38^{a}
Chara braunii (n = 48)	457.71±4.22^{a}	292.92±4.17^{a}	7.21±0.06^{a}	65.1±0.82^{c}	215.21±3.09^{a}	157.2±1.80^{a}	47.06±0.60^{ab}
Chara "*connivens*" (n = 62)	528.06±3.52^{b}	292.26±2.38^{a}	10.45±0.09^{c}	51.21±0.64^{a}	271.13±2.65^{c}	181.38±1.85^{b}	51.34±0.35^{c}
Chara globularis (n = 94)	694.57±4.72^{c}	377.98±3.38^{b}	11.52±0.10^{d}	59.63±0.63^{b}	340.32±3.6^{d}	184.79±1.77^{bc}	48.99±0.37^{b}

Chara braunii

Oospores of the *Chara braunii* analyzed in this study were ellipsoidal in shape and elongated (prolate). Their color was mostly dark brown, to black – from RAL 8016 (mahogany brown) to RAL 8022 (black-brown), rarely RAL 9011 (graphite black) (Fig. 3D). The membrane was light brown and granular. A total number of 48 oospores was measured and included in the analysis, 28 from the vicinity of Bela Crkva (pond LO1) and 20 from the SNR Gornje Podunavlje (GPL3).

Oospore parameters of *Chara braunii* in relation to localities where this taxon was recorded are shown in Figure 6. Analysis described 22% variability in our data, and was significant (F = 1.3, P = 0.002). Variability between populations in remote localities was detectable, and all oospore parameters (except ANI and ISI) correlated with the locality in the vicinity of Bela Crkva (LO1), meaning that the oospores from this locality were distinctively longer and wider, with wider fossa. ISI showed positive correlation only with the locality in SNR Gornje Podunavlje (GPL3), while ANI did not show correlation to any of the sites.

Chara baueri

Oospores of *Chara baueri* measured in this study were ellipsoidal in shape, elongated (prolate) to strongly elongated (perprolate) and black – from RAL 9011 (graphite black) to RAL 9005 (jet black) (Fig. 3C). The membrane was brownish and finely granulated. In total, 127 oospores were measured and included in the analysis, all of them from the SNR Gornje Podunavlje (GPL1 and GPL2). A detailed description of the oospores of this species is available in Trbojević *et al.* (2020b).

Comparative analysis of oospores of *Chara baueri* and *Chara braunii*

In Figure 7, *Chara baueri* and *Chara braunii* are shown in relation to the measured oospore parameters. The analysis (F = 6.3, P = 0.002) described 27% variability in our data. Oospore parameters AND, LPA, ISI and number of ridges were positively correlated with *Chara baueri*, while LED and width of fossa showed positive correlation with *Chara braunii*, meaning that *Chara baueri* is characterized by longer, somewhat narrower, and more prolate oospores compared to those of *C. braunii*. Also in *C. baueri* the number of ridges was higher and they were more compactly arranged (narrower fossa). The correlation of ANI was almost nonexistent when both species were considered.

Cluster analyses of oospore parameters

Cluster analysis results presented in Figure 8 confirm the potential of applying oospores and their features in species delineation by means of unsupervised machine learning.

The results of hierarchical clustering that was applied on the sample median values for all oospore parameters also confirmed the separation of samples into four separate clusters that matched individual taxa (Fig. 8).

DISCUSSION

Our results show that although variability within one species populations in fact exists, oospore parameters are clearly distinctive between the morphologically similar taxa analyzed in this study.

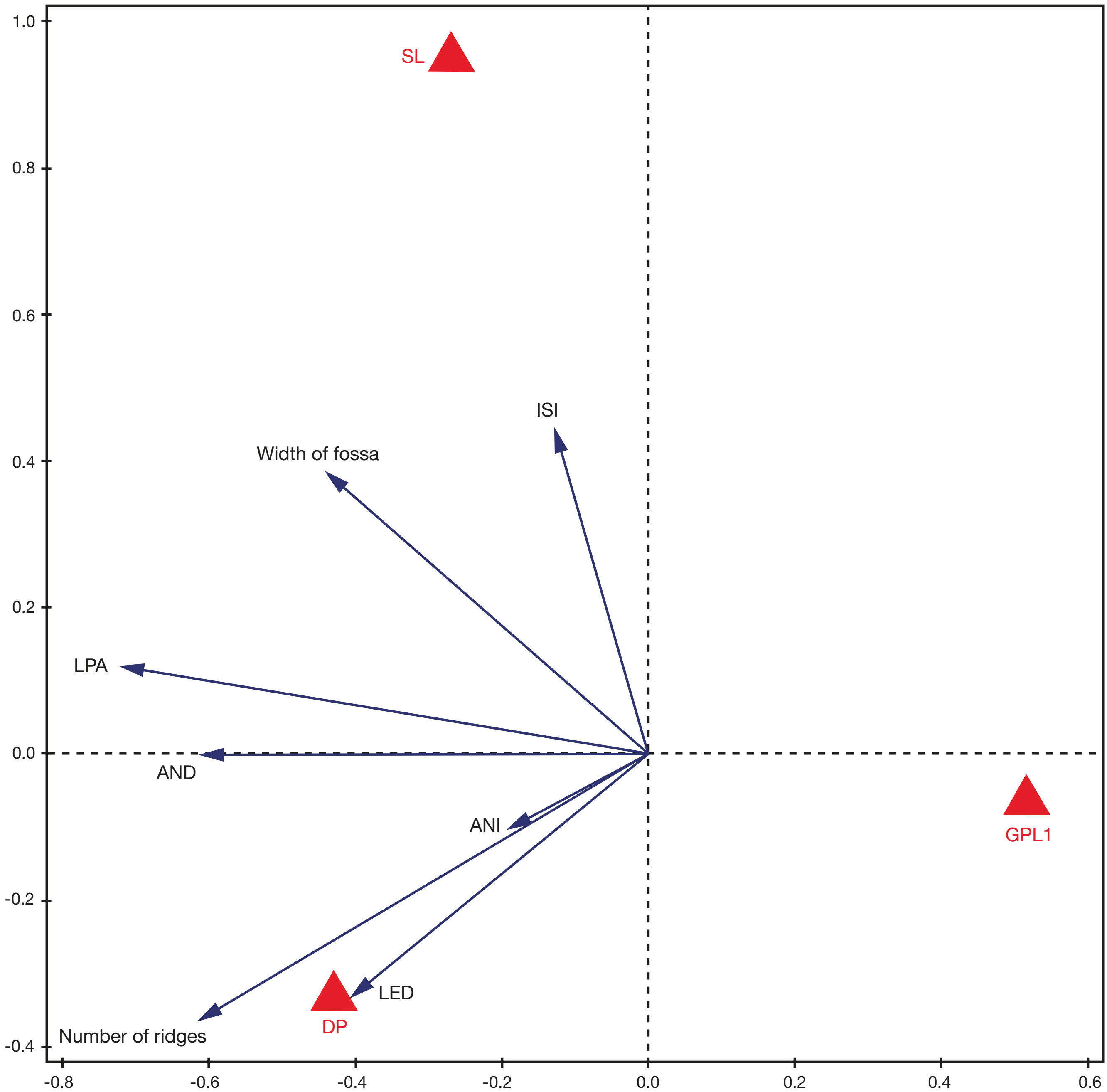

Fig. 4. — Oospore parameters of *Chara globularis* Thuil. in relation to localities where this species was found. Redundancy analysis (RDA). Abbreviations: see Material and methods.

In our study, interpopulation variability of oospores parameters was detected in the case of both *C. globularis* and *C. braunii* (Figs 4; 6). This is not surprising, considering the results from Blume *et al.* (2009), who showed that the variations in oospore parameters among populations of the same taxon were high when considering *Chara baltica* Bruzelius "species complex". Pukacz *et al.* (2012) pointed out that the data describing the relationship between oospore morphometry and habitat conditions are usually lacking in literature, but that they are most likely responsible for interpopulation differentiation. Our results showed that oospores of *C. globularis* differed in populations from SL, DP and GPL1 (Fig. 4), where the water chemistry also differed (Table 1). Populations of *C. braunii* also showed differences in oospore parameters in geographically remote localities – measured values were higher in LO1, a permanent fluvial pond with high TP concentration, in comparison to GPL3, a small field pond characterized by high concentrations of nitrogen compounds (Table 1). Although the physical and chemical parameters of water are not the only ecosystem features responsible for observed interpopulation variability, our data represent a valuable contribution to the interpretation and potential explanation of this phenomenon. Casanova (1997)

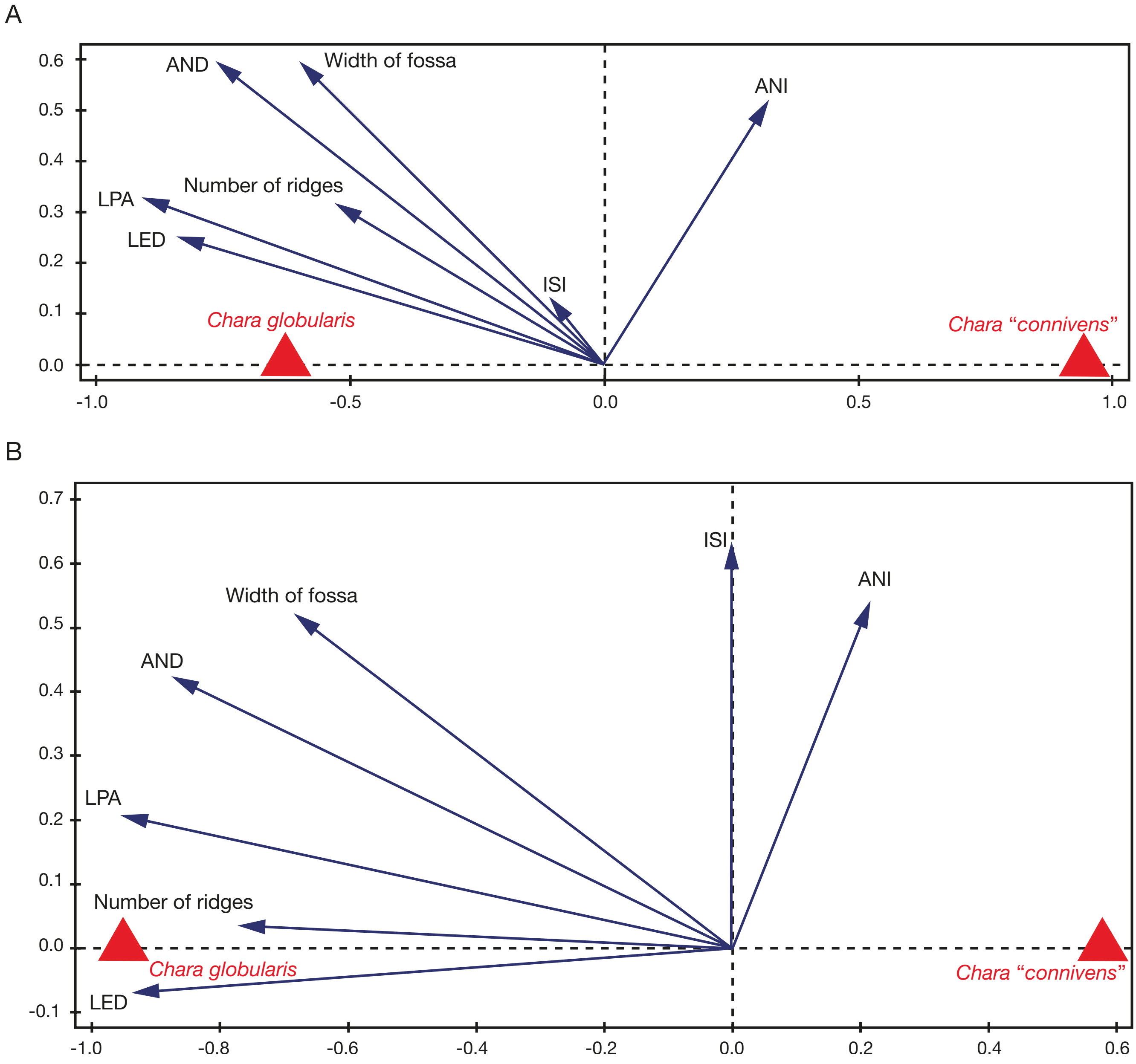

Fig. 5. — The relationship between oospore parameters of *Chara* "*connivens*" P.Salzmann ex A.Braun and *Chara globularis* Thuil., and nominal variable referring to these two species: **A**, considering all sampling localities; **B**, referring to these two species considering Dulin pond locality only. Redundancy analysis (RDA). Abbreviations: see Material and methods.

suggested that oospores of charophytes in temporary habitats might be subject to the strong influence of natural selection for size, thus leading to uniformity of the oospores in the populations inhabiting such habitats, relative to the species in permanent habitats, where the oospore size is expected to be more variable. We couldn't establish a clear difference in oospore variability between populations of *C. globularis* and *C. braunii* in temporary and permanent habitats (or between investigated species), but our results showed that oospores of both species were overall smaller in small and shallow habitats prone to drying in the SNR Gornje Podunavlje (Figs 4; 6) in comparison to the permanent and larger water bodies in the vicinity of Bela Crkva. Further investigations should be undertaken to determine the relative roles of environmental parameters and hydrological regime in water bodies on oospore variation within charophyte populations.

Although the intraspecific variation in *C. globularis* oospores exists (Fig. 4), when its oospores are compared to those of *C.* "*connivens*", the difference between these two species is clear and uniform (Fig. 5A, B). Urbaniak & Gąbka (2014) stated that *C. globularis* is the only species similar to *C. connivens*. Although some differences exist on the morphology of branches, bract cells and bracteoles, if the individuals of these two species inhabit the same water body (as in our research, in Dulin pond) and are sterile (*C. globularis* is monecious and *C. connivens* dioecious), they cannot be distinguished. As previously mentioned, we used a specific freshwater *Chara* "*connivens*" (typically *C. connivens*

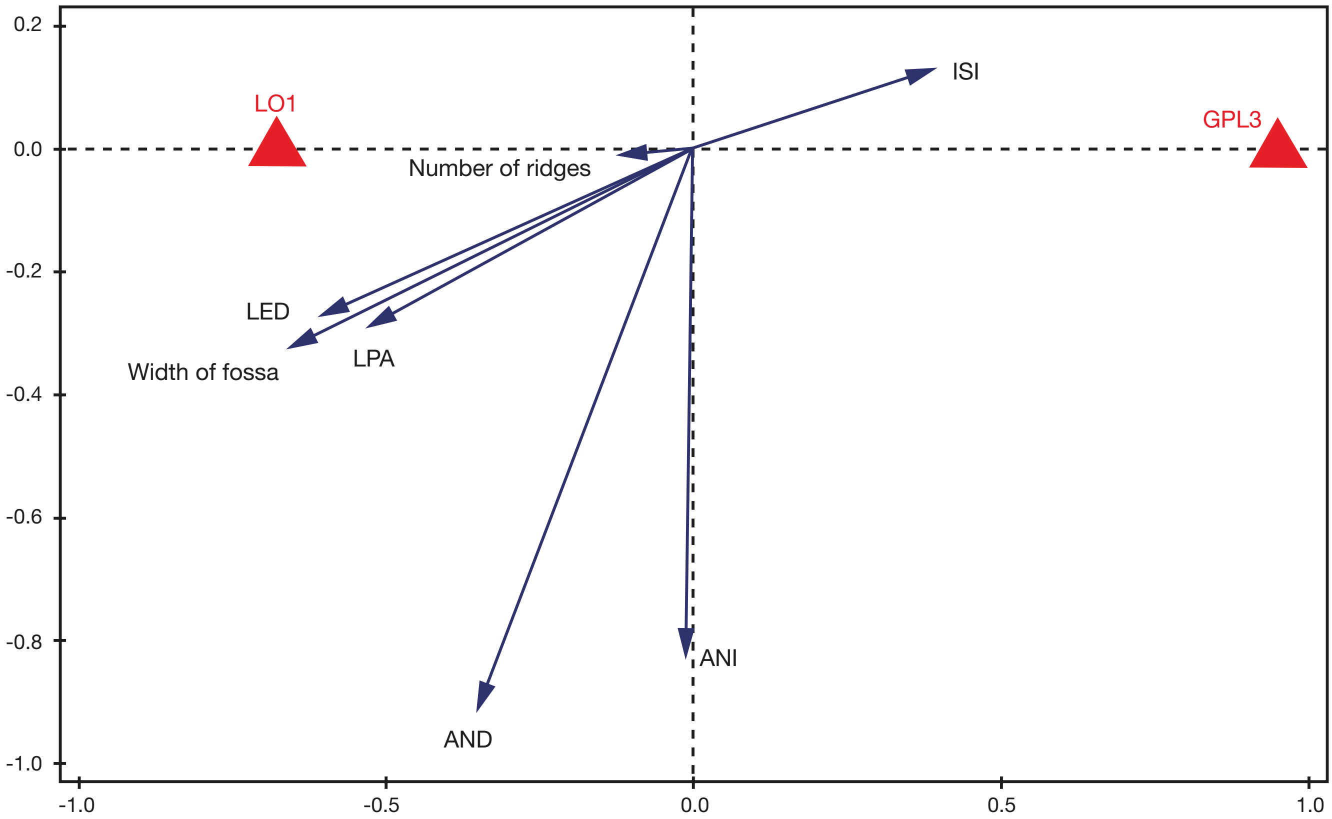

Fig. 6. — Oospore parameters of *Chara braunii* C.C.Gmel. in relation to localities where this species was found. Redundancy analysis (RDA). Abbreviations: see Material and methods.

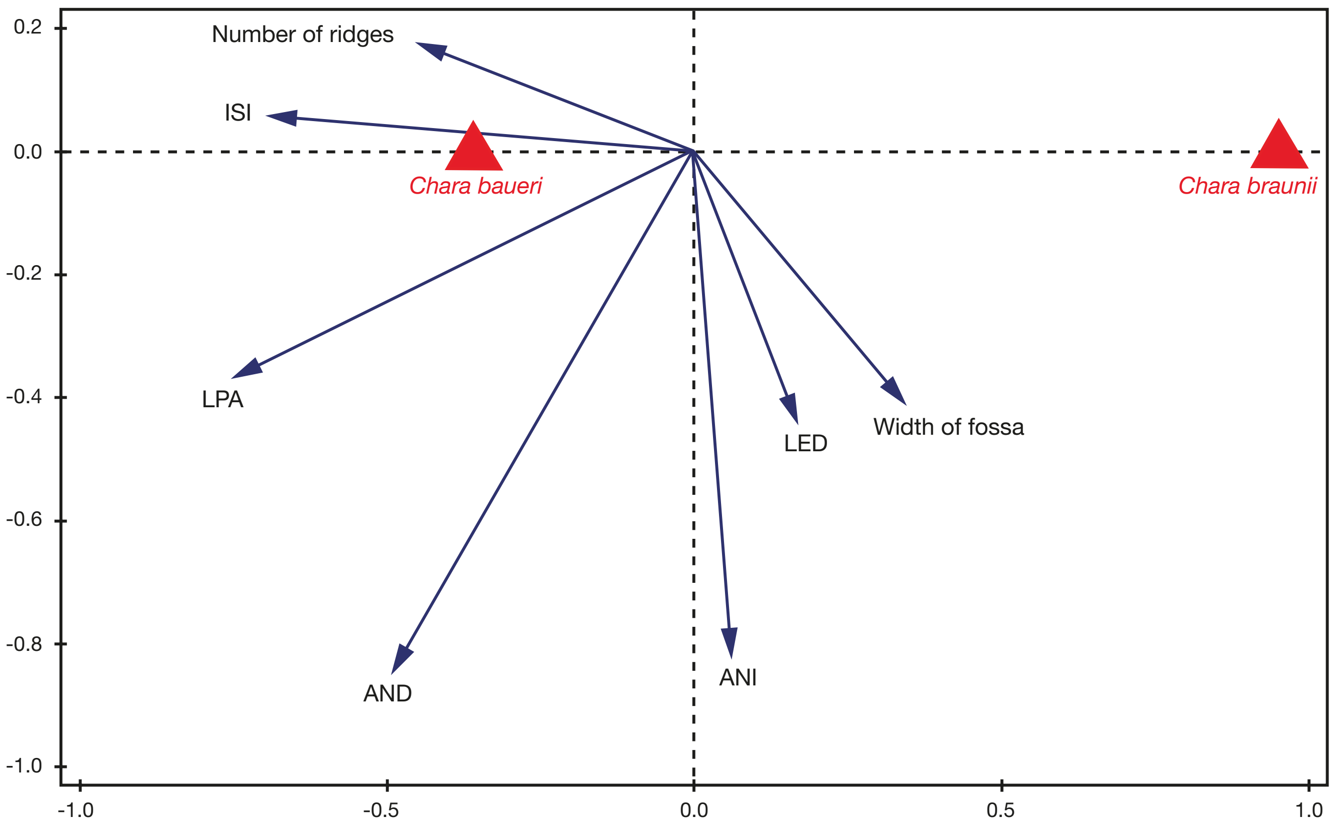

Fig. 7. — The relationship between oospore parameters of *Chara baueri* A.Braun and *Chara braunii* C.C.Gmel., and variable that represents these two species from all sampling localities. Redundancy analysis (RDA). Abbreviations: see Material and methods.

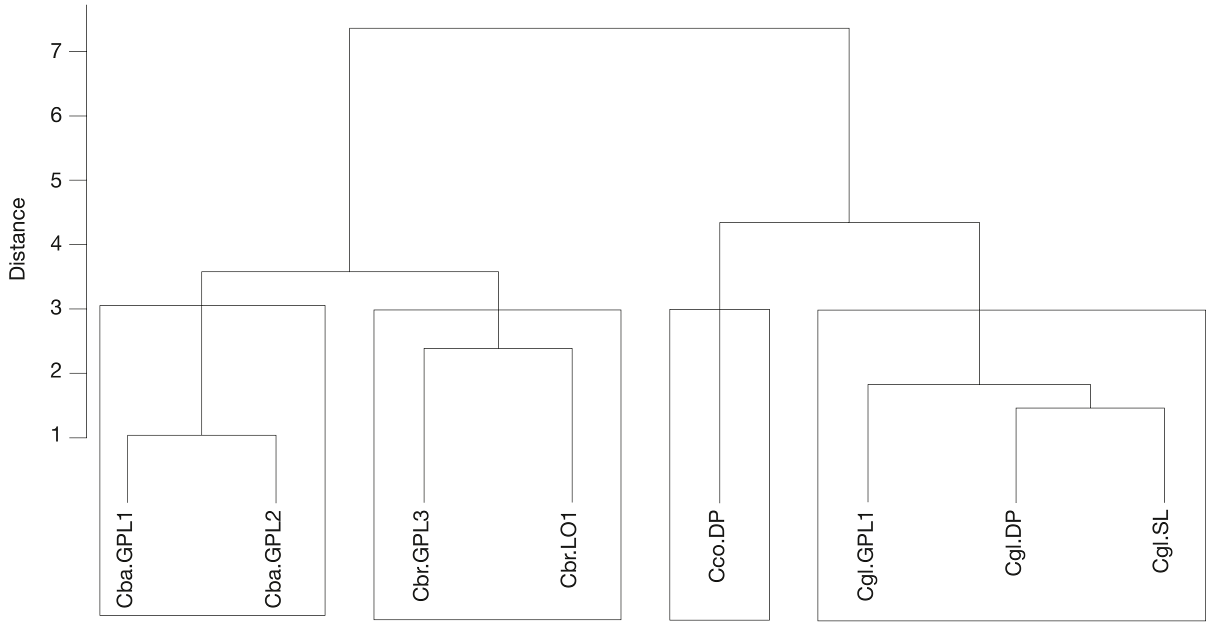

Fig. 8. — Hierarchical cluster dendrogram showing Euclidian distances of sample median values for oospore parameters. Species: **Cba**, *C. baueri* A.Braun; **Cbr**, *C. braunii* C.C.Gmel.; **Cco**, *C.* "*connivens*" P.Salzmann ex A.Braun; **Cgl**, *C. globularis* Thuil.

is a brackish species) from Dulin pond whose status has yet to be determined, since it formed a separate cluster grouped with monoecious *C. globularis* specimens using barcoding of *matK* (Trbojević *et al.* 2020a). Despite the aforementioned grouping with *C. globularis*, the results of our study show that there is a distinctive difference between *C. globularis* and *C.* "*connivens*" on the oospore level, meaning that these species could possibly be distinguished from oospores in a diaspore bank, even if only sterile (or no) specimens would be found in the field study. The question of whether differentiation of oospore features *C.* "*connivens*" from Dulin pond and regular *C. connivens* is possible remains unanswered.

A comparative analysis of oospores of *Chara baueri* and *Chara braunii* also suggests that the oospores of these two morphologically similar species could be separated – oospores of *C. baueri* were longer, narrower, and more prolate, with more ridges and a narrower fossa than those of *C. braunii* (Fig. 7). According to Krause (1997), *C. braunii* and *C. baueri* are closely related taxa, although this has still not been confirmed/refuted using modern methods such as DNA barcoding, probably due to the rarity of *C. baueri*. While *C. braunii* has been described as a cosmopolitan species (Krause 1997), *C. baueri* is considered to be one of the rarest charophytes in the world (Trbojević *et al.* 2020b and references therein). *C. baueri* distribution is still being updated, with only a few known localities in Europe and Asia (Trbojević *et al.* 2020b and references therein). Contrary to our results, Soulié-Märsche (1989) described oospores of these two species as morphologically similar. However, her conclusions are based primarily on the comparison of qualitative characteristics (e.g. sporostine appearance, apical and basal pole similarity), while we present the importance of biometric characteristics, without neglecting the qualitative ones (such as the general appearance of the oospores and their shape and color). In Serbia, these two species have been found at the same locality – a small field pond (Trbojević *et al.* 2020b). Bearing in mind the aforementioned similarity of these taxa and thus the high possibility of misidentification, the need for a complete description of the distinguishing features is emphasized – including the differences on the oospore level. Our study is the first one describing these differences and proving the potential to distinguish these morphologically similar species using oospores only.

During her attempt to determine the level of oospore variation within species and populations of three species of genus *Chara*, Casanova (1997: 278) concluded that "in many cases, oospores alone can be used to distinguish species of *Chara*" which is especially important if the oospores are the only material that is available for study, "as in seed bank or paleobotanical sediment studies". On the other hand, Blume *et al.* (2009: 999) do not share this optimistic opinion, as their results indicate that "a distinction among closely related taxa by means of oospores alone may turn out to be impossible within the genus *Chara*". Still, our results (although obtained upon the limited number of samples all originating from Serbia) clearly confirm the potential of applying oospores and their features in species delineation by means of unsupervised machine learning (Fig. 8). The relatively low intraspecific variability found in our study, compared to other investigations which cover a larger geographical region (Casanova 1997; Blume *et al.* 2009) could fit in Proctor's context of the declining trend of cross-fertility within the same species with increasing distance. This ultimately raises the question to what extent are the geographically remote populations really conspecific (Proctor 1975 and references therein). We suggest changing perspectives when perceiving spatial components, i.e., not looking at Europe as a whole, but as separate regions corresponding to geographic, geologic and climate conditions (because, for the sake of illustration, according to Schneider *et al.* 2013 and references therein, Europe is divided in as much as 35 different climate areas). Few identification keys for charophyte oospores have already been developed on a regional scale (e.g. Haas (1994) for Central Europe; Vedder (2004) for the Baltic Sea; de Winton *et al.* (2007) for New Zealand). Holzhausen *et al.* (2015) also confirmed that there are differences in oospore dimensions at the regional level. Similarity patterns considering environmental features should be followed and locally suitable oospore identification keys that will have a practical purpose in identification of oospores and improving their usage as a restoration measure should be developed. Of course, far more information on oospore morphometry is needed, from both local and regional scales, so that our results and suggestions could be proved and applicable.

Our results contribute to the more precise description of the oospores of a selected charophyte species in Serbia (Vojvodina), but also indicate the potential of the oospores in distinguishing species similar in their thalli characteristics. Diaspore bank surveys are another major reason why the potential of oospore based charophyte identification should be further explored. Diaspore banks consist of seeds and charophyte oospores trapped in sediment, and are seen as a potential maintainer of plant diversity (Ferreira *et al.* 2016). They also offer the possibility for the recovery of native diversity (Nishihiro *et al.* 2006; Amano *et al.* 2008) and assess the changes in species composition over time (Lu *et al.* 2012). Charophyte oospores can be the numerically dominant constituents of diaspore banks (Grillas *et al.* 1993; Bonis & Grillas 2002; Nowak *et al.* 2017). Diaspore bank activation is of great importance in habitat restoration and biodiversity revitalization projects, since it contains autochthonous seeds and oospores, which reduces the possibility of gene pool contamination characteristic for other restoration measures, such as transplantation (Holzhausen *et al.* 2017 and references therein). In order for the diaspore bank to be described more precisely, a more accurate identification of charophyte oospores is needed. The results obtained in our study bring us a step closer to achieving this goal.

It can be concluded that, in our study, intraspecific variability of the oospore parameters among different *C. globularis* and *C. braunii* populations was detected, as well as interspecific differences between morphologically similar taxa – *C. globularis*/*C.* "*connivens*" and *C. baueri*/*C. braunii*. The observed interspecific differences indicate a potential for clear and uniform separation of the oospores of the morphologically similar taxa. This is important not only from the taxonomy perspective, but also for the potential application in diaspore bank characterization and subsequent use of the results in habitat restoration projects. It also justifies the existence of oospore identification keys at the local level and even promotes the idea of developing them for the regions where they are lacking, including Serbia. However, in order to bring a complete and safe conclusion regarding this research question, more information is needed, and future studies should be focused on explaining intraspecific variability of oospore features, as well as collecting more information about the oospore morphometry in general.

Acknowledgements

This work was supported by the Rufford foundation (Grant no. 25789-1), and Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant no. 451-03-68/2022-14/200178). The authors are sincerely grateful to Milica Petrović Đurić for the technical support, expertise and commitment in material processing. We extend our gratitude to Ms Nadežda Buntić for English language editing. We thank two anonymous reviewers for their careful reading of our manuscript and their insightful comments and suggestions.

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Submitted on 22 December 2021;
accepted on 16 August 2022;
published on 16 November 2022.