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SEM study of oospore characteristics in endemic and endangered Balkan Charophytes

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Abstract – The ultrastructural features of oospore wall ornamentation in endemic and critically endangered Balkan Charophytes were examined using scanning electron microscopy (SEM). The present study shows for the first time, in this amount of detail, the interspecific variations in the oospore of endemic and rare Charophytes in the genus *Chara* from the Balkan Peninsula, which will assist in the correct determination and taxonomy of this genus. Oospore features are presented in detail as the background for some additional tips, including the length and width of the oospore, the fossa width, the number of striae and the length to width ratio. Based on oospore ultrastructure, the taxonomic relationships between the endemic *Chara conniventi-fragilis* and *C. connivens*, *C. visianii* and *C. intermedia* species are discussed.

Charophyta / Characeae / *Chara* / *Nitella* / oospore wall / morphology / ultrastructure / endemic plants / Europe / Balkans

Résumé – Étude au MEB des caractéristiques de l'oospore chez des Characées endémiques ou menacées des Balkans. L'ornementation de la paroi de l'oospore de Characées endémiques et/ou fortement menacées dans les Balkans a été examinée au microscope électronique à balayage (MEB). Cette étude présente, pour la première fois, une quantité de détails sur la variation interspécifique des oospores chez ces Characées endémiques et rares, ce qui peut être utile pour la détermination correcte et la taxinomie du genre *Chara*. L'ultrastructure de l'oogone est présentée en détail, ainsi que des caractères observés au microscope optique tels que longueur et largeur de l'oospore, largeur des fosses, nombre des stries et rapport de la longueur à la largeur. Les relations taxinomiques entre l'endémique *Chara conniventi-fragilis* et *C. connivens*, *C. visianii* et *C. intermedia* sont évaluées sur la base de l'ultrastructure des oospores.

Charophytes / Characées / *Chara* / *Nitella* / paroi de l'oospore / morphologie / ultrastructure / endémiques / Europe / Balkans

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INTRODUCTION

The Balkan Peninsula is one of the richest and most diverse regions of Europe (Stevanovi, 1999). It is rich not only in fauna and flora, but is also distinguished by rich phytoplankton and macroscopic algae such as Charophytes. This is a result of evolutionary changes on the Earth during the past several million years that resulted in changing habitat conditions. The most important change in this case was isolation by glacial ice during the Tertiary ice age. Glaciation did not affect the Balkans as seriously as it did the rest of Europe, and thus some species of flora and fauna only survived in the Balkans, resulting in the presence of a diverse range of endemic species in this part of Europe (Blaženčić *et al*., 2006; Keddy, 2007).

Charophytes (stoneworts) are a group of macroscopic algae that are commonly found in diverse types of water ecosystem and are an important component of the underwater vegetation where they sometimes form dense underwater meadows. They are common in all Balkan countries, and about 47 species of the six charophyte genera have been recorded in this region (Blaženčić *et al*., 2006).

All members of the Charophytes (order Charales, family Characeae) produce a thick-walled zygote – the female sex organ – surrounded by five sinistrally wound spiral cells. After fertilisation, rapid changes occur in the walls of the newly formed zygote and the ensheathing sterile cells. These changes result in the formation of a thick wall, which is multilayered, pigmented and often overlaid by calcite. This structure is commonly known as the spore, oosporangium or oospore (John *et al*., 1990). The outermost layer of the wall, known as the ornamentation layer or the ectosporine, is deposited by the spiral cells, and has chemical properties similar to sporopollenin (Horn af Rantizien, 1956; Leitch, 1989). Differential deposition in this layer of the wall is frequently sculptured and forms specific oospore wall ornamentation (Ray *et al.,* 2001). Of the different oospore characters, the ornamentation pattern is considered to be an important taxonomic marker because of its conservative nature (Ray *et al.,* 2001). Dyck (1970) and Frame (1977) were the first to consider that the ultrastructural details of both the ornamentation and oosporangial wall layers provide useful characteristics for distinguishing four species of *Chara*, and documented the ornamentation pattern of a large number of species, mostly of *Chara* and *Nitella*.

The aims of this study were to determine the type and extent of oospore variation in the different endemic and rare Balkan species using SEM microscopy; to document the size of the oospore; and to show how the differences between them can be helpful in taxonomy and identification (Urbaniak, 2009). To date, the only paper dealing with the oospore wall ornamentation of species from the Balkans was published by Mandal *et al.* (2002). However, they only included a limited number of species in their study. The present paper provides more details about the oospore wall ornamentation of rare and endemic Balkan Charophytes, and discusses some taxonomic problems in the light of these findings.

MATERIALS AND METHODS

The examined oospores (about 25-35 per population) were collected from dried specimens from natural populations or in several cases from the Herbarium of the Faculty of Biology at the Institute of Botany and Botanical

Table 1. Species and their population studied including data collection, and location (herbarium). IUNC criteria after Blaženčić *et al.*, (2006): CR (glob)- globally critically endangered, (CR Balk)- critically endangered in the Balkans, (EN Balk)- endangered in the Balkans

Species studied	*Population examined*	*Location (herbarium)*
C. conniventi – fragilis CR (glob)	Lake Skadar, Montenegro; leg. J. Blaženčić (21.08.1980)	BEOU, fresh water
C. corfuensis CR (glob)	Lake Desne, Croatia; leg. J. Blaženčić (09.08.1989)	BEOU, fresh water
C. kokeilii CR (glob)	Lake Skadar, Montenegro; leg. J. Blaženčić (20.08.1980)	BEOU, fresh water
C. ohridiana CR (glob)	Lake Skadar, Montenegro; leg. J. Blaženčić (20.08.1980)	BEOU, fresh water
C. visanii CR (glob)	Krka river (Skradin cascades); leg. J. Blaženčić (22.07.1990	BEOU, fresh water
N. hyalina (CR Balk)	Lake Ohrid, Macedonia; leg. J. Urbaniak (20.09.2009)	J. Urbaniak - private collection, fresh water
T. prolifera (EN Balk)	Serbia, Dunav; leg. J. Blaženčić (15.04.1999)	BEOU, fresh water

Garden of the University of Belgrade (BEOU) (Table 1). The species names written on the herbarium sheets and the identification of the recently collected specimens were verified according to Blaženčić & Randjelovič (1994) and Krause (1990, 1997). Species names are given after Krause (1997) with more common synonyms proposed by Wood (1962). Additional data on the oospore length, width, number of striae and fossa width from several populations of *C. connivens* Salzm. ex A. Braun, *C. delicatula* Agardh, *C. globularis* Thuillier, *C. hispida* L. and *C. intermedia* A. Braun, collected in Poland, were used as an outgroup for comparison in multivariate analysis in order to check the relationship between *C. conniventi-fragilis* Hy and *C. connivens, C. visianii* Blaz. & Randj. and *C. hispida* or *C. intermedia.*

Separation of the mature oospores (black, dark brown or brownish) from herbarium sheets or from the lake sediment was carried out carefully using a needle and forceps. The oospores were then treated using the method of Ray *et al.* (2001), including an acetolysis procedure similar to that described by Erdtman (1960) for the preparation of pollen grains for light microscope or SEM investigation. All chemicals used were of analytical grade (sulphuric acid, acetic acid, hydrochloric acid, ethanol – POCH, acetic anhydride – Merck).

Spores were mounted on brass stubs with double-sided adhesive tape, sputter-coated with gold, and viewed with an LEO 435 VP (ZEISS) or HITACHI S-4700 scanning electron microscope. The oospore wall ornamentation pattern was described according to the terminology used by John & Moore (1987), John *et al.* (1990), Leitch *et al.* (1990) and Ray *et al.* (2001). The length, width, number of striae and fossa width of all available oospores per population were measured before SEM imaging under a light microscope (Zeiss Axioscope), with the exception of the projections and striae width, which were measured during the SEM analysis. Cleaned oospores mounted on brass stubs are available from J. Urbaniak.

In order to reveal morphological differences between oospores (length, width, number of striae and fossa width) from different taxa and single populations, basic statistics (min.-max. range, mean and standard deviation), scatter

plot analysis and cluster analysis (Ward method, Euclidean distance) and discriminant analysis were undertaken. The differences were tested using the non-parametric one-way ANOVA Kruskal-Wallis test and K-W multiple comparisons of mean ranks. All statistical analyses were conducted using the Statistica 9.0 package (StatSoft, 2009).

RESULTS

The results of the investigation of oospore wall ornamentation in Charophyte species are shown in Table 2 and Figs 1-18. The measurements and statistical analysis of the investigated oospores are presented in Tables 3-4 and Figs 19-20. The oospores were measured after cleaning/acetolysis pretreatment (for SEM); however, cleaning/acetolysis may have a similar effect on oospore size as drying. Blume *et al.* (2009) found that dry oospores were about 5% shorter and 27% narrower than wet ones. The best way to compare acetolysed oospores with those that have not been cleaned remains unclear. Comparison of data is only possible when oospores are treated in the same way: 'acetolysed/cleaned' or 'not acetolysed/not cleaned'. Unfortunately, there is no information on the influence of different methods of oospore preparation. A separate study on this subject should be conducted, especially when data are intended to be used for making taxonomic comparisons. As many oospores as possible should be examined, but according to Blume *et al.* (2009) about 50 oospores per population seems to be sufficient for analysis. In some of the studied species, it was not possible to find a sufficient number of oospores for statistical analysis and the analyses were run with a smaller number of measurements. It is well known that if the number of oospores is insufficient or only one or a few populations from each species are examined, there is a high risk that differences among populations may be erroneously interpreted as 'interspecific' differences. However, in the case of *C. conniventi-fragilis* and *C. connivens*, the small number of measured oospores was sufficient to show a close taxonomic relationship between these two species (Fig. 20).

DISCUSSION

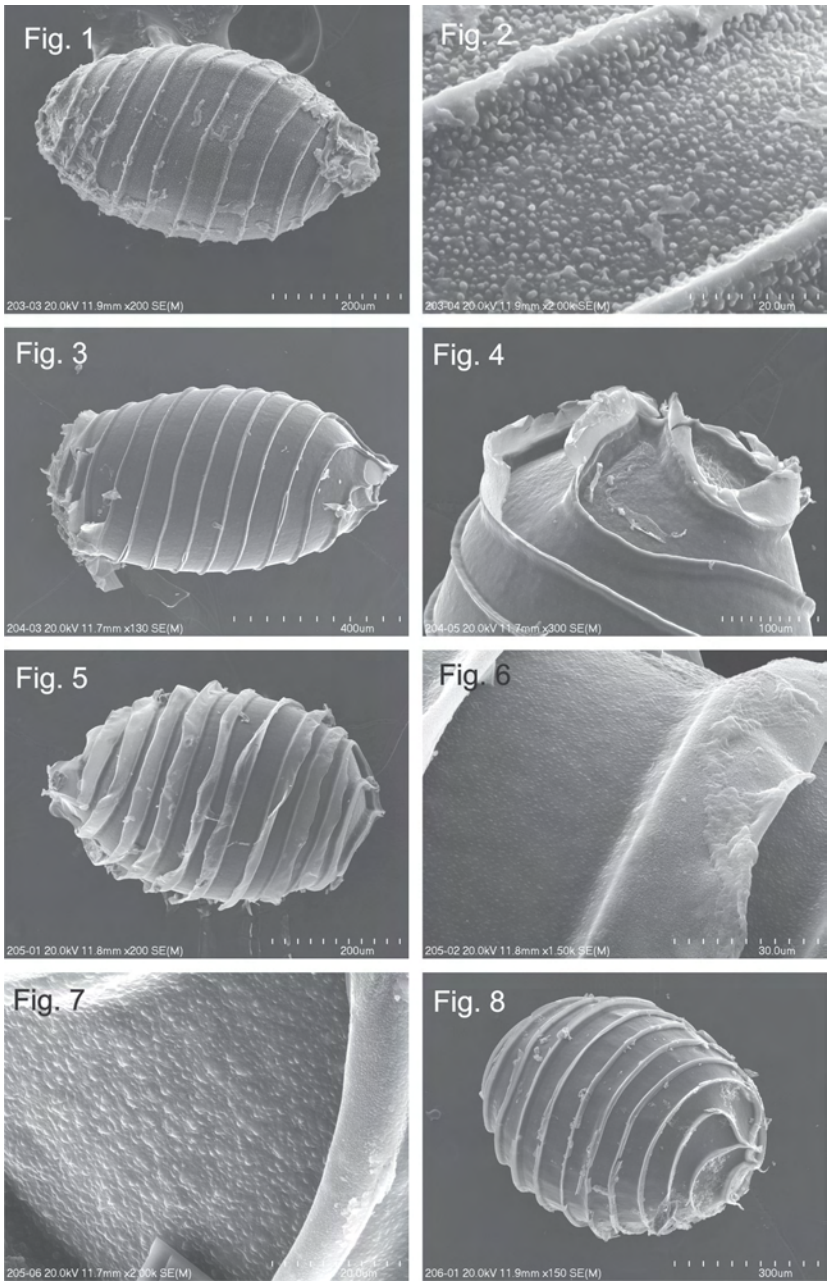
The results of numerous studies (John & Moore, 1987; John *et al*., 1990; Leitch *et al.,* 1990; Ray *et al.,* 2001; Urbaniak, 2009; 2011a; b) indicate that in many cases, oospores alone can be used to distinguish many *Chara* and *Nitella* species. This is especially important when oospores are the only material available for study, as when using seed banks, or in palaeontological sediment studies (Wood & Imahori, 1965; Soulie-Märsche, 1989; Casanova, 1997; Ray *et al.,* 2001).

The oospore wall ornamentation of *C. conniventi-fragilis* is clearly granulate. Similar ornamentation was found on *C. connivens* by Leitch *et al.* (1990) and John *et al.* (1990) and in specimens from Poland (Figs 1-2, 18) which had densely granulate, elongated (papillate) projections on their surface. This indicates that these two species are very similar, or have a close taxonomic relationship. Using median values of oospore length, width, and the length to width ratio, *C. conniventi-fragilis* and two populations of *C. connivens* collected

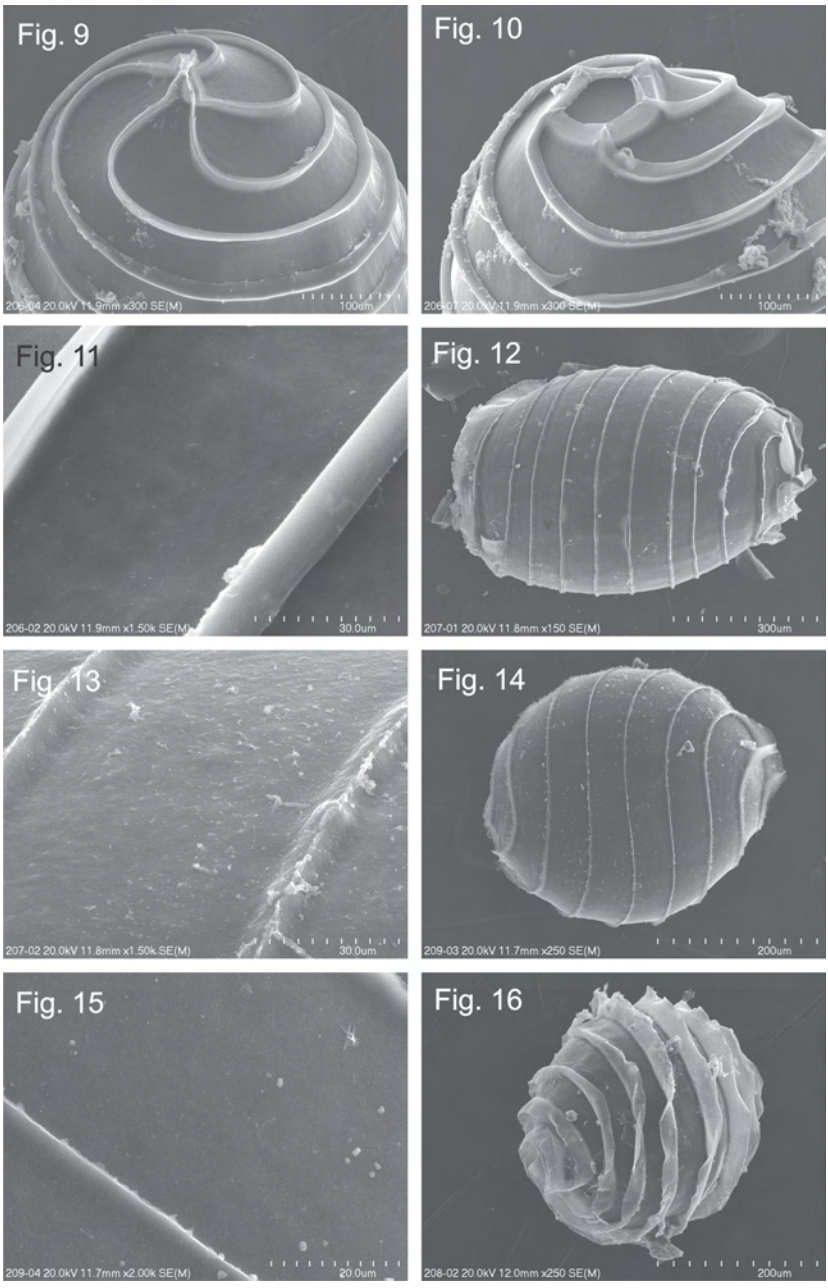
Table 2. Summary of species oospore wall ornamentation

Population studied (figures number)	*Oospore wall ornamentation at different magnifications*
	***C. conniventi – fragilis* Hy**
Figs 1-2	200×, 2000×: oospore wall had densely granulate ornamentation, the projections range from about 0.6 – 4 μm in diameter and number about 30- 45 across the fossa wall; the striae vary from 8-11 in number and was about 6.4-7.1 μm wide at base; a ribbon-like structure was not observed on the striae of any of the oospores examined, the granulate projections are also present on the lower part of ridges
	***C. corfuensis* J. Gr. ex Fil.**
Figs 3-4	130×, 300×: oospore had a smooth ornamentation; the well developed striae were about 4.0-5.1 μm wide and 10-12 μm height; on internal and external surface of the striae, smooth ornamentation was found; a delicate ribbon-like structure aroused on the ridge of each striae
	***C. kokeilii* A. Braun 1847** *C. globularis* var. *kokeilii* (A. Braun) Wood 1962
Figs 5-7	200×: undefined ornamentation, 1500× and 2000×: pustular ornamentation; the striae (10-12) were well developed with a prominent flange (23-36 μm high, 2-3 μm wide); both, striae and ribbon-like structure were covered with granular ornamentation on their surface
	***C. ohridiana* Kostić 1936** *C. dissoluta* var. *ohridiana* Kostić 1936
Figs 8-11	130×, 300× and 1500×: the oospore surface had a smooth ornamentation; the well developed striae were 9 to 11 in number; a well developed smooth ribbon-like structure aroused on the striae
	***C. visanii* J. Blaz. *et* V. Randj.**
Figs 12-1 3	150×, 1500×: the oospores studied had a smooth ornamentation; the well developed striae were about 6.1-9.4 μm wide and are 9 to 12 in number; a delicate, small and slightly curved ribbon-like structure was found on the striae (often become completely or was partially detached during cleaning)
	***N. hyalina* (DC) Ag.**
Figs 14-15	250×: oospores examined were almost round in shape and had a smooth ornamentation, 2000×: slightly roughened ornamentation due to the presence of numerous irregular pits and pores, the weakly developed striae were about 2-2.4 μm wide; a delicate and small ribbon–like structure was found on the striae, but often become completely or was partially detached during cleaning, the projections were absent on the striae
	***Tolypella prolifera* (Ziz ex A. Braun) Leonhardi 1863** *T. prolifera* var. *intricata* f. *prolifera* (Ziz ex A. Braun) Wood 1962
Figs 16-17	250×, 1000×: oospores were smooth and featureless; the striae were well developed and without any type of ornamentation

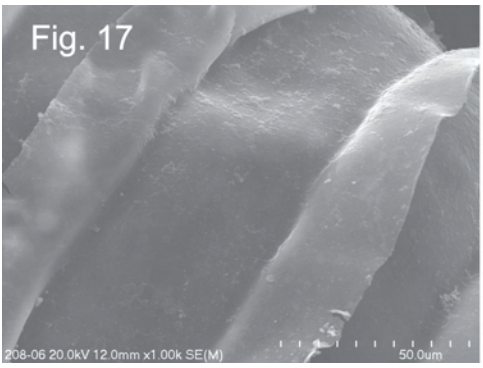
from Poland were placed close together by cluster (Fig. 19) and discriminant analysis (Fig. 20). These two species were well separated from populations of *C. delicatula* and *C. globularis*, which were used as an outgroup for statistical analysis (Fig. 19). This could indicate that these species are taxonomically closely related: they live in similar ecological conditions: *C. connivens* is normally found

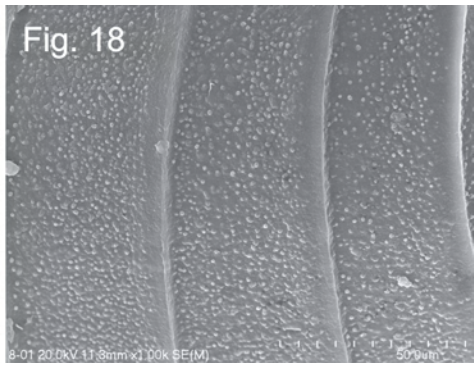


Figs 1-8. **1.** A general view of an oospore showing well developed striae and granulate ornamentation on the oospore wall (*C. conniventi - fragilis*). **2.** Granulate ornamentation of the oospore (*C. conniventi - fragilis*). **3.** General view of the oospore with its smooth ornamentation (*C. corfuensis*). **4.** Apical part of the smooth oospore wall (*C. corfuensis*). **5.** A general view of an oospore showing thick, prominent and flanged striae (*C. kokeili*). **6.** Pustular ornamentation on the oospore wall; note the pustular ornamentation covering the the striae (*C. kokeiili*). **7.** Details of the pustular ornamentation on the striae (*C. kokeilii*). **8.** A general view of an oospore with showing well developed, flanged striae (*C. ohridiana*).



Figs 9-16. **9.** Basal part of the oospore with smooth ornamentation (*C. ohridiana*). **10.** Apical part of the smooth oospore with well developed, flanged striae (*C. ohridiana*). **11.** Apical part of the smooth oospore fossa wall (*C. ohridiana*). **12.** A general view of an oospore showing weakly developed striae and smooth ornamentation on the surface (*C. visanii*). **13.** Details of the oospore with smooth ornamentation type (*C. visanii*). **14.** Smooth oospore wall and weakly developed striae (*N. hyalina*). **15.** A portion of the oospore without any visible ornamentation (*N. hyalina*). **16.** Oospore with its well developed and flanged striae and smooth ornamentation (*T. prolifera*).





Figs 17-18. **17.** Part of the oospore without any visible ornamentation (*T. prolifera*). **18.** Part of the oospore wall ornamentation of *C. connivens* from Poland.

Table 3. Oospore details from studied populations: min. max. (μm), median ± standard error

n.	*Length (μm)*	*Width (μm)*	*Number of striae*	*Fossa width (μm)*	*Length/width ratio*
			C. conniventi – fragilis		
17	504 – 588	294 – 325	8 – 11	45 – 55	1.64 – 1.93
	546 ± 24.6	304 ± 8.5	10 ± 0.6	55 ± 3.1	1.73 ± 0.07
			C. corfuensis		
18	745 – 871	388 – 462	10 – 12	70 – 100	1.65 – 2.13
	829 ± 31.3	430 ± 22.2	11 ± 0.6	80 ± 6.7	1.95 ± 0.11
			C. kokeilii		
16	441 – 556	304 – 451	10 – 12	40 – 50	0.97 – 1.71
	472 ±27.9	315 ± 36.3	10 ± 0.6	45 ± 3.5	1.46 ± 0.16
			C. ohridina		
6	661 – 682	472 – 493	9 – 11	60 – 70	1.34 – 1.44
	682 ± 11.1	483 ±10.5	10 ± 0.8	65 ± 3.5	1.41 ± 0.05
			C. visanii		
13	651 – 787	399 – 493	9 – 12	15 – 80	1.44 – 1.66
	719 ±	472 ± 23.8	11 ± 0.9	72 ± 17.4	1.54 ± 0.06
			N. hyalina		
13	346 – 420	315 – 336	7 – 8	35 – 60	1.06 – 1.25
	330 ± 17.8	294 ±8.8	6 ± 0.5	45 ± 6.2	1.16 ± 0.05
			T. prolifera		
5	294 – 346	280 – 304	6 – 8	40 – 55	1.03 – 2.50
	378 ± 20.9	325 ± 9.7	7 ± 1.1	50 ± 5.7	1.72 ± 0.29

in brackish water (1-8 psu) (Georg & Torn, 2003) but also in fresh water, whereas *C. conniventi-fragilis* was found in the freshwater lake Skadar (Montenegro). It is difficult to determine the taxonomic relationship between these species, but certain close similarities, such as the morphology of the thalli, similar oospore wall ornamentation, ecological conditions, oospore length and width contradict the statement that there is a very close relationship between these species.

Table 4. Details of statistical analysis of Kruskal-Wallis test and test for multiply comparisons of mean ranks. The mean values that are not significantly different from each other are labeled with the asterix

Species	*n*	*Length*	*Width*	*Length / width ratio*
		$X^2 = 121.3$ $P < 0.001$	$X^2 = 98.6$ $P < 0.001$	$X^2 = 83.2$ $P < 0.001$
C. conniventi - fragilis	17	*	*	*
C. connivens 1	27	*	*	
C. connivens 2	23	*	*	
C. delicatula 1	43			*
C. delicatula 2	26	*		*
C. globularis 1	31			*
C. globularis 2	28		*	*

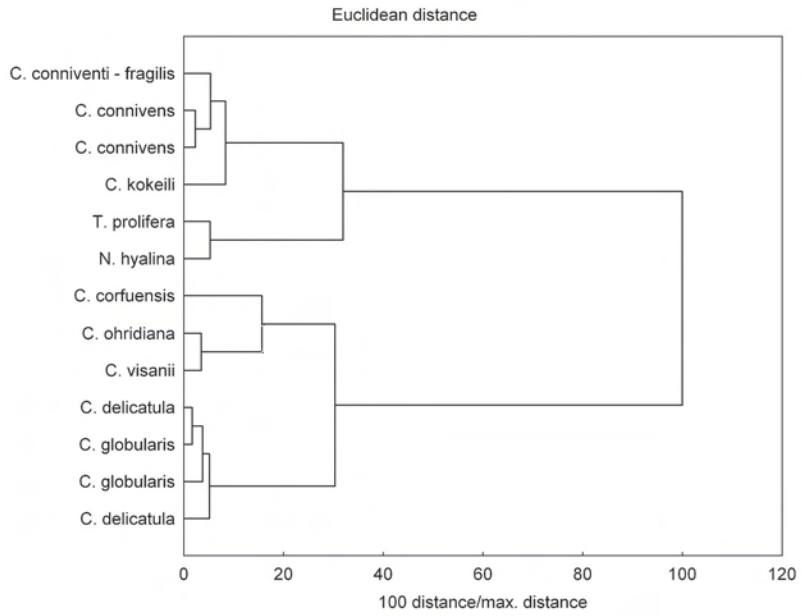


Fig. 19. Cluster analysis showing distances of oospore populations for oospore length, width and length to width ratio.

The oospore wall of *C. corfuensis* J. Gr. *ex* Fil. shows smooth ornamentation (Figs 3-4) as do the oospore walls of C. *ohridiana* Kosti (Figs 8-11). *C. kokeili* A. Braun (Figs 5-7) oospores were undefined when examined at a magnification of 200× and clearly pustular when investigated at 2000×. A ribbon-like structure, covered with granular ornamentation, was present on all investigated oospores. The length and width of the *C. kokeili* oospores corresponded closely with data presented by Krause (1990).

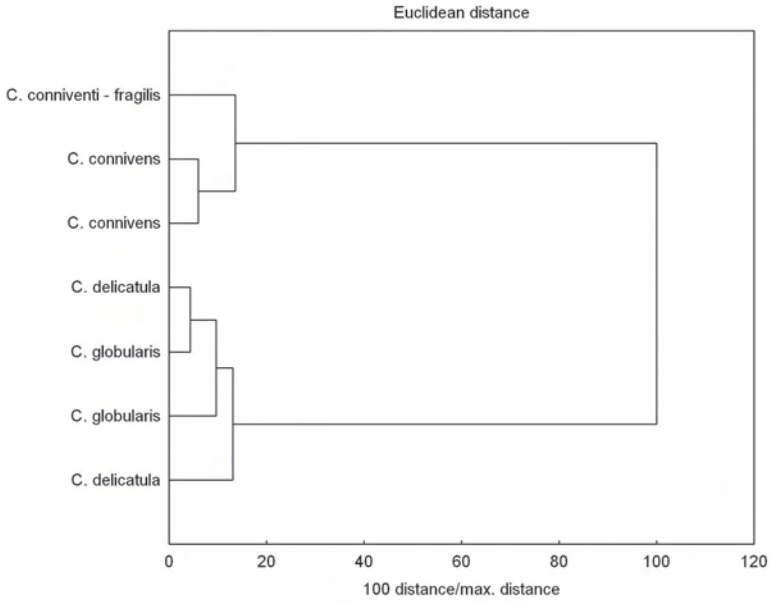


Fig. 20. Discrimination analysis for oospore length, width and length to width ratio for *C. conniventi – fragilis*, *C. connivens*, *C. delicatula* and *C. globularis* populations. Close relationships in between *C. conniventi – fragilis* and *C. connivens* are presented as a group in the circle.

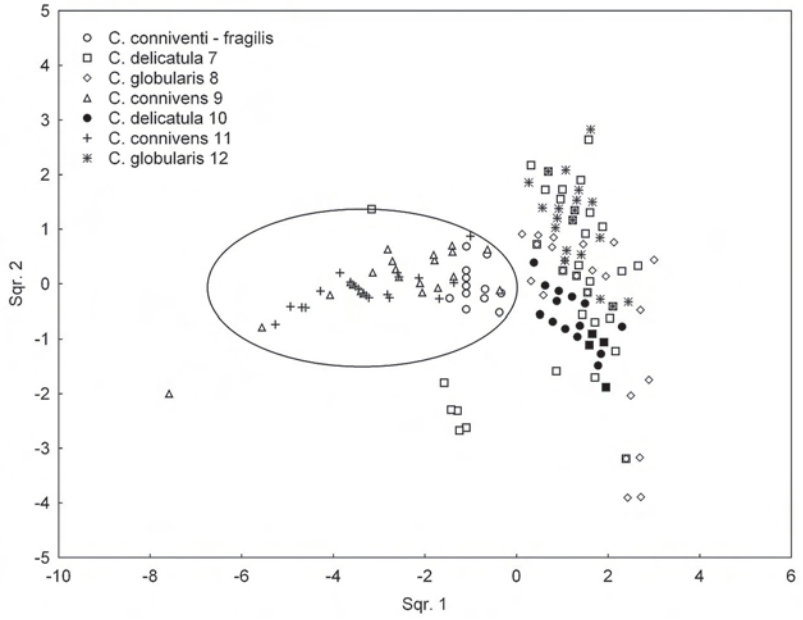


Fig. 21. Discrimination analysis for oospore length, width and length to width ratio for *C. conniventi – fragilis*, *C. connivens*, *C. delicatula* and *C. globularis* populations.

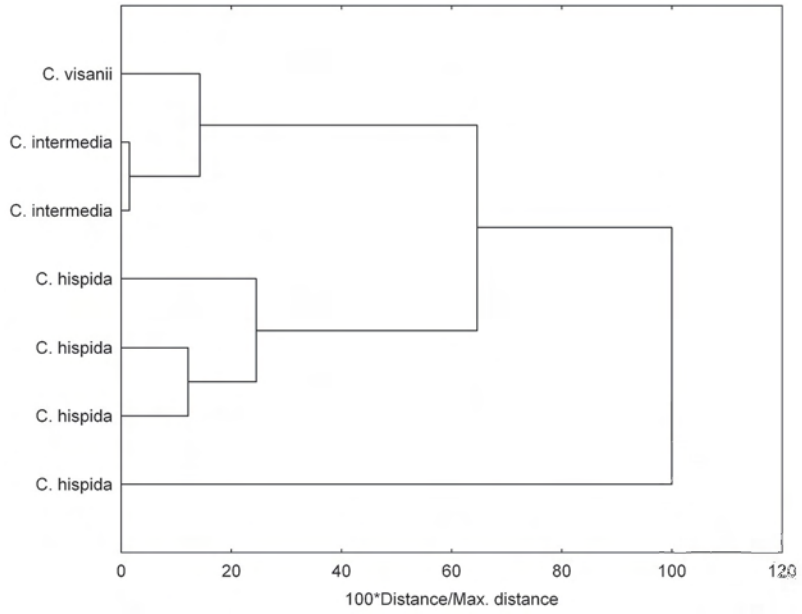


Fig. 22. Cluster analysis showing distances of oospore populations for oospore length, width, number of striae and fossa width for *C. conniventi – fragilis*, *C. connivens*, *C. delicatula* and *C. globularis*.

C. visianii oospores are smooth, with no ornamentation on the wall. According to Blaûeni & Randejelovi (1994), this species belongs to the *C. hispida* complex and is similar to both aulacanthous *C. hispida* and thylacanthous *C. intermedia*. The authors of this paper observed significant differences between *C. visianii* and other closely related species, based mostly on the general appearance of the specimens, the flat coronula of the oospore and ecological conditions. During cluster analysis based on oospore length, width, fossa width and number of striae, we contradict that close relationships exist between *C. visianii* and *C. hispida* as well as *C. visianii* and *C. intermedia* (Fig. 19). Unfortunately the SEM analysis was not particularly helpful in this taxonomic analysis. Species that belong to the broad *C. hispida* complex of species have a smooth oospore wall with no ornamentation (Urbaniak, unpublished results).

The oospore wall in *N. hyalina* (DC) Ag. (Figs 14-15) is smooth, with no ornamentation on the wall surface. Cirujano *et al.* (2008) reported a similar ornamentation pattern for a Spanish population. In *T. prolifera* (Figs 16-17), the oospore wall is smooth and without ornamentation, as reported by Cirujano *et al.* (2008) for populations from Spain.

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