Chara canescens part I: Oospore differentiation of parthenogenic and dioecious strains and salt-dependent oospore sizes

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Abstract

Chara canescens Loisel. is one of two European species of the section Desvauxia R.D. Wood of the genus *Chara* L. Whereas most populations of *C. canescens* reproduce parthenogenetically, a few sites with sexual reproducing populations are known. Studies of European *C. canescens* oospore morphology led to open questions about the taxonomic status. Here we investigated nearly 1000 oospores from 16 European populations originated from plant release, sediments, germination experiments and herbaria and two Asian populations resulting in a regional determination key for studied populations as well as important database implications regarding origin, oospore plant position and equipment used. The impact of salinisation on oospore morphology were tested by artificial salt levels. The longest but smallest oospores were formed at 3 PSU, whereas the widest at 0.1 PSU and the shortest at 5 PSU. Basal width and shape, on the other hand, seem to be only affected by higher salt contents. This study contributes the lacking oospore-information for both reproduction modes of European of *C. canescens* – populations.

Key words charophytes, section Desvauxia, oospores, wall ornamentation, salinisation

1. Introduction

Charophytes (Characeae) are one of the three lineages (Charophyceae, Zygnematophyceae and Coleochaetophyceae; Streptophyta) that form, together with land plants, the monophyletic group Phragmoplastophyta (Buschmann and Zachgo 2016). These macroscopic multicellular complex algae inhabit freshwater, as well as brackish and saline environments worldwide (Khan 1991). The relevance of Charophyceae for evolutionary and applied research areas, such as ecophysiology, paleoecology, ontogenesis, molecular and comparative genomics (Beilby 2019, Vicente et al. 2019, Holzhausen et al. 2022b, Quade et al. 2022) peaked in the establishment of the freshwater alga *Chara braunii* C.C. Gmel. 1826 as a model organism for water-land-transition of plants after publication of its draft genome (Nishiyama et al. 2018).

Within Characeae, the halotolerant species *Chara canescens* Loisel. (former *C. crinita* Wallroth 1815) is in three respects particular. First, it only can reproduce via oospores, secondly, it is the one of two European species of the section Desvauxia R.D. Wood and third, *C. canescens* is capable to reproduce via parthenogenesis, being a remarkable exception known among charophytes so far. This reproduction mode which is called apogamia in spermatophytes is a well-known phenomenon within flowering plants (Carman 1997, Hand and Koltunow 2014, Underwood et al. 2022) and described in detail for *C. canescens* by Ernst (1918) although the underlying molecular mechanisms that leads to geographic parthenogenesis of *C. canescens* are completely unknown so far (Schaible 2006).

Lessing seems to be the first who collected male plants from a bisexual population in the Caspian Sea and described them as a distinct species, *Chara karelinii* Lessing 1835 (Komarov Botanical Institute of the Russian Academy of Sciences, LE; Romanov 2021). Different opinions of leading botanist's resulted in an incorporation of this dioecious population into the parthenogenetic *C. crinita* due to the absence of male species in field excursions over Central Europe (Braun 1857). Outside Europe, several monoecious and dioecious species from the section Desvauxia were described from Asia and North America. For example, T.F. Allen described monoecious *Chara evoluta* T. F. Allen and *C. hirsuta* T.F. Allen 1900 for North America, whereas monoecious *C. altaica* A. Braun in Braun and Nordstedt (1882) , *C. sibirica* Migula 1904 were described from Siberia, monoecious *C. abnormiformis* Vilhelm 1928 from Central Asia, monoecious *C. piniformis* W.Q. Chen and F.S. Han in Chen et al. 1990 and *C.* F.S. Han 1964 from China, as well as dioecious Hollerb. in Hollerb. et Krassavina 1983 from Central Asia, *C.* Y.J. Ling et al. 1991, and *C. shanxiensis* Y.J. Ling 1985 from China. Male plants are known for *C. canescens* and *C. canescentiformis* but not found in case of *C. shanxiensis* and *C. pseudocanescens*.

Still, until the discovery of *C. altaica*, found in two localities at the south of Eastern Europe (Romanov and Vishnyakov, in press), only one species of the section Desvauxia, i.e., *C.*

canescens, was known from Europe. This species is represented in Europe mostly by parthenogenetic populations with a few sexually reproducing ones found in France, Italy (Sicily, Sardinia), Greece, Austria, and Serbia (Krause 1997, Nowak et al. 2019, Sabovljević et al. 2022). Outside Europe, sexually reproducing populations are reported from North Africa (Morocco), Central Asia, West Siberia, and China (Hollerbach and Krassavina 1983, Fushan and Yaoying 1994, Nowak et al. 2019, Romanov unpubl. data).

For Europe, the Austrian dioecious population is investigated in more detail. Decreased sodium chloride concentrations and increased concentrations of soda and sulphate are reported as decisive for their restriction (Krause 1997). Further, ecological conditions such as light irradiance and salinity do not have an impact on the occurrence of male *C. canescens* (Schaible and Schubert 2008).

The conservation of parthenogenic *C. canescens* strains require reliable taxonomic determination for both cases. If alga material is present, differentiation by use of *rbc*L and *atp*B genes can be used as successful solution (Schaible et al. 2009, Nowak et al. 2019). But parthenogenetically and sexually reproducing species are well known from temporary habitats in Spain, Austria, and Italy. The changes caused by climate change, such as longer and more frequent periods of drought, require the identification of oospores from sediments.

Taxonomical use of oospores is challenging (Holzhausen et al. 2023). One solution for a) identification of regional differences, b) equipment and storage-based differences and c) linkage between oospores and environmental data is the buildup of an worldwide oospore database (currently: Rostocker oospore-charophyte database, Anja Holzhausen) as recommended by Holzhausen et al. (2015) and described in Holzhausen et al. (*In Press*). This combines morphology data of oospores and alga, environmental data and few genetic analyses to assess those open fundamental questions and allow re-evaluation by storage of oospores and herbarium material.

The present study resolves the question of oospore differences by contribution of lacking oospore-information for both reproduction modes of European *C. canescens* – populations. The discovery of another member of the section Desvauxia in Europe raises the question of the origin of the parthenogenetic taxon.

2. Materials and methods

Nearly 1000 oospores from 16 European populations and one Asian population of *C. canescens* were analysed for quantitative and qualitative morphological parameters including the structure of the oospore wall. Table 1 lists all populations examined, 10 out of 16 were obtained from fresh plant material, oospores were harvested after release (Austria, Germany, France, Kazakhstan). Five populations were obtained from laboratory germination experiments

(Austria, Germany, France, Spain), one from a sediment sample (Germany) and one from herbarium (France). Additionally, oospores of one *C. altaica* population from Kazakhstan was included (fresh material). For light microscopy (LM), oospores were placed on microscope slides with an adhesive surface and documented with a stereomicroscope in lateral, apical and basal view (Olympus SZX16 or Leica DM2500). Oospore colour, shape and appendixes were determined. Parameters such as oospore length, oospore width, fossa width, basal width and angle of striae to the longitudinal axis were measured using ImageJ (v. 1.50i).

Scanning electron microscope analysis (SEM) of the oospore wall structure were done at the National History Museum London by M. T. Casanova. Oospores were dried by lyophilization and sputter-coated with gold but not treated with surface cleaning agents. SEM images of the surface of oospores and fossa walls were produced according to Wilbraham and Casanova (2019).

Statistical analyses were performed by using R (Team R 2022). Data were tested for normal distribution by Shapiro-Wilk test from the EnvStats package and for homogeneity of variance across groups with Levene's test from car package (Fox and Weisberg 2019). ANOVA were performed for normal and homogeneous distributed data and Kruskal-Wallis rank sum tests for non-Gaussian (not normal) distributed data or data with heterogeneous variances. For data with significant ANOVA results Tukey's HSD post-hoc tests were performed. In case of significant results from the Kruskal-Wallis tests multiple comparisons were done by Dunn's test (Dinno 2017).

Box plot visualisation was conducted by the R packages ggplot2 and ggstatsplot (Wickham 2016, Patil 2021). Principal component analysis was performed and visualised using the R packages ggbiplot and corrplot (Wei et al. 2017).

3. Results

In a first step, morphological oospore differences possibly caused by their origin (oospores from sediments vs. oospores from habitat plants vs. in vitro germination experiments), the microscope used or the whorl number at which the oospores are grown were tested.

Differences between oospores from sediments, the field and *in vitro* germination experiments

Oospore length (p.adj. = 5.33e-16), number of striae (p.adj. = 1.74e- 2), colour (p.adj = 0.00387), mean basal width (p.adj. = 1.42e- 8) and length/width ratio (p.adj. = 4.02e- 7) of oospores from sediment samples of lake Borken differ significantly from oospores from in vitro germination experiments. These differences are mainly caused by broadened size ranges of oospores from sediment samples, except for the number of striae (Tab.3).

In contrast to this, no significant differences were found between oospores from field material and the corresponding in vitro germination experiment, except for oospore length (p.adj. = 2.732e-2) and the calculated length to width ratio (p.adj. = 8.75e-3). Despite small differences in size ranges, the calculated means of both oospore populations are consistent for all other oospore characters.

Salt-dependent oospore differences

Oospores formed in medium with artificially increased salt content show clearly significant differences between the salt levels and to the field population (S-Tab.1).

For all characters, except the oospore colour (RAL9005), significant differences were found for at least two of the three salt levels (0.1 PSU, 3 PSU, 5 PSU, S-Tab.1) and the corresponding reference group (Can_Slp_G). Salinisation has the greatest effect on oospore length, oospore width, fossa width and number of striae. Figure 1 showed boxplots for these parameters and all groups. The longest but smallest oospores were formed at 3 PSU, whereas the widest at 0.1 PSU and the shortest at 5 PSU (Tab. 4). Basal width and shape, on the other hand, seem to be only affected by higher salt contents (S-Tab.1). Only oospores from 5 PSU showed significant differences to 0.1 PSU (basal width p.adj. = 8.45e-3, shape p.adj. = 1.5e-4), 3 PSU (basal width p.adj. = 3.3e-4, shape p.adj. = 8.9e-8) and the reference group (basal width p.adj. = 7.7e-4, shape p.adj. = 7.9e-6). Below this salt boundary, no differences in shape or basal width can be detected. The two axes of the principal component analysis determine 96% of the cumulative variation of oospores (eigenvalue 1 = 1319.56, eigenvalue 2 = 324.37) and were determined by the characters of oospore length and oospore width (Fig. 1E).

Differences between oospores photographed with different microscopes

To exclude differences between microscopes used, oospores from Angersdorf and Neusiedler See were photographed with two different microscopes.

Anova results showed few significant differences between both microscopes and populations. Oospores from Neusiedler See Laken differ significantly in the oospore width (p.adj. = 2.3e-05), basal width (p.adj. = 6.1e-07) and striae (p.adj. = 8.3e-11), oospores from Angersdorf show differences in the oospore width (p.adj. = 5.7e-04), basal width (p.adj. =3e-03) and fossa width (p.adj. <2e-16). However, the size ranges and the results of the PCA clearly showed that the oospores of the two microscopes overlapped completely for both Angersdorf and Neusiedler See (Tab. 5). These statistical differences are caused by higher numbers of oospores photographed with microscope 1 resulting in broader size ranges (Tab. 5). Comparing mean values, no differences can be detected by use of different microscopes.

Differences between whorls

Oospores of *C. canescens* from Plóvan (France) were tested for oospore differences regarding their whorl position. For this population, no significant differences could be found that initiate position-dependent oospore sizes.

Differences between monospecific and parthenogenetic strains

Based on the results above, two monospecific and eight parthenogenetic strains obtained from field samples were included to test whether morphological differences exist between oospores of monospecific and parthenogenetic strains. The results showed that there is no individual oospore character that allow the differentiation between both reproduction modes by oospores. However, using a model that combines morphological characters, it is possible to successfully separate any population of the data used in this study (Figure 2).

However, the two axes of the principal component analysis determine 99.2% of the cumulative variation of oospores (eigenvalue 1 = 1.18e+04, eigenvalue 2 = 1.09e+03) and were determined by the characters of oospore length and oospore width (Figure 3).

The ratio of these two morphological characters allow to distinguish a group of oospores from German brackish lagoons (Poel, Fastensee and Haide) from all other populations in a first step. Simultaneously, this group of oospores from German brackish lagoons could be separated by the mean value of the five lines surrounding the pentagonal basal impression. Finally, by a combination of the characters length to width ratio, oospore length and oospore width, number of striae and the mean value of the five lines surrounding the pentagonal basal impression successfully separated oospores from the Austrian dioecious population (Neusiedler Lacken) from all parthenogenetic populations.

In addition, oospores of parthenogenetic strains from marine brackish waters and lagoons (Poel, Haide, Fastensee) significantly differ from enclosed waters such as inland brackish waters (Angersdorf, Weißsee). The shape of oospores from marine conditions changes from ellipsoid prolonged to rounded (Tab.6).

The oospore wall ornamentation of oospores investigated is listed in Table 7. Three European *C. canescens* populations (Austria, France and Spain) and *C. altaica* from Kazakhstan showed a pustular wall ornamentation, although differences in the shape and regularity of pustules exist. A difference between ornamentation pattern and salinity could not be found. Rather, oospores from salinity experiment showed the same sparsely pustular ornamentation whereas oospores from field samples showed a densely pustular ornamentation.

Parthenogenetic strains from Austria and Germany and herbarium sample from France showed a smooth ornamentation without pustules.

4. Discussion

The halotolerant species *C. canescens* is one of two European species of the section *Desvauxia* and characterised by its capability to reproduce parthenogenetically and sexually. In Europe, monoecious and dioecious populations of *C. canescens* are described (Comelles 1986, Schaible and Schubert 2008, Nowak et al. 2019), monoecious *C. altaica* populations were recently found in South Europe (Romanov and Vishnyakov *In Press*). In contrast to

Europe, different monoecious and dioecious species are described for Asia and North America such as *C. hirsuta*, *C. evoluta* or *C. sibirica* (Allen 1882, 1900, Migula 1904).

Phylogenetic studies of European and Asian populations of *C. canescens* and *C. altaica* using available *rbc*L and *mat*K gene sequences from Austria, Germany, Greece, Italy, Japan, Kazakhstan, Spain and Sweden have revealed that bisexual specimens from Austria and Italy can be separated from parthenogenetic strains, *C. altaica* from Japan and Kazakhstan clustered within the group of parthenogenetically reproducing specimens from Germany, Greece, Spain and Sweden (Kato et al. 2010, Schneider et al. 2016, Langangen et al. 2019, Nowak et al. 2019).

In cases of absent vegetation or restauration purposes, taxonomic determination of oospores is performed via the stored oospore pool in sediments (Holzhausen 2018). Despite overlapping size ranges the determination of species groups is possible (Haas 1994). Furthermore, the lack of available information on oospore handling of previous studies aggravates the comparison of these data. Here we use nearly 1000 oospores sampled between 2004 and 2016, stored for re-evaluation in the Rostocker oospore-charophyte database, linked with plant material, genetics and available environmental data as well as further dry and wet stored material in a living oospore database, allowing a comprehensive regional evaluation resulting in a regional determination key for populations investigated and decisive implication for the used database.

As common for alga/plant determination keys, the use of characters combined is successfully used to differentiate the Austrian dioecious population from parthenogenetic strains. Here we used the combination of length to width ratio, the mean value of the five surrounding lines of the basal impression, the oospore length, oospore width and number of striae (Fig. 4). The oospores from Bódon Blanco are excluded. For this site, Comelles described the co-existence of the parthenogenetically and dioecious strain (Comelles 1986). Although in subsequent germination experiments only parthenogenetically reproducing alga emerged, bisexual origin could not be excluded. Comparing oospore morphology with existing phylogenetic analysis (Kato et al. 2010, Schneider et al. 2015, Langangen et al. 2019, Nowak et al. 2019), differences between oospores of parthenogenetically reproducing populations could be not reflected by sequence data of the plastid genes although the Austrian dioecious population can be separated by character combination.

Furthermore, our results suppose a strong ion influence on oospore morphology features. Environmental influence on oospore morphology was hypothesized by different authors (Soulié-Märsche 1989, Casanova 1997, Holzhausen et al. 2022a, V. et al. 2022). Here, oospores of parthenogenetic populations from high fluctuating habitats as well oospores of the salt-stress-germination experiment differ from temporary populations. Combining ontogenesis

and physiological studies, an accumulation of sucrose as direct storage is presumable (Holzhausen et al. 2017) increased sucrose concentrations at times of gametangia formation and maturation could explain the increased oospore volume at brackish sites with a salinity above 7.

This analysis of *C. canescens* oospores show that the oospore data base as supposed in (Holzhausen et al. 2015) and described in (Holzhausen et al. *In Press*) has the potential to elucidate regional differences in a worldwide assay.

Data on oospore origin and treatment are essential as shown by the results. Oospore sizes of field material and in vitro germination assays are comparable and could be combined consequently in analyses whereas oospores from sediment samples should be treated as separate group. Diaspore banks are known as storage pools of oospores from various previous years whereas oospores from plant material and germination assays display a time restricted period with known environmental conditions.

In contrast, our studies on oospores also have shown that no position dependency for oospores from Plovan (France) exist. The results regarding the microscope used, are in accordance with (Soulié-Märsche and Joseph 1991) who found small differences in oospores length and width for *Sphaerochara prolifera*, *S. davidii*, *Nitellopsis etrusca* and *Chara vulgaris*. In our study, these results should not be over-intellectualised, size ranges of both microscopes overlap completely. Position dependency is only known for oogonia and antheridia sizes (Ernst 1918, Calero and Rodrigo 2022). For *C. canescens* from France, no position dependency could be identified for matured oospores.

The comparison with available traits of oospores of Eurasian populations of *C. altaica*, *C. canescentiformis*, *C. evoluta*, *C. longiarticulata*, *C. pseudocanescens*, *C. piniformis*, and *C. shanxiensis* (Hollerbach and Krassavina 1983, Han and Ling 1994, Romanov 2011) yielded that all size values are mostly overlapping. Oospore surface of *C. canescens* and *C. altaica* viewed in SEM seems to be similar although variable between populations (Ray et al. 2001, Kato et al. 2010, Romanov 2011, Urbaniak 2011), plants from the same population or even within the same plant (Romanov, unpubl. data). It is described as sparsely papillate or minutely granulate to indistinctly pustular and pustular. In our study, oospores of parthenogenetic strains from Germany and Austria have shown a smooth ornamentation whereas *C. altaica* and dioecious *C. canescens* population from Austria showed a (sparsely) pustular ornamentation.

C. canescens oospores from France are pustular or smooth and the oospores from Spain also showed pustulars. Based on these results, only regional differences on oospore ornamentation can be fixed, a correlation between salinity and ornamentation per se do not exist. Further studies are essential to pinpoint abiotic and biotic causes for ornamentation pattering.

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Table 1. Summary of *C. canescens* and *C. altaica* populations used. Listed are the sampling site, habitat (temporary (t), permanent(p)), geographic coordinates (latitude, longitude), number of analysed oospores (N), the kind of material as well as the oospore-database ID (living material).

location	oospore database ID	habitat	geographic coordinates	N (oospores)	origin of material
Neusiedler Seewinkel upper part (Austria)	Oo-AHO-00206 to Oo-AHO-00236	t		40	released from alga
Neusiedler Seewinkel lower part (Austria)	Oo-AHO-00193 to Oo-AHO-00207	t	47.801337°N 16.785191°E	64	released from alga
	Oo-AHO-00313	t		40	germination experiment
	Oo-AHO-001279 to Oo-AHO-001320	t		121	salt experiment
Weißsee (Austria)	Oo-AHO-001321 to Oo-AHO-001339	p	47.716667°N 16.816667°E	40	released from alga
Angersdorf (Germany)	Oo-AHO-00138 to Oo-AHO-00142, Oo-AHO-00314	p	51.333889°N 11.906481°E	102	released from alga
Haide (Germany)	-	p	54.47665°N 13.14442°E	75	released from alga
Fastensee (Germany)	Oo-AHO-00356 to Oo-AHO-00395	p	54.512241°N 11.035908°E	40	released from alga
Island Poel (Germany)	Oo-AHO-00396 to Oo-AHO-00400	p	53.958717°N 11.447594°E	40	released from alga
lake Borken (Germany)	Oo-AHO-00144 to Oo-AHO-00172 / Oo-AHO-00808	p	51.024947°N 9.270591°E	40	sediments
	Oo-AHO-00173 to Oo-AHO-00189, Oo-AHO-00312	p		40	germination experiment
Montpellier (France)	Oo-AHO-00315	-[1]	-[1]	22	herbarium
Plovan (France)	-	t	47.911555°N -4.376621°E	22	released from alga
Le Boucaud 5 (France)	Oo-AHO-001340 to Oo-AHO-001344	t	46.996000°N, -2.243500°E	42	germination experiment
Valencia (Spain)	Oo-AHO-00319 to Oo-AHO-00325	t	-	105	released from alga
Bódon Blanco (Spain)	Oo-AHO-00190 to Oo-AHO-00192; Oo-AHO-00316 to Oo-AHO-00318, Oo-AHO-001140 to Oo-AHO-001143	t	41.249531°N 4.67089°W	33	germination experiment
C. canescens Lake Zerenda (Kazakhstan)	Oo-AHO-00076 to Oo-AHO-00115	p	52.922358°N 69.149730°E	41	released from alga
C. altaica Lake Zerenda (Kazakhstan)	Oo-AHO-00116 to Oo-AHO-00137	p		43	released from alga

[1] Coordinates could not be given. The former site is not known anymore.

Table 2. Physico-chemical parameter of *C. canescens* sampling sites.

Site	pH	salinity (PSU)	conductivity (µS/cm)	oxygen (mg/L)	T (°C)
Angersdorf	-	7	62.4	-	12.6
lake Borken	8.43	0.41	710	8.40	-
Fastensee (Fehmarn)	8.3	15.9	20120	7.81	7.9
Haide	-	9.7	-	-	-
Poel	-	13.5	-	-	-
Neusiedler Seewinkel, lower part	9.65	5.19	8710	8.96	15.8
Neusiedler Seewinkel, upper part	9.57	4.6	6960	8.46	15.7
Weißsee	8.63	1.32	2270	8.28	18.5

Table 3. Overview of oospore characteristics of *C. canescens* populations from sediment samples, in vitro germination experiments and the field.

variable		can_BS_S	can_BS_G	Slp_H	Slp_G	Sup
site		lake Borken	lake Borken	Neusiedler Lacken lower part	Neusiedler Lacken lower part	Neusiedler Lacken upper part
origin		sediment	in vitro germination experiment	field	in vitro germination experiment	field
n		40	40	64	40	40
length (µm)	min	405.341	358.068	383.202	383.356	421.930
	max	746.421	529.241	491.172	512.949	560.121
	mean	565.277	422.440	434.260	456.929	468.795
	sd	77.057	37.001	22.129	27.240	32.144
width (µm)	min	129.021	146.001	191.163	200.126	180.050
	max	238.683	275.158	256.690	256.860	288.979
	mean	185.672	202.545	229.894	223.595	246.717
	sd	31.203	22.065	17.049	14.452	25.409
striae	min	11	9	7	7	7
	max	12	12	10	11	10
	mean	11.925	10.25	8.562	8.825	8.475
	sd	0.267	0.630	0.732	1.035	0.816
colour	RAL9005	100 %	90 %	100 %	100 %	100 %
	RAL8011	-	10 %	-	-	-
	RAL8007	-	-	-	-	-
	sd	0.000	0.304	0.000	0.000	0.000
shape	ellipsoid prolonged	100 %	100%	55 %	52.5 %	47.5 %
	ellipsoid rounded	-	-	45 %	47.5 %	52.5 %
	rounded	-	-	-	-	
	sd	0.000	0.000	0.473	0.506	0.506
angle(°)	min	52.003	64.541	54.025	58.425	60.754
	max	89.677	83.991	82.594	82.638	82.803
	mean	70.575	71.535	70.833	71.091	73.544

	sd	8.652	4.988	5.492	4.522	5.434
fossa width(µm)	min	23.746	27.461	32.464	32.853	32.223
	max	51.411	44.834	51.362	55.931	48.002
	mean	35.860	35.832	40.045	39.397	39.612
	sd	6.599	3.386	3.887	4.634	3.745
basal width (µm)	min	NA	31.435	30.614	42.181	27.111
	max	NA	50.125	63.976	59.722	67.489
	mean	NA	39.841	49.644	48.733	48.011
	sd	NA	4.243	7.161	3.928	6.867
length/width	min	2.410	1.636	1.538	1.782	1.589
	max	4.424	3.004	2.313	2.446	2.637
	mean	3.089	2.110	1.898	2.049	1.920
	sd	0.438	0.306	0.149	0.145	0.240

Table 4. Overview of morphological characters of *C. canescens* oospores from an in vitro germination experiment using different salt levels.

variable		Can.Slp.G.0.1	Can.Slp.G.3	Can.Slp.G.5
site		Neusiedler Lacken lower part	Neusiedler Lacken lower part	Neusiedler Lacken lower part
origin		in vitro germination experiment	in vitro germination experiment	in vitro germination experiment
salinity (PSU)		0.1	3	5
n		40	39	41
length (µm)	min	392.945	447.614	327.521
	max	511.919	538.426	500.801
	mean	448.498	492.224	416.560
	sd	27.790	23.858	27.798
width (µm)	min	217.481	198.664	206.071
	max	272.717	287.471	264.233
	mean	245.177	232.796	235.227
	sd	14.467	21.680	12.237
striae	min	8	10	8
	max	12	12	11
	mean	10.050	10.897	9.366
	sd	0.986	0.754	0.915
colour	RAL9005	100 %	100 %	100 %
	RAL8011	-	-	-
	RAL8007	-	-	-
	sd	0.000	0.000	0.000
shape	ellipsoid prolonged	50 %	64.1 %	9.8 %
	ellipsoid rounded	42.5 %	35.9 %	70.7 %
	rounded	7.5 %	-	19.5 %
	sd	0.636	0.486	0.539
angle(°)	min	65.444	63.419	67.493
	max	87.094	82.802	84.462
	mean	73.994	73.489	74.796
	sd	4.784	4.400	4.678

fossa width(µm)	min	26.896	29.055	25.158
	max	41.341	36.449	40.743
	mean	33.537	32.247	32.530
	sd	3.251	1.741	3.484
basal width (µm)	min	38.414	39.514	37.532
	max	57.201	58.674	50.917
	mean	47.775	49.234	45.340
	sd	4.624	4.405	3.350
length/width	min	1.473	1.618	1.470
	max	2.172	2.587	2.135
	mean	1.836	2.136	1.774
	sd	0.162	0.259	0.130

Table 5. Overview of morphological characters of *C. canescens* oospores from Neusiedler Lacken and Angersdorf photographed with two different microscopes. M1 = Olympus SZX16, M2 = Leica DM2500.

variable		Can.Slp.M1	Can.Slp.M2	Can.A.M1	Can.A.M2
site		Neusiedler Lacken lower part	Neusiedler Lacken lower part	Angersdorf	Angersdorf
origin		field	field	field	field
microscope		Olympus SZX16	Leica DM2500	Olympus SZX16	Leica DM2500
n		40	24	73	22
length (µm)	min	393.461	383.202	338.030	348.123
	max	476.917	491.172	461.176	424.493
	mean	431.720	438.493	404.465	387.454
	sd	22.641	21.028	42.640	21.211
width (µm)	min	191.163	217.365	136.150	179.281
	max	253.035	256.690	204.391	213.852
	mean	223.233	240.995	215.166	197.584
	sd	16.485	11.387	32.585	9.407
striae	min	7	7	7	9
	max	10	9	11	12
	mean	8.562	8.375	8.945	10.545
	sd	0.732	0.647	0.705	0.671
colour	RAL9005	100 %	100 %	100 %	100 %
	RAL8011	-	-	-	-
	RAL8007	-	-	-	-
	sd	0.000	0.000	0.000	0.000
shape	ellipsoid prolonged	55 %	87.5 %	100 %	100 %
	ellipsoid rounded	45 %	12.5 %	-	-
	rounded	-	-	-	-
	sd	0.473	0.338	0.000	0.000
angle(°)	min	54.025	62.485	63.430	65.908
	max	81.314	82.594	83.830	79.202

	mean	71.307	70.043	72.687	73.171
	sd	5.618	5.295	4.996	3.679
fossa width(μm)	min	32.464	37.315	34.625	26.930
	max	51.362	47.478	35.504	32.992
	mean	39.596	40.794	31.454	30.064
	sd	4.370	2.841	4.509	1.579
basal width (μm)	min	30.614	47.417	30.920	37.684
	max	63.976	62.887	55.666	46.355
	mean	46.620	54.684	45.155	42.872
	sd	7.106	3.524	5.193	2.090
length/width	min	1.667	1.538	1.597	1.715
	max	2.313	2.040	2.648	2.211
	mean	1.941	1.825	1.899	1.965
	sd	0.139	0.138	0.196	0.140

Table 9. Overview of morphological oospore characters from European and Asian bisexual and parthenogenetic *C. canescens* strains as well as one Asian *Chara altaica* strain.

variable		Can.Neus Lacken	Can.W	Can.F	Can.P	Can.H	Can.A	Can.Pl	Can.V	Can.LZ	Alt.LZ
site		Neusiedler Lacken lower + upper part, Austria	Weißsee, Austria	Fastense e, Germany	Poel, Germany	Haide, Germany	Angersdo rf, Germany	Plován, France	Valencía, Spain	Lake Zerendo, Kasachstan	Lake Zerendo, Kasachstan
origin		field	field	field	field	field	field	field	field	field	field
reproduction mode		bisexual	partheno genese	partheno genese	partheno genese	partheno genese	partheno genese	parthenogenes e	parthenogenese	parthenogenese	parthenogenese
n		104	40	40	40	75	95	22	105	40	43
length (µm)	min	383.202	355.970	399.431	526.009	468.220	338.030	354.533	348.363	501.997	506.794
	max	560.121	523.500	575.234	611.739	603.950	661.176	579.474	439.668	639.344	690.466
	mean	447.543	414.774	494.982	571.050	550.456	400.526	486.087	393.426	574.754	577.817
	sd	31.237	44.859	33.162	23.108	18.468	39.309	43.386	19.741	34.990	36.513
width (µm)	min	180.050	167.620	276.825	348.207	359.610	136.150	203.285	157.847	232.009	232.054
	max	288.979	307.260	376.258	446.466	473.830	404.391	293.195	227.577	357.821	376.005
	mean	236.364	224.597	322.811	407.758	410.205	211.095	244.931	197.861	294.992	315.003
	sd	22.134	31.682	22.529	24.374	18.100	29.810	19.847	13.955	30.702	30.857
striae	min	7	7	7	6	7	7	6	6	8	8
	max	10	11	8	9	10	12	11	10	11	11
	mean	8.529	8.950	7.900	7.825	8.800	9.316	10.273	8.333	9.900	9.628
	sd	0.763	0.959	0.304	0.675	0.615	0.970	1.279	0.840	0.778	0.900
colour	RAL9005	100 %	100 %	100 %	92.5 %	98.7 %	100 %	100 %	100 %	100 %	97.7 %
	RAL8011	-	-	-	7.5 %	-	-	-	-	-	-
	RAL8007	-	-	-	-	1.3 %	-	-	-	-	2.3 %
	sd	0.000	0.000	0.000	0.267	0.231	0.000	0.000	0.000	0.000	0.305
shape	ellipsoid	-	-	-	-	-	-	18.2 %	-	100 %	100 %

	ellipsoid prolonged	59.6 %	37.5 %	5 %	-	-	100 %	81.8 %	100 %	-	-
	ellipsoid rounded	40.4 %	62.5 %	55 %	25 %	-	-	-	-	-	-
	rounded	-	-	40 %	75 %	100 %	-	-	-	-	-
	sd	0.493	0.490	0.580	0.439	0.000	0.000	0.395	0.000	0.000	0.000
angle (°)	min	54.025	66.280	64.426	67.564	70.500	63.430	58.392	60.495	65.847	60.739
	max	82.803	82.230	82.504	84.894	89.580	83.830	88.717	81.034	79.372	81.607
	mean	71.875	75.023	74.837	76.826	80.116	72.799	69.696	72.409	73.412	72.696
	sd	5.602	3.733	4.219	4.742	3.933	4.710	6.369	4.301	3.090	4.974
fossa width (µm)	min	32.223	26.371	43.142	43.630	54.451	26.930	31.941	29.649	37.957	32.131
	max	51.362	52.924	59.321	68.074	84.215	55.504	46.539	47.737	52.465	56.315
	mean	39.879	37.078	49.751	55.726	64.198	38.816	37.953	39.006	44.380	45.309
	sd	3.821	5.099	3.598	4.971	5.231	6.282	3.294	3.561	3.635	5.503
basal width (µm)	min	27.111	27.571	59.867	59.110	65.174	30.920	37.965	38.060	51.357	38.266
	max	67.489	58.147	80.543	100.596	97.064	55.666	52.892	57.022	72.161	69.948
	mean	49.016	41.903	69.820	85.052	78.313	44.626	44.136	45.743	60.618	59.381
	sd	7.061	6.666	4.738	8.388	4.994	4.751	3.200	3.816	5.419	6.813
length/ width	min	1.538	1.571	1.340	1.254	1.130	1.597	1.620	1.690	1.608	1.556
	max	2.637	2.416	1.824	1.580	1.450	2.648	2.229	2.416	2.393	2.338
	mean	1.906	1.862	1.536	1.404	1.343	1.914	1.988	1.996	1.961	1.849
	sd	0.189	0.185	0.090	0.081	0.054	0.186	0.152	0.147	0.153	0.188

Table 7. Summary of oospore wall ornamentation of *C. canescens* and *C. altaica*.

species	location	wall ornamentation
C. canescens	Neusiedler Lacken, Austria	pustular, pustules in irregular linear shape
	Neusiedler Lacken 0.1 PSU, Austria	sparsely pustular, irregular in shape
	Neusiedler Lacken 5 PSU, Austria	sparsely pustular, irregular in shape
	Weißsee, Austria	smooth
	Montpellier, France	smooth
	Kermadec, France	pustular, pustules of irregular lined and dotted shape
	lake Borken, Germany	smooth
	Darss Zingster bodden chain, Germany	smooth
	Angersdorf, Germany	smooth
	Bódon Blanco, Spain	pustular, pustules with regular dotted shape
C. altaica	Lake Zerenda, Kazahkstan	pustular, pustules regularly dotted

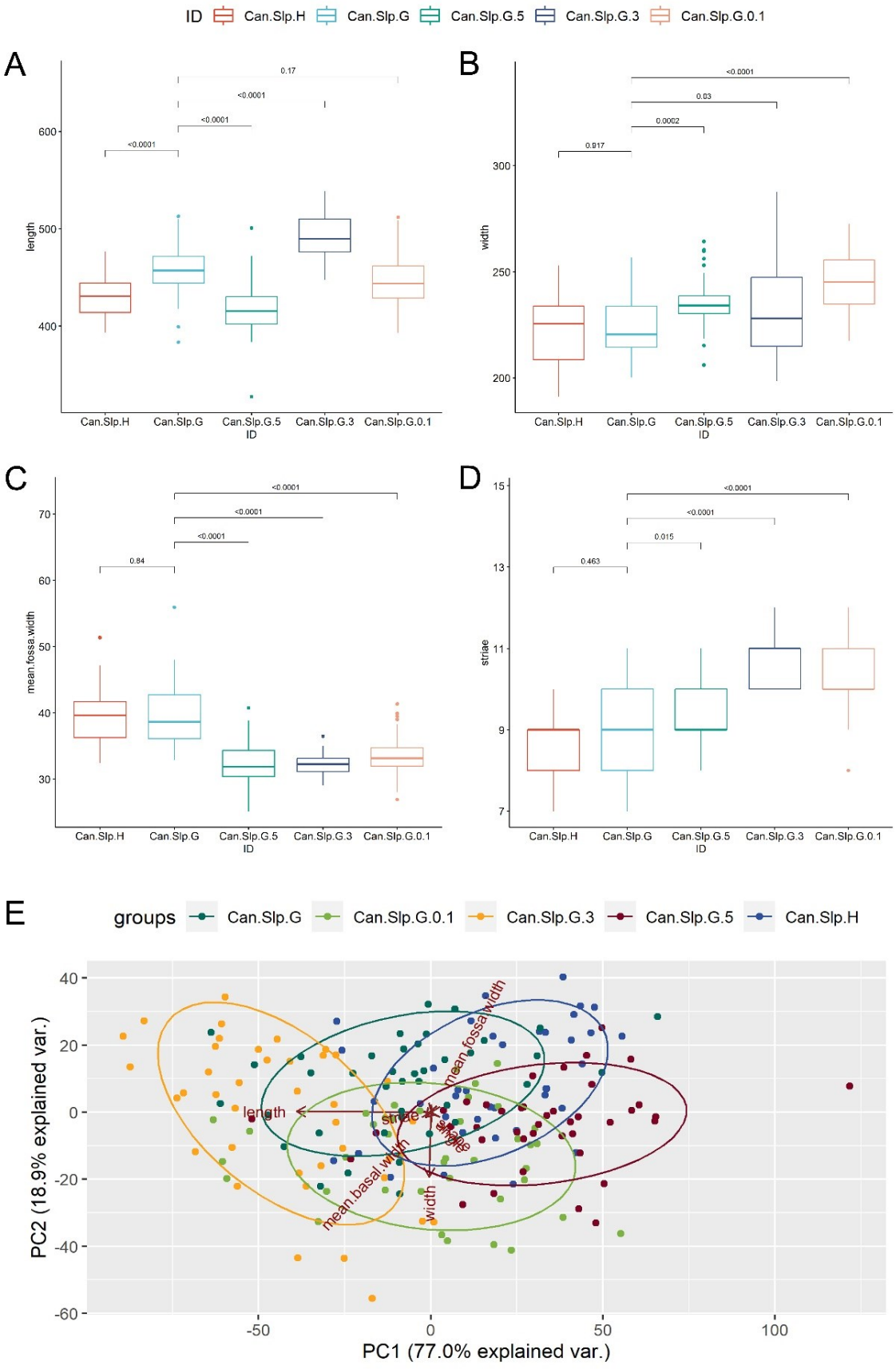

Figure 1. Visualisation of results for the comparison of C. canescens oospores from Neusiedler Lacken fresh material and germination experiments with different artificial salt levels. A-D.

Visualisation of oospore length (A), oospore width (B), mean fossa width (C) and number of striae (D) for the five tested groups. E. Principal component analysis of oospores.

1 oospore length to oospore width ratio
- a. < 1.6 ..... **2**
- b. > 1.6 ..... **3**

2 mean value of the five lines surrounding the pentagonal basal impression
- a. 60 - 70 µm ..... **Can.F**
- b. 70 - 80 µm ..... **Can.H**
- c. > 80 µm ..... **Can.P**

3 oospore length and oospore width
- a. < 400 µm long, < 200 µm wide ..... **Can.V**
- b. 400 - 450 µm long, 200 - 250 µm wide **4**
- c. 450 - 500 µm long, 200 - 250 µm wide ..... **Can.PI**
- d. > 500 µm long, 250 - 300 µm wide ..... **Can.LZ**
- e. > 500 µm long, 300 - 350 µm wide ..... **Alt.LZ**

4 number of striae
- a. 8 - 9 ..... **5**
- b. 9 - 10 ..... **Can.A**

5 mean value of the five lines surrounding the pentagonal basal impression
- a. < 45 µm ..... **Can.W**
- b. > 45 µm ..... **Can.Neus.Lacken**

Figure 2. Determination key for geographically separated *C. canescens* populations of dioecious and parthenogenetic strands.

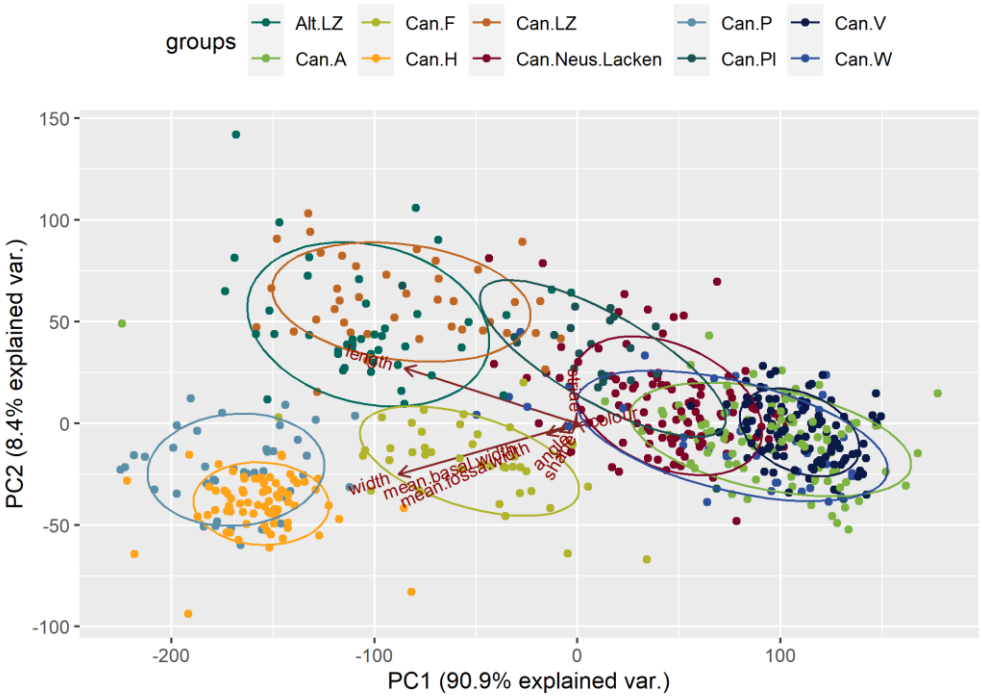

Figure 3. Principal component analysis of *C. canescens* populations of dioecious and parthenogenetic strands.

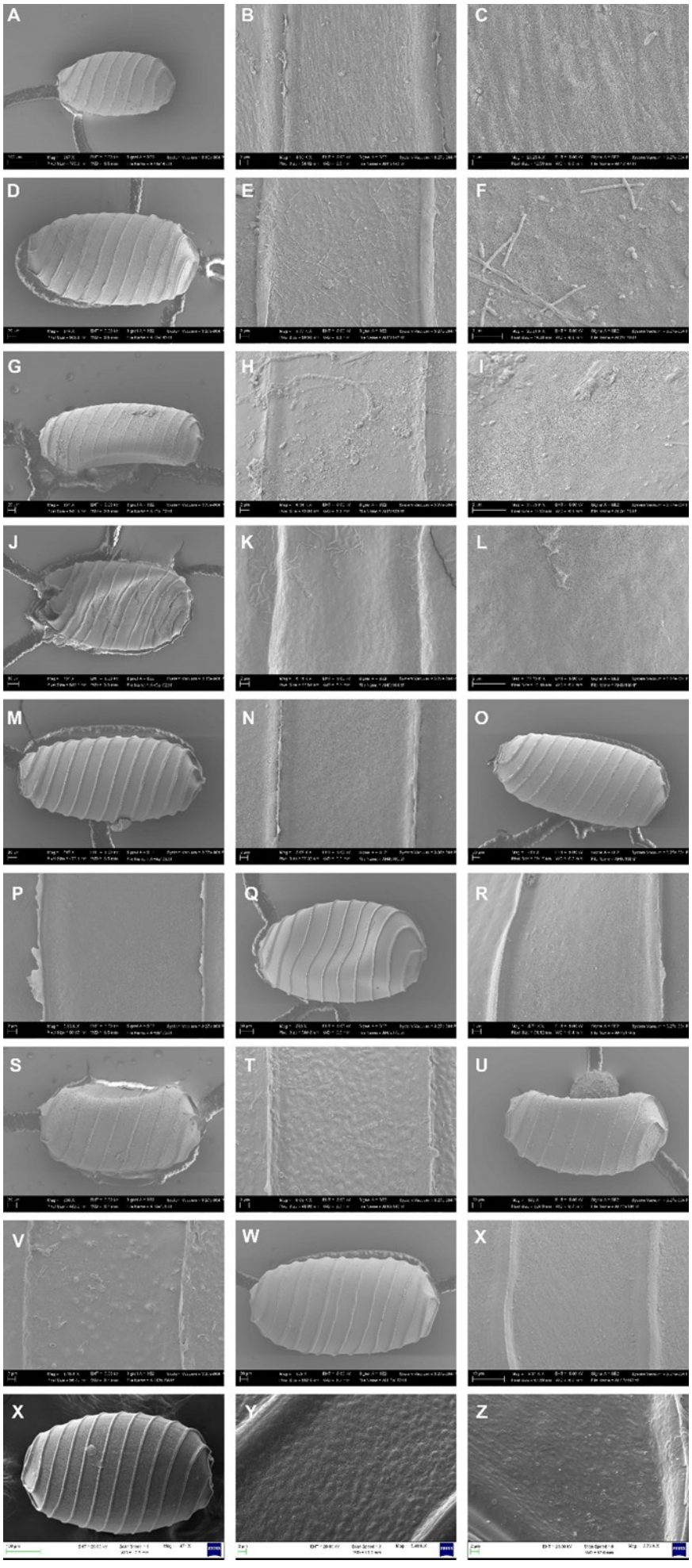

Figure 4. Oospores of *Chara canescens* and *Chara altaica* obtained from different populations. Listed were oospore-IDs (Holzhausen et a. 2024, *In Press*) and location. Images by M.T. Casanova. A-C Can13, Neusiedler Lacken, Austria. A) lateral view, B) fossa and ridges, C) fine details of oospore wall. D-F BB5-5N, Bodón Blanco, Spain. D) lateral view, E) fossa and ridges, F) fine details of oospore wall. G-I Can08, 0.1PSU, Neusiedler Lacken, Austria. G) lateral view, H) fossa and ridges, I) fine details of oospore wall. J-L BS56 Can02, Lake Borken, Germany. J) lateral view, K) fossa and ridges, L) fine details of oospore wall. M-N Darss Zingster bodden chain, Germany. M) lateral view, N) fine details of oospore wall. O-P Can04, Angersdorf Germany O) lateral view, P) fine details of oospore wall. Q-R Can57, Weißsee, Austria Q) lateral view, R) detail of oospore wall. S-T FR-E02 C1, Kermadec, France S) lateral view, T) details of oospore wall. U-V Can04 PSU, Neusiedler Lacken, Austria U) lateral view, V) details of oospore wall. W-X Montpellier, France W) lateral view, X) details of oospore wall. Y-AB *Chara altaica*, Lake Zerenda, Kazakhstan. Y) lateral view, Z-AB) details of oospore walls.