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Codium fragile in Norway: subspecies identity and morphology

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Abstract: The green alga *Codium fragile* consists of 10 subspecies, of which subspecies *fragile* is a well-known invasive seaweed. Morphological work carried out in the 1950s suggested that there were three subspecies along the Norwegian coast: subsp. *fragile*, subsp. *atlanticum* and subsp. *scandinavicum*. However, more recent molecular data have shown the existence of only two subspecies and that these are frequently misidentified. The aims of the present study were therefore to verify which subspecies occur in Norway using the *rpl*16-*rps*3 chloroplast marker, to ascertain their likely time of arrival and to compare their morphology to their genetic identity. DNA sequences were obtained for 60 thalli from 18 sites along the coast (57–69° N) and 10 herbarium specimens (1902–1950). The sequences indicated that both subsp. *fragile* and subsp. *atlanticum* occur at present and have been in Norway since at least 1932 and 1948, respectively. The subspecies co-occurred at one site, but in general, subsp. *atlanticum* appears to have a narrower distribution than subsp. *fragile*, both geographically and in terms of habitat. Importantly, mucron length, other utricle features, or habitat were not always sufficiently reliable to give an accurate subspecies identification, demonstrating the necessity of DNA sequencing for the identification of these subspecies.

Keywords: *Codium atlanticum*; *Codium fragile*; herbarium samples; introduced species; morphology.

Introduction

Codium fragile (Bryopsidales, Chlorophyceae) is a siphonous green alga with a NW Pacific origin (Trowbridge 1998). This taxon is presently divided into 10 subspecies (Brodie et al. 2007), one of which is a well-known introduced seaweed: *C. fragile* subsp. *fragile* (Suringar) Hariot (previously *C. fragile* subsp. *tomentosoides*; Provan et al. 2008). This subspecies has good dispersal and establishment abilities (Nyberg and Wallentinus 2005) and has spread worldwide over the last 200 years (Provan et al. 2008). In new habitats, it can have ecological and economic impacts; for example, it may compete with native kelps or fucoids (Scheibling and Gagnon 2006, Armitage et al. 2014), influence seaweed-associated fauna composition (Schmidt and Scheibling 2006, Drouin et al. 2011, Armitage and Sjøtun 2016), negatively affect commercial bivalve beds (summarised in Trowbridge 1998) and impact ecosystem services (Vilà et al. 2010). In Norway, *C. fragile* subsp. *fragile* has been classified as a high-impact non-native species due to its widespread distribution, long expected population lifetime and moderate ecological impact (Gederaas et al. 2012). In some regions, it can become locally abundant, growing in patches in the upper subtidal and infralittoral zones of sheltered and moderately wave-exposed locations, especially with boulder/cobble substratum (Armitage et al. 2014).

According to Silva (1955, 1957), *C. fragile* subsp. *atlanticum* (Cotton) Silva and subsp. *scandinavicum* Silva were already present in Norway in 1946 when subsp. *fragile* was first recorded, with the first records of subsp. *atlanticum* from 1895 and subsp. *scandinavicum* from 1929. These identifications were based on observations of utricle morphology; in particular, utricle dimensions and mucron shape and length have been used to separate the subspecies (e.g. Silva 1955, 1957, Trowbridge and Todd 1999a,b, Brodie et al. 2007). The subsp. *scandinavicum* was hypothesised to be a northern-adapted subspecies of *C. fragile* potentially originating from Siberia (Silva 1957), whereas subsp. *atlanticum* had a more southern distribution, and is listed as observed in Norway, the British Isles, France, the Netherlands, Spain and the Azores (Guiry and Guiry 2015).

However, the use of molecular methods has shown that the status and distribution of the subspecies needs re-examination. Subsp. *atlanticum* is genetically distinct from subsp. *fragile* according to a marker in the plastid genome (*rpl*16-*rps*3) and has been verified as present in the British Isles (Provan et al. 2008), but there is no genetic confirmation of its distribution in other countries to the authors' current knowledge. Furthermore,

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sequences of this marker have revealed the type material of subsp. *scandinavicum* to be the same as subsp. *fragile*, uncovering no evidence of the existence of a separate subspecies (Provan et al. 2008). More recently, a comparison of several *C. fragile* subspecies (including subsp. *fragile*, but not *atlanticum*) has suggested that subsp. *fragile* could be a separate species (Verbruggen et al. 2017).

The introduced subsp. *fragile* has often been misidentified (Provan et al. 2008). Rojo et al. (2014) found two morphological groups of *C. fragile* in NW Spain that they initially assigned to subsp. *atlanticum* and subsp. *fragile*, but the *rpl16-rps3* sequences indicated that all were subsp. *fragile*. Hubbard and Garbary (2002) and Kusakina et al. (2006) also found morphologically distinct populations of *C. fragile* in Canada, supported by genetic differences in ISSR nuclear markers (Kusakina et al. 2006), and they suggested that one might be subsp. *atlanticum*. However, later sequencing of *rpl*16-*rps*3 indicated that these were two variants of subsp. *fragile* (Benton 2014). In Norway, molecular work has been done on only a few samples of *C. fragile*: Provan et al. (2008) sequenced one sample which was assumed to be subsp. *scandinavicum*, and Armitage et al. (in press) sequenced 11 samples from the Bergen area, but all of these turned out to be subsp. *fragile*.

The morphological variability of subsp. *fragile* and the common misidentification of *C. fragile* subspecies could imply two things for Norwegian records. The first is that the arrival of the non-native subsp. *fragile* in Norway could be much earlier than the first record according to Silva (1955, 1957). Observations from the 1930s describe a dramatic and obvious increase of *Codium* (notes on University Museum of Bergen herbarium specimens include: "found drifting everywhere in great quantities in the sounds in Austevoll", collected by K. Fægri in 1933; "has spread profusely in Norway in the last few years, earlier nearly unknown here" (translated), collected by H.H.H. Heiberg in 1936). This potentially reflects a rapid expansion of subsp. *fragile*, years before the first official collection (also see Fægri and Moss 1952). The second is that subsp. *atlanticum* may not actually be present in Norway, given that misidentification is common and that subsp. *atlanticum* is only confirmed from the British Isles (Provan et al. 2008).

The aims of this study were therefore (1) to sequence historical collected specimens of *C. fragile* in order to find the most likely time that subsp. *fragile* spread to Norway, (2) to check which subspecies of *C. fragile* are present in Norway, and if more than one is found, (3) to assess whether currently used micro-morphological characters are reliable for their identification. This was done by sequencing the *rps*3 – *rpl*16 region of the plastid genome in samples of *C. fragile* and examining the utricle morphology of the subspecies.

Materials and methods

Sampling

Fresh samples of *C. fragile* were collected along the coast of Norway between 57° and 67° N during 2014–2015 (Table 1). *C. fragile* has been recorded along the coast of Norway north to around 70° N (Stellander 1969), but is relatively rare in the southeast (Husa et al. 2013), so there were no samples from this area. Two clean branch tips around 3 cm in length from each thallus were dried in silica gel, except when the whole thallus was collected and dried as a herbarium specimen. Samples from Norwegian herbarium collections were also taken (Table 2). Herbaria contacted included the University Museum of Bergen (BG), the Botanical Museum in Oslo (O), the Norwegian University of Science and Technology Museum (TRH) and the Adger Museum of Natural History and Botanical Garden (KMN). Samples were taken of thalli which were identified as subsp. *atlanticum* in the collections (which looked in reasonable condition), along with other specimens if they were from geographical areas with poor coverage from the fresh material, or from early dates.

Sequence data

The molecular work in this study was done using methods described by Provan et al. (2008). The primers of Provan et al. (2004) were used to amplify and sequence the *rpl*16-*rps*3 region of the plastid genome, which is suitable for indicating evolutionary units within the genus *Codium* (Verbruggen et al. 2007) and allows identification of subspecies of *C. fragile* using four single nucleotide polymorphisms (SNPs) (Provan et al. 2008). The UCP6 set encompasses ca. 450 bp, and three sets of primers (UCP61, 2 and 3) divide this up into smaller fragments to allow sequencing of potentially poor-quality herbarium DNA (Provan et al. 2008).

All laboratory work was carried out at the Biodiversity Laboratories (BDL, DNA section) at the University Museum of Bergen/Department of Biology (University of Bergen). DNA was extracted from a small (0.5–1 cm) section of the dried *C. fragile* using a Qiagen DNeasy

Table 1: *Codium fragile*: sequenced fresh samples from the coast of Norway (collection locations listed south to north, counties in bold).

Collection location		Date	Collector[a]	Remarks by collector	Sample code
Vest Agder					
Lillehavn	57.99302, 7.090001	Aug 2015	VH	In a harbour	70 : 1
Kilen	58.05488, 7.09849	Aug 2015	VH	On a floating dock	71 : 1
Øksnes	58.05799, 7.11044	Aug 2015	VH	On a floating dock	72 : 1
Rogaland					
Nord Talgje	59.22836, 5.78642	Jun 2014	SØ	Subtidal, fairly sheltered location	56 : 1, 56 : 3, 56 : 4, 56 : 6, 56 : 8
Hordaland					
Bømlo, Tjongspollen	59.674, 5.238	Oct-Nov 2014	KS, CSA	Thalli fertile, subtidal. Collection site is one of the few Norwegian locations where *Codium vermilara* grows (Heggøy 2000)	65 : 2, 65 : 3, 65 : 4
Austevoll, S. Huftarøy	59.99667, 5.26100	Aug 2014	SØ	Subtidal, sheltered location	63 : 1, 63 : 4
Austevoll, Rostøy	60.09187, 5.20770	Sep 2015	CSA	Thallus < 11 cm, growing on a semi-exposed vertical rock face. In a turf of *Corallina officinalis* and *Bonnemaisonia hamifera* around low water	14 : 1 (V) [KX755326]
Austevoll, Kubbholmen	60.14918, 5.16932	Mar 2014	CSA	Infralittoral/subtidal dominant patch, stony cobble substratum. Thalli ca. 20 cm, often large holdfasts (≥ 5 cm)	18 : 2, 18 : 5, 18 : 6, 18 : 7, 18 : 8
Stora Karlsøy	60.11325, 5.06491	Apr 2014	CSA	Collected from intertidal rock- pools, fairly wave exposed site. Thalli 10–15 cm, holdfasts ca. 1 cm	54 : 1, 54 : 2
Bjorøy	60.30122, 5.16673	Sep 2014	CSA	Around chart datum, patchy growth of thalli on stony cobble substratum	52 : 1 (V) [KX755329]
Lindås, Lygra	60.69869, 5.10828	Aug 2014	CSA	Floating thalli	64 : 1, 64 : 2, 64 : 3
Baløy/Nordre Sævrøyna	60.80528, 4.80860	Mar 2014	CSA	Subtidal, thalli ca. 20 cm, holdfasts often > 1 cm	53 : 9, 53 : 10, 53 : 11, 53 : 12, 53 : 13
Sogn og Fjordane					
Flora, East of Stavøya	61.54699, 5.17723	Aug 2014	MHE	Sheltered location. Some collected from a floating dock, always ca. 15 cm deep, thalli 10–15 cm. Others collected nearby, also subtidal	58 : 2, 58 : 3, 58 : 4, 58 : 5
Møre & Romsdal					
Runde, Måkeneset	62.38484, 5.60889	Jul 2014	AC	Collected from two intertidal rockpools. Small thalli (around 10 cm) arising from a basal filamentous mat	57 : 1, 57 : 4, 57 : 5, 57 : 8, 57 : 10, 57 : 12
Sør Trøndelag					
Frøya, Titran	63.66618, 8.30521	Nov 2014	OV	Grew on the shore between the quay and floating dock at Titran, in a very limited area. Samples taken within a radius of 5 m	67 : 1, **67 : 2**, 67 : 3, **67 : 4**, 67 : 5
Frøya, Hellskjæret	63.75801, 8.90768	Nov 2014	OV	–	**68 : 1, 68 : 2, 68 : 3, 68 : 4**
		June 2015	BTH	Growing in a shallow rockpool (ca. 15 × 20 m, 30–40 cm deep) with sandy/shell sand bottom on the southern tip of island. Not very abundant compared to other species, growing with *Ascophyllum nodosum*. Thalli 23–33 cm long	**68 : 8, 68 : 9, 68 : 10** (V) [KX755328]

Table 1 (continued)

Collection location		Date	Collector[a]	Remarks by collector	Sample code
Frøya, Kya	63.77967, 8.35347	June 2015	BTH	Located in shallow rockpool (ca 3 m × 1 m, 20–30 cm deep). Bedrock. *Codium* quite abundant together with coralline Rhodophyta and filamentous Chlorophyta. Thalli 15–23 cm long	**69 : 1, 69 : 2, 69 : 3** [KX755327]
Nordland					
Godøystraumen (east of Bodø)	67.24016, 14.71148	Oct 2014	KR	Sheltered site, but with current (in a channel). Thalli in a low density patch on rock, around 65 cm deep. Thalli ca. 10 cm long	66 : 1, 66 : 2, 66 : 3, 66 : 4, 66 : 5

[a]VH, Vivian Husa; SØ, Siri Ødegaard; KS, Kjersti Sjøtun; CSA, Caroline S. Armitage; MHE, Mari Heggernes Eilertsen; AC, Annelise Chapman; OV, Ola Vie; BTH, Barbro Taraldset Haugland; KR, Katrin Reiss.
The sample code is made up of "site number: sample number from that site", with sample codes in bold representing subsp. *atlanticum*, and normal font representing subsp. *fragile*. "V" indicates that thallus is stored as a herbarium specimen, and GenBank accession numbers are written in square brackets directly after the samples which they were taken from.

Plant Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. Prior to PCR amplification, the DNA extractions of the fresh samples were diluted by 50, and the herbarium samples by 10. The PCR reaction mix (25 µl total) contained 1 µl 10 µmol forward primer, 1 µl 10 µmol reverse primer, 1 µl DNA, 2 µl dNTP, 2.5 µl 10× PCR buffer, 17.35 µl ddH_2O, and 0.15 µl TaKaRa Taq Hot Start version (Takara Bio Inc., Otsu, Japan). The PCR was done under the following thermal settings: initial denaturation at 94°C for 5 min, 5 cycles with denaturation at 94°C for 1 min, annealing at 45°C for 90 s, and extension at 72°C for 90 s, followed by 35 cycles with 94°C for 1 min, 50°C for 90 s and 72°C for 1 min, then a final extension for 5 min at 72°C. Positive and negative controls were routinely used.

Gel electrophoresis was used to check the PCR products. A 1% agarose gel made with 1×TAE buffer (Tris base, acetic acid, EDTA) and containing GelRed (Biotium, Hayward, CA, USA) was loaded with a mix of 4 µl PCR product and 1 µl loading buffer. FastRuler DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA) and images taken with GeneSnap (SynGene, Cambridge, UK) were used to assess DNA size and quantity. PCR products were then purified in 10-µl reactions, containing 8 µl of PCR product, 0.1 µl exonuclease 1 (EXO, 10 U μL^{-1}), 1.0 µl shrimp alkaline phosphatase (SAP 10 U μL^{-1}) and 0.9 µl ddH_2O. Incubation at 37°C for 15 min was followed by an inactivation step at 85°C for 15 min. The BigDye (v3.1) method was used to sequence the DNA, using an Applied Biosystems 3730XL Analyzer (Thermo Fisher Scientific) at the Sequencing Facility, Molecular Biology Institute, University of Bergen (Norway).

The programme Geneious (v. 6.1, Biomatters Ltd., Auckland, New Zealand) was used to check sequences, assemble contigs and align the sequences using MUSCLE (multiple sequence comparison by log-expectation). These data were then used to ascertain subspecies identity by comparison with the sequences of Provan et al. (2008) and Benton (2014).

Morphological data

Eleven samples were examined microscopically. These came from six sites: two in Trøndelag (Hellsjæret and Titran) and four in Hordaland (Stora Karlsøy, Bjorøy, Baløy and Tjongspollen; Table 1). Dried tissue was rehydrated in seawater, and utricle morphology was examined approximately 2 cm from branch tips (the area normally used for identification and considered the most consistent; Silva 1957, Dromgoole 1975, Trowbridge 1998). Mucron length and shape was recorded for 16–20 utricles per thallus; these were selected by preparing a slide and measuring the first 20 mucrons that could be clearly seen and were not distorted. Utricles with no mucron at all were not included. Since the starting point for measuring mucron length is not clearly described in every publication, we measured it both from the inner cell wall [hereafter referred to as "length *a*", used by Kusakina et al. (2006)] and from the "shoulder" of the utricle ("length *b*"; see Supplementary Figure S1 for clarification). Utricle shape, length and width, hair scar distance to apex, and whether gametangia were present were also recorded for samples which rehydrated well, for up to 10 utricles per sample. Measurements and images

Table 2: *Codium fragile*: Norwegian herbarium samples from which sequence data could be obtained, listed by date (oldest to most recent) and with herbarium code (Herb. code).

Originally identified as	Identification by	Place of collection	Date	Collector	Herbarium code	Sample code
–	–	Møre og Romsdal, Ålesund	Aug, 1902	N. Wille	O	*OM6, OM7*
subsp. *atlanticum*	T. Levring (1937) as *f. atlanticum*	Hordaland, Herdla, Raugnøpollen	27th Jul 1932	G. Hygen, I. Jorde	BG	BM5
–	–	Vest Agder, Farsund, Vesthassel	22nd Aug 1934	O. Håversen	O	OM2
subsp. *scandinavicum* > *atlanticum*	P.C. Silva (1956)	Hordaland, Smekkevik, south of Brattholmen, Fjell	10th Jul 1948	E. Moss	BG	BM10
subsp. *scandinavicum* > ("slightly") *tomentosoides*	P.C. Silva (1956)	Sogn and Fjordane, Kinn (south side of inner Hovdevåg)	21st Aug 1948	E. Moss	BG	BM11
subsp. *scandinavicum*	P.C. Silva (1956)	Sogn and Fjordane, Solund, Aspø	11th Aug 1948	E. Moss	BG	**BM12**
subsp. *scandinavicum*	P.C. Silva (1956)	Nord Trøndelag, Ulsund, Ytra Vikna, Vikna	14th Jul 1949	E. Moss	BG	BM15
subsp. *scandinavicum*	P.C. Silva (1956)	Nord Trøndelag, Lovunden, Lurøy	20th Jul 1949	E. Moss	BG	BM16
subsp. *scandinavicum*	P.C. Silva (1956)	Vest Adger, Mandal	2nd Jul 1950	E. Moss	BG	BM3

Samples in bold are those genetically identified as subsp. *atlanticum*, those in normal font as subsp. *fragile*, and those in italics are neither or are doubtful (see Results).

were taken using Leica application suite software (v.4.5), Leica DFC450 camera and DM2000 LED microscope (Leica microsystems, Wetzlar, Germany).

Results

Sequence data

The sequences of the collected samples confirmed that both *C. fragile* subsp. *fragile* and subsp. *atlanticum* currently inhabit the coast of Norway. Although all of the samples from the south, southwest and northern coastlines were subsp. *fragile*, the islands around Frøya in midwest Norway support populations of subsp. *atlanticum* (Figure 1A). This included one sampling site where both of the subspecies were growing together within a radius of around 5 m (site 67, Titran; Table 1).

Sequences of herbarium samples also showed that nearly all were subsp. *fragile*, including the earliest sequence, which was from a thallus from Hordaland in 1932 (BM5). One thallus, collected from Solund in 1948 (BM12) and originally designated as subsp. *scandinavicum* by Silva, was genotyped as subsp. *atlanticum* (Table 2, Figure 1B).

It was not possible to get sequences from a number of the herbarium samples. This included the earliest *C. fragile* in the collections (1890, Bomlø, Hordaland, B. Hansteen, O) and a floating specimen found on Jan Mayen (1930, J. Lid, O). Short sequences were obtained for the herbarium specimens OM6 and OM7, collected in Ålesund in 1902, but with unexpected results; the sequence for OM7 (136 bp) was most similar to that of *Codium vermilara*, but had four single nucleotide differences and one 3-nucleotide difference compared to the reference sequences deposited by Verbruggen et al. (2007). The OM6 sequence was short and based on only one strand, which showed double peaks at many of the sites where *C. vermilara* differs from *C. fragile*; this may be a result of contamination over the years in the herbarium.

For both subspecies, there is a single-base pair discrepancy between the sequences in the present study and the representative sequences of Provan et al. (2008) to which they were compared (GenBank accession numbers EU045560 for subsp. *fragile* and EU045559 for subsp. *atlanticum*); it has been ascertained that these are misreads in the original Provan et al. (2008) sequences (Benton 2014, personal communication J. Provan). Representative sequences from the present study have been uploaded to GenBank; subsp. *fragile* as accession number KX755326, subsp. *atlanticum* as accession number KX755327, along

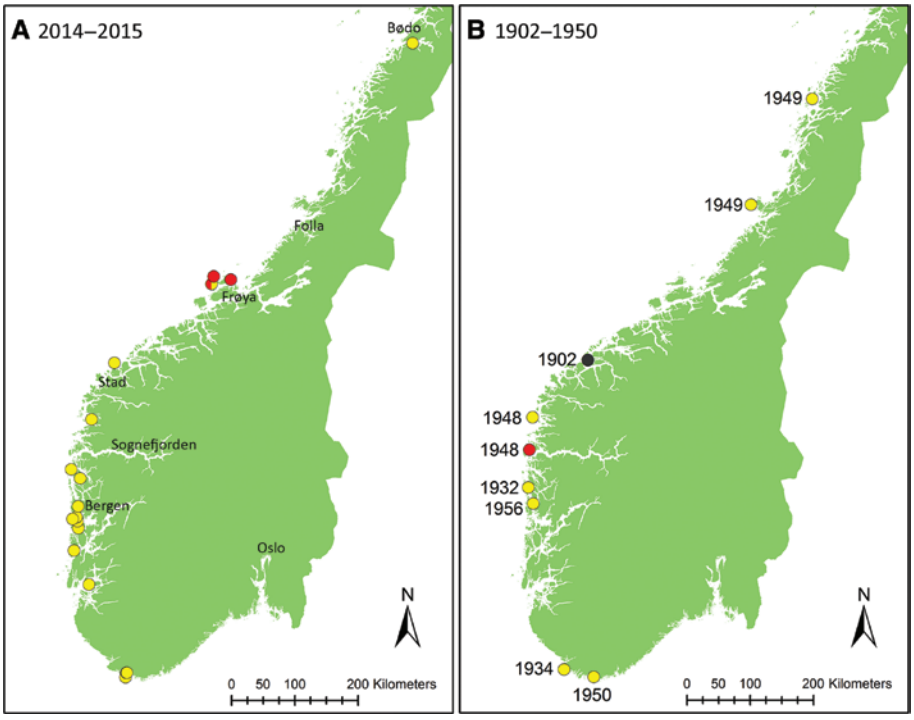

Figure 1: *Codium fragile*: Sampling sites along the coast of Norway (excluding northern Norway), showing subspecies identity at each location (subsp. *fragile* in yellow; subsp. *atlanticum* in red; uncertain or not *C. fragile* in grey) according to the *rpl*16-*rps*3 genetic marker. Map (A) shows samples from 2014 to 2015, and place names mentioned in the text; map (B) shows herbarium specimens 1902–1950 and their date of collection.

with sequences from two additional voucher specimens (whole dried thallus; Table 1).

Morphological data

In the thalli we measured, there were no consistent differences in mucron length between subsp. *fragile* and subsp. *atlanticum* (Figure 2). The seven individuals of subsp. *fragile* had a mean mucron length *a* of 14 µm (with individual thallus means ranging from 9 to 18 µm), whereas the four *atlanticum* thalli had a mean of 14 (13–18) µm. Using mucron length *b* also gave similar results for the two subspecies: 19 (15–28) µm for subsp. *fragile* and 18 (14–23) µm for subsp. *atlanticum*. With regard to mucron shape, subsp. *atlanticum* tended to have fewer pointed mucrons than subsp. *fragile*, but this character also overlapped between the two subspecies (Figures 3 and 4). In addition, both subspecies could have mucrons with fine striations (Figure 4).

Utricle widths were similar between the subspecies, at 271 µm (with individual thallus means ranging from

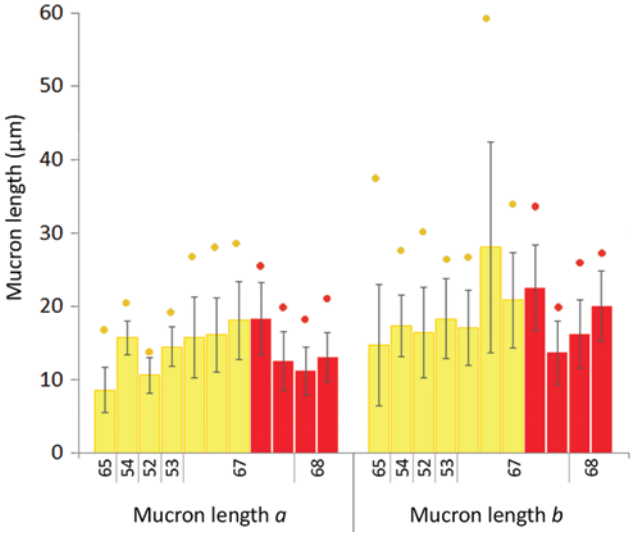

Figure 2: *Codium fragile* mucron lengths, as measured from the cell wall (length *a*) and the shoulder of the utricle (length *b*). Each bar represents the mean mucron length in one thallus, with standard deviation (bars) and maximum lengths (circles) shown (n = 16–20 mucrons per thallus). Site number is labelled below the bars, ordered from south to north (Table 1). Subsp. *fragile* is displayed in yellow, and subsp. *atlanticum* in red.

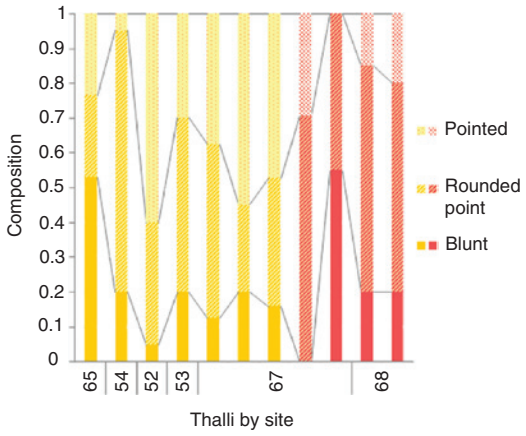

Figure 3: *Codium fragile*: The frequency of different mucron shapes observed in subsp. *fragile* (yellow) and subsp. *atlanticum* (red) thalli (n = 16–20 mucrons per thallus).
Each bar represents one thallus. Site number is labelled below the bars, ordered from south to north (Table 1). Mucron shapes are clarified in Figure 4.

241–309 µm) for subsp. *fragile* and 274 (248–299) µm for subsp. *atlanticum*. However, the utricles were slightly shorter in subsp. *fragile*, which had a mean utricle length of 634 (586–694) µm whereas *atlanticum* had a mean length of 711 (680–760) µm (Figure 5). The standard deviations of these measurements for each thallus were quite large, indicating much variation. Both subsp. *atlanticum* and *fragile* could display a constriction in the middle of the utricle (Figure 4A and B). Gametangia were present in only a few individuals; these were 351 (332–374) µm long in subsp. *atlanticum* (3 thalli, 5–10 measured per thallus), but only one mature gametangium was seen in the utricles measured for subsp. *fragile*, which was 256 µm long (see Supplementary Table S1 for full data).

There appeared to be a difference in the timing of fertility in the area where both subspecies were present. In the thalli sampled from Frøya (sites 67 and 68) in November 2014, all four subsp. *atlanticum* thalli had mature

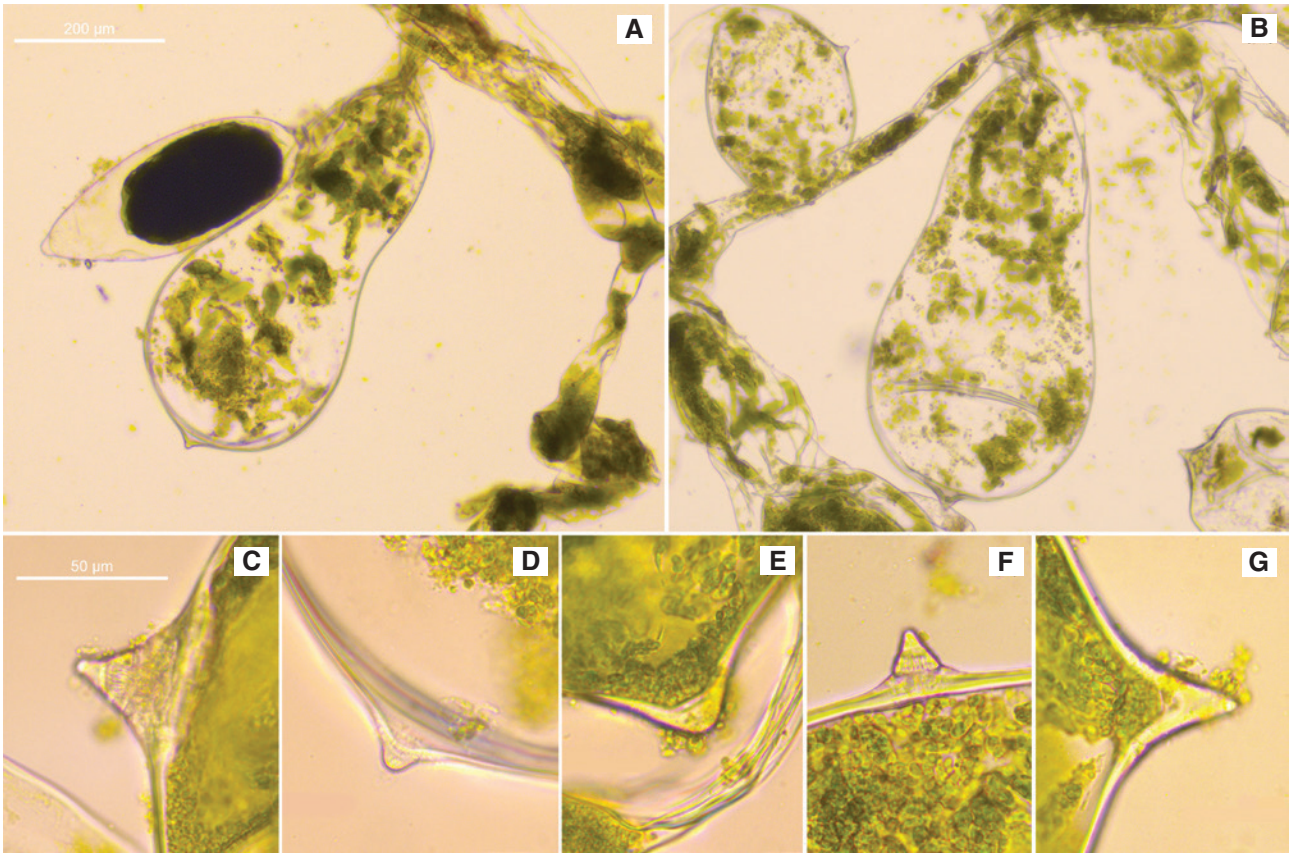

Figure 4: *Codium fragile*: Photographs of mucrons and utricles of subsp. *atlanticum* (A, C and D), and subsp. *fragile* (B, E, F and G), collected from Titran on Frøya, Norway (63.66618, 8.30521) in November 2014.
Mucrons (C) and (G) were categorised as pointed; mucrons like (E) or flatter were categorised as "blunt"; mucrons like (D) and ranging in pointedness towards (F) were categorised as "rounded points".

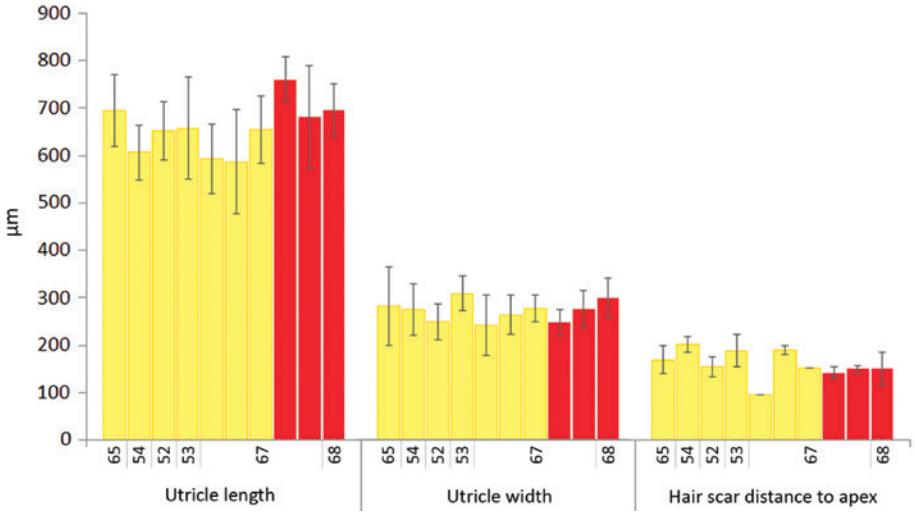

Figure 5: *Codium fragile* utricle measurements. Each bar represents the mean measurement in one thallus, with standard deviation (for utricle length and width, n = 10 utricles per thallus, except for the thallus 67 : 3, where n = 3). "Hair scar distance" refers to the distance of the hair scar from the apex of the utricle (n = 1–9 utricles per thallus). For more details of all measurements, see Supplementary Table S1. Site number is labelled below the bars, ordered from south to north (Table 1). Subsp. *fragile* is displayed in yellow, and subsp. *atlanticum* in red.

gametangia, whereas the three subsp. *fragile* thalli were infertile.

Discussion

Distribution of *Codium fragile* subspecies in Norway

By genotyping of herbarium material, the presence of *C. fragile* subsp. *fragile* was confirmed for Hordaland by 1932 and for Vest-Adger by 1934, setting back the first collection of this non-native subspecies in Norway by 14 years. However, as these two locations are approximately 300 km apart, it suggests that subsp. *fragile* had already been spreading for some time before 1932. This is supported by the fact that subsp. *fragile* had spread through the majority of its current Norwegian distribution by 1950 (approximately 1100 km of coastline), and the fact that subsp. *fragile* was already present in the Orkneys (N. Scotland) in 1891 (Provan et al. 2008). It seems likely that the dramatic increase in *Codium* along the Norwegian coast in the early 1930s (Fægri and Moss 1952) was a rapid expansion of *C. fragile* subsp. *fragile*, which was also spreading quickly in parts of Ireland during the same decade (see Trowbridge et al. 2013).

In contrast, only a few specimens of subsp. *atlanticum* were identified in the present study. This subspecies has been present in Ireland since at least 1845 (Provan et al. 2008) and is thought to have spread northwards through Scotland on north-flowing currents (Trowbridge 1998). The distribution in Norway found in the present study is consistent with this mode of dispersal, as the current that flows northwards past the Scotland and the Shetlands tends to hit the Norwegian coast around Stad (Brattegard 2011). This current accounts for a large portion of the marine species which spread naturally to the Norwegian coast (Brattegard 2011). *C. fragile* is clearly capable of floating long-distances on currents, as shown by the drift specimen found on Jan Mayen (collected in 1930, O), which is approximately 1300 km from the Scottish mainland and 900 km from the nearest point in Norway.

Why subsp. *atlanticum* has only been found in a fairly limited region of Norway (61–64° N) compared to subsp. *fragile* has a number of possible explanations. It may be that subsp. *atlanticum* is relatively rare here, and that more samples will reveal a wider distribution. Subsp. *atlanticum* is also more uncommon and restricted in distribution than subsp. *fragile* in the British Isles, being absent from areas such as the English Channel (Brodie et al. 2007, Trowbridge and Farnham 2009). Another alternative is that subsp. *atlanticum* may require higher winter temperatures for survival than subsp. *fragile*, as seawater temperatures in winter are highest in Norway between Stad and Folla (Brattegard 2011). However, the sample from 1948 just north of Sognefjorden does not fit this pattern. Another possibility is that spread from initial colonisation sites may have been easier for the non-native subsp. *fragile* than subsp. *atlanticum*. If subsp. *atlanticum* spread to mid-Norway from the

British Isles, to expand into southern Norway, it would have to disperse against the north-flowing Norwegian coastal current and across several fjord outflows (Bakketeig et al. 2016). On the other hand, some of the earliest findings of subsp. *fragile* in the present study were from southern Norway; from here, expansion northwards along the coast could be easily achieved by drifting on the coastal current. Subsp. *fragile* can also be spread by human vectors such as boat traffic (Trowbridge 1998) and can reproduce parthenogenetically and from fragments (Churchill and Moeller 1972, Ramus 1972, Dromgoole 1975, Prince and Trowbridge 2004), meaning a only a small portion of one thallus needs to be transported to start a new population.

In the present study, the subsp. *atlanticum* samples were from rock-pools on relatively exposed islands, and the shore of a more sheltered location. No subtidal subsp. *atlanticum* was found. This fits with observations from the British Isles, where subsp. *atlanticum* has been reported to grow mainly in mid-low intertidal rock-pools (Burrows 1991) or high pools at exposed locations (Trowbridge and Todd 1999a). On the other hand, subsp. *fragile* can be found in nearly all types of habitat: the subtidal, low intertidal (around or just below mean low water), and rock-pools at both sheltered and more exposed locations. The lack of either subspecies in the mid or high intertidal is unsurprising given that occurrence on emergent substrata is relatively rare for both subspecies in areas where winter freezing occurs (Trowbridge 1998). Because subsp. *fragile* can be found in a wide range of habitats, we suggest that using tidal position or habitat for subspecies identification can be unreliable (with the possible exception of the subtidal for subsp. *fragile*, pending further investigation). The observations also suggest that subsp. *atlanticum* has a more restricted habitat than subsp. *fragile* in Norway, in addition to the more restricted geographic distribution discussed above. However, sampling at more locations is necessary to confirm this, particularly in mid-Norway and on islands at the outer edge of the coastline. Whether subsp. *atlanticum* is native or introduced is somewhat uncertain (Trowbridge 1998) but it does not appear to possess the same invasiveness as subsp. *fragile* on the Norwegian coast.

Morphology of *Codium fragile* subspecies

The results indicate that utricle morphology is not a particularly reliable character for separating *C. fragile* subsp. *fragile* and *atlanticum* in Norway. There are some trends in the utricle characters which could be related to genetic identity, but there is clearly much overlap and individual variation, making it difficult to use these characters for identification guidelines. Regarding the mucrons, most of the thalli had mean and maximum mucron lengths between 15 and 30 μm, which is intermediate between typical values used for identification of subsp. *atlanticum* and *fragile*. Only one extremely long mucron (>40 μm) was seen, and the subsp. *fragile* mucrons were frequently shorter and blunter than expected (Silva 1957). Thus using commonly applied mucron characters for identification of these thalli would lead to misidentifications; for example that mucrons are <15/20 μm long in subsp. *atlanticum* (Silva 1957, Burrows 1991, Brodie et al. 2007), mucrons are sharp in subsp. *fragile* (Silva 1957), and that subsp. *fragile* has fine concentric striations on the mucrons (Burrows 1991).

Utricle widths were similar between the two subspecies when from the same site, and both could display a constriction (normally only attributed to subsp. *fragile*; Silva 1957). Distance of the hair scar from the utricle apex was also quite similar: whereas the subsp. *atlanticum* samples were all within the 130–200 μm range described for subsp. *atlanticum* and below the range of 160–260 μm range for subsp. *fragile*, some of the subsp. *fragile* samples were also below 160 μm. In addition, although the subsp. *atlanticum* utricles tended to be slightly longer than the subsp. *fragile* utricles, they were generally shorter than as described for the subspecies (780–1100 μm). Their length and length/width ratio was more typical of subsp. *fragile* or subsp. "*scandinavicum*" (550–1050 and 480–850 μm, respectively; Silva 1957). There were not enough gametangia in the samples to justify a comparison in size or position between the subspecies, and all those observed were either female or, in most cases, indistinct. Determination of the mode of reproduction (parthenogenetic or sexual) has been used as a method of separating subspecies in some studies (e.g. Trowbridge and Todd 1999a) but was not investigated here as most samples were dried and/or without gametangia.

Morphological characters between the diagnostic values for each subspecies are not uncommon in Scandinavian *C. fragile*, as discussed by Silva (1957). Hybridisation has been proposed as one explanation for "intermediate" morphologies (e.g. Silva 1957, Trowbridge 1998, Kusakina et al. 2006). Theoretically, the two subspecies may be able to hybridise if a male gamete of subsp. *atlanticum* fused with a female gamete (normally parthenogenetic) of subsp. *fragile* (Trowbridge 1998). Around Frøya, the subsp. *atlanticum* thalli were fertile in November whereas the subsp. *fragile* thalli were not, suggesting reproductive separation in time – but, it is unknown whether an overlap might have occurred before sampling.

However, when considering diagnostic values, it should be taken into account that subsp. "*scandinavicum*" is likely conspecific with subsp. *fragile* (Provan et al. 2008). If so, this would mean that the described morphological differences between them may be largely due to environment (as proposed by Fægri and Moss 1952, and discussed in Silva, 1957). If this is the case, it would partially explain Silva's observation that "intergrades" are fairly common in Norway, lying between the two "morphological plateaus" of subsp. *fragile* and subsp. "*scandinavicum*" (Silva 1957), and would imply that the original morphological description of subsp. *fragile* is too narrow, in particular with regard to mucron length which can be much shorter within individuals of subsp. *scandinavicum* (described as up to 20 μm; Silva 1957). Some "intermediate" characters between subsp. *fragile* and subsp. *atlanticum* may therefore actually lie within the normal range of subsp. *fragile*, rather than being a product of hybridisation. Large morphological variability in subsp. *fragile* has also been highlighted in recent work by Armitage et al. (in press) in New Zealand.

The macro-morphology of the two subspecies was not examined here, but some observations were made. It is sometimes stated that the holdfast of subsp. *fragile* is small (usually <1 cm; Brodie et al. 2007) compared to that of subsp. *atlanticum*, but personal observations of holdfast size at sites where all sequenced samples were genetically determined as subsp. *fragile* (e.g. site 18) indicate that the holdfasts can often be much larger than 1 cm in diameter and can spread out in a mossy, undifferentiated way (Supplementary Figure S2). Differences in thallus size and number of dichotomies (e.g. Trowbridge and Todd 1999b) should also be used with caution when the samples are not from the same site. The subsp. *fragile* found in the present study could occur in patches where all were only around 10 cm long, whereas subsp. *atlanticum* could be longer than the typical 25 cm (Silva 1957). A difference that may be worth further investigation is that the subsp. *atlanticum* observed in the present study seemed to have blunter branch tips than subsp. *fragile*, which had more pointed tips (see Supplementary Figures S2 and S3), but it is unknown if this is influenced by environment.

Conclusion

Both *C. fragile* subsp. *fragile* and subsp. *atlanticum* have been growing in Norway since at least 1932 and 1948, respectively, and are still present today. The distribution of subsp. *atlanticum* is consistent with spread by currents from the British Isles, and it appears to have a more restricted distribution than subsp. *fragile*, both geographically and in habitat, but more extensive sampling is needed to confirm this. There are indications of some potential differences between the subspecies in tidal position and timing of fertility, but this also needs further investigation. The results indicate that using micro-morphological or habitat characters to identify subsp. *fragile* and subsp. *atlanticum* can easily lead to misidentifications in some locations. Because the morphological characters can overlap between the subspecies, molecular identification is recommended. Genetic identification may allow future studies of the ecology and morphology of these subspecies to reveal further and more reliable differences.

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