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ARTICLE

Charophytes in warm springs on Svalbard (Spitsbergen): DNA barcoding identifies *Chara aspera* and *Chara canescens* with unusual morphological traits

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ABSTRACT

The Troll springs are warm springs on Svalbard (Spitsbergen). Charophytes were collected in the years 1910, 1912, 1958, 1992/1993, and 2018. However, since the *Chara* samples showed unusual morphological traits, there were doubts with respect to species identity. We here use DNA barcoding to show that there occur two *Chara* species in the Troll springs: *Chara aspera* and *C. canescens*.

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Svalbard; Spitsbergen; *Chara*; matK; barcoding

1. Introduction

In 1910 and 1912, the Norwegian scientist Adolf Hoel collected a number of algae in the Troll-springs, a group of warm springs in Bockfjorden (79°25′N, 13°17′E) on Spitsbergen, Svalbard (Hoel and Holtedahl 1911; Strøm 1921) (Figure 1). One *Chara* taxon was found in the springs, and sent to professor Otto Nordstedt in Lund, Sweden, one of the most renowned charophyte experts at that time. He determined the species as *Chara aspera* and described it (invalidly) as *C. aspera* f. *spitsbergensis* (Hoel and Holtedahl 1911). The valid description of *Chara aspera* Willd. f. *spitsbergensis* Nordstedt, based on material collected in 1912, was given in Strøm (1921).

In 1958, Niels Foged collected specimens of *Chara* in the same springs (Foged 1964). They were determined by the Swedish charologist Henning Horn af Rantzien, who "*considered it as a rather peculiar form of C. canescens Lois., but at the same time he said he felt uncertain about the definition*" (Foged 1964). This finding is described in Langangen (1979), where the taxon is stated to be *Chara canescens*.

In 1992 and 1993 charophytes were again collected from the springs by Sissel Aarvik from the Governor of Svalbard. In this sampling, two *Chara* taxa were found, which both were suggested to be different forms of *Chara canescens*. They were described and discussed in Langangen (2000). In this work, the taxon *C. aspera* f. *spitsbergensis* Nordstedt was given the new combination *C. canescens* f. *spitsbergensis* (Nordstedt) Langangen (nom. inval.). The other taxon was given the name *C. canescens* subsp. *hoelii* Langangen, and described as a subspecies. We here describe and interpret the results of a new collection of *Chara* material from the Troll springs in 2018. Our aim was to confirm and if necessary, correct the species identity using DNA barcoding.

2. Material and methods

2.1 Study site and sampling of chara material

According to Hoel (1914) the Troll-springs have 14 individual ground-water sources and consist of large sinter terraces. Charophytes were found and collected in two springs named Spring 4 and Spring 6 (Hoel 1914). Spring 4 is the largest of the springs, approximately 11 m long, 7 m wide and 2 m deep. Two photos from spring 4, taken in 1912 and 2018, respectively, illustrate that the Troll springs have changed very little in more than 100 years (Figure 2). Spring 6 is approximately 3 m long, 2 m wide and 1 m deep. There is a considerable flow of water from this spring, estimated by Hoel (1914) to be at least 100 l per minute. The outlet of spring 6 was overgrown by filamentous algae in 2018, and the spring itself was dominated by reproductive *Chara canescens*.

2.2 Physical and chemical characteristics of the Troll springs

Water temperature, conductivity and Ca-content were measured in the springs, using the following methods; specific conductivity was measured in 1992–93 with a Hach conductivity meter (Model 44600/CND/TDS) and in 2018 with a Milwaukee SM 301 ECmeter (range 0–1990 µS/cm). Calcium was measured in 1992–93 with Aquamerck 11110 Calcium test and in 2018 with the Calcium MColortest from Merck. Temperature was measured in 1912 and 1992–93 with unknown types of

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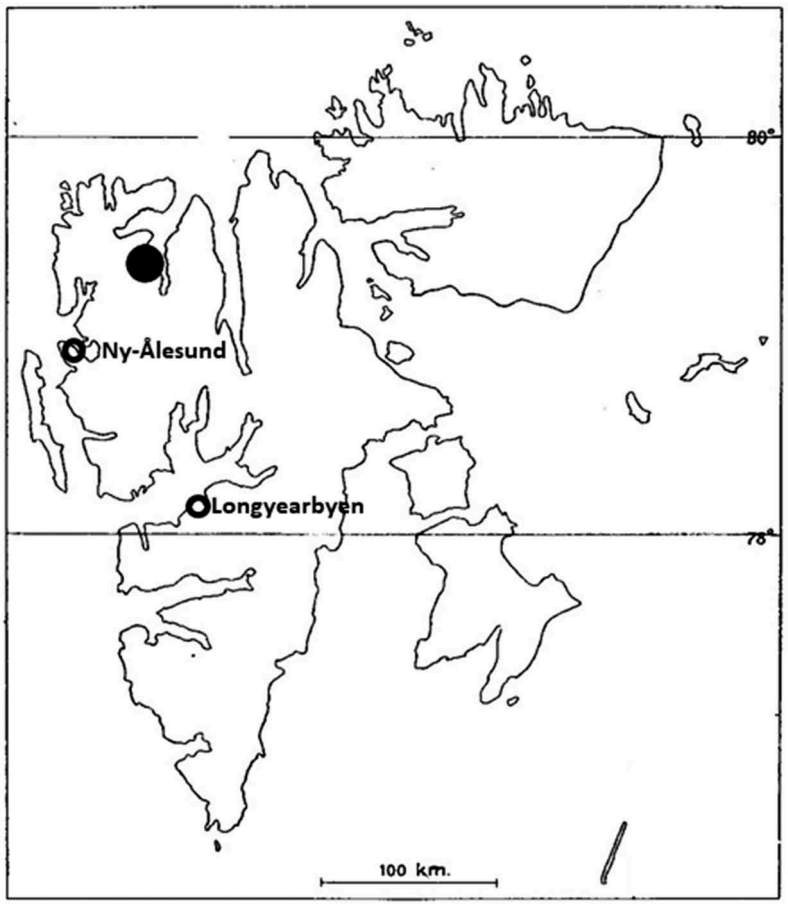

Figure 1. Location of the troll-springs on Svalbard.

thermometers. In 2018 we used a Ziel Mercury L0110/10 305 mm yellow back thermometer.

2.3 DNA barcoding

Two different methods were used to isolate genomic DNA from *Chara* samples investigated in this study. An overview over which samples were analyzed with which method is given in Table 1. The sequence data were deposited in the European Nucleotide Archive (ENA) under the accession numbers given in Table 1.

Method A: Genomic DNA from *Chara* material was isolated after Schneider et al. (2016). PCR for the matK gene was performed on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Oslo, Norway) using the iProof High-Fidelity PCR Kit (Bio-Rad Laboratories, Oslo, Norway). Amplification of the matK gene region was conducted using the primers F-Chara (agaatgagcttaaacaaggat) and R-Chara (acgatttgaacatccactataata). The following cycling protocol was used: one cycle of 5 min at 94°C, and then 35 cycles each consisting of 10 s at 94°C, 20 s at 62°C, and 20 s at 72°C, followed by a final elongation step of 72°C for 5 min. PCR products were visualized by 1.5% agarose gel electrophoresis with GelRed staining and UV illumination. For sequencing the same primers and the intermediate primers charaintF (gatggctattcaagcagga), charaintR (ctaccgataagttcgtcct), charaBt2f (datatggcaacaycaaaagac) and charaBT2R (atacagaccatgcagcytt) were used. Sequences were analysed and aligned using Seqassem (version 04/2008) and Align (version 03/2007) MS Windows-based manual sequence alignment editor (SequentiX – DigitalDNA Processing, Klein Raden Germany) to obtain DNA sequence alignments, which were then corrected manually. For each PCR product, both strands were sequenced on an ABI 3730 Avant genetic analyser using the BigDye terminator V.3.1 cycle sequencing kit (Biosystems (Applied Biosystems, Thermo Fisher Scientific Oslo, Norway) according to the manufacturer's instructions.

Method B: Preparation of total DNA was performed using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. Amplification of the *matK* gene region was performed with a Taq PCR Master Mix (Qiagen, Hilden, Germany), using the primers matK-F2 (aatgagcttaaacaaggattc) and matK-R1b (gcagccttatgaattggatagc). The following PCR protocol was used: 10 cycles of 1 min each at 94° C, 55° C, and 72° C, followed by 1 min each at 94° C, 52° C, and 72° C for 25 cycles. The amplified DNA was purified with the Biometra-innuPrep Gel ExtractionKit (Analytik Jena, Jena, Germany) according to the manufacturer's instructions and was sequenced directly on a 3130 × L GeneticAnalyzer (Applied Biosystems, NY, USA)

Figure 2. Troll-spring 4 in 3 August 1912 (top), and 15 August 2018 (bottom). The picture from 1912 was taken during a second visit, 2 years after the first collection of algae. Picture taken by Adolf Hoel, Norsk Polarinstitutt (top) and Gunhild Lutnæs (bottom).

using the BigDye terminator V.1.1 cycle sequencing kit (Applied Biosystems, Thermo Fisher Scientific, Darmstadt, Germany). Sequencing primers were identical to the primers that were used for the PCR reactions. Achieved sequences were proofed and manually edited using the BioEdit programme (Hall 1999).

2.4 Phylogenetic analysis

Segments with highly variable and ambiguous regions and gaps, making proper alignment impossible, were excluded from the analyses. In addition to two samples collected in the Troll springs in 2018, a matK set containing 38 other *Chara* sequences (Table 1), including a sample of the 1992 sampling in the Troll-springs, and 1023 nucleotide positions was used for phylogenetic analysis. *Nitellopsis obtusa* (AY170447) was used as an outgroup taxon in the matK tree. The dataset was analyzed using the maximum likelihood (ML) algorithm in MEGA version 7 (Kumar et al. 2016). The method selected GTR+G as the best-fitting evolutionary model for the matK gene region. ML analyses were performed with 1000 bootstrap replicates in MEGA version 7 (Kumar et al. 2016).

3. Results

3.1 Physical and chemical characteristics of the troll springs

Water temperature was around 20 °C in spring 4, and around 26 °C in spring 6 in all years (Table 2). Conductivity varied slightly but was above 1300 µS/cm in all years. This indicates slightly brackish water. Calcium concentrations of 100 mg/L and above indicate hard water in both springs. All water samples are from August in the respective years, and we do not have any information on water chemistry from other months.

Table 1. List of 40 *Chara* individuals (and one *Nitellopsis obtusa*) used in the present study. "Method A and B" refers to the method used for DNA-sequencing described in 2.3. Samples from the Troll springs are shaded.

Identification	Field ID	Genbank access number	method	coll. year	country	author
C. aspera	MB67	LR134032	A	2005	UK	Willd. 1809
C. aspera	S117	LR134033	A	2018	Norway (Svalbard)	
C. aspera	MB23	LR134034	A	2005	Sweden	
C. aspera	MB14	LR134035	A	2000	Germany	
C. aspera	MB11	LR134036	A	2001	Germany	
C. aspera	M8	LR134037	A	2008	Norway	
C. aspera	GJ53	LR134038	B	2009	Sweden	
C. aspera	CS31	LR134039	B	2012	France	
C. aspera	DH1b	LR134040	B	2011	UK	
C. aspera	Zi08_F2	LR134041	B	2010	Germany	
C. aspera	L1_1	LR134042	B	2015	Germany	
C. aspera	R1_3	LR134043	B	2014	Germany	
C. aspera	MB10	LR134044	A	2000	Germany	
C. aspera	MB73	LR134045	A	2001	France	
C. aspera	MB75	LR134046	A	2001	France	
C. aspera	MB76	LR134047	A	2001	France	
C. canescens	SR49	LR134049	B	2010	Spain	Desv. et Loisel. 1810
C. canescens	SR53	LR134050	B	2010	Spain	
C. canescens	SR56	LR134051	B	2010	Spain	
C. canescens	SR72	LR134052	B	2010	Spain	
C. canescens	GeSa18	LR134053	B	2008	Italy (Sardinia)	
C. canescens	15KW03_10	LR134054	B	2015	Italy (Sardinia)	
C. canescens	AL02	LR134055	A	1992	Norway (Svalbard)	
C. canescens	GeSa19	LR134056	B	2015	Italy (Sardinia)	
C. canescens	U47	LR134057	B	2014	Germany	
C. canescens	U84	LR134058	B	2014	Germany	
C. canescens	CCDZ01	LR134059	B	2011	Germany	
C. canescens	SV22	LR134060	B	2003	Sweden	
C. canescens	MB21	LR134061	A	2005	Greece	
C. canescens	S118	LR134062	A	2018	Norway (Svalbard)	
C. connivens		AY170442				Salzm. ex A. Braun 1835
C. contraria	M17	LR134063	A	2008	Norway	A. Br. ex Kütz. 1845 s. str.
C. galioides	MB77	LR134048	A	2001	France	De Candolle 1813
C. globularis	16	LR134067	A	2009	Macedonia	Thuillier 1799
C. hispida	MB6	LR134064	A	2004	Germany	(L.) Hartm. 1820
C. longifolia		AY170444				(Rob.) R.D.Wood 1965
C. strigosa	KR12_11	LR134068	B	2011	Germany	A. Braun 1847
C. tomentosa	MB7	LR134066	A	2004	Germany	L. 1753
C. virgata	GJ43	LR134069	B	2009	Sweden	Kütz. 1834
C. vulgaris	MB53	LR134065	A	2001	France	L. 1753
Nitellopsis obtusa		AY170447				(Desvaux) J. Groves 1919

3.1 Barcoding results

Chara aspera and *Chara canescens* were separated into two monophyletic groups supported by bootstrap values ≥99 (Figure 3). All other taxa used in the present study were clearly separated from these two large groups. Sample S117 from spring 4 clustered with *C. aspera*, while sample S118 from spring 6, and sample AL02 which was sampled in spring 6 in 1992, clustered with *C. canescens*. Despite the well supported clusters, both *C. aspera* and *C. canescens* exhibited some degree of variability in the matK sequences (Figure 3). However, sample S117 collected in the Troll springs had identical sequences to samples of *C. aspera* from Sweden, Norway, the UK, Germany and France, while samples S118 and AL02 were identical to samples of *C. canescens* collected in Sweden, Germany, Spain and Italy (Sardinia).

Table 2. Physical and chemical characteristics of the Troll-springs 4 and 6, where charophytes were found. The water sample taken from spring 4 in 1993 was damaged during transport from Svalbard. The data from 1912 were taken from Hoel (1914).

	3.8.1912	28.8.1992	16.8.1993	15.8.2018
Spring 4				
conductivity [µS/cm]		1620		1380
Ca^{2+} [mg/L]		125		100
Temp. [°C]	21		19	20
Spring 6				
conductivity [µS/cm]		1600	1470	1480
Ca^{2+} [mg/L]		122	130	110
Temp. [°C]	26		25	27

4. Discussion

4.1 Implications for taxonomy

The barcoding results clearly indicate that there are two *Chara* species in the Troll springs on Svalbard: *Chara aspera* and *Chara canescens*. The samples collected from Svalbard were genetically identical to other samples of the same species from several countries in Europe. This has the following implications for taxonomy:

4.1.1. Not accepted taxa

4.1.1.1. Chara aspera Willd. f. spitsbergensis Nordstedt in Strom (1921). Nordstedt (in Strøm 1921) gives a latin diagnosis of the new forma. The

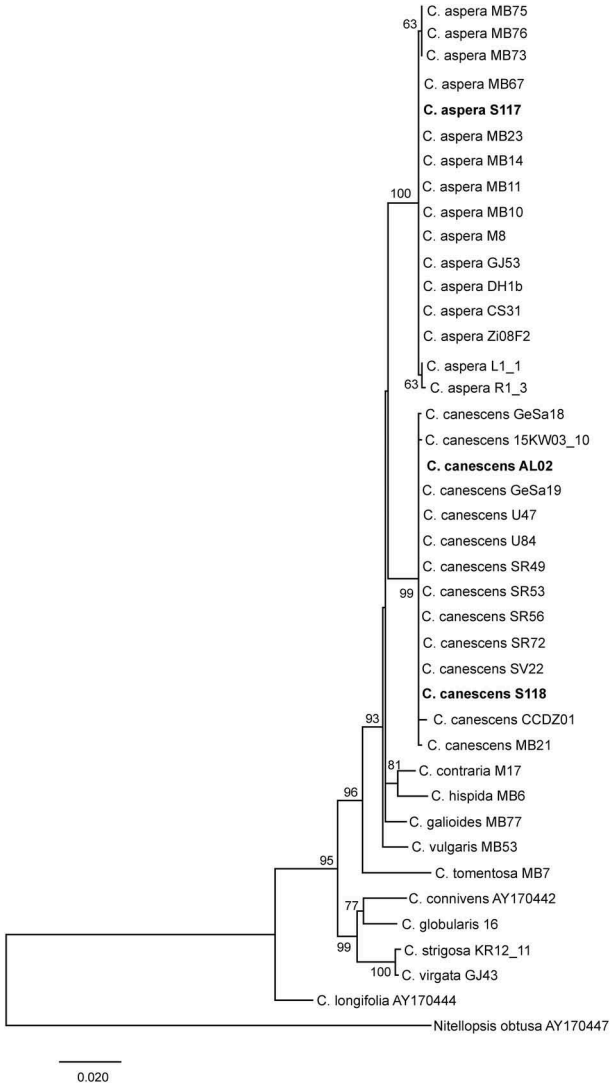

Figure 3. Maximum Likelihood tree of the matK gene of *Chara* spp. Bootstrap values above 50 are included. The scale bar indicates 2% sequence divergence. Sample S117 is from spring 4, and sample S118 is from spring 6. Sample AL02 was sampled from spring 6 in 1992, and sequences were obtained from herbarium material.

Figure 4. *Chara canescens*. Specimens of different lengths from spring 6. The left specimen is covered by a brown clayish coating. The picture is from 1992, but the specimen from the 2018 collection looked the same. Picture taken by A. Langangen.

description is a combination of characters from what we now know are two species, *C. aspera* and *C. canescens*. This also agrees with the fact that the original material, found in 1912 is a mixture of both taxa. We designate a lectotype as the part of the original collection which matches with what we now know is *Chara aspera* and which consists of only sterile specimens: Svalbard, Bockfjorden, the Troll springs, 1912-08-03, A. Hoel, coll. (O, p.p.). This taxon is regarded as a synonym to *Chara aspera*.

4.1.1.2. Chara canescens subsp. hoelii Langangen.

This taxon is described and discussed in Langangen (2000). According to our barcoding results, this is *Chara aspera*. We regard the taxon as an aberrant form of *Chara aspera*, due to both the morphology and the missing support for a subspecies in the genetic analysis (Figure 3). This taxon is regarded as a synonym to *Chara aspera*.

4.1.2. Accepted taxa

*4.1.2.1. Chara canescens Desv. & Loisel. (**Figure 4**).*

Chara canescens is an exceptionally variable species, and many forms have been described (Schubert and Blindow 2004). The specimen we found in the Troll springs in 2018 looked the same as those found in 1992/1993 (Langangen 2000). Therefore, the morphology of this species in the Troll springs seems to be stable. For the sake of completeness, we here repeat (in condensed form) the description given in Langangen (2000).

Plants were unbranched to strongly branched, only slightly encrusted in part of the whorls. The axes were 400 to 750 µm in diameter, and the internodes 2 to 15 mm long, 1 to 4 times the length of the branchlets. The stem cortex was regularly haplostichous in younger internodes,

Figure 5. *Chara aspera*. Habitus of three specimens. The picture is from 1992, but the specimen from the 2018 collection looked the same. Picture taken by A. Langangen.

and irregular or absent from older internodes. Spine cells were acute, often short but in some cases up to 1.5 times the diameter of the axes. Stipulodes were in 1–2 tiers, 2 per branchlet. Branchlets were 7–9 in a whorl, up to 4 mm long, slightly connivent, with 3–4 segments, and with end segments of up to 3 ecorticate cells. The end segments were up to 2 mm long, and longer than the corticate segments. The branchlet cortex was more or less regular. Bract cells were verticillate and ca. 500 µm long. Bracteoles were up to 1 mm long. The whorls were often "nestlike" (Figure 4), consisting of relatively short branchlets filled with oogonia and black ripe oospores. These whorls were 3.2–5.0 mm wide. The plants were dioecious, and only oogonia were found. Oogonia (675–825 µm long, 275–450 µm wide, with 9–10 convolutions, coronula 50 µm long and 125 µm wide) were found adjacent to both corticate and ecorticate internodes, but were most common on the two lowest branchlet nodes. Oospores were black, ovoid to elliptical (475–600 µm long to 325–400 µm wide, and with 9 ridges). The oospores were extremely abundant.

*4.1.2.2. Chara aspera Willd. (**Figure 5**).* *Chara aspera* from the Troll springs is difficult to determine morphologically, because the plants generally are ecorticate and sterile. Plants were up to 14 cm long, mildly to strongly encrusted with calcium carbonate. Axes to 350 µm in diameter, with internodes up to 2 cm long, ecorticate. Stipulodes were not observed. Branchlets 7–9, up to 15 mm long, with 5–10 segments. Cortex of branchlets mostly rudimentary or missing, often with cortical cells standing out from the branchlet internodes. Branchlets were tipped with 2–3 ecorticate cells. In some whorls, accessory branchlets are found in rows above and/or below the primary branchlets. Gametangia have not been found. Bulbils were one-celled, acute, ovoid, 750–1500 µm long and up to 600 µm wide. They occurred as solitary bulbils, in pairs or in groups of three or four.

The specimens collected in the Troll springs were genetically identical to specimens of *C. aspera* collected in several countries in Europe (Figure 3). We, therefore, regard the specimens from the Troll springs as aberrant forms of *Chara aspera*, likely caused by the "extreme" environment (warm springs in a polar environment).

4.2 Species distribution and possible survival in an extreme Northern environment

Both taxa, *C. aspera* and *C. canescens* are mesohaline species (Krause 1997), and this matches well with the conductivity measured in the Troll springs (Table 2). The occurrence of two *Chara* species so far north is, however, remarkable. The closest known locality of *Chara aspera* is at the Norwegian coast approximately 900 km south of the Troll springs, while the closest known locality of *Chara canescens* is approximately 1000 km south of the Troll springs (Langangen 2007). The closest currently known localities where both species occur are Alstahaug municipality in Nordland county (Gaarder et al. 2012), at a distance of approximately 1300 km from the Troll springs on Svalbard, and Eide municipality in Møre and Romsdal county (Langangen et al. 2001), at a distance of approximately 1800 km from the Troll springs (Figure 6). Although this must remain speculative, both species may have arrived in the Troll springs via long-distance dispersal by birds. Several species of geese, e.g. barnacle goose (*Branta leucopsis*), have migration routes from Scotland and South Norway, with resting places in Nordland county before breeding on Svalbard, including the area around the Troll springs (Griffin et al. 2011). Figure 6 shows that Eide and Alstahaug, where both *C. aspera* and *C. canescens* occur, are on the migration route of barnacle goose to breeding places on Svalbard.

It also is remarkable that the *Chara* species survive in the harsh Northern environment. In this area, the polar night (i.e. the time of darkness during which the sun never is above the horizon) lasts from October 26 to February 15 (data for Longyearbyen). The polar night is black in cloudy weather but can be surprisingly bright in clear weather and when there is moonlight. Several studies analysed the light dependency of *Chara* photosynthesis, growth, or reproduction (e.g. Blindow and Schütte 2007; Schaible and Schubert 2008; Schneider et al. 2015), but to our knowledge, no studies on how charophytes may survive several months of almost complete darkness exist. To our

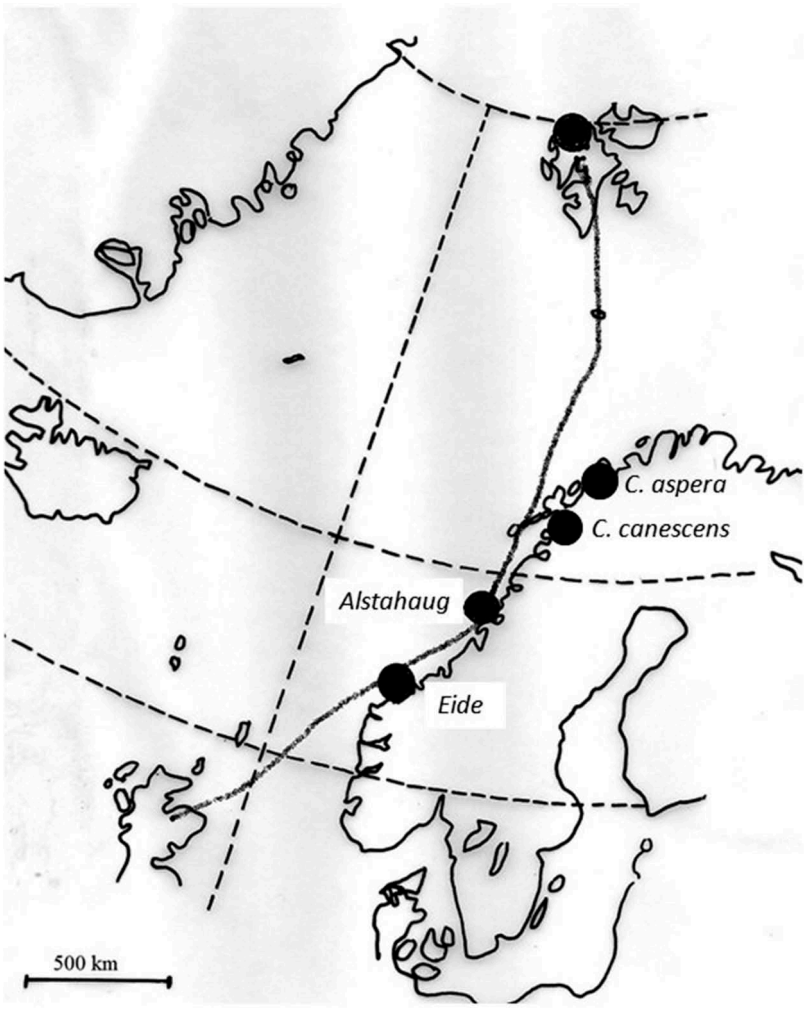

Figure 6. Migration route for barnacle goose (*Branta leucopsis*) and the three localities where both, *Chara aspera* and *C. canescens* occur (Eide, Alstahaug and the Troll springs).

knowledge, nobody has ever collected samples in the Troll springs during winter. Consequently, it is unknown if the charophytes survive winter as green plants. Whether or not moonlight may be sufficient to sustain *Chara* photosynthesis is, to our knowledge, unknown. According to local, unpublished observations, water temperature in the Troll springs does not fall below zero, i.e. the springs stay, at least in parts, ice-free. On the one hand, the lack of any sunlight over a period of about 4 months should make a vegetative survival difficult. On the other hand, however, *Chara* species can in Nordic lakes survive vegetatively for 4 months underneath snow-covered ice (which almost completely blocks sunlight; own observations). However, charophytes are well-known pioneer plants, which may persist during unfavourable conditions, e.g. the desiccation of temporal lakes, in form of resting stages. Charophytes are known to be able to quickly regrow from oospores, bulbils, or starch reserves in axial nodes (Krause 1997).

Chara canescens, as the only parthenogenetic charophyte taxon (Schaible et al. 2008), generally produces a large number of oospores, and this was also the case in the samples from the Troll springs. After the oospores ripen, *C. canescens* plants usually degenerate (Schubert et al. 2016), and it, therefore, is assumed that this species generally regrows each year from oospores. It is unknown if this also occurs in the Troll springs on Svalbard, but the large number of oospores which occurred on the *C. canescens* samples indicate that this may be the case. However, no oospores were observed on *C. aspera* from the Troll springs, neither in 2018, nor in 1992/1993 or in 1912 (Langangen 2000). *C. aspera*, therefore, seems to either regrow from bulbils or axial nodes in spring, or survives 4 months of almost complete darkness during the polar night in vegetative form.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Anders Langangen is cand.real. from the University of Oslo with a thesis on Norwegian charophytes, and a retired lecturer from Oslo Cathedral school. He has worked with freshwater algae and specially with charophytes since 1968. Contribution: study design, manuscript writing.

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Susanne C. Schneider is a senior scientist at the Norwegian Institute for Water Research, and adjunct professor at the Norwegian University of Life Sciences. She is mainly interested in the ecology of macrophytes and benthic algae. Contribution: manuscript writing.

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