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A review on the distribution and habitat features of *Chara canescens* in the Iberian Peninsula: sexual populations revisited

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ABSTRACT

A review on the distribution and habitat features of *Chara canescens* in the Iberian Peninsula: sexual populations revisited.

Chara canescens, mainly distributed in Europe and the African Mediterranean coast, is the only Charophyceae species capable of reproducing parthenogenetically, the asexual populations being common; however, the sexual ones are rare. In this study we compile all the literature and herbaria data concerning the presence of *C. canescens* in the Iberian Peninsula and the Balearic Islands over several decades, analysing the historical and spatial distribution of the species and the limnological characteristics of its habitats. A decline in the number of records in the literature and herbarium sheets since 2010 was detected. Most of the populations are concentrated in Castilla-La Mancha, Andalucía and Castilla-León. The species lives in brackish waterbodies, both in coastal areas and endorheic shallow small lakes, and the majority of these locations do not exhibit a good conservation status. The only three locations with sexual populations previously cited in the literature (Bodón Blanco –Valladolid- and Las Eras –Segovia- lakes and Lucio Largo –Doñana-) were revisited in June 2023. Sexual populations of *C. canescens* in the two former lakes occurred, but the lake in the Doñana National Park was completely dry. Two new sexual populations of the species were found in La Iglesia and Caballo Alba lakes (Segovia), increasing the citation to five sexual populations. A description of population (coverage, male:female ratio) and individual (reproductive features) variables for the four *C. canescens* sexual populations are provided. The analysed limnological variables (water chemistry, other hydrophytes and marginal vegetation) showed that the lakes harbouring these populations exhibited signs of pollution, as well as altered and unstructured marginal vegetation. The genetic diversity of the species depends on the sexual populations, which are very rare in Europe, and are mainly concentrated in Spain. Therefore, the preservation of these inner brackish-water habitats is essential for the implementation of transnational effective conservation measures to protect this relevant and particular species.

KEY WORDS: Characeae, limnological characterisation, Iberian Peninsula, geographic parthenogenesis, sexual propagation, brackish aquatic ecosystems, aquatic macrophytes.

RESUMEN

Revisión de la distribución y de las características del hábitat de **Chara canescens** *en la península Ibérica: revisitando las poblaciones sexuales.*

La especie Chara canescens*, principalmente distribuida en Europa y la costa africana mediterránea, es la única de la clase Charophyceae capaz de reproducirse partenogenéticamente, y posee poblaciones asexuales que son comunes pero las sexuales son raras. En este estudio reunimos datos de la literatura y de herbarios sobre la presencia de* C. canescens *en la península Ibérica y las Islas Baleares en las pasadas décadas, analizando su distribución histórica y espacial y las características limnológicas de sus hábitats. Se ha detectado un descenso del número de registros en la literatura y en herbarios desde 2010. La mayoría de las poblaciones se concentran en Castilla-La Mancha, Andalucía y Castilla-León. La especie vive en masas acuáticas salobres tanto en las zonas costeras como en lagunas endorreicas pequeñas y someras, y la mayoría de ellas no muestran un buen estado de conservación. Revisitamos las tres únicas localizaciones con poblaciones sexuales previamente citadas en la bibliografía (lagunas Bodón Blanco –Valladolid-, Las Eras –Segovia- y Lucio Largo –Doñana-) en junio de 2023. Encontramos poblaciones sexuales en las dos primeras lagunas, pero la del Parque Nacional de Doñana estaba completamente seca. Se descubrieron dos nuevas poblaciones sexuales para la especie en las lagunas La Iglesia y Caballo Alba (Segovia), aumentando así la cifra a cinco poblaciones sexuales. Se aporta la descripción de variables poblacionales (cobertura, proporción machos:hembras) e individuales (características reproductivas) para las cuatro poblaciones. Las variables limnológicas analizadas (hidroquímica, otros hidrófitos y vegetación marginal) indican que las lagunas con dichas poblaciones muestran signos de contaminación y de vegetación alterada y desestructurada. La diversidad genética de la especie depende de las poblaciones sexuales, las cuales son muy raras en Europa, y están concentradas principalmente en España. Por tanto, la preservación de estos hábitats salobres de interior es esencial para la implementación de medidas efectivas transnacionales para proteger a esta particular y relevante especie.*

PALABRAS CLAVE: *Characeae, caracterización limnológica, Península Ibérica, partenogénesis geográfica, propagación sexual, ecosistemas acuáticos salobres, macrófitos acuáticos.*

INTRODUCTION

Charophyta are a group of macroscopic green algae (Streptophyta infrakingdom, family Characeae) that play a fundamental role in the ecosystems in which they develop because they control and provide a multitude of ecological functions and ecosystem services (Coops & Doef, 1996, Meerhoff et al., 2003), acting, for example, as ecosystem engineers. However, the level of knowledge about this group of algae is far from that of terrestrial vascular plants and has traditionally been scarcely studied in Portuguese and Spanish botanical surveys, with their records often being secondary and included in more general studies (Cirujano et al., 2007). Among the difficulties in the study of these algae is the lack, in many cases, of specimens preserved in public accessible herbaria to confirm their identity and to contrast the information contained in bibliographical references. These circumstances, combined with a high phenotypic variability, complicate their taxonomy and cause some complex nomenclatural problems (Cirujano et al., 2007).

Moreover, many species of charophytes are seriously threatened by the consequences of global change: enhanced eutrophication, an increase in temperatures, desiccation and other disturbances that are affecting many waterbodies, especially in the Mediterranean region (Álvarez-Cobelas et al., 1991, Blindow, 1992, Jeppesen et al., 2011, Calero et al., 2015). Related to this, the studies assessing the distribution of the species from a historical perspective are very useful, and can help us to understand how the species, their ecological niches and the ecosystems that they inhabit have evolved over time, which may contribute to developing effective conservation measures (Chauvel et al., 2006, Nelson et al., 2013, Mosena et al., 2018, Büttner et al., 2022, Buldrini et al., 2023).

The focus of the present study, is *Chara canescens* Desv. & Lois. (1810), a species mainly distributed in Europe and the African Mediterranean coast, which has its highest prevalence in the coastal areas near the Baltic Sea, with occasional localities in Australia, North America and Asia (Schubert et al., 2024). The Iberian populations are distributed throughout the peninsula, although they are scarcer in the western half (Cirujano et al., 2007, Schubert et al., 2024). Some populations were also recorded in the Balearic Islands in the past (Margalef, 1952, 1953; Comelles, 1984, 1986; Martínez-Taberner & Pericàs, 1988;

Martínez-Taberner et al., 1995). This species is unique among charophytes because it shows a combination of characteristics not found in any other species: on one hand, a combination of complete haplostic cortication and dioeciousness (Corillion, 1975) and, on the other, the occurrence of parthenogenetic reproduction, in which females produce oospores without fertilisation (Ernst, 1920). It has been described as an annual species, although overwintering individuals have been found in the Mediterranean region (Calero & Rodrigo, 2018). *C. canescens* inhabits brackish waters, often temporary, being unable to grow in freshwaters or marine conditions and it is also a species with a multitude of varieties and forms (Blindow & Schubert, 2004).

Clearly, the most notorious characteristic of the species is its ability to reproduce parthenogenetically (Proctor, 1980). Parthenogenesis has been recorded in other organisms inhabiting temporary (Schmit et al., 2013) or brackish water bodies (Nascetti et al., 2003), and it has been well-documented in various species of zooplankton with similar habitats (Birky & Gilbert, 1971, De Meester, 1996, Serra et al., 2019). Parthenogenetic reproduction is not common in aquatic plants and does not occur in any other species of the genus *Chara*, nor in other charophytes. It has been suggested that this kind of reproduction may increase fitness, especially in marginal, stressful or extreme habitats with a high degree of subdivision, recolonization and local extinction (Haag & Ebert, 2004), being common in invasive species which are favoured by the fragmentation and reduction of the habitat caused by humans.

Schaible and Schubert (2008) put forward some hypotheses for the success of this reproductive strategy in *C. canescens*, where parthenogenetic populations seem to have evolved from the sexual ones and to have surpassed them in distribution range. These hypotheses are: higher probability for asexuals to propagate in pioneer habitats, higher energetic costs of sexual reproduction, and differences in ecophysiological acclimation abilities between male and female organisms in general, or between sexually and asexually propagating populations. Currently, sexual populations are rare throughout the world, they are restricted to Europe, with the Mediterranean region standing out, where five sexual populations were recorded over several decades: two in Italy (Becker, 2019, Troia, 2020) and three populations in Spain (Comelles, 1986, García-Murillo et al., 1993). This reproductive strategy in *C. canescens* seems to follow the ecological pattern of "geographical parthenogenesis" (Vandel, 1928), where parthenogens occupy a wider geographic range while their close sexual relatives are restricted to a core area, presenting different distribution with potential overlap (Hörandl, 2009), like the sympatric population of *C. canescens* which was confirmed in Austria by Schaible et al. (2011).

The present study reviews bibliography and herbarium data concerning *Chara canescens* in order to establish the historical distribution and the main limnological features of the habitat of its parthenogenetic and sexual populations in the Iberian Peninsula and the Balearic Islands. Moreover, the sexual populations described in the literature have been revisited to gain information about population and individual characteristics of this species. The main aim of this work is to contribute to the establishment of the scientific and conceptual basis to protect the genetic diversity of *C. canescens* and the necessary gene flow between populations through the implementation of transnational effective conservation measures.

MATERIALS AND METHODS

Revision of bibliography and herbarium data concerning *Chara canescens* and its habitat

In order to list and catalogue the populations of *C. canescens*, the information contained in bibliographical references has been collected. Following the study by Cirujano et al. (2008) as a basis, the references citing *C. canescens* present in ResearchGate, Google Scholar and several other webpages have been reviewed. Moreover, additional bibliographical material (reports, books and thesis) has been also accessed.

Two herbaria were reviewed: the Royal Botanical Garden of the Madrid Herbarium (MA), in Spain, and the Botanical Garden of the National History and Science Museum of the Lisboa Herbarium (LISU), in Portugal. In the MA-Algae, 27 sheets and 46 specimens preserved in conservative solution were re-examined. Observations were made using a stereo-microscope. In the LISU, Dra. Ana Isabel Correia checked for the existence of *C. canescens*. The identification number of the material reviewed can be consulted in Table S1 of Supplementary Material (available at https://www.limnetica.net/en/limnetica).

From the bibliography, the considered limnological features of the habitat were mainly water pH, conductivity, chloride and ammonium concentrations. Only the limnological data related to the occurrence of *C. canescens* in the same study are presented. The information about the current conservation status of the *C. canescens* locations has been obtained from the data gathered by Fundación Global Nature (2024) from the Spanish Inventory of Wetlands and the Autonomous communities' reports.

The sites of the occurrence of historical sexual populations

The three sites where *C. canescens* sexual populations were cited in the bibliography were visited. Namely: Bodón Blanco Lake (Bocigas) in Valladolid, Las Eras Lake (Villagonzalo de Coca) in Segovia (Comelles, 1984, 1986) and Lucio Largo in Doñana National Park (Huelva) (García-Murillo et al., 1993) (Fig. 1). Las Eras and Bodón Blanco were sampled on 13th and 14th June 2023, respectively. Lucio Largo was visited on 21st May 2023, but the site was completely dry. In Doñana National Park, the rainfall for the September 2022-August 2023 hydrometeorological period was below-average for over more than a decade, this cycle being described as "very dry". Furthermore, the highest temperatures for the entire historical series were reached in those years in the area. The combination of high temperatures and low rainfall, as well as the increase in nearby groundwater abstractions for intensive agriculture and human supply have seriously affected the Doñana's water bodies system, with a severe lack of water (De Felipe et al., 2023).

Two other shallow endorheic lakes were visited during the sampling in Segovia on 14th June 2023, La Iglesia Lake (Villagonzalo de Coca) and Caballo Alba Lake (Villeguillo) where parthenogenetic populations were previously recorded by Comelles (1984). These lakes (together with Bodón Blanco and Las Eras lakes) are included in the Natura 2000 network (ZEC - ES4160062) called Lagunas de Coca y Olmedo

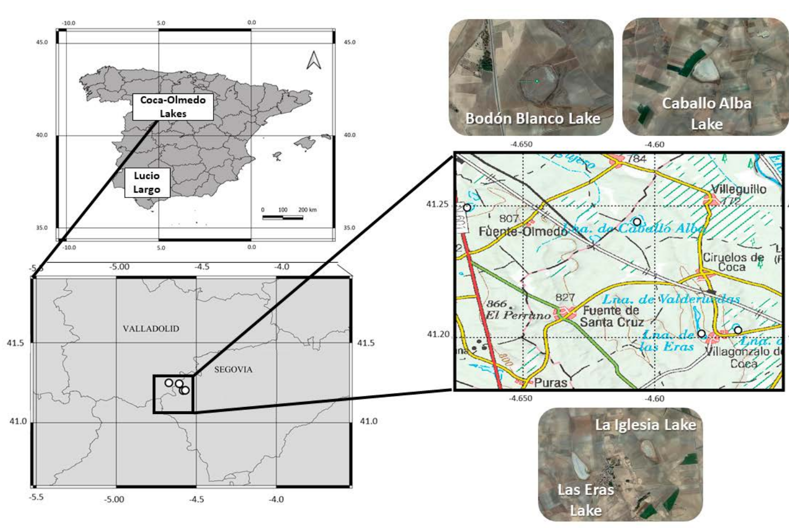

Figure 1. Location and aerial view of the four sites studied in the complex "Lagunas de Coca y Olmedo" located in the inner Iberian Peninsula (provinces of Segovia and Valladolid) and Lucio Largo (Doñana National Park), in the SW Spanish littoral. The locations are also indicated with a white dot in the Spain map. Photographs taken from Google Earth. *Localización y vista aérea de las cuatro localizaciones estudiadas en el complejo "Lagunas de Coca y Olmedo" situado en el interior de la Península Ibérica (provincias de Segovia y Valladolid) y El Lucio Largo (Parque Nacional de Doñana), en el litoral SW de España. La situación se indica en el mapa de España. Fotografías tomadas de Google Earth.*

and catalogued as "LIC" (site of communitarian importance for the Mediterranean biogeographic region). In this area, several wetlands between the provinces of Valladolid and Segovia are located. They are also important areas for the migration of birds. Nevertheless, these ecosystems have been affected by human activities, such as agriculture, especially cereal crops, the exploitation of pines for resin and pine nuts, the exploitation of the aquifer as well as other activities that have modified the habitat and affected the water available for aquatic macrophytes, such as charophytes (LIFE MedWetRivers, 2013).

Limnological characterization of the *Chara canescens* sexual population sites

Water temperature (ºC), conductivity (mS/cm), salinity (g/L), total dissolved solids (TDS) (g/L), pH and dissolved oxygen (mg/L and % saturation) were measured in situ by means of appropriate sensors in each lake. Water samples were taken by hand using a jar and kept in plastic bottles for further chemical analyses in the laboratory. Calcium carbonate, chloride, ammonium and silica concentrations (mg/L) were measured following A.P.H.A (2012).

A study of the vegetation and plant communities surrounding the lakes was also carried out for the four locations. The communities were identified using the phytosociological methodology of the Zurich-Montpellier school (Braun-Blanquet, 1979), although it should be noted that the recognition of the different communities was difficult due to the profound alteration of the local vegetation cover, which caused the absence of relevant species, as well as the coverage and frequency in which they were present. For this reason, some studies carried out in the area, such as the one by Ladero-Álvarez et al. (1984), were used as a reference. For vascular plants, the nomenclature of the Flora Ibérica (Castroviejo et al. (eds.), 1986-2021) was followed.

Sampling and analysis of populations and individuals of *Chara canescens*

In each small lake, the sampling of *C. canescens* individuals was done in ten different sites separated by two meters along a transect, from the shoreline to the deeper part of the waterbody. A round sampling unit with a surface area of 10 cm^2 was used to sample each site of the transect. In the field, the maximum % coverage of the *C. canescens* meadow and the male:female ratio were determined visually (males are easily distinguishable by their large red antheridia). This ratio was confirmed later in the laboratory from observation of the collected specimens.

The fresh hand-collected material was transported to the laboratory in a portable cooler inside plastic bags. In the laboratory, the material was washed to remove the organic matter attached, and the individuals were measured from the apex to the beginning of the rhizoids using a ruler. Mean lengths were calculated for male and female individuals separately.

The reproductive organs, oogonia and antheridia, were counted and expressed per fertile branchlet. An Olympus SZ61 (Shinjuku, Tokyo, Japan) stereomicroscope equipped with a camera (Model LC20-Olympus Soft Imaging Solutions GMBH) was used. The antheridia, oogonia and oospores were measured following the methodology established by Soulié-Märsche & García (2015). The length (longest polar axis, LPA) and width (largest equatorial diameter, LED) were measured for the female organs and the isopolarity index (ISI =LPA/LED×100) was calculated. The diameter for the male organs was also determined. The measurements were performed on photographs taken by the camera attached to the stereomicroscope using the LC micro measuring programme.

Some *C. canescens* specimens collected that were not used for other scientific purposes were deposited in the University of Valencia Herbarium "(VAL-Algae)" with the corresponding numbers: 3063, 3064, 3065, 3066, 3067, 3068, 3069, 3070, 3071.

RESULTS AND DISCUSSION

The historical distribution of *Chara canescens*

Chara canescens Desv. & Loisel In Loisel., Not. Fl. France : 139 (1810)

= *Chara crinita* Wallr., Annus Bot. 190 (1815)

All the found citations of this species (under its different names) in the Iberian Peninsula and the Balearic Islands up to the present time are gathered in Table S1 (Supplementary material).

In Portugal, the presence of the species was confirmed in the Herbarium of the Botanical Garden of the National History and Science Museum of Lisboa (LISU), with only one record: LISU-P45314, from Costa da Trafaria in 1843, F. Welwitsch. In Spain, the first records of *C. canescens*, date back to 1908 by E. Reyes-Prósper. Female individuals are represented in an herbarium sheet under the name of *Chara crinita* Wallr. var. *toletana* Prósper. from Quero (province of Toledo) and preserved in the cryptogamic collection of the Royal Botanical Garden of the Madrid Herbarium (MA-Algae). In 1910, Reyes-Prósper published the volume “The charophytes of Spain”, with detailed information and other records of *C. crinita*. Later, Caballero (1922,1929), carried out some other studies on Spanish charophytes; however, there is little literature recording *C. canescens* before 1950. Between 1950 and 1979, new records of this species appeared, but the locations from the previous period were not reconfirmed (Fig. 2A), and there is no herbarium material available. Most of the references for this species belong to the 1980-2010 period, when the maximum number of recorded locations was assessed, and some of the previous populations were reconfirmed (Fig. 2A). The increase in the number of *C. canescens* records after the 1980s is probably related to the increase in ecological studies, as well as social awareness regarding environmental matters, biodiversity, and conservation management (see Cirujano et al., 2007). Particularly relevant are the studies by Cirujano et al. (1980, 1982,1988, 1990, 1993, 2002, 2007, 2008, 2015), Comelles (1984,1986) and García-Murillo (1993, 1995). The number of herbarium specimens preserved during these years is also the highest recorded. Despite nearly two decades having passed, the number of records since 2010 has not yet surpassed the previous period (Fig. 2A).

Regarding the spatial distribution of *C. canescens* in the Iberian Peninsula and the Balearic Islands, the maximum number of localities re-

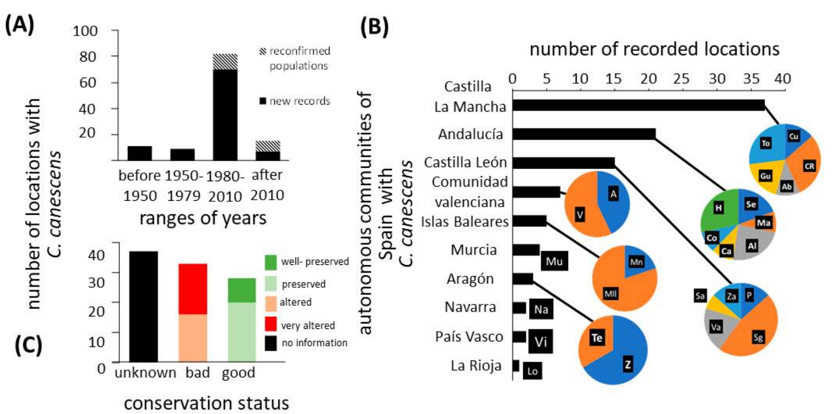

Figure 2. (A) Number of locations recorded in the literature with *Chara canescens* sorted by year periods including new records for each period and reconfirmed ones (B) Distribution of the number of locations with *C. canescens* within the Autonomous Communities of Spain and Provinces of each Community ordered by decreasing number of locations. The provincial codes used in the list are those used by Cirujano et al., (2008): see the list below. (C) Number of locations with *C. canescens* for each category of conservation status of the waterbody. (*A) Número de localidades con presencia de* Chara canescens *registradas en la literatura en periodos de años incluyendo los nuevos registros para cada periodo y los reconfirmados (B) Distribución del número de localidades con presencia de* C. canescens *dentro de las Comunidades Autónomas de España y las provincias de cada Comunidad ordenadas de mayor a menor número de localidades. Los códigos provinciales usados son los establecidos por Cirujano* et al., *2008: A=Alicante, Ab=Albacete, Al=Almería, Ca=Cádiz, CR=Ciudad Real, Co= Córdoba, Cu= Cuenca, Gu=Guadalajara, H=Huelva, Lo= Logroño, Ma=Málaga, Mll=Mallorca, Mn=Menorca, Mu=Murcia, Na=Navarra, P=Palencia, Sa=Salamanca, Sg=Segovia, Se=Sevilla, Te=Teruel, To=-Toledo, V=Valencia, Va=Valladolid, Vi=Álava, Za=Zamora, Z=Zaragoza. (C) Número de localidades con* C. canescens *para cada categoría de estado de conservación de los cuerpos de agua.*

corded with parthenogenetic populations of the species corresponds to the central and southern regions of the peninsula: Ciudad Real and Toledo provinces, belonging to the Castilla-La Mancha autonomous community, followed by the communities of Andalucía and Castilla León (Fig. 2B). Sexual populations of *C. canescens* also occurred in Castilla-León (Comelles, 1986) and Andalucía regions (García-Murillo et al., 1993) (Fig. 3).

Despite most sites with populations of *C. canescens* are protected by the Natura 2000 network or other protection schemes, many of them have a bad conservation status (Fig. 2C). In Spain, the main threats that affect the wetlands are the transformation of the land, pollution and the climate-change consequences (MITECO, 2023). In addition, inner brackish water sites, which host *C. canescens*, are especially sensitive to the artificial freshwater inputs performed for other human purposes and economic interests. Some of the Spanish locations where *C. canescens* were recorded have been dried, drained, cultivated (Gobierno Vasco, 1994), with their original hydrographical net modified (Álvarez-et al., 2013) and affected by sewage treatment plants and by grazing and slurry (Cirujano et al., 2015). The ignorance and lack of interest in aquatic vegetation causes the poor conservation status of habitats that host *C. canescens*, thus its presence (like other aquatic macrophytes) is only briefly mentioned in studies focused on other ecological aspects or taxonomic groups (Cirujano et al., 2008).

Limnological features of the habitat

C. canescens is a representative species for waters of moderate salinity (Cirujano, 1990) being one of the few true brackish water species of charophytes, neither occurring in freshwater nor under fully marine conditions (Schubert et al., 2024). In the Iberian Peninsula, the habitat of *C. canescens* is characterized by neutral-basic pH values, above 7, reaching 9 in some lakes (Table 1). *C. canescens* populations inhabit waterbodies with a wide range of conductivity values, but most of them are between 4 and 20 mS/cm (Table 1). Chloride concentrations are often higher than 1000 mg/L, while ammonium concentrations are lower than 1.5 mg/L, despite this value is not often measured in the studies assessing *C. canescens* populations (Table 1).

Sexual populations of *Chara canescens* in the revisited sites

Previously recorded sexual populations in Bodón

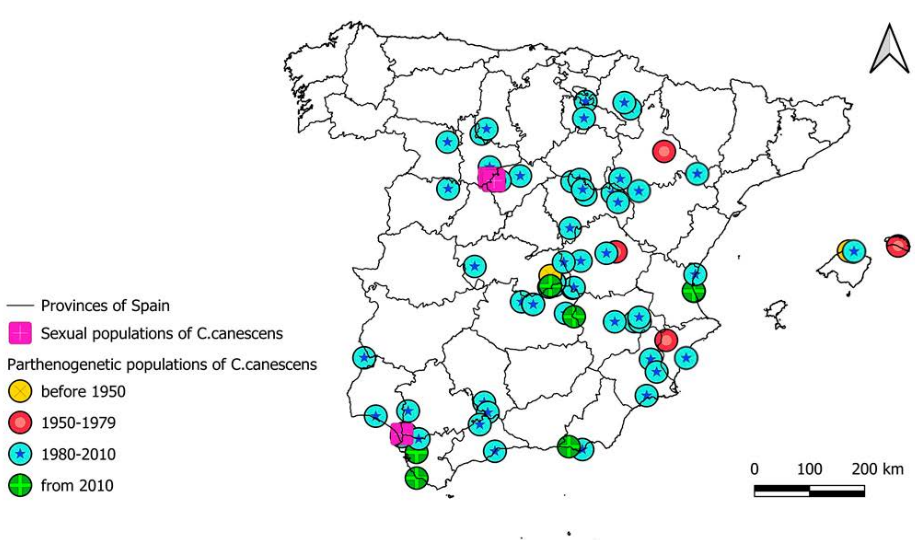

Figure 3. Historical distribution map of *C. canescens* populations in the Iberian Peninsula and Balearic Islands. The period of the cited location is indicated. *Mapa de distribución histórica de las poblaciones de* C. canescens *en la Península Ibérica y las Islas Baleares. Se señala la época a la que corresponde la cita de la localización.*

Table 1. Limnological variables measured for different water bodies that host *Chara canescens* populations in the Iberian Peninsula. The reference for each data is indicated. *Variables limnológicas medidas para diferentes masas de agua que albergan poblaciones de* C. canescens *en la Península Ibérica. Se indica la referencia de la que proviene cada dato.*

Name of the lake/area	pH	Cond. (mS/cm)	Cl^- (mg/L)	NH_4^+ (mg/L)	Referemce
Casa Bomba		20.2			García-Murillo et al. (1995)
Casa Nueva 2		10.0			Cirujano (1990)
Chica de Quero		4.7	700		Cirujano et al. (2002)
Corral Rubio	9.8	9.4	1967		Cirujano (1990) ; Cirujano et al. (2002)
Dehesilla	7.8	21.9	5160		Cirujano (1980) ; Cirujano et al. (2002)
El Taray		3.8	430		Cirujano et al. (2002)
Hoya Husilla		5.0			Cirujano (1990)
Laguna Chica (Villafranca)		4.1	429.1		Cirujano et al. (2002)
Laguna de Musco	7	4.3	297	0.1	Gobierno Vasco (1994)
Las Eras/Bodón Blanco			1100-2500		Comelles (1986)
Lucio de los Patos Reales		30			Espinar et al. (1997)
Mallada “Canescens”	7.7	7.2	2110	0.3	Calero & Rodrigo (2018)
Manjavacas		22.0-47.9	8329 -11 350		Cirujano et al. (2002)
Nava Grande Malagón		14.8			Cirujano et al (1993)
Navahornos	7.6-8.9	0.3-1.0	8.0-34.0		Cirujano et al. (2015)
Murcia	7.4	10.5	2370.1		Aboal (1985)
Salinas San Rafael		30.1			Cirujano et al. (1993)
Puzol, Sagunto	7.4-8.1	5.0-9.3	1750-2320	0.1-1.1	Carretero (1993)

Blanco and Las Eras endorheic shallow lakes (Comelles, 1986) were reconfirmed during the samplings in June 2023. For the same dates, male individuals were found in the populations of *C. canescens* occurring in Caballo Alba and La Iglesia lakes. These latter lakes have not been previously recorded as hosting sexual populations until the present study, but exclusively parthenogenetic populations (Comelles, 1984). Probably, the male individuals could have passed unnoticed in past surveys, since, in most cases, the male:female ratio is clearly biased to females (see below).

The studied lake complex was previously highlighted by its uniqueness with respect to other lake complexes in Spain, or even in Europe, by authors such as Velasco et al. (2004) and Cabestrero (2018), because of its exceptionally high alkalinity, its saline hydrochemistry, the precipitation of rare minerals and high pH values.

We corroborated the high water pHs in June 2023 (values ranging from 9 to 11) with the lowest in Bodón Blanco and the highest in Las Eras Lake (Table 2), These pH values are much higher than the ones measured for most of waterbodies where the parthenogenetic populations of *C. canescens* occur in Spain, which ranged from 7 to 8 (Cirujano, 1980, Aboal, 1985, Carretero, 1993, Gobierno Vasco, 1994, Cirujano et al., 2002, Cirujano et al., 2015, Calero & Rodrigo, 2018) (Table 1). Regarding water conductivity, its values ranged from 4 to 9 mS/cm, and the highest values were measured in Las Eras Lake (Table 2). These values are within the range measured in the waterbodies of parthenogenetic populations of the same species (Table 1). With respect to the values of other compounds, the greatest ammonium concentration in June 2023 was found in Bodón Blanco Lake, which might indicate a higher pollution in these waters than in the other ones from the same area, probably due to the wide area of agriculture crops surrounding the lake (Table 2). Compared to the concentrations in the parthenogenetic sites (Table 1), the values are similar, with the exception of some sites visited by Carretero

(1993) in the Valencian Community, where they reached more than 1 mg/L. Despite the relative proximity of the four lakes, they differ considerably in silicate concentrations, with Bodón Blanco Lake standing out with the highest concentration and Caballo Alba Lake with the lowest value (Table 2).

The vegetation within the water bodies and their surroundings was highly altered due to the impact of agricultural crops surrounding the shallow lakes and also, in some of them, due to the discharge of waste water into the basin. This was reflected in the observed plant communities, which appeared quite disorganized, particularly the helophyte communities. Under these circumstances, finding the organizational patterns that allow them to be assigned to phytosociological categories was a complicated task.

In Bodón Blanco Lake, the submersed vegetation formed meadows of aquatic macrophytes with an uneven coverage; there were patches of around 1 m^2 with total coverage; however, in other sites the coverage was only 20% (for the same surface area). The western, central, and the south-eastern shore areas were the most covered, without following a clear pattern. The predominant species was *C. canescens* (covered by a thin layer of epiphyton), although *Zannichellia pedunculata* Reichemb. (1830) was also abundant, particularly in the western and south-eastern areas, where it reached 100% coverage. In addition, some isolated individuals of *Ruppia drepanensis* Tineo ex Guss. (1845) were present, especially in the central area of the lake. Both *Z. pedunculata* and *R. drepanensis* showed flowers and fruits at the time of sampling. This community can be assimilated into the phytosociological association *Rupietum drepanensis* Brullo &. Furnari 1976 (Fig. 4A). The water-margin vegetation was completely unstructured with only isolated and incomplete patches, remains of original plant communities that formed the vegetation belt around the lake (Table 3). In some cases, the crops that surrounded the lake extended almost to the edge. Only in the south-eastern part of the lake, a more developed portion of helophytic vegetation was found (10-15 m). The communities were organized according to the time passed since the area was flooded and the soil moisture (Fig. 4A and Table 3). *Riella helicophylla* (Bory & Mont.) Mont. (1852) and *Tolypella salina* Corillion. (1960) had also been cited from this lake (Cirujano et al., 1988; Comelles, 1984); however, they were not observed during the 2023 sampling. In Las Eras Lake, the vegetation structure was similar to the one described for Bodón Blanco Lake, although with a higher degree of alteration. The hydrophytic vegetation was less diverse than in the previous lake and consisted basically of *C. canescens* meadows. Additionally, *Z. pedunculata* and, more rarely, *R. drepanensis* were present.

Table 2. Limnological variables measured for each lake studied in the Valladolid-Segovia area. *Variables limnológicas medidas para cada laguna estudiada en el área Valladolid-Segovia.*

	Bodón-Blanco	Las Eras	La Iglesia	Caballo Blanco
Water temperature (°C)	20.8	22.6	21.5	24.6
Conductivity (mS/cm)	4.2	8.9	5.9	8.5
Salinity (g/L)	2.7	5.0	3.8	5.6
TDS (g/L)	2.2	5.8	3.2	4.7
pH	9.6	10.7	10.1	10.3
Dissolved O_2 (mg/L)	4.7	14.0	12.6	13.0
O_2 saturation (%)	57.2	165.8	146.1	160.2
$CaCO_3$(mg/L)	1680	1990	1940	810
Cl^- (mg/L)	590	1440	1330	1670
NH_4^+ (mg/L)	0.19	0.02	0.01	0.01
SiO_2 (mg/L)	1.62	0.41	0.17	0.06

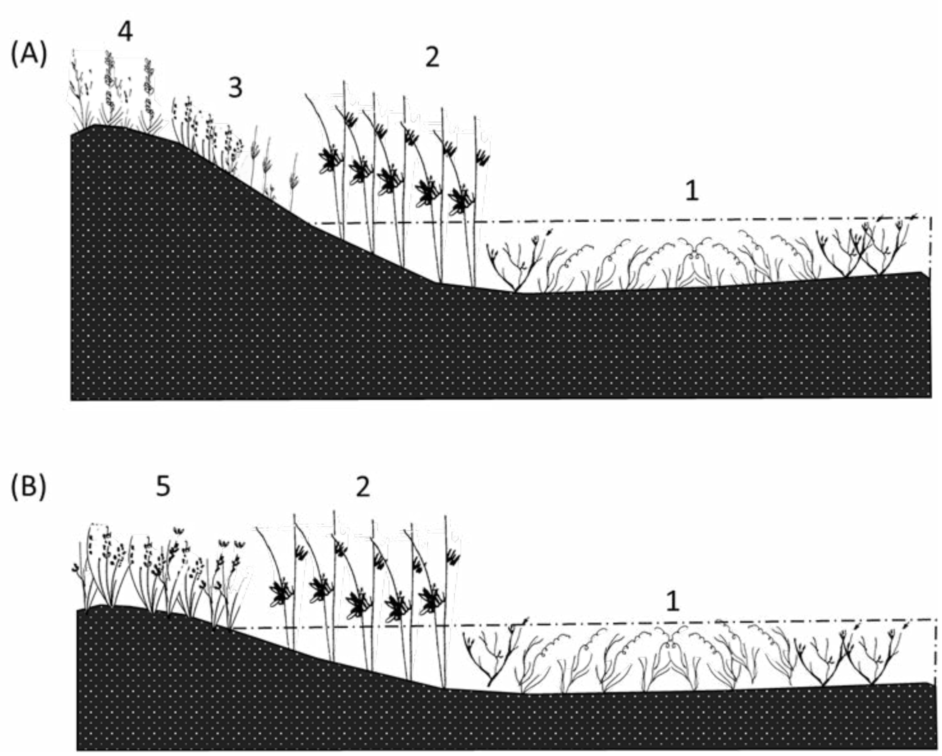

Figure 4. Vegetation catena corresponding to A) Bodón Blanco Lake (Bocigas, Valladolid) and B) La Iglesia Lake (Villagonzalo de Coca, Segovia). Catena de vegetación correspondiente a A) laguna Bodón Blanco (Bocigas, Valladolid) and B) laguna La Iglesia (Villagonzalo de Coca, Segovia). 1. *Rupietum drepanensis.* 2. *Scirpetum compacto-littoralis*. 3. *Bupleuro tenuissimi-Juncetum gerardii.* 4. *Aeluropo littoralis-Puccinelletum fasciculatae*. 5. *Soncho crassifolii-Juncetum maritime*.

Both *Z. pedunculata* and *R. drepanensis* had flowers and fruits at the time of sampling. This community also belonged to the association *Rupietum drepanensis*. The palustrine marsh vegetation in this lake was sparsely developed, forming a 5-m wide ring around the waterbody in the most developed parts, but fragmented. The composition of this ring (Table 3) can be interpreted in the same way as in the case of Bodón Blanco Lake, although it was impossible to distinguish communities here.

The vegetation in La Iglesia Lake (Table 3) showed signs of alteration caused by the agricultural crops that surround the water body. Despite this fact, this lake was, of the four visited, the one with the best-preserved vegetation. The submersed vegetation was dominated by *C. canescens* meadows with an estimated 100% coverage in the central zone of the lake. Furthermore, patches of *Potamogeton pectinatus* L. (1753) were observed in the central area and isolated plants of *Z. pedunculata* and *R. drepanensis*. The three aforementioned species had flowers at the time of sampling, and the last two, also had fruits. Likewise, this community corresponds to the association *Rupietum drepanensis* (Fig. 4B). In the north-western sector of the lake, the helophytic vegetation was highly developed (around 20 m wide), and two main communities could be distinguished: the inner belt, in a 5-10 cm deep area a dense monospecific belt of *Bolboschoenus maritimus* (L.) Palla (1905) occurred, reaching 100% coverage. All the plants were flowered. The community can be included in the association Scirpetum compacto-littoralis BR.-BL. 1931 (corr. Castroviejo, Valdés-Bermejo, Rivas-Martínez & Costa, 1980) (Fig. 4B). The outer belt, the area with wet soils, but not flooded, had a community that can be assimilated to the association *Soncho crassifolii-Juncetum maritime* Braun-Blanquet &

Table 3. Aquatic and marginal vegetation observed in the Valladolid-Segovia lakes and their surroundings in June 2023. *Vegetación acuática y marginal observada en las lagunas de Valladolid-Segovia y su entorno en junio de 2023.*

	Bodón Blanco	Las Eras	La Iglesia	Caballo Alba
HYDROPHYTES				
Chara canescens Desv. & Lois.	x	x	x	x
Potamogeton pectinatus L.			x	
Ruppia drepanensis Tineo ex Guss.	x	x	x	
Zannichellia pedunculata Reichemb.	x	x	x	x
WATER-MARGIN VEGETATION AND OTHERS				
Atriplex patula L.	x			x
Bartsia trixago L.	x			
Bolboschoenus maritimus (L.) Palla.	x	x	x	
Carex divisa Hudson, Fl. Angl.	x		x	
Centaurea calcitrapa L.	x			
Chenopodium rubrum L.				x
Convolvulus lineatus L.	x			
Dactylis glomerata L.	x			x
Elymus curvifolius (Lange) Melderis.		x	x	
Hordeum marinum Huds.	x			x
Juncus acutus (L.) Torr. ex Retz.	x			
Juncus gerardii Loisel.	x	x	x	
Juncus maritimus Lam.	x	x	x	
Lactuca saligna L.	x	x		x
Papaver rhoeas L.	x			
Petrorhagia prolifera (L.) P.W. Ball & Heywood.	x			
Plantago coronopus L.		x		x
Plantago maritimus L.	x			
Poa bulbosa L.	x			
Polypogon maritimus Willd.	x			x
Puccinellia fasciculata (Torr.) E.P. Bicknell.	x	x		x
Spergularia media (L.) K. Presl.	x	x		x
Tragopogon dubius Scop.	x			
Trifolium campestre Schreb.	x			

O. Bolòs 1958 (Fig. 4B).

Finally, Caballo Alba Lake was the most altered of all the studied waterbodies, due to the impact of the crops that surround the lake (practically reaching the shore), the sewage water discharged into the basin, and the solid waste (plastics, remains of pipes) that could be seen everywhere. This resulted in very unstructured vegetation. *C. canescens* was practically the only hydrophyte present and nearly covered the whole lake basin. The helophytic vegetation was almost absent as crops and weed communities reached the shore, hence only some isolated plants could be seen. As indicated above, the four sampled lakes presented monospecific meadows of charophytes, *C. canescens* being the only charophyte present, with coverages ranging from 80% (Bodón Blanco Lake) to 100% (Las Eras and La Iglesia lakes). Monospecific stands of *C. canescens* have been also reported for a sexual population of this species in the National Park system between Neusiedler See (Austria) and the Hungarian border (Schaible & Schubert, 2008). Regarding the presence of males within the meadows, the highest

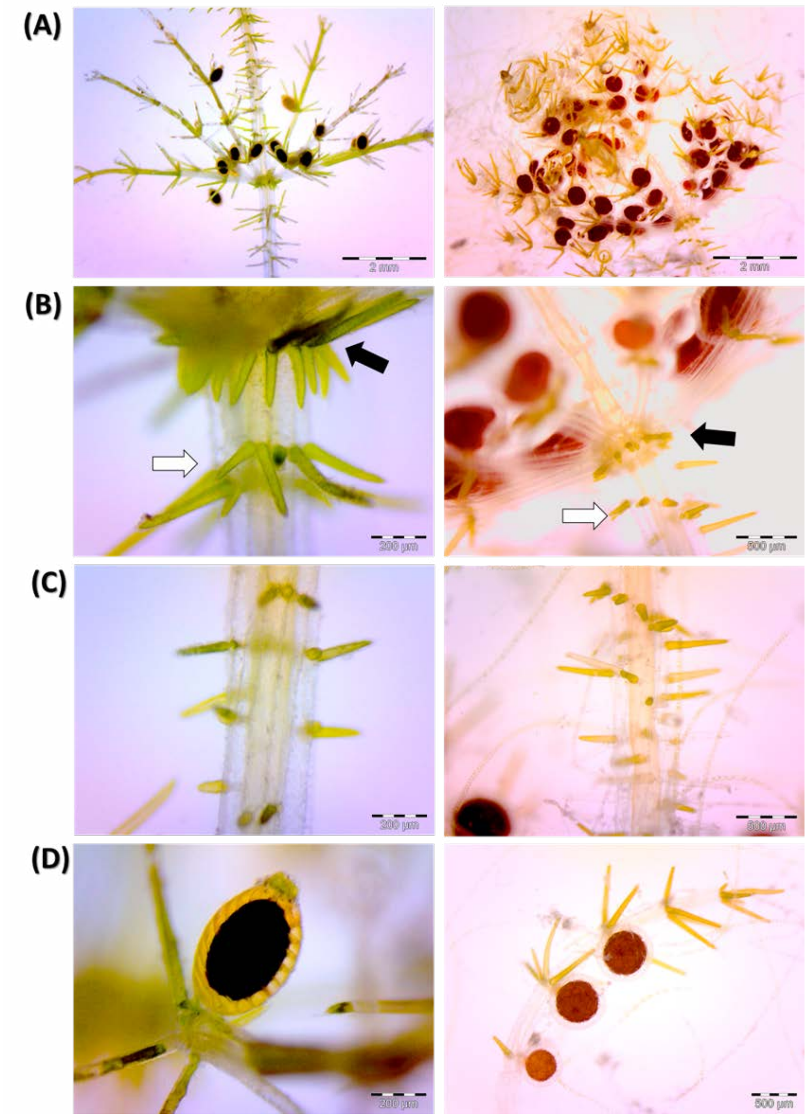

Figure 5. *Chara canescens* female individual from Bodón Blanco Lake (left) and *C. canescens* male individual from Las Eras Lake (right). A) general aspect of whorls. B) Detail of the stipulodes (black arrows) and spine-cells (white arrows) C) Axes with presence of spine-cells D) Detail of the sexual organs: oogonium with the ripe oospore inside (left) and a branchlet with antheridia (right). *Individuo hembra de* Chara canescens *de la laguna Bodón Blanco (izquierda) e individuo macho de* C. canescens *de Laguna de Las Eras (derecha). A) Aspecto general de los verticilos. B) Detalle de los estipuloides (flechas negras) y de las acículas (flechas blancas) C) Ejes con presencia de acículas D) Detalle de los órganos sexuales: oogonio con la oospora madura en su interior (izquierda) y un filoide con anteridios (derecha).*

male:female ratio was found in Las Eras Lake and the lowest in Bodón Blanco Lake (Table 4). No previous information was found to compare the current ratios of sexual individuals in the studied lakes.

Regarding the *C. canescens* sexual populations, the collected individuals presented hyaline colouring of the internodal cells of the thallus and yellowish green in the central cell. The cortication was haplostic and regular, and the diameter of the axis was around 500 µm. The distribution of spine-cells was sparse, being mostly solitary, their length was up to 500 µm, with a diameter of 100 µm approximately, although there was much variability in length within the same individual. The stipulodes were diplostephanous, being in

Table 4. *Chara canescens* population variables determined for each lake and some individual variables determined for 10 specimens (Mean ± S.D. for isopolarity index). LPA is the longest polar axis; LED is largest equatorial diameter. ISI is the isopolarity index (LPA/LED)*100. *Variables poblacionales de* C. canescens *determinadas para cada laguna y algunas variables individuales determinadas en 10 ejemplares (Media ± D.S. para el índice de isopolaridad). LPA es el eje polar más largo; LED es el diámetro ecuatorial más ancho. ISI es el índice de isopolaridad (LPA/LED)*100*

	Bodón Blanco	**Las Eras**	**La Iglesia**	**Caballo Alba**
POPULATION				
male:female ratio	5:95	60:40	25:75	10:90
maximum coverage (%)	80%	100%	100%	90%
INDIVIDUAL				
antheridia/branchlet	3 (2-3)	3 (2-5)	2(2-3)	3 (2-4)
antheridia diameter (µm)	338-600	469-642	305-376	317-454
oogonia/branchlet	3 (2-5)	4 (2-5)	2(1-2)	3 (2-4)
oospore size (µm): LPAxLED	297-554x207-340	315-537x175-343	313-434x219-288	428-508x178-309
Isopolarity index (ISI)	155±14	179±27	153±9	204±38

some cases reduced, whose length was less than that of the spines. The branchlets consisted of five segments, all of which were corticated. The number of branchlets per whorl numbered up to 8. Anterior bracts were 350-450 µm long, and the posterior bracts from 400 µm to over 1 mm, with a lot of variability in length within an individual, but generally over a millimetre long (Fig. 5, Table 4).

With respect to sexual organs, the male gametangia were located in the corticated nodes of the branchlets, with a maximum of five antheridia per fertile branchlet (the case of individuals taken from Las Eras Lake; Table 4) and often found in pairs as Comelles (1986), but no antheridia were found at the base of the whorls, in contrast to the findings of this author. The diameter of the antheridia ranged from 300 to 650 µm, which is more similar to the data provided by Corillion (1975) than the measurements performed by Comelles (1986) for the same lakes. Regarding female plants, the ripe oospores, bright black, ranged between 300-550 µm long and between 170-345 µm wide, being longer than the oospores studied by Comelles (1986).

With these data we confirm two new sexual populations with the presence of males for Spain. However, sexual and parthenogenetic populations of *C. canescens* can be sympatric (Schaible & Schubert, 2008), thus, further genetic analyses will play a key role in cataloguing these sites as strict sexual populations or sympatric ones.

FINAL REMARKS AND CONCLUSIONS

As pointed out by Martínez-Abraín et al. (2022) management decisions and population projections are commonly based on snapshots and present observations. To avoid short-sighted forecasts, it is important to develop studies assessing the distribution of the species from a historical perspective. For this reason, we have reviewed the historical and spatial distribution of *Chara canescens* in the Iberian Peninsula and Balearic Islands. *C. canescens* is not evenly distributed in the Iberian Peninsula, and the known sexual populations (the former ones and the new two sites discovered in this study) are concentrated mainly in Castilla-León. Up to now, Spain is the country with the highest number of cited sexual populations in Europe. These findings are very important because the genetic diversity of the species depends on this type of populations. Unfortunately, the conservation status of the inner brackish-water habitats harbouring these populations is not good. Understanding the distribution of different reproducing-populations of *C. canescens* may help us to develop further optimal conservation strategies for this and other similar species, and to have an overview of the evolutionary constrains that outlined the present distribution. This is particularly relevant in the current context of global change, which causes an important affectation (pollution from new agriculture practices, reduced rainfall and warmer temperatures which cause desiccation, etc.) to aquatic ecosystems and, therefore to the aquatic vegetation. To better understand this matter, we need to increase knowledge about the limnological features of the habitat where the species develops. *C. canescens* is mainly distributed in Europe and the African Mediterranean coast, thus the implementation of transnational effective conservation measures to protect this relevant and particular species is essential. This study contributes to this enormous task.

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CONFLICT OF INTERESTS

The authors declare not to have any competing interests.

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