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Morphological differentiation of *Chara aspera* Detharding *ex* Willdenow and *Chara galioides* De Candolle under different environmental variables

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Abstract — *Chara aspera* Detharding *ex* Willdenow and *Chara galioides* De Candolle are two taxa that show close morphological similarities, making it difficult to determine their taxonomic position. Their identification is primarily limited to one reproductive character: the diameter of the mature antheridia, which is larger in *C. galioides*. We attempted to identify other differentiating characters and assess the validity of considering them as one or two different species. Both taxa were cultured under controlled environmental conditions. In the indoor experiment, 216 plants of both taxa were incubated under three different combinations of salinity, light and temperature. The measurements taken during and after the experimental period (9 weeks) included shoot elongation rate, the diameter of the main axis, length and width of spine cells, and length and width of anterior and posterior bract cells. According to our results, the morphometric data acquired from these measurements allow discrimination of *C. aspera* and *C. galioides*. These parameters are useful characters to differentiate these taxa and support the view that they represent independent species.

Charophytes / *Chara aspera* / *Chara galioides* / Algae cultures / Morphology / Ecology / Salinity / Light / Temperature / Spain

Résumé — *Chara aspera* Detharding *ex* Willdenow et *Chara galioides* De Candolle sont deux taxons morphologiquememt très proches, ce qui rend difficile la détermination de leur statut taxonomique. Leur distinction s'appuie en général seulement sur le diamètre des anthéridies mûres qui est plus grand chez *C. galioides*. Le présent travail a pour but de trouver d'autres caractères distinctifs afin de tester les arguments qui les feraient considérer soit comme une seule espèce, soit comme deux espèces différentes. Des expériences sous

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conditions contrôlées ont été effectuées. 216 plantes de chaque taxon ont été cultivées au laboratoire en faisant varier différemment la salinité, la lumière et la température. Le taux d'élongation des plantes, le diamètre de l'axe principal, la longueur et la largeur des acicules et celles des cellules bractées antérieures et postérieures sont mesurées pendant et à la fin de la période d'expérimentation (9 semaines). Les résultats permettent de différencier *Chara aspera* de *C. galioides*. Ces caractères semblent donc utiles pour séparer ces deux taxons et parlent en faveur de leur séparation en tant qu'espèces.

Charophytes / *Chara aspera* / *Chara galioides* / cultures d'algues / Morphologie / écologie / salinité / lumière / température / Espagne

INTRODUCTION

The family Characeae includes green, complex algae that usually form submerged meadows in aquatic systems. The development of these algae depends on the geological substratum, the depth, insolation, temperature, salinity and nutrients in the water (Margalef, 1983; Comelles, 1985; Llimona *et al.*, 1985; Coops, 2002). They are an important component of aquatic ecosystem dynamics, as they are primary producers. In addition, their rhizoids prevent the suspension of sediment particles; therefore they keep the water column clear. They are a very important component of trophic networks and serve as an inhabited refuge where different groups of organisms, such as zooplankton, macroinvertebrates and fish, can reproduce (García, 1990; van den Berg & Coops, 1999; Coops, 2002; Schwarz *et al.*, 2002; Cirujano, 2003).

Species delimitation in the Characeae can be controversial. (Forsberg, 1963; Wood & Imahori, 1964; Wood, 1965; Margalef, 1983; Martín-Closas, 1985; Soulié-Märsche, 1989; Bonis *et al.*, 1993; Blazencic, 1995; Cirujano & Medina, 2002). This is because the morphological variability in response to different and changing ecological water variables is high (Olsen, 1944; Corillion, 1957; Pal *et al.*, 1962; Wood & Imahori, 1964; Forsberg, 1965; Wood, 1965; Margalef, 1983; Llimona *et al.*, 1985; Krause, 1997; Meiers *et al.*, 1997; Cirujano & Medina, 2002; Blindow *et al.*, 2003; Cirujano, 2003).

The species studied in this paper are *Chara aspera* Detharding ex Willdenow and *Chara galioides* De Candolle (De Candolle, 1813; Wood & Imahori, 1964; Wood, 1965), which are distributed in the Northern Hemisphere. These two taxa present a set of similarities and differences that have caused taxonomic controversy. The most obvious difference between them lies in the diameter of the antheridia. Antheridia of *C. galioides* have always been listed as larger (ranging from 700 to 1100 µm) than those of *C. aspera* (which are mostly in the range of 400 to 700 µm). Other differences noted in previous studies are the presence of spherical and whitish bulbils in *C. aspera*, while *C. galioides* does not have these structures. In addition, the spine cells are clearly larger in *C. aspera* (Migula, 1897; Reyes-Prósper, 1910; Groves & Bullock-Webster, 1924; Verdam, 1938; Zaneveld, 1940; Gonçalves de Cunha, 1942; Olsen, 1944; Feldmann, 1946; Corillion, 1952; 1957; Pal *et al.*, 1962; Forsberg, 1963; Wood, 1965; Corillion & Guerlesquin, 1967; 1972; Comelles, 1982; 1985; 1986; Moore, 1988; Bonis *et al.*, 1993; Krause, 1997; Olivares, 1998; Cirujano & Medina, 2002). Depending on the importance given to these morphological differences, the two taxa have been considered two varieties of the same species (Hy, 1913; Feldmann, 1946; Corillion,

1952; Wood, 1965; Comelles, 1985; 1986), one species with a wide ecological scope (Langangen, 1974), or two different species (Corillion, 1957; 1975; Comelles, 1982; Bonis *et al.*, 1993; Cirujano & Medina, 2002; Cirujano, 2003). Recently, genetical investigations showed a clear split between *C. aspera* and *C. galioides* by using AFLP analysis and the total height and length of spine cells (Mannschreck, 2003).

C. aspera is a very common species in Europe, Africa, Asia and N. America (Olsen, 1944). It is present in almost all the communities of Spain and the south of Portugal (Cambra *et al.*, 1998). In contrast, *C. galioides* is less common. It has a mediterranean distribution and it is exclusively found in brackish waters (Corillion, 1952; 1957; Comelles, 1982; 1985; 1986; Cambra *et al.*, 1998; Cirujano & Medina, 2002).

The main objective of this work is to discern which environmental variables affect the morphological characteristics of these two taxa, and to attempt to establish if they are the same species or two different taxonomic entities.

MATERIALS AND METHODS

Site description

Plants of *C. aspera* and sediment for culture were collected in the small pond of Basturs (Isona, Catalonia, Spain. UTM: 31 TCG 3566) on 9 May 2004. *C. galioides* was collected in the Sancho Gómez shallow lagoon (Mota del Cuervo, Cuenca, Spain. UTM: 30 SWJ 1645) on 15 May 2004.

The two Basturs ponds, which are 220m apart, are located in an agricultural zone. The small pond, where the plants of *C. aspera* were found sterile and in a monospecific population at 1.5 or 2 m maximum depth, supported almost constant water level throughout the year. At the time of collection, the conductivity was 0.45 mS/cm and the temperature at 2 m was 12.8ºC. The *C. aspera* specimens were collected in deep, oligotrophic and cool alkaline waters.

The Sancho Gómez shallow and saline lagoon forms part of a bigger complex called Manjavacas. It is subjected to peaks of eutrophy due to human activity and to seasonal changes, since it is a temporary environment that is usually dry in summer. Such oscillations in water level induce chemical changes that directly affect the biodiversity of the lagoon. Field ecological data were measured: the conductivity was 5.18 mS/cm and temperature was 20ºC. The plants of *C. galioides* were found at about a maximum depth of 20 cm and were representative of a population living in shallow, brackish and warm water.

Culture experiment description

Growth experiments were carried out in a culture chamber located at the "Serveis de Camps Experimentals" (Faculty of Biology, University of Barcelona). The controlled variables in the cultures were salinity (**S**), light (**L**) and temperature (**T**). For each one of these three variables, three categories were chosen:

S1 (0 PSU)	**L1** (5-10 $\mu mol.m^{-2}.s^{-1}$)	**T1** (15ºC)
S2 (7.5 PSU)	**L2** (15-20 $\mu mol.m^{-2}.s^{-1}$)	**T2** (20ºC)
S3 (20 PSU)	**L3** (50-60 $\mu mol.m^{-2}.s^{-1}$)	**T3** (25ºC)

PSU: Practical Units of Salinity (Equivalent to g/l or ‰)

The three levels of salinity were prepared by mixing distilled water with different proportions of the salts NaCl, Na_2SO_4, $MgCl_2$, $MgSO_4$, $CaCl_2$ and KCl, in order to obtain the composition of macroconstituents in natural seawater (1: 19.8 g Cl^-, 11.0 g Na^+, 2.8 g SO_4^{2-}, 1.3 g Mg^{2+}, 0.43 g Ca^{2+}, 0.41 g K^+) (Blindow *et al.*, 2003). In the case of the lowest salinity treatments (S1), nine tanks were filled with distilled water only. Light levels were varied by covering the twelve lamps of the culture chamber with different types of plastic mesh, and the photoperiod was 12:12. The T2 and T3 temperature treatments were obtained by introducing a small element heater (220 Volts and 35 Watts) into each one of the nine corresponding tanks. Each element heater set was regulated by an independent thermostat and the culture chamber was adjusted to the minimum experimental temperature (T1).

The combination of the three levels of the three different variables produced a total of twenty-seven experimental conditions (Table 1). Each experiment was carried out in a glass culture vessel (closed tank) of approximately 4 dm^3 which acted as a microcosm.

Specimens used for the *C. aspera* cultures were selected as the most representative in size and condition of the natural population. They were cut into segments of 3 cm, leaving one or two nodes and the end portion of the last internodal cell free. This group was buried into the sediment (van den Berg *et al.*, 1998; Blindow *et al.*, 2003). The *C. galioides* plants used were small specimens. They were selected to be as homogenous as possible. However, in this case, the material used for the cultures were individuals, which were planted with their own rhizoidal apparatus, to correct for their smaller size (an average height of 1.3 cm).

Four 250 ml cylindrical plastic containers were introduced into each tank. We placed one specimen of each taxon into each of these containers, planting the thalli into the substratum to a depth of 1.5 cm (Corillion, 1957). The substratum had previously been mixed and sieved (2 mm mesh size). In total, 216 apical sections of both species were planted (4 replicates/taxon for each one of the 27 microcosms).

In addition, to prevent a possible shading effect among the different tanks or culture vessels, the

Table 1. Combination of the three levels of the three variables forming a total of 27 different conditions

Tank	*S1*	*S2*	*S3*	*L1*	*L2*	*L3*	*T1*	*T2*	*T3*	*Conditions*
1										S1 L1 T1
2										S1 L1 T2
3										S1 L1 T3
4										S1 L2 T1
5										S1 L2 T2
6										S1 L2 T3
7										S1 L3 T1
8										S1 L3 T2
9										S1 L3 T3
16										S2 L1 T1
17										S2 L1 T2
18										S2 L1 T3
10										S2 L2 T1
11										S2 L2 T2
12										S2 L2 T3
13										S2 L3 T1
14										S2 L3 T2
15										S2 L3 T3
22										S3 L1 T1
23										S3 L1 T2
24										S3 L1 T3
25										S3 L2 T1
26										S3 L2 T2
27										S3 L2 T3
19										S3 L3 T1
20										S3 L3 T2
21										S3 L3 T3

tanks were placed under a rotation controlled system. To avoid the stratification of water, it was mixed by a plastic tube system with an end diffuser, all connected to only one air pump.

Controlled measurements

The experiment ran for 63 days (25 May-26 July, 2004), during which time two types of measurements were taken in a total of fourteen non-consecutive days of lab measurements. These were repeated measurements and non-repeated measurements. Repeated measurements were taken one or two times per week. They included: shoot elongation, number of whorls, number of branchlets/whorl and generation of lateral branches (the last three observations were not treated statistically). The non-repeated measurements were taken at the end of the experiment, after the plants were taken out of the containers and from the units in tanks 13, 14 and 15 (the plants that had reproductive structures). These tanks only differed in temperature, with increasing growth when temperature increased. The non-repeated measurements were: the size of spine cells (length and width to the base); the diameter of the main axis and the anterior and posterior bract cells measurements (length and width to the base).

Statistical analysis

Statistical analyses were performed with SPSS 10.0 software. Several types of analyses of variance were applied. In the case of shoot elongation measurements, data were analyzed according to the linear model with repeated measures. Single factor ANOVA analyses were used for the case of the rest of the variables (length and width of spine cells and of anterior and posterior bract cells and main axis diameter).

RESULTS

Main shoot elongation rate

In a general context, without considering the precise effect of each one of the levels of the three variables, both taxa presented different shoot elongation tendencies throughout the experiment (Fig. 1).

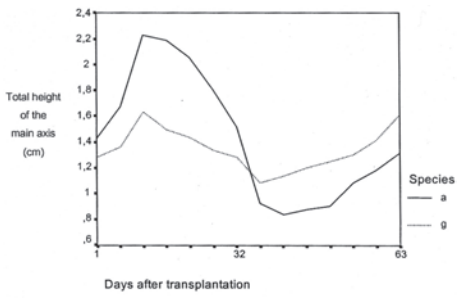


Fig. 1. Differential growth of *Chara aspera* (**a**) and *Chara galioides* (**b**) throughout the culture period.

Despite the fact that *C. aspera* was planted without its own rhizoidal apparatus, it showed a higher growth rate, mainly at the beginning of the culture. There was a sharp decline in its growth rate between the 3rd and 8th day of measurement, from 13 to 36 days after transplantation. This coincided with the demise of all the specimens subjected to the highest salinity (S3) at midday on the 8th day of measurement. From the 8th day on, the growth values recovered gradually, due to the growth of lateral branches and the resprouting of others. *C. aspera* responded fast and radically to different culture conditions. *C. galioides*

presented a more constant and relatively progressive increase in growth rate over time. Growth declined from the 8th day of measurement (36 days after transplantation). However, this decline was not as pronounced as that of *C. aspera*, and *C. galioides* made a significant recovery later on.

Each one of the three experimental variables caused differences in the growth of the shoots. In both species, these differences were mainly dependent on the salinity treatment (Tab. 2). The optimum salinity for the growth of both taxa was clearly treatment S2, particularly in the case of *C. aspera* plants, followed by S1. The treatment S3 was lethal from the 7th to the 8th day of measurement (Fig. 2).

The three levels of the variable temperature caused similar tendencies in the growth rate of both taxa. However, in *C. aspera*, an increase in temperature diminished the growth. In contrast, the growth rate of *C. galioides* increased significantly when the temperature increased (Fig. 3).

The levels of light used were varied enough to show significant differences in the growth rates of both taxa. *C. aspera* presented greater growth as the levels of light increased. *C. galioides* did not show a direct pattern, since it grew more in the L3 treatment, followed by L1 and L2 (Fig. 4).

The interaction that had the most significant results on the different measured parameters was salinity-light (Tab. 2). An increase in the light variable at medium salinities, led to an increase in the shoot elongation rate of the main axis in both taxa. However, the effect on *C. galioides* was greater than on *C. aspera*. The plants could not develop under S1 and S3 (Fig. 5).

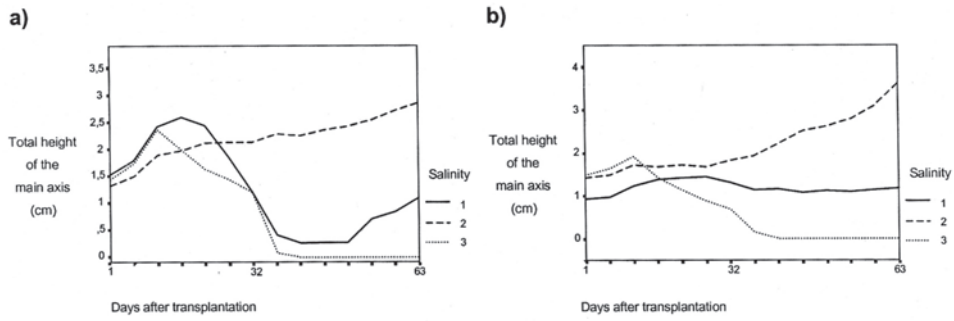


Fig. 2. Height evolution of the plants of *Chara aspera* (**a**) and *Chara galioides* (**b**) in response to salinity (**S1**: 0 PSU, **S2**: 7.5 PSU, **S3**: 20 PSU).

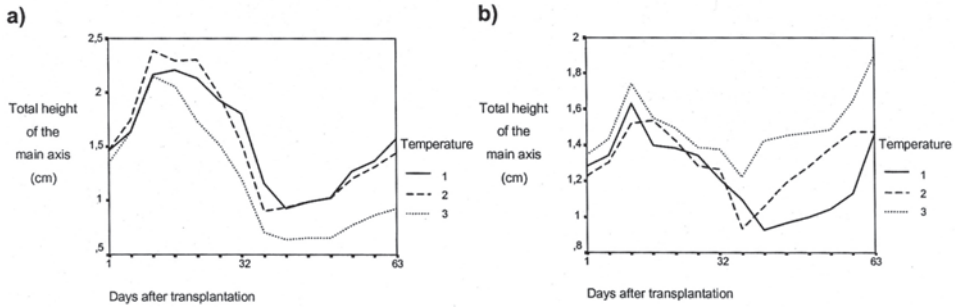


Fig. 3. Height evolution of the plants of *Chara aspera* (**a**) and *Chara galioides* (**b**) in response to temperature (**T1**: 15ºC, **T2**: 20ºC, **T3**: 25ºC).

Spine cells (length and width to the base)

We observed differences in the length and width of the spine cells. They were clearly longer and wider in the case of *C. aspera* (Figs 6-7). The three levels of the variable temperature significantly affected the development of the length of *C. aspera*'s spine cells: the greatest development occurred in T2, followed by T1 and then T3 (Tab. 2, Fig. 8). On the other hand, the three levels of the temperature variable did not significantly affect the development in the length of *C. galioides* structures (Tab. 2, Fig. 8). However, the three levels of temperature significantly affected the width of spine cells in both taxa: spine cells were narrower at higher temperatures (Tab. 2, Fig. 9).

Main axis diameter

The species factor was the only one that caused significant differences in the diameter of the main axis. This was clearly larger in the case of *C. galioides* (Tab. 2, Figs 6-7). This parameter could therefore be used to distinguish between *C. aspera* and *C. galioides*. The increase in temperature led to a clear development pattern of *C. aspera*'s main axis diameter. The value slightly decreased with the increasing temperature (Fig. 10).

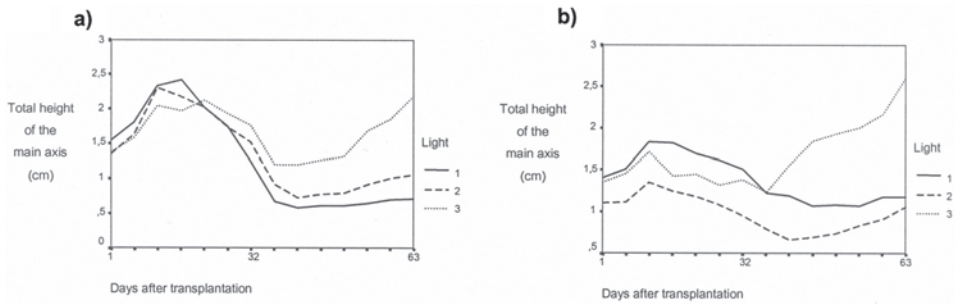


Fig. 4. Height evolution of the plants of *Chara aspera* (**a**) and *Chara galioides* (**b**) in response to light (**L1**: 5-10 µmol.m^{-2}.s^{-1}, **L2**: 15-20 µmol.m^{-2}.s^{-1}, L3: 50-60 µmol.m^{-2}.s^{-1}).

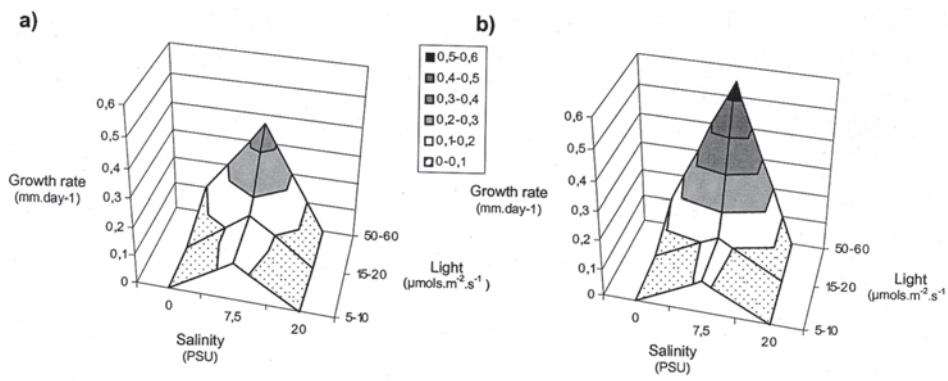
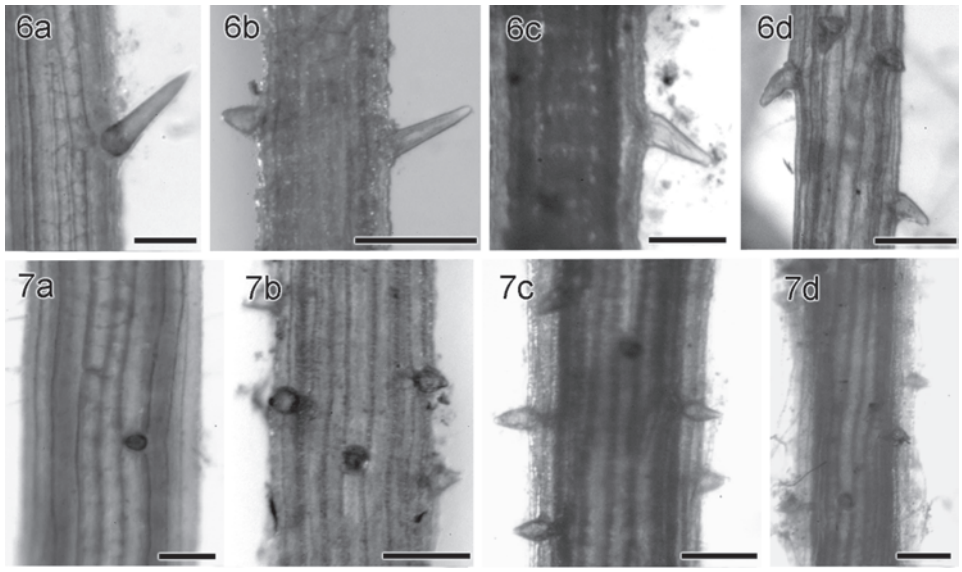


Fig. 5. Shoot elongation rate of *Chara aspera* (**a**) and *Chara galioides* (**b**) in response to different salinity and light treatments.



Figs 6-7. **6.** *Chara aspera* main axis and spine cells at the beginning of cultures (**a**) and at the end of the experimentation period [Tank **13** (**b**), Tank **14** (**c**) and Tank **15** (**d**)]. **7.** *Chara galioides* main axis and spine cells at the beginning of cultures (**a**) and at the end of the experimentation period [Tank **13** (**b**), Tank **14** (**c**) and Tank **15** (**d**)]. Scale bars: 125 µm.

Table 2. Main ANOVA results: significances (P-values) of the effects of salinity, light, temperature, their interactions and the species factor on different parameters ($\alpha = 0.05$) (**Ca**: *Chara aspera*; **Cg**: *Chara galioides*; **n.s.**: test not significant)

Parameter	*Species*	*Salinity*	*Light*	*Temperature*	*S × L*	*S × T*	*L × T*	*Species factor*
Shoot elongation rate	Ca	0.000	0.005	0.015	0.004	n.s.	n.s.	
	Cg	0.000	0.000	n.s.	0.000	0.014	0.000	
Spine cells length	Ca			0.000				n.s.
	Cg			n.s.				n.s.
	Both			0.033				0.000
Spine cells width	Ca			0.035				n.s.
	Cg			0.000				n.s.
	Both			n.s.				0.000
Main axis diameter	Ca			n.s.				n.s.
	Cg			n.s.				n.s.
	Both			n.s.				0.000
Anterior bract cells length	Ca			0.000				n.s.
	Cg			0.015				n.s.
	Both			0.000				0.000
Anterior bract cells width	Ca			0.006				n.s.
	Cg			0.001				n.s.
	Both			0.000				0.018
Posterior bract cell length	Ca			0.024				n.s.
	Cg			0.000				n.s.
	Both			0.000				0.034
Posterior bract cells width	Ca			n.s.				n.s.
	Cg			n.s.				n.s.
	Both			n.s.				n.s.

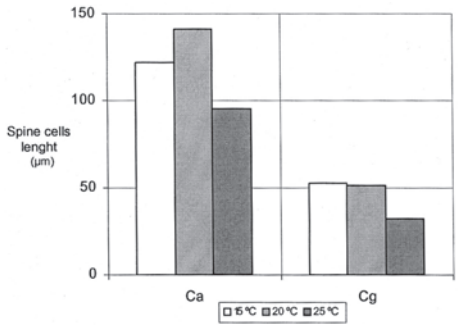


Fig. 8. Spine cells length at the end of the experimental period and for the different temperature treatments (**Ca**: *Chara aspera*; **Cg**: *Chara galioides*).

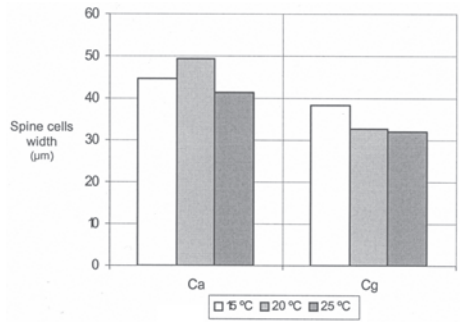


Fig. 9. Spine cells width at the end of the experimental period and for the different temperature treatments (**Ca**: *Chara aspera*; **Cg**: *Chara galioides*).

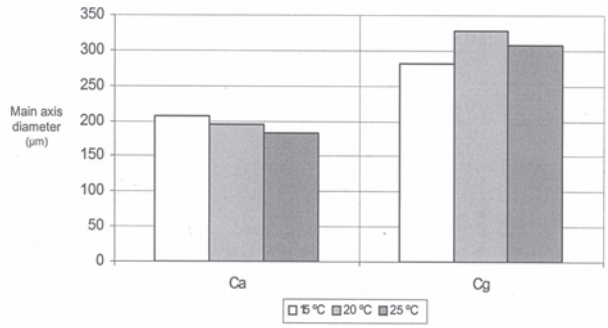


Fig. 10. Main axis diameter at the end of the experimentation period and for the different temperature treatments (**Ca**: *Chara aspera*; **Cg**: *Chara galioides*)

Anterior and posterior bract cells (length and width)

The temperature variable affected significantly the development of the bract cells, although it had no effect on the posterior bract cells width (Tab. 2). The length of the posterior bract cells decreased with increasing temperature, though less in *C. aspera* than in *C. galioides*, and similarly happened but to a lesser degree, in the case of the width (Figs 11-12). The length of the anterior and posterior bract cells and the width of the anterior bract cells were also significant characters to distinguish between these two taxa (Tab. 2).

DISCUSSION

Light has often been proposed as the most significant factor in determining the vertical zonation and dynamics of characean populations and possible polymorphisms. However, temperature, water and soil characteristics are also very important (Zaneveld, 1940; Corillion, 1957, 1975; Forsberg, 1963; Dale, 1986; van den Berg *et al.*, 1998; Coops, 2002; Schwarz *et al.*, 2002; de Winton *et al.*, 2004). At the same time, we consider that the growth rate is not constant, and is dependent on the different light conditions at different depths. The results of this study are

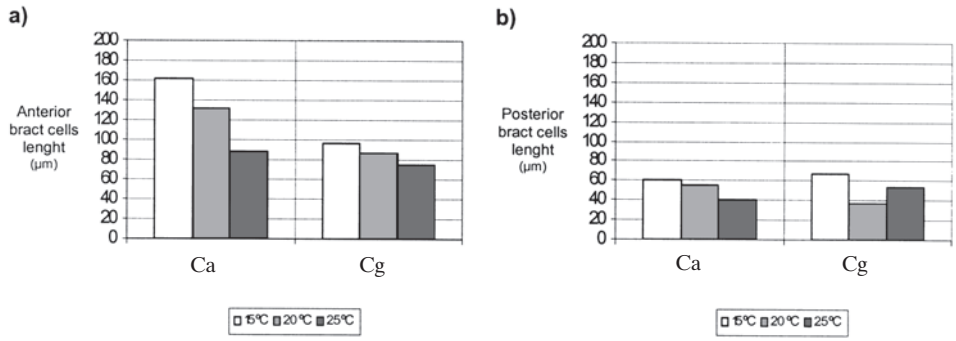


Fig. 11. Anterior (**a**) and posterior (**b**) bract cells length at the end the experimental period and for the different temperature treatments (**Ca**: *Chara aspera*; **Cg**: *Chara galioides*).

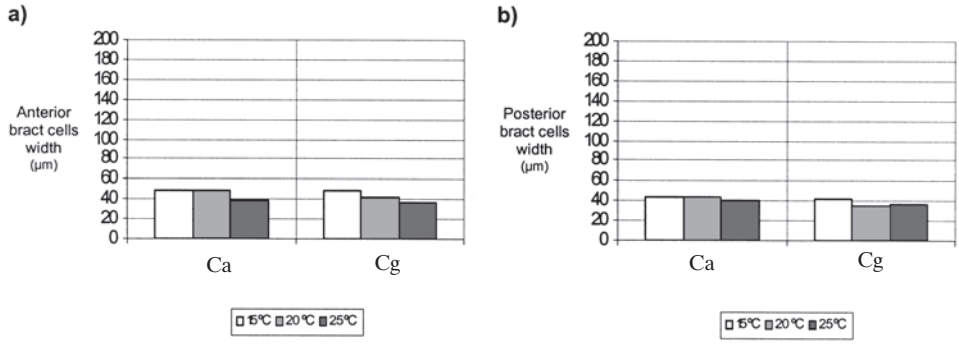


Fig. 12. Anterior (**a**) and posterior (**b**) bract cells width at the end the experimental period for the different temperature treatments (**Ca**: *Chara aspera*; **Cg**: *Chara galioides*)

consistent with the literature for both taxa in terms of the three light levels. These results allow us to distribute both taxa throughout a gradient of vertical zonation that coincides with previous bibliographical data. In such data, *C. aspera* shows a preference for living in deeper, clearer and cooler alkaline waters (van den Berg, 1999). *C. galioides* seems to prefer mainly temporary, shallow and relatively brackish waters, that warm up very quickly (Corillion, 1975; Coops, 2002).

The maximum depth for macrophytes is limited by hydrostatic pressure, light and temperature. Thus, when turbidity does not limit the light penetration and the temperature at depth is enough to allow vegetal growth, the plants that are adapted to depth are usually characean (Dale, 1986). *C. aspera* likes deeper waters and grew more at the lowest temperatures in our cultures. In contrast, *C. galioides* grows better at higher temperatures and is adapted to grow more precociously, due to the swiftly changing conditions of the shallow habitats where it usually grows.

The temperature in aquatic systems is considered more homogeneous than for terrestrial habitats. However, thermic microchanges are important to understand the species and the distribution of characeae communities (Corillion, 1957). This fact serves to justify that, even though the range of the three temperature levels in our cultures is not sufficiently wide, the results, show different growth rates in the specimens.

The intermediate level of salinity (S2), in which the two species present the maximum growth, could be considered as the highest limit of salinity tolerance for *C. aspera*. This is established at values between 8-15 PSU (Blindow, 2000). However, it has also been observed in much higher salinities: 19.5-25 PSU (Blindow, 2000). In the same way, Corillion (1975) classifies *C. aspera* as a species that is able to live in habitats where the water is slightly brackish (accidental halophilous species), where it may have morphological variations. Therefore, the S2 level of salinity could be considered as an underestimated highest limit for *C. galioides*, since this species is considered a true halophile. It is often found in coastal, brackish shallow lakes, where it tolerates salinities of 14 PSU. It has been recorded in localities with salinity levels as high as 20 to 40 PSU (Corillion, 1957; 1975). Meanwhile, the highest level of salinity (S3) is too far above the intermediate level, and it masks the two species' true highest limit of tolerance to salinity.

Blindow *et al.* (2003), showed that specimens of *C. aspera* that are common in brackish waters survived at the same highest salinity as one of our cultures (20 PSU). However, the plants reduced their biomass and gametangia production. This indicates that higher salinity is a stress factor for this taxon. In contrast, the units of *C. aspera* from freshwater could not acclimate to the same conditions, as in our experiment. Blindow *et al.* (2003) also affirmed that plants of *C. aspera* collected from freshwater performed best. Such plants increased their daily rate of growth at low salinity. Plants collected from brackish water were better adapted to intermediate salinities. Our results, like those of Bonis (1993), indicate that under the three levels of salinity used, both species present a similar dynamic trend in the daily rate of growth. Therefore, if we only look at the salinity factor, it is possible to consider that they can coexist in the same habitat.

Characeae osmoregulation occurs by means of K^+ accumulation. Other algal groups use the alternative mechanism of Na^+ accumulation (Blindow *et al.*, 2003). The K^+ accumulation mechanism is present in different euryhaline characean taxa, suggesting that the mechanism was already in place before the main actual lineage existed. The brackish characean species (i.e. *C. galioides*) may have originated from those euryhaline forms, which lost their ability to regulate the osmoregulation completely. In contrast, the mesohaline species (i.e. *C. aspera*) are not able to maintain the same regulation rate of turgidity, because of the increment of Na^+ at elevated salinities. Therefore, they are restricted to salinities of 15-20 PSU by their incomplete regulation (Winter *et al.*, 1987; Winter & Kirst, 1990; 1992; Winter *et al.*, 1996; Winter *et al.*, 1999; Blindow *et al.*, 2003). The difference in the capability of the species of Characeae to regulate their turgidity partly explains their distribution in the world and has effects on growth, cellular division and cell elongation (Winter & Kirst, 1992). Blindow *et al.* (2003) concluded that genetic differences, more than physiological acclimatization to the different conditions, are the true causes of the different optimal levels of salinity in two populations of *C. aspera*.

The investment in structures for generative reproduction supposedly increases under stressfull conditions (de Bakker *et al.*, 2001). However, Blindow *et al.* (2003) observed that the number of gametangia diminished when salinity increased. We only found non-mature gametangia in the algae subjected to the intermediate salinity level. This was the level that allowed a greater growth in the height of the units and the consequent passage from vegetative to reproductive stage (Winter & Kirst, 1992). Many authors differentiate between *C. galioides* and *C. aspera* on the basis of the diameter of the mature antheridia only. They avoid other possible differentiating vegetative and ecological characters that have been

shown to be equally valid for separating the two species. The attempt to delimit taxonomic units and understanding of phylogenetic relationships within the *Characeae* requires interdisciplinary collaboration, including genetic molecular sequencing, culture studies and crossing experiments. The work of Corillion (1957) suggested that both taxa in this study should be cultured, particularly by the germination of oospores of *C. aspera* in brackish conditions and those of *C. galioides* in freshwater conditions.

Some authors do not clarify their position on whether they consider *C. galioides* as a different species to *C. aspera*, indicating only that these taxa are morphologically similar (Olsen, 1944). These two taxa have mainly been differentiated on the basis of: the diameter of mature antheridia (bigger in *C. galioides*); the presence or not of bulbils in the rhizoidal apparatus (*C. galioides* hardly produces bulbils and when it does, they are transparent; *C. aspera* frequently produces bulbils and they are spherical and whitish); and the dimensions of the bract cells and the spine cells (larger and wider in *C. aspera*) (Reyes-Prósper, 1910; Groves & Bullock-Webster, 1924; Zaneveld, 1940; Gonçalves de Cunha, 1942; Olsen, 1944; Corillion, 1957; Wood & Imahori, 1964; Wood, 1965; Corillion & Guerlesquin, 1967; 1972; Comelles, 1982; 1985; 1986; Moore, 1988; Bonis *et al.*, 1993; Krause, 1997; Olivares, 1998; Cirujano & Medina, 2002). Recently, Mannschreck (2003) has listed total height and length of spine cells as morphological characters that can be used to distinguish *C. aspera* from *C. galioides*. Some of these characters, for example, antheridia diameter or spine cells size, could be more or less developed depending on the salinity of the environment (Corillion, 1957; Comelles, 1986; Bonis *et al.*, 1993). If, due to osmotic phenomena, the salinity of the water causes changes in the morphometry of these structures, the supposed halotolerancy of *C. galioides* could not be considered a consistent criterion for differentiation and would not have any systematic meaning. In addition, in some environments the two taxa have been found together with their traditional morphological features (Rodríguez Femenías, 1904; Margalef, 1952; Corillion, 1977; Guerlesquin, 1980; Asensi & Nieto, 1981; Cirujano, 1982; Velayos *et al.*, 1989; Cirujano *et al.*, 1992; Bonis *et al.*, 1993; Cirujano & Medina, 2002). Thus it could be assumed that they are reproductively isolated. However, some studies corroborate the reliability of using the diameter of antheridia to distinguish both taxa, and have enzymatic results to confirm this (Bonis *et al.*, 1993). In addition, according to the data in this experiment, the dimensions of the spine cells, the diameter of the main axis, the length of the anterior and posterior bract cells and the width of the anterior bract cells, could be considered as valid vegetative characteristics for the differentiation of *C. aspera* and *C. galioides*.

CONCLUSIONS

The variables salinity, light and their interaction were the main factors that determined the growth rate for both taxa, which presented the highest growth rate at the intermediate salinity level. *Chara aspera* presented an increase in its growth rate when the level of light increased. This fact reflects the preference of this taxon for clear waters. In contrast, it also grew more when the temperature diminished, showing its preference for deeper, cooler waters. *Chara galioides* did not present a clear pattern of growth in relation to light, but responded well to

high light conditions as would be expected in shallow environments. It also seems that it is able to tolerate shaded places. Simultaneously, it grew more when the value of the variable temperature increased, showing a thermophilous characteristic pattern. Temperature significantly affected the spine cell development of both taxa, especially the width and development in length of *C. aspera*. The species factor showed significant differences with respect to the main diameter axis for both taxa. This was clearly wider in the case of *C. galioides*. The temperature significantly affects the development of the bract cells, although less in the case of the width of the posterior bract cells. From the results obtained here, *C. aspera* and *C. galioides* appear to display systematic differences in the diameter of the main axis, the dimensions of the spine cells, the length of the anterior and posterior bract cells, and the width of the anterior bract cells. As these characters were maintained under identical culture conditions, they are likely to represent genetic differences and support the separation of *C. aspera* and *C. galioides* as distinct species.

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