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A molecular survey of *Ulva* L. in Scandinavia

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Abstract

The species composition and distribution of marine green macroalgae in Scandinavia are poorly understood as historical records are based on morphological characters which are unsuitable to identify many species. This is especially true of the ecologically and economically important genus *Ulva*, the taxonomy and systematics of which have been rewritten with the use of molecular methods. Additionally, the European seaweed industry purports to sell species which are likely misidentified. To investigate the diversity, distribution and systematics of *Ulva* in Scandinavia, ca 500 specimens of green macroalgae were collected along the coasts of Sweden, Norway, and Denmark in the summer months of 2020 during the peak of green algae vegetation. Specimens or sequences from biomass sold by three European companies were also obtained. After genomic DNA extraction, the plastid *tuf*A gene was amplified and sequenced, followed by Maximum Likelihood phylogenetic analysis. *tuf*A barcoding was effective in identification of *Ulva* spp. However, all taxa could not be delimited to species level, and the method is limited both by a lack of reference sequences and misidentification of voucher material. The results were compared with and found to differ from historical species inventories and distribution records. Fewer and different species were detected, including at least three introduced species identified in this study and related field collections. The distribution of *U. fenestrata* (previously known as *U. lactuca*) was different from historical records, in part due to salinity related morphological variation of *U. compressa*, which has led to past misidentifications. *U. laetevirens* is likely a common species sold in Europe, both harvested from the wild and cultivated. More data is needed to confirm and expand upon the results regarding species occurrences and distributions. On a larger scale, the taxonomic information in online repositories needs to be updated and expanded with new and more reliable reference sequences.

Keywords: *Ulva*, DNA barcoding, *tuf*A, phylogeny

Abstrakt

Artsammansättningen och distributionen av marina, gröna makroalger i Skandinavien är dåligt förstådda eftersom historisk information är baserad på morfologiska karaktärer som inte är lämpliga för identifiering av många arter. Detta gäller särskilt för det ekologiskt och ekonomiskt viktiga släktet *Ulva*, vars taxonomi och systematik har reviderats med hjälp av molekylära metoder. Den europeiska makroalgsindustrin påstår sig dessutom sälja arter som sannolikt är felaktigt identifierade. För att undersöka diversiteten, distributionen och systematiken av *Ulva* i Skandinavien samlades ca 500 exemplar av gröna makroalger in längs Sveriges, Norges och Danmarks kuster under sommarmånaderna 2020 när grön makroalgsvegetation är som störst. Exemplar eller DNA-sekvenser från biomassa såld av tre europeiska företag införskaffades också. Efter genomisk DNA-extraktion amplifierades och sekvenserades kloroplastgenen *tuf*A. Därefter utfördes fylogenetisk Maximum Likelihood-analys. DNA-streckkodning med *tuf*A var effektiv för identifiering av arter av *Ulva*, men alla taxa kunde inte bestämmas till artnivå, och metoden begränsas av såväl brist på referenssekvenser som felidentifierade beläggexemplar. Resultaten jämfördes med, och fanns skilja sig från, historiska artinventeringar och uppskattningar av arternas distribution. Färre och andra arter upptäcktes, inklusive åtminstone tre introducerade arter som identifierats i den här studien och relaterade fältinsamlingar. Distributionen av *U. fenestrata* (tidigare känd som *U. lactuca*) skilde sig från historiska uppskattningar, delvis på grund av salinitetsrelaterade morfologiska variationer av *U. compressa*, vilket har lett till felidentifieringar. *U. laetevirens* är sannolikt en vanlig art som säljs i Europa, både vilt skördad och odlad. Mer data behövs för att bekräfta och utveckla resultaten angående arters förekomst och distribution. På en större skala behöver taxonomisk information i databaser uppdateras och expanderas med nya, mer pålitliga, referenssekvenser.

Nyckelord: *Ulva*, DNA-streckkodning, *tuf*A, fylogeni

1 Introduction

Macroalgae, more commonly known as seaweeds, is a collective term for a polyphyletic group of organisms that share a similar form and lifestyle. It includes members of three distantly related clades – Phaeophyta (brown algae), Rhodophyta (red algae), and Chlorophyta (green algae) – which are united by being macroscopic, aquatic and photosynthetic. Macroalgae play crucial roles as habitat formers and food resource for countless organisms and thus contribute majorly to the biodiversity of our oceans. *Ulva* L. is a morphologically and ecologically diverse genus of green macroalgae (Chlorophyta, Ulvales, Ulvaceae) that are ubiquitous inhabitants of marine, brackish, and fresh waters all over the world. They play many important roles to humans and in the larger environment and have a complicated taxonomic and systematic history.

1.1 Biology of *Ulva*

The genus *Ulva* consists of 84 currently accepted species (Guiry & Guiry 2021) which exhibit two typical morphological forms: foliose (sheet-like) and tubular. Foliose forms are usually distromatic with two adhering cell layers, while tubular forms consist of two cell layers separated by a cavity (Brodie et al. 2007), though monostromatic sheets of a typically tubular species have been observed in the Finnish Baltic (Bäck et al. 2000). They are often attached to a substrate by rhizoids but can also be free-floating.

The diploid sporophyte and haploid gametophyte of *Ulva* spp. are isomorphic. Gametes develop in gametangia which can form in almost any cell in the gametophyte thallus. On rare occasion, gametophytes can also form so-called parthenospores, diploid swarmers which look like gametes but develop parthenogenetically (Föyn 1962). The diploid sporophyte genome contains a mix of both parents' DNA, but the chloroplasts contain a single genome inherited from only one parent (Bråten 1973; Kagami et al. 2008). Therefore, the nuclear DNA of an interspecific hybrid is expected to reflect the genomes of both hybridizing species, while the chloroplast DNA only reflects one of the species (Fort et al. 2020b).

For normal morphological development to occur in *Ulva*, the presence of a minimum of two bacterial strains which excrete certain regulatory and growth promoting factors are necessary (Spoerner et al. 2012). The composition of the microbiome is not restricted to specific species. Instead, a combination of the functional properties of at least two strains induces normal morphogenesis (Ghaderiardakani et al. 2017). In axenic cultures, *Ulva* cells remain undifferentiated and single strains induce cell differentiation but lead to deficient thalli (Ghaderiardakani et al. 2017; Spoerner et al. 2012). These obligatory cross-kingdom interactions make *Ulva* an example of an algal holobiont (Barott et al. 2011; Egan et al. 2013).

1.2 Importance and applications of *Ulva*

1.2.1 Green tides

Free-floating forms of several *Ulva* species can generate mass proliferations known as "green tides" which have substantial ecological and economic consequences. A major economic sector affected by green tides is tourism (Charlier et al. 2008; Smetacek & Zingone 2013). Environmental problems include anoxia and the subsequent release of hazardous gases caused by sulfate-reducing bacteria during decomposition (Nedergaard et al. 2002; Wang et al. 2011) and negative impacts on biodiversity (Mackenzie 2005). Algal mats formed by green tides are generally considered to be deleterious but may be beneficial for a few tolerant or opportunistic species, such as the gastropod *Peringia ulvae* (Pennant) and the polychaetes *Hediste diversicolor* (Müller) and *Capitella capitata* (Fabricius) (Cardoso et al. 2004). Mats of *Ulva* spp. have been observed to outcompete the seagrass *Zostera marina* L. both in the wild (den Hartog 1994) and experimentally (Bittick et al. 2018). In the former case, a monitored 10-hectare seabed of *Z. marina* and *Z. noltei* Hornemann on the south coast of England, disappeared entirely one year after the appearance of *U. radiata* (J. Agardh). *Z. marina* forms shallow seagrass beds which provide spawning grounds, shelter, habitat, and food for numerous species (Cole & Moksnes 2016; Hughes et al. 2009). The habitat is under threat and sensitive to disturbance and is currently the focus of restoration projects in Sweden (Jahnke et al. 2020).

Environmental factors that contribute to green tides include high nutrient levels and high temperatures. The phenomenon is predicted to increase as an effect of continued climate change, especially if nitrate levels cannot be controlled (Gao et al. 2017). Recently Fort et al. (2020c) demonstrated that strains of various green tide-forming *Ulva* species exhibit metabolic and growth characters different from non-green tide strains of

the same species. They hypothesise a genetic component to green tides and a selection pressure for tide-forming strains, which could further exacerbate the issue in the future.

1.2.2 Uses and applications

Ulva is not only important because of the nuisance of green tides, but also because of a wide range of current and potential uses and applications. In Brittany, France, large quantities of beached *Ulva* resulting from green tides are harvested annually, and methods for sustainable use of this biomass are being developed, such as stabilization or methanization for use as fertilizer and biofuel respectively (Charlier et al. 2008). Rather than using beached biomass, cultivation is a more reliable source. Lehahn et al. (2016) have estimated that offshore biomass cultivation of *Ulva* has the potential to contribute greatly to a decrease of new CO_2 emissions and the use of arable land by providing food, feed, chemical and fuel products. *Ulva* species have been identified as good candidates for biofuel production and protein animal feed through biorefinery processes (Bikker et al. 2016). They also contain bioactive compounds like antioxidants (Holdt & Kraan 2011), and some have been shown to have antialgal (Sun et al. 2018) or antibacterial properties (Ismail et al. 2018). The polysaccharide ulvan is especially interesting as a potential high-value product due to a wide range of pharmaceutical properties (Abd-Ellatef et al. 2017; Adrien et al. 2019; Kim et al. 2011; Leiro et al. 2007). *Ulva* also has a well-established role in bioremediation, especially as biofilters in aquaculture (e.g. Cohen & Neori 1991; Neori et al. 1991; Jiménez del Río et al. 1996). As simple biofilters, they are used to remove nutrients from wastewater. In integrated multi-trophic aquaculture (IMTA) this can be combined with using *Ulva* biomass as feed, something which has proven successful for fish (Shpigel et al. 2017), abalone (Bolton et al. 2009), and sea urchins (Shpigel et al. 2018). A related bioremediation use is for bisorption of heavy metals (Ibrahim et al. 2016; Wahlström et al. 2020). Finally, *U. compressa* var. *mutabilis* has become a model organism for studying, for example, the evolution of multicellularity and cross-kingdom interactions (De Clerck et al. 2018; Wichard et al. 2015).

1.2.3 Seaweed industry in Europe and the world

According to Food and Agriculture Organization of the United Nations (FAO 2020), in 2018 97.1 % of the total world production of macroalgae was a result of cultivation rather than wild harvesting, and most of that cultivation takes place in Asia. Between 2000 and 2018, total global production grew from 10.6 to 32.4 tonnes – in large part due to Indonesian cultivation of seaweeds used to produce carrageenan – but growth has been slow in recent years, and in 2018 there was a global decline of 0.7 % (FAO 2020). In contrast to global production, most European seaweed is harvested from the wild, either mechanically or by hand, and European seaweed production declined by almost one third in the first ten years of the 2000s (Netalgae 2012). However, the seaweed sector is receiving attention for applications as outlined above.

Currently, macroalgae aquaculture, which is usually based in the ocean, mostly use the biomass for volume and result in products where the concentration and composition of various compounds is not a main factor (Hafting et al. 2015). Almost all European seaweed biomass, most of which consists of brown algae, goes toward production of alginates, animal feed and fertilizer, which require large quantities but are low-value products. For *Ulva* spp., the main product categories are fertilizer and sea vegetables (Netalgae 2012). High-value markets for bioactive compounds, on the other hand, will require specialized, standardized, and traceable products. Levels of various compounds of the biomass (including sugars and proteins) can fluctuate depending on season, location, depth, strain, and other factors, which limits the usefulness of sea farm aquaculture (Hafting et al. 2015). However, this can be addressed by land-based aquaculture where the environment can be controlled. Selection of specific strains and manipulation of environmental variables have the potential to maximize the desired properties for a specific purpose. Olsson et al. (2020) and Toth et al. (2020) have recently demonstrated that abiotic factors during cultivation influence the composition of the high-value monosaccharides that make up ulvan, as well as growth and the content of e.g. fatty acids and proteins. A recent analysis by van den Burg et al. (2016) suggests large-scale offshore production is currently not economically feasible in the North Sea, but using controlled systems may make cultivation more profitable.

Despite the current uses and great promise of *Ulva*, which species are being sold, whether harvested or cultivated, is poorly known. Species are marketed which likely do not occur in European waters, and others are almost impossible to identify morphologically. The properties and compounds differ between species, meaning species identification in the seaweed industry is highly relevant no matter where the biomass comes from or what it is used for.

1.3 Taxonomy, identification, and distribution of *Ulva*

The taxonomic and nomenclatural history of the Ulvaceae family is complicated and currently in flux. The global algal database AlgaeBase lists 407 species names and 200 infraspecific names for *Ulva*, but only 84 accepted species names (Guiry & Guiry 2021). Traditionally, and in contrast to Linnaeus' original description (1753), tubular taxa have been placed in the genus *Enteromorpha* Link whereas *Ulva* contained the foliose species. Today, it has not only been established that tubular and foliose species are not separate, monophyletic groups (Hayden et al. 2003), but that some individual species exhibit both morphologies (Steinhagen et al. 2019b; Steinhagen et al. 2019d).

1.3.1 Recent changes in taxonomy

In 2019, a molecular analysis by Hughey et al. found that the name *U. lactuca* L., which has been assigned to most foliose specimens in the cold temperate waters of northern Europe since Linnaeus' days, is in fact the senior synonym of the warm temperate and tropical species *U. fasciata* Delile, leaving *U. fenestrata* Postels & Ruprecht as the oldest available name for the cold temperate species. This study will use *U. fenestrata* when referring to what has previously been called *U. lactuca* in the region. Type material of *U. tenera* Kornmann & Sahling was also found to be misidentified and in fact be a junior synonym of *U. fenestrata* (Steinhagen et al. 2019b). Another recent taxonomic change pertains to the model organism *U. mutabilis* Föyn. The type strains of the species have been maintained in culture since the species was described in 1958, and were recently found to be conspecific with, and thus a junior synonym of, *U. compressa* by molecular methods as well as morphological patterns and the generation of fertile offspring (Steinhagen et al. 2019a).

1.3.2 Morphological identification

Long before the taxonomic upheaval caused by molecular methods, identification of *Ulva* (and former *Enteromorpha*) species based morphological characters was considered notoriously difficult. Some characters that have often been used include thallus shape and texture, branching patterns, and cytological characters such as cell size and shape, chloroplast position and number of pyrenoids in the chloroplast. It has been known that these characters can be highly plastic due to age, season and environmental conditions for a long time (Bliding 1963; Brodie et al. 2007). An illustrative example of the problems of morphological identification is the species pair *U. compressa* and *U. intestinalis* L. The two species are usually separated by branching patterns in the literature, the former being branched and the latter unbranched, despite known exceptions for both species (Bliding 1963). Later experiments (and field collections, see below) have confirmed that branching can be induced in unbranched specimens by extreme salinity and sudden changes in salinity (Reed & Russell 1977) as well as low light intensity (De Silva & Burrows 1973).

1.3.3 Molecular identification and distribution patterns

Based on the issues with morphological identification, historic species inventories and observed and estimated distribution patterns would not be expected to be consistent with new inventories using molecular identification methods. This was confirmed by Steinhagen et al. (2019b) in a molecular survey of the German coastline, which found several recently introduced species, very different distribution patterns compared with historical records, and numerous misidentifications of historical vouchers. Similarly, historic tubular samples assigned to *Enteromorpha compressa* (Linnaeus) Nees have been molecularly identified as *U. intestinalis* and *U. linza* L, and historic foliose samples assigned to *U. compressa* as *U. fenestrata* (Steinhagen et al. 2019d).

A recent example of distribution patterns being misunderstood despite molecular identification methods is an investigation of the distribution and genetic variation of *U. intestinalis* and *U. compressa* in the Baltic and North Seas (Leskinen et al. 2004). No records of the latter were found in the Baltic Sea (salinities < 15 ppt) and they concluded the distribution of *U. compressa* was more restricted than previously reported. However, a molecular analysis by Steinhagen et al. (2019d) found *U. compressa* in salinities as low as 9. The confusion resulted from sampling bias, as Leskinen et al. (2004) only sampled tubular specimens. Steinhagen et al. (2019d) confirmed that tubular specimens of the species were only found at higher salinities (> 17), but also found foliose specimens in lower salinities. The foliose form was found in higher salinities as well, but only in environments with extreme fluctuations in salinity and temperature. The original descriptions of branching

patterns still hold mostly true for normal marine conditions, but even moderately reduced salinity will produce different branching patterns (Leskinen et al. 2004).

Despite the progress that has been made on the systematics and identification of *Ulva* spp. with the help of molecular methods, much remains ambiguous. Some species cannot be confidently delimited even using molecular characters and are sometimes referred to as species complexes (e.g. *U. californica–flexuosa, U. linza–prolifera*), and some phylogenetic relationships within the order are still uncertain (Hayden & Waaland 2002; Hiraoka et al. 2017; Steinhagen et al. 2019b).

1.4 Molecular methods: DNA barcoding and phylogenetic analysis

1.4.1 History and principles of DNA barcoding

DNA sequencing was first pioneered in the 1970s on microorganisms. The use of DNA sequencing as a way to investigate the evolutionary relationships between organisms followed soon thereafter. Since then, both methods and applications have evolved dramatically, and in 2003, the modern concept of DNA barcoding was introduced by Hebert et al. (2003). DNA barcoding is meant to be a method of identifying distinct molecular operational taxonomic units (MOTUs) which can – but do not necessarily – represent species. In an ideal world this could be done using a universal genetic marker with a sufficient interspecific and small enough intraspecific difference between and within all taxa; this difference is referred to as the "barcode gap". The barcode gap is reflective of a sequence with a high enough substitution rate for a difference to become apparent in recently diverged species, but also with high conservation within a species. Unfortunately, no marker capable of distinguishing all organisms is known; instead, different markers have been identified for different groups. Several factors are important for successful DNA barcoding. A suitable genetic marker (with universal primer regions) needs to be identified, and reliable reference sequences to check the unknown specimen against are required. A marker suitable for most metazoan taxa (the mitochondrial DNA sequence cytochrome c oxidase subunit 1, COI) was identified in the original publication by Hebert et al. (2003). Unfortunately, it is not appropriate for other organism groups, including Chlorophyta.

1.4.2 Molecular studies and DNA barcoding of Ulva

In earlier studies of *Ulva*, the plastid rubisco large subunit (*rbc*L) and the nuclear Internal Transcribed Spacer 1 and 2 (ITS1 and ITS2) have been the markers of choice, but Saunders and Kucera (2010) found in an evaluation of several markers that the plastid elongation factor Tu, *tuf*A was the best universal marker for Chlorophyta (with the exception of Cladophoraceae). Species discriminatory power was similar for *rbc*L and *tuf*A, but the amplification success was much higher for *tuf*A, presumably due to the presence of introns in *rbc*L (Saunders & Kucera 2010). While *tuf*A thus seems to be the preferable marker for DNA barcoding of *Ulva*, that does not necessarily mean it is the most appropriate sequence for phylogenetic analysis. This is in part because a good barcoding sequence is by definition short (so as to be able to be sequenced in a single read). Depending on the time scale of the evolutionary relationships in question it may also desirable to use sequences with slower substitution rates, because several substitution rates at the same site cannot be detected accurately.

Scandinavia has a rich history of phycological studies. Despite this, identification is still based on outdated species concepts and identification keys, and few molecular studies on the diversity of marine green algae in the region have been performed. This study is part of a larger project on the diversity of green algae in the region. Three introduced species have already been detected using DNA barcoding, and a new understanding of the occurrence and distribution of species is beginning to take form.

2 Aims

The aim of this study was to investigate the diversity of *Ulva* in Scandinavia with DNA barcoding and to delimit species via phylogenetic analyses using the *tuf*A marker. Originally, the intention was to use an additional marker, *rbc*L, partly to compare the results and partly because there are more reference sequences available of *rbc*L than *tuf*A. Unfortunately, this proved too ambitious and had to be left out because of time constraints. In addition to the molecular work, I aimed to perform a review of historical records of Ulvales and Ulotrichales in the region and evaluate against recent taxonomy and the molecularly identified species of

this project. Finally, I wanted to review European seaweed companies to determine which species and products of *Ulva* are reportedly being sold and compare the results with DNA barcoding of biomass from some of those companies. More specifically, I aimed to answer the following questions:

- Using *tuf*A, which species can be detected in the region, and what are the phylogenetic relationships between them?
- Are historical records consistent with molecularly detected species and their distributions?
- Which *Ulva* spp. are reportedly cultivated and sold in Europe and are they correctly identified?

The detected species and their distributions were predicted to differ from historical inventories, specifically regarding introduced species and different distribution patterns for *U. fenestrata* and *U. compressa*. Inaccuracies in species identification in European *Ulva* production were also expected.

3 Methods

3.1 Historical records and nomenclature

A historical survey of the presence and distribution of marine Ulvales and Ulotrichales in Scandinavia was performed by reviewing the following works: *Die Chlorophyceen der Schwedischen Westküste* (Kylin 1949), *Norsk Algeflora* (Rueness 1977), *Marina grönalger vid svenska västkusten* (University of Gothenburg 1988, updated 2010), *Ostsee-Algenflora* (Pankow 1990), and *Ecology and taxonomy of* Enteromorpha *species in the vicinity of the Forsmark nuclear power plant (Bothnian Sea)* (Snoejis 1992). Combined, they cover the entire geographic region and more than sixty years. All species listed in these works were compiled with notes on their distributions. Subsequently, the species names were looked up on AlgaeBase. The recent validity and nomenclatural history (author, basionym, heterotypic and homotypic synonyms) of all species names was recorded. For the most up-to-date official information on *Ulva* in Sweden, the genus was accessed on the Swedish taxonomical database Dyntaxa (dyntaxa.se) and data on species names and occurrences were noted.

3.2 Study specimens

3.2.1 Field sampling

The specimens used in this study were collected at 149 sites during the summer of 2020 along the coasts of Sweden (n = 56), Norway (n = 27) and Denmark (n = 66) by Dr. Sophie Steinhagen (Figure 1). The collection sites ranged in salinity from 3,5 in the northern Baltic to fully marine environments in the North Sea and included a wide variety of habitats. Salinity, temperature and O_2 levels were recorded, as was growth substrate. Brief notes were taken on gross morphology (e.g. thallus shape and branching patterns) but no detailed morphological identification was performed. Vouchers were prepared to be deposited at the Natural History Museum of Denmark in Copenhagen.

3.2.2 Seaweed companies

Specimens or sequences from biomass sold by three European companies (Kosteralg, Sweden; Dansk Tang, Denmark; and Investalga, Spain) were acquired through different means. Sequences from Kosteralg's tank aquaculture were available at NCBI (National Center for Biotechnology Information) (Toth et al. 2020). Specimens from a site where Dansk Tang are believed to harvest their biomass were collected in the field. Fresh samples

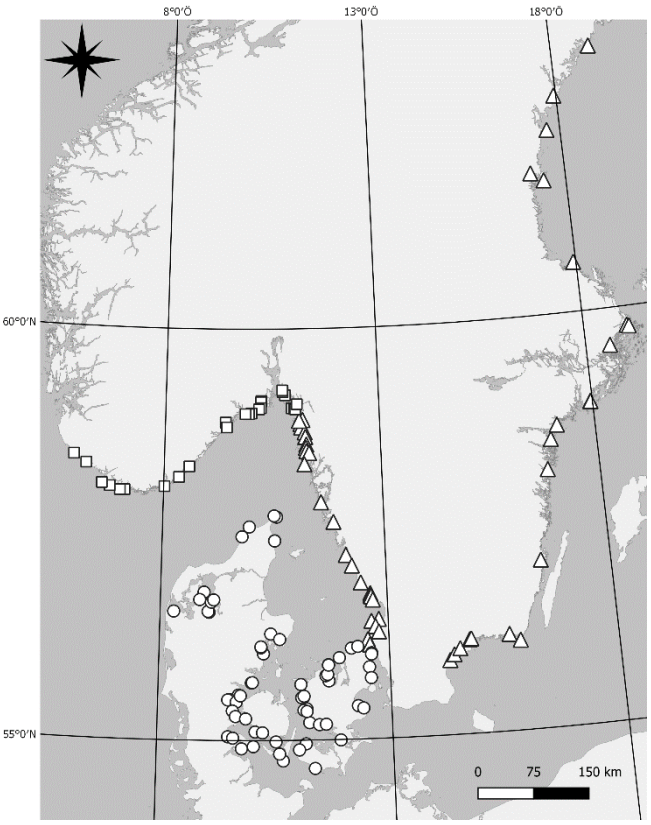

Figure 1 Sampling sites in Norway (squares), Sweden (triangles), and Denmark (circles).

from Investalgas's mixed tank aquaculture were provided from the company.

3.3 DNA extraction, amplification, and sequencing

519 lyophilized specimens were ground to powder by hand. Genomic DNA extraction was performed using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol with some small modifications. During cell lysis (step 8), incubation was increased from 15 to 20 minutes and mixing was performed by vortex instead of inverting the tube. A second elution (step 19) was omitted. Following extraction, the *tuf*A marker was amplified by PCR (polymerase chain reaction) with the tufGF4 (Saunders & Kucera 2010) and tufAR (Famà et al. 2002) primers and the following procedure:

1. Initial denaturation for 4 minutes at 94° C
2. 37 cycles of denaturation, annealing, and polymerization at 94° C for 60 seconds, 55° C for 30 seconds and 72° C for 60 seconds
3. Final extension at 72° C for 7 minutes

Gel electrophoresis on 2 % agarose gel was performed on the PCR product to determine amplification success, after which the amplicons were sent to Eurofins Genomics in Germany for Sanger sequencing using their LightRun service. Some DNA extracts, where the returned sequences were of poor quality and/or specific interest, were amplified a second time and purified using the QIAquick PCR purification kit (Qiagen), following the manufacturer's protocol. If this was not successful, or in the case of mistakes during the extraction process, back-up specimens (when available) of the same thallus were re-extracted, amplified and sequenced following the same methods. In total, ca 27 % of the specimens were re-sequenced.

3.4 Molecular examination

3.4.1 DNA barcoding

Upon return the raw sequences were run through NCBI's Basic Local Alignment Search Tool (BLAST) and preliminarily identified based on the best hit.

3.4.2 Reference sequences

Reference sequences for use during sequence editing and phylogenetic analysis were downloaded from NCBI. When available, peer reviewed sequences from European material were used. For species where such sequences were not available, other sequences were selected in the following order: peer reviewed sequences from other northern temperate seas; peer reviewed sequences from the rest of the world; non-peer reviewed sequences from European or northern temperate seas; other non-peer reviewed sequences. Later, some reference sequences were replaced or supplemented when better alternatives became available through personal communication or recent publications.

3.4.3 Sequence editing and alignment

A representative number of high-quality sequences for each species from the three countries were edited by eye using BioEdit (Hall 1999). The edited sequences were aligned with reference sequences using ClustalW Multiple Alignment in BioEdit.

3.4.4 Phylogenetic analysis

Neighbour joining (NJ) analysis with 1000 bootstrap replications was performed in MEGA X (Kumar et al. 2018) for a preliminary phylogeny which was used for final preparations of the alignment (e.g. removal of superfluous sequences, tentative naming of clades that could not be identified to species level). The MEGA X model selection test was used to determine the most suitable substitution model to use for Maximum Likelihood (ML) analysis. Based on the Akaike Information Criterion corrected for small sample size (AICc) this resulted in the use of a General Time Reversible model with Gamma distributed rates and Invariant sites (GTR + G + I). The ML analysis was also performed in MEGA X with 1000 bootstrap replications. The analysis included 170 sequences and was 832 bases long. A separate ML analysis of 46 sequences was performed on the sequences obtained from the seaweed companies using the same method but without invariant sites, as suggested by the model selection test. The initial tree was made automatically using NJ/BioNJ. Nearest-Neighbour-Interchange (NNI) was used for tree inference. The phylogenetic trees resulting from the NJ and ML analyses were visualized and edited using InkScape (2020) and rooted by

Blidingia marginata (J. Agardh) P.J.L. Dangeard ex Bliding (NCBI accession no. MH538543.1, HQ610237) and *Kornmannia leptoderma* (Kjellman) Bliding (NCBI accession no. MH720542.1, HQ610252.1).

3.5 Distribution

Species distributions were visualized using QGIS3 (2020) and included all specimens with an identification value of > 95 %. Specimens belonging to clades which could not be delimited to species level were assigned a name (*Ulva* sp. 1, *Ulva* sp. 2 etc.) based on clustering in the phylogenetic analysis.

3.6 Seaweed company survey

Combinations of different search terms were used to find companies selling macroalgae – and specifically *Ulva* spp. – in Europe. These included (but were not limited to): seaweed, Ulva, Ulva lactuca, sea lettuce, company, price, product, cultivation, aquaculture, farm, sea farm, wholesale, fresh, frozen, dried, the names of different countries/nations (e.g. Sweden, Denmark, Norway, Ireland, England, Scotland, France, Spain, Netherlands). Due to the large number of companies the search was eventually limited to companies selling *Ulva* spp. Data collected included company information (name, website, country), which taxa they listed as being for sale, the source of the biomass (wild harvest, aquaculture), the product categories for sale, and prices per kg on fresh and dried biomass. Price inquiries were made to companies which did not have price information on their websites. Very late in the project, a new database (Phyconomy, https://phyconomy.net/) of European seaweed companies was brought to my attention, which I compared my own findings with.

4 Results

4.1 Taxonomy

The complete taxonomic history of the region's *Ulva* species, based on data from AlgaeBase [compiled July–August 2020], can be found in Table 4. For many species, the history was complicated and included numerous synonyms. For a few species (*U. curvata* [Kützing] De Toni 1889; *U. pseudorotundata* Cormaci, G. Furnari & Alongi 2014; *U. kylinii* [Bliding] H. S. Hayden, Blomster, Maggs, P. C. Silva, Stanhope & Waaland 2003) the taxonomy was straightforward. For example, being a tubular species, *U. kylinii* has been described as *Enteromorpha kylinii* Bliding 1948 but had no other synonyms. On the other side of the spectrum species like *U. clathrata* (Roth) C. Agardh 1811 were found, with a total of 35 homotypic and heterotypic synonyms.

In total, the reviewed literature contained 23 names of *Ulva* and *Enteromorpha* on species level (Table 1). Due to changes in taxonomy, they correspond to 14 currently valid species names. Excluding Dyntaxa, only five species were listed in at least one source using the currently valid name. The University of Gothenburg identification key contained notes on synonymy added in the updated 2010 version, which were at times incorrect. *E. clathrata* (accepted name *U. clathrata*) was listed as a synonym of *E. flexuosa* (accepted name *U. flexuosa*). *E. procera* (accepted name *U. prolifera*) was listed as a synonym of *E. ahlneriana* (accepted name U. *linza*). *U. rigida C.* Agardh, and *U. laetevirens* were keyed out as different species but noted as synonyms later in the key. Other notes were accurate, such as *U. scandinavica* being a synonym of *U. rigida*, and *E. muscoides* being the same species as *E. ramulosa*, though the accepted name is *U. clathrata*. Dyntaxa contained none of the introduced species and four outdated species names which have been reduced to synonymy.

4.2 DNA barcoding and phylogenetics

The species that were detected in this study and the species which should be present according to the reviewed literature are presented in Table 2. Eight species could be identified using DNA barcoding, seven expected and one introduced (Table 2). Three more species, two of which introduced, have previously been detected in field collections from 2018 and 2019 (Dr. Sophie Steinhagen, personal communication). Between two and six other MOTUs could not be identified to species level with the methods used here. The best BLAST hits for these sequences were *U. gigantea* (Kützing) Bliding), *U. shanxiensis* L. Chen, J. Feng & S. L. Xie, *U. flexuosa* Wulfen, and *Ulva* sp., but none of the sequences clustered with those species in the phylogenetic analysis.

Table 1 Species names of *Ulva* sensu lato found in the reviewed literature (excluding Snoejis 1992) and on Dyntaxa and their current validity. Names in bold indicate the currently valid species name was used in any of the reviewed literature (excluding Dyntaxa). Starred checkmarks indicate notes on taxonomic changes in the 2010 update of the University of Gothenburg (UoG) identification key (1988).

Species name used in the literature	UoG	Kylin	Pankow	Rueness	Dyntaxa	Accepted species name
Ulva clathrata	✓	—	—	—	✓	*Ulva clathrata*
Enteromorpha clathrata	✓*	✓	✓	✓	—	
Enteromorpha muscoides	✓*	—	—	—	—	
Enteromorpha ramulosa	✓*	—	✓	✓	—	
Enteromorpha complanata	—	✓	—	—	—	*Ulva compressa*
Enteromorpha compressa	✓	✓	✓	✓	—	
Ulva compressa	—	—	—	—	✓	
Ulva curvata	✓	—	✓	✓	✓	*Ulva curvata*
Ulva fenestrata	—	—	—	—	—	*Ulva fenestrata*
Ulva lactuca	✓	✓	✓	✓	✓	
Enteromorpha flexuosa	✓	—	✓	✓	—	*Ulva flexuosa*
Ulva flexuosa	—	—	—	—	✓	
Enteromorpha intestinalis	✓	✓	✓	✓	—	*Ulva intestinalis*
Ulva intestinalis	—	—	—	—	✓	
Ulva kylinii	✓	—	—	—	✓	*Ulva kylinii*
Enteromorpha kylinii	—	✓	—	✓	—	
Ulva laetevirens	✓*	—	—	—	✓	*Ulva laetevirens*
Enteromorpha ahlneriana	✓*	✓	✓	✓	—	*Ulva linza*
Enteromorpha linza	✓	✓	✓	✓	—	
Ulva linza	—	—	—	—	✓	
Enteromorpha flexuosa var. *pilifera*	✓	—	—	—	—	*Ulva pilifera*
Ulva pilifera	—	—	—	—	—	
Enteromorpha procera	✓*	—	—	—	—	*Ulva prolifera*
Ulva procera	—	—	—	—	✓	
Enteromorpha prolifera	✓	✓	✓	✓	—	
Ulva prolifera	—	—	—	—	✓	
Enteromorpha simplex	✓	—	—	—	—	
Ulva simplex	—	—	—	—	✓	
Ulva pseudorotundata	—	—	—	—	—	*Ulva pseudorotundata*
Ulva rotundata	—	—	—	✓	—	
Ulva rigida	✓	—	✓	✓	✓	*Ulva rigida*
Ulva scandinavica	✓*	—	—	✓	✓	
Ulva torta	✓	—	—	—	✓	*Ulva torta*
Enteromorpha torta	✓	—	✓	—	—	

Final sequencing success was acceptable with 92,6 % of specimens having an identity match of > 95 %, with some taxa being more reliable than others. *U. intestinalis*, *U. australis* Areschoug, and *U. linza* were unproblematic, forming clear clusters with reference sequences and being correctly named when using BLAST on the raw sequences. *U. fenestrata*, *U. compressa*, *U. torta* and *U. laetevirens* also formed clear clusters with reference sequences but had some taxonomic or other issues on NCBI. *U. fenestrata* and *U. compressa* were often identified by the invalid species names *U. lactuca* and *U. mutabilis* respectively. The

Table 2 Currently accepted species present according to the reviewed literature and detected by DNA barcoding. Species in bold indicate introduced species. Starred checkmarks indicate species detected in field collections from 2018 and 2019 but not in 2020. Light grey rows indicate species with no available ***tuf***A reference sequences. Dark grey rows indicate species with no available reference sequences for any gene.

Species	Historical presence	DNA barcoding detection
Ulva australis	—	✓
Ulva californica	—	✓*
Ulva clathrata	✓	—
Ulva compressa	✓	✓
Ulva curvata	✓	—
Ulva fenestrata	✓	✓
Ulva flexuosa	✓	✓*
Ulva gigantea	—	✓*
Ulva intestinalis	✓	✓
Ulva kylinii	✓	—
Ulva laetevirens	✓	✓
Ulva linza	✓	✓
Ulva pilifera	✓	—
Ulva prolifera	✓	✓
Ulva pseudorotundata	✓	—
Ulva rigida	✓	—
Ulva torta	✓	✓

best hit for *U. torta* was frequently *Ulva* sp. due to sharing the highest similarity with a misannotated sequence. *U. laetevirens* was sometimes identified as *U. rigida*.

4.2.1 Reference sequences

Four of the species present in the region according to the literature (*U. clathrata*, *U. curvata*, *U. kylinii* and *U. pilifera* (Kützing) Škaloud & Leliaert 2018) lacked *tuf*A reference sequences. Of those, one species (*U. kylinii*) lacked reference sequences for any gene (Table 2).

4.2.2 Phylogenetic analysis

The phylogram of the main ML analysis (Figure 3) shows two main branches. One consisted of two sister clades. One clade contained *U. intestinalis* and *U. compressa*, the other one included *U. fenestrata*, *U. australis* and four species from other parts of the world. The other main branch contained a larger number of species as well as the clades which could not be delimited to species level. One of these clades, named *Ulva* sp. 2, was retrieved as the sister species of the rest of the branch, from which it had a considerable evolutionary distance. The remaining species formed two clades. One consisted of the closely related *U. flexuosa* and *U. californica* Wille and their sister species, *U. torta*, and the sister species *U. prolifera* and *U. linza*. Two lone sequences were noticeable on this branch: SV353 (from previous field collections in Sweden) and NO137. The second clade was divided in two branches with the type sequence of *U. shanxiensis* as their sister species. One branch consisted of the closely related *U. laetevirens* and *U. rigida*, and the three southern species *U. lactuca* (annotated as and formerly known as *U. fasciata*), *U. ohnoi*, and *U. gigantea*, the last of which has been introduced in Europe including Sweden. The other branch consisted of sequences which could not be delimited to species level, and two reference sequences annotated as *U. shanxiensis*. While the branch as a whole had low bootstrap support, four distinct MOTUs with higher support were retrieved, named *Ulva* sp. 3, *Ulva* sp. 4, *Ulva* sp. 5, and *Ulva* sp. 6.

The topology of the separate tree of the smaller ML analysis with sequences from the seaweed companies (Figure 4) was similar but with some differences. Where the topology differed, the bootstrap support was consistently lower than in the first tree.

4.3 Occurrence and distribution of species

As mentioned previously, based on historical data, 14 species were expected to occur in the region. Of these, only seven were detected in this survey. Three introduced species (*U. australis*, *U. californica* and *U. gigantea*) have previously been detected using DNA barcoding. Of these, only one (*U. australis*) was detected in this study on the south coast of Norway (Figure 2). At least two unidentifiable

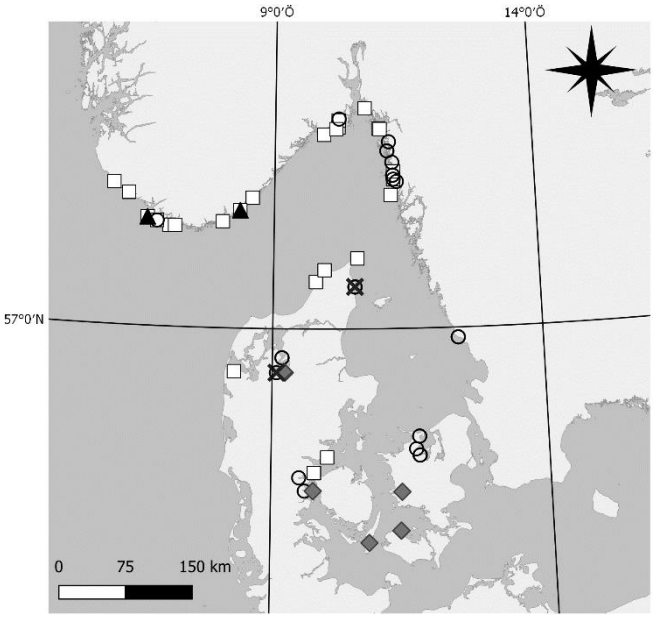

Figure 2 Presence of foliose *Ulva* detected by *tuf*A barcoding. *U. australis* (black triangles), *U. fenestrata* (white squares), *U. laetevirens* (transparent circles), and foliose *U. compressa* (grey diamonds).

MOTUs of *Ulva* were also detected (Figure 5).

The historical estimated distribution of *U. fenestrata* included most of the region and did not match the detected distribution. The combined distributions of *U. fenestrata*, *U. laetevirens* and foliose *U. compressa* covered a larger area (Figure 2).

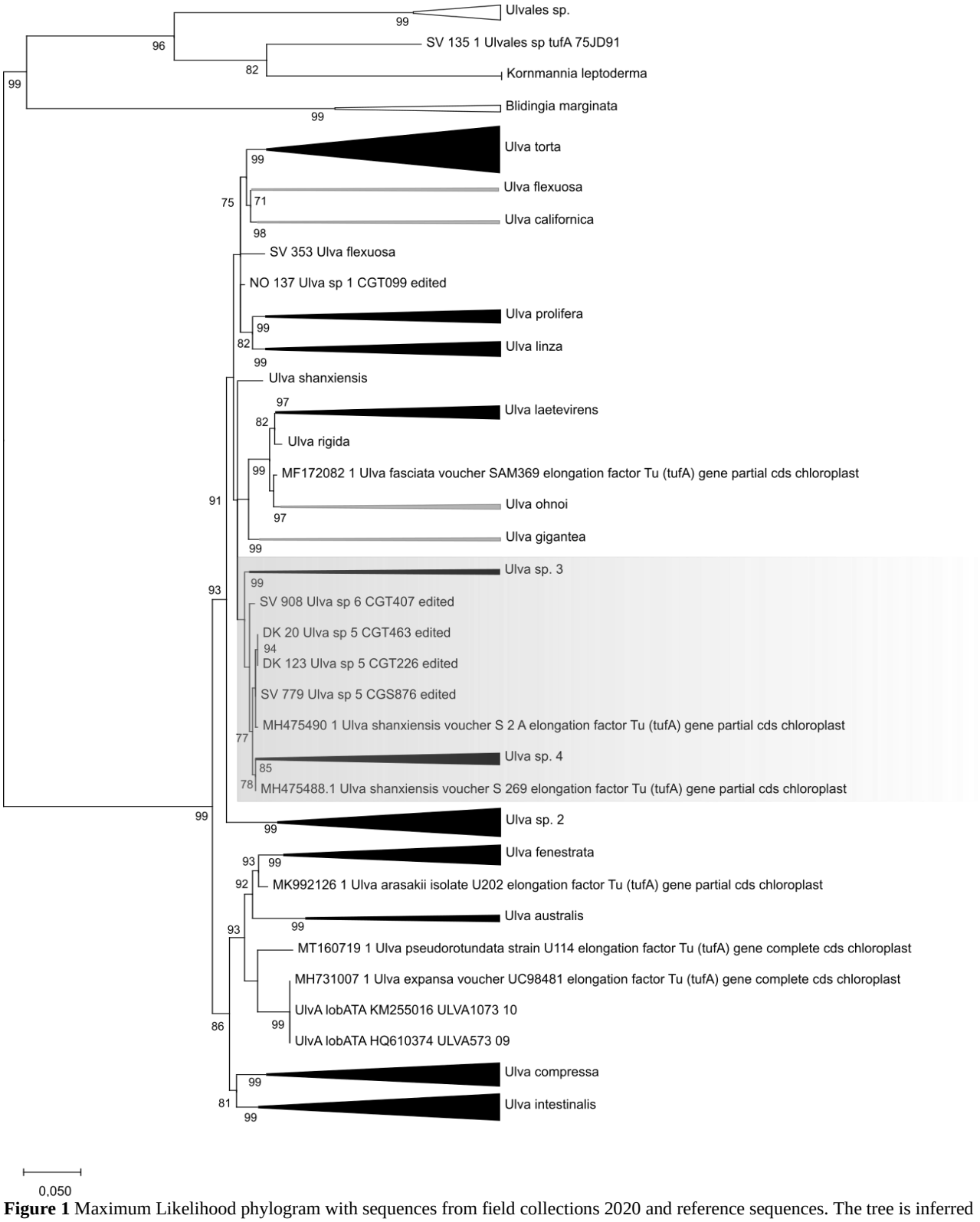

Figure 1 Maximum Likelihood phylogram with sequences from field collections 2020 and reference sequences. The tree is inferred from *tuf*A sequences using a General Time Reversible model with Gamma distribution, invariant sites and 1000 bootstrap replications. Numbers at nodes refer to bootstrap values. Bootstrap values of < 70 were removed. The tree is drawn to scale with branch lengths measured in the number of substitutions per site as indicated by the scale bar. This analysis involved 170 nucleotide sequences and 832 positions. Black and shaded clades indicate taxa detected in this study. Grey bars indicate other *Ulva* species. White bars indicate the outgroup.

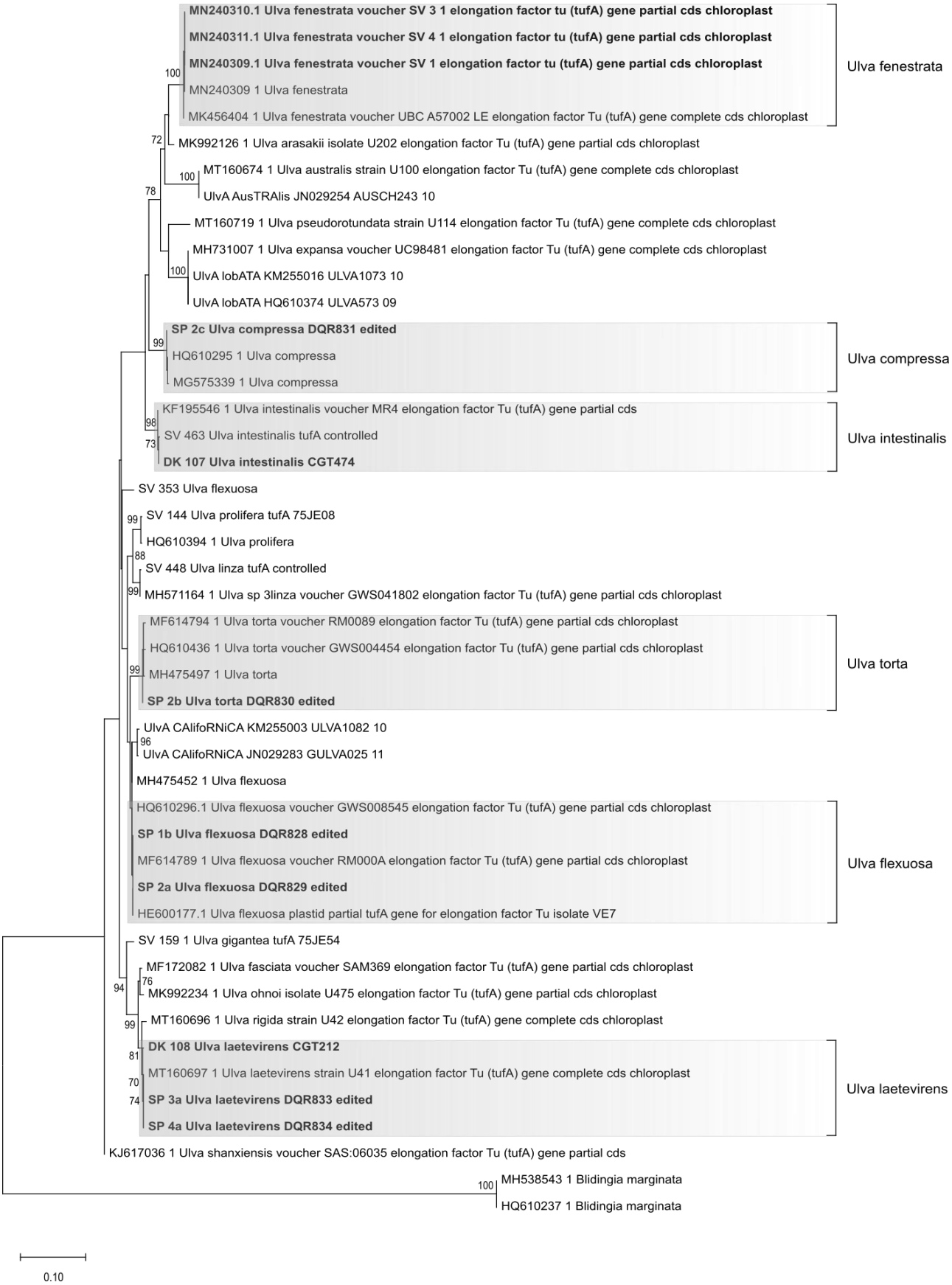

Figure 2 Maximum Likelihood phylogram with sequences from seaweed companies and reference sequences. The tree is inferred from *tuf*A sequences using a General Time Reversible Model with Gamma distributed sites and 1000 bootstrap replications. Numbers at nodes refer to bootstrap values. Bootstrap values of < 70 were removed. The tree is drawn to scale with branch lengths measured in the number of substitutions per site as indicated by the scale bar. The analysis involved 46 nucleotide sequences and 837 positions. Shaded clades indicate species detected in this study. Names in bold indicate sequences from one of the seaweed companies.

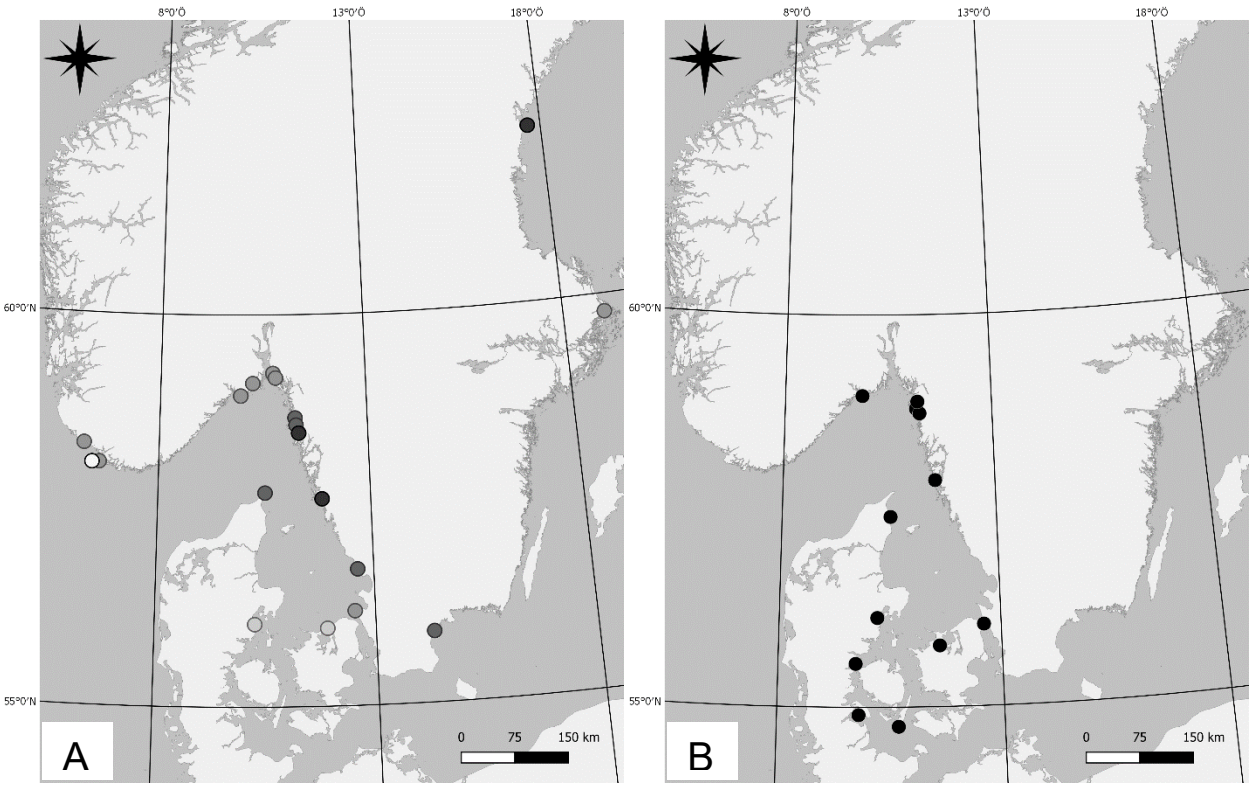

Figure 5 Presence of clades that could not be identified to species level detected by *tuf*A DNA barcoding. (A) From light to dark: *Ulva* sp. 1, *Ulva* sp. 3, *Ulva* sp. 4, *Ulva* sp. 5 and *Ulva* sp. 6. (B) *Ulva* sp. 2.

U. rigida, which is very similar in appearance to *U. laetevirens*, and which was consistently recorded in the region after it was first found in Sweden in 1964 (Rueness 1977), was not detected. *U. laetevirens*, on the other hand, was only mentioned in the University of Gothenburg identification key, where, as previously mentioned, it was given as a synonym of *U. rigida*. Only two species were found to be causing green tides during this survey: *U. compressa* and *U. laetevirens*. Two green tides were noted (Figure 2).

Snoejis (1992) found three *Ulva* species in the Bothnian Bay near Forsmark nucelar powerplant ca 130 km north of Stockholm. According to the taxonomy used at the time, they were the following: *Enteromorpha ahlneriana*, *E. clathrata* and *E. intestinalis*. *E. ahlneriana* has since been synonymized with *U. linza*. This survey detected two identifiable species in the Baltic east of Denmark: *U. intestinalis* and *U. linza* (Figure 6), and 1–2 of the MOTUs (Figure 5). *U. compressa* has previosuly been described as occuring in the northern Baltic (Hällfors et al. 1987) but was not detected in this study.

U. kylinii was noted as occurring in Norway and as being common at various localities near Kristineberg. *U. curvata* was noted by Rueness (1977) as being a southern species first found on the Swedish west coast in 1977, while Pankow (1990) described it as being present in the Western Baltic. Rueness included *U. pilifera* but did not describe its distribution. Rueness included *U. pseudorotundata* as another southern species which had only been recorded in Troms at the time. *U. rigida* was also noted as a southern species with its first record on the west coast of Sweden in 1964, and Pankow described its distribution as the Western Baltic. A notable absence was *U. flexuosa*, which has been molecularly detected in Sweden before.

U. torta was found to be fairly widely distributed despite only being present in the Western Baltic according to Pankow and mentioned as either unconfirmed, in adjacent waters, or found drifting, in the updated version of the University of Gothenburg identification key (Figure 7). The distribution of *U. prolifera* reached further east according to the literature than what was detected here (Figure 8).

4.4 Morphology

Based on the non-exhaustive morphological observations made in the field, which need to be complemented with further examinations, only two exclusively foliose species (*U. fenestrata* and *U. australis*) were detected. One known species, *U. prolifera*, was found to be exclusively tubular, as were all of the uniden-

tified taxa. *U. compressa* exhibited mixed morphology related to salinity, with the exception of one site (Figure 9). Two specimens of monostromatic, foliose specimens identified as *U. intestinalis* were detected. Six branched specimens identified as *U. intestinalis* were found in Sweden and Norway in salinities ranging from 3,8 to 32. *U. linza* also exhibited mixed morphology. One specimen each identified as *U. torta* and *U. laetevirens*, collected at the same location, exhibited unexpected morphology.

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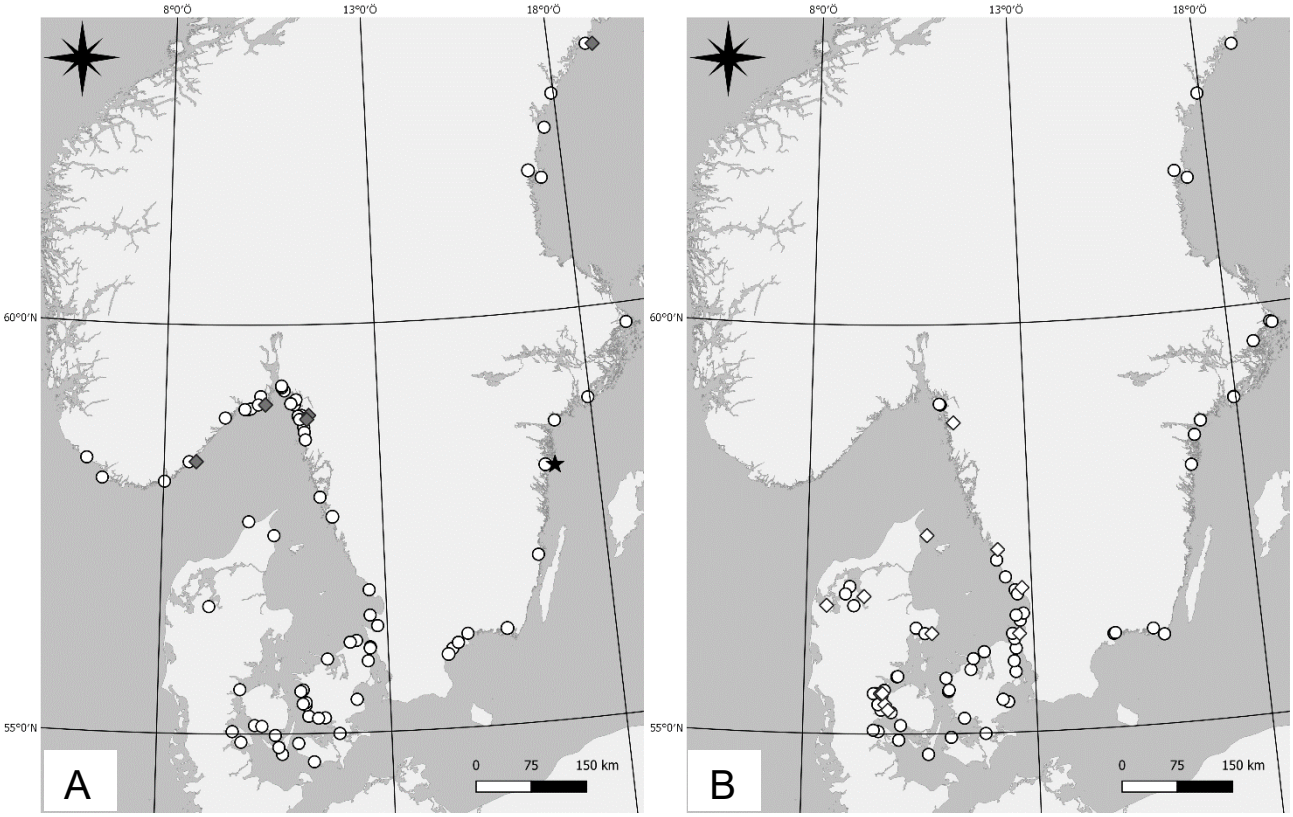

Figure 6 Presence of *Ulva* species detected in the Baltic detected by *tuf*A DNA barcoding. (A) *U. intestinalis*. Unbranched specimens indicated by white circles. Atypical, branched specimens indicated by grey diamonds. Black star indicates monostromatic sheets. (B) *Ulva linza*. Tubular spcimens indicated by white circles. Foliose or uncertain specimens indicated by white diamonds.

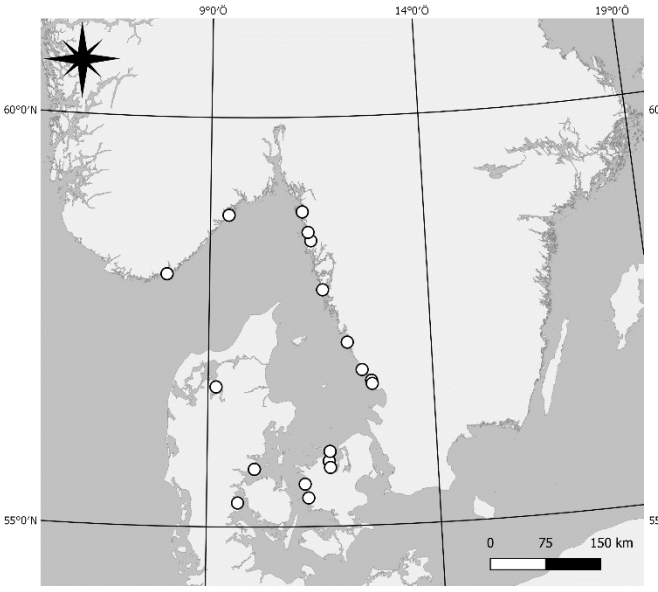

Figure 7 Presence of *Ulva torta* detected by *tuf*A DNA barcoding.

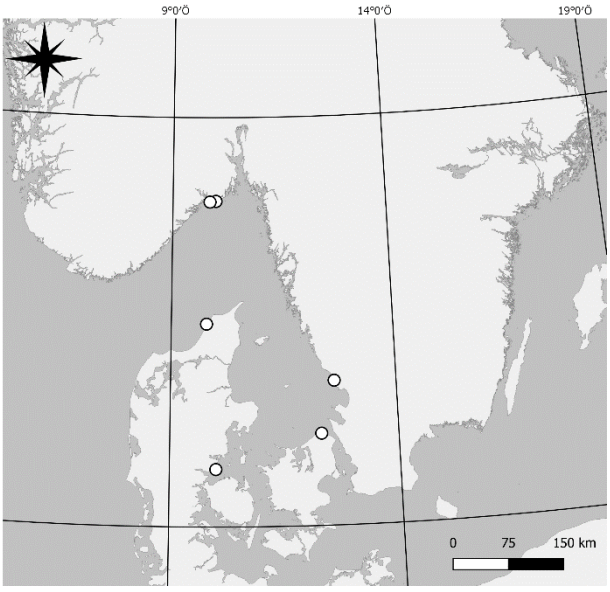

Figure 8 Presence of *Ulva prolifera* detected by *tuf*A DNA barcoding.

4.5 Seaweed company survey

Of the 31 companies first investigated, 14 had at least one species of *Ulva* for sale (Table 3). Nine of them sold wild harvested *Ulva*. Of the remaining five, two used tank-based aquaculture, one land-based basins, one was a coastal sea farm and one an integrated fish farm. Some generic names were used for products. For *Ulva* (or possibly other green macroalgae), these were "aonori" and "sea greens". The most commonly named species was *U. lactuca*, though more sold unspecified *Ulva* species.

The database Phyconomy included a total of 218 European seaweed production companies, 37 of which had *Ulva* production. 25 of the companies used wild harvest; one had an unspecified production process; two used a mix of harvesting and aquaculture; and nine companies utilized aquaculture, using a mix of off-shore, coastal and land-based cultivation methods. Only seven of the fourteen companies I had compiled were present in the Phyconomy database (Table 3).

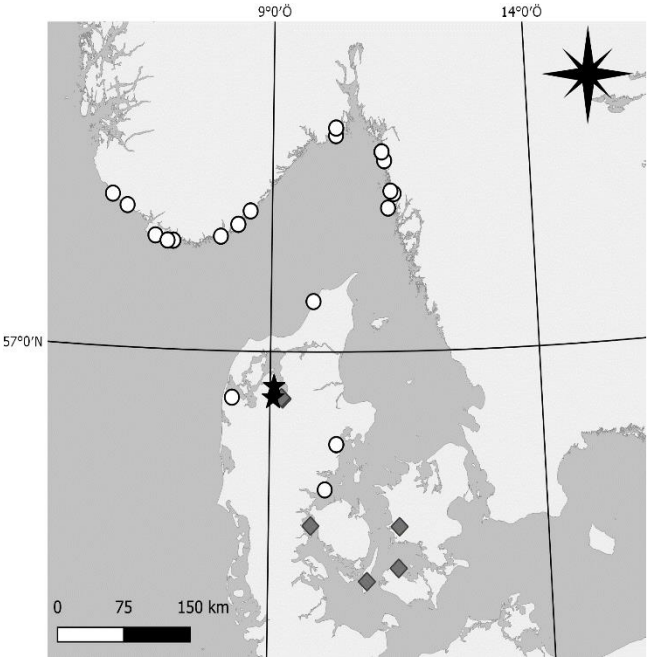

Figure 9 Presence of *Ulva compressa* detected by *tuf*A DNA barcoding. White circles indicate tubular specimens. Grey diamonds indicate foliose specimens. Black stars indicate foliose specimens in salinity > 20.

Prices ranged wildly, from ca 60–760 Euro/kg for the dried product and 10–30 Euro/kg for fresh product. Three companies provided no pricing information on their websites or after price inquiries. Prices are from July 2020 and were obtained either from the company website or personal communication.

4.5.1 DNA barcoding

The cultivated algae from Kosteralg were identified as *U. fenestrata*. At the time of data collection [May 2020], the website listed *U. lactuca* as the species for sale. The specimens collected in the field from the Dansk Tang location were identified as *U. laetevirens*, *U. torta* and *U. intestinalis*. *Ulva s*pecimens from the tank aquaculture of Investalga were mixed and included *U. laetevirens*, *U. torta*, *U. flexuosa* and *U. compressa*. Other chlorophytes were also present, morphologically identified as *Cladophora* sp. and *Chaetomorpha* sp., the former in large amounts.

Table 3 European seaweed companies which sell macroalgae. Starred company names indicate companies which did not occur in the Phyconomy database.

Company	Country	Species	Production process
AlgAran	Ireland	*Ulva lactuca* var. *spiralis*	Wild harvest
Algorythme*	France	Aonori, *Ulva* sp.	Wild harvest
Caledonian Seaweeds	Scotland	*Ulva* sp.	Wild harvest
Connemara Organic Seaweed Company*	Ireland	*Ulva lactuca*	Wild harvest
Cornish Seaweed Company*	England	Sea greens	Wild harvest
Dansk Tang	Denmark	*Ulva* sp.	Wild harvest
Emerald Isle Seaweed*	Northern Ireland	*Ulva lactuca*, *Ulva lactuca* var. *spiralis*	Wild harvest
Investalga	Spain	*Ulva* sp.	Tank aquaculture
Kosteralg	Sweden	*Ulva lactuca*	Tank aquaculture
Ocean Forest	Norway	*Ulva lactuca*	Integrated fish farm
Scandsea *	Sweden	*Ulva intestinalis*	Wild harvest
Waddenwier*	Netherlands	*Ulva* sp.	Land-based basins
Wild Irish Seaweeds*	Ireland	*Ulva intestinalis*, *Ulva lactuca*	Wild harvest
Zeewar	Netherlands	*Ulva* sp.	Coastal seafarm

Table 4 Nomenclatural history of *Ulva* species present in the region according to the reviewed literature.

Accepted species name	Homotypic synonyms	Heterotypic synonyms	Basionym
Ulva clathrata (Roth) C.Agardh 1811	*Conferva clathrata* Roth 1806*;* *Enteromorpha clathrata* (Roth) Greville 1830; *Enteronia clathrata* (Roth) Chevallier 1836	*Enteromorpha ramulosa* var. *robusta* Hauck; *Enteromorpha paradoxa* var. *tenuissima* Kützing; *Scytosiphon clathratus* var. *uncinatus* Lyngbye; *Conferva crinita* Roth 1797; *Ulva muscoides* Clemente 1807; *Ulva ramulosa* Smith 1810; *Enteromorpha crinita* Nees 1820; *Enteromorpha clathrata* var. *uncinata* (Lyngbye) Greville 1830; *Enteromorpha ramulosa* (Smith) Carmichael 1833; *Enteronia rigidula* Chevallier 1836; *Ulva linkiana* (Greville) Trevisan 1842; *Zignoa ramulosa* (J.E.Smith) Trevisan 1842; *Enteromorpha ramulosa* var. *spinosa* Kützing 1845 *Enteromorpha complanata* var. *crinita* (Nees) Kützing 1845; *Enteromorpha clathrata* var. *ramulosa* (J.E.Smith) Rabenhorst 1847; *Enteromorpha gelatinosa* Kützing 1849; *Enteromorpha clathrata* var. *linkiana* (Greville) Areschoug 1850; *Enteromorpha clathrata* var. *mucosa* P.Crouan & H.Crouan 1852; *Enteromorpha spinescens* Kützing 1856; *Ulva clathrata* f. *prostrata* Le Jolis 1863; *Ulva clathrata* f. *gracilis* Le Jolis 1863; *Enteromorpha welwitschii* J.Agardh 1883; *Enteromorpha clathrata* var. *crinita* (Nees) Hauck 1884; *Enteromorpha clathrata* var. *prostrata* (Le Jolis) Batters 1890; *Enteromorpha clathrata* var. *gracilis* (Le Jolis) Batters 1902; *Enteromorpha ramulosa* var. *spinescens* (Kützing) Chalon 1905; *Enteromorpha ramulosa* var. *tenerrima* Schiffner 1931; *Enteromorpha ramulosa* f. *minima* Schiffner 1933; *Enteromorpha clathrata* f. *gracilis* (Le Jolis) V.J.Chapman 1937; *Enteromorpha clathrata* f. *linkiana* (Greville) V.J.Chapman 1937; *Enteromorpha prolifera* var. *crinita* (Nees) V.J.Chapman 1956; *Enteromorpha muscoides* (Clemente) Cremades 1990	*Conferva clathrata* Roth 1806

Ulva compressa Linnaeus 1753	*Conferva compressa* (Linnaeus) Roth 1797; *Tubularia compressa* (Linnaeus) Roussel 1806 *Scytosiphon compressa* (Linnaeus) Lyngbye 1819; *Enteromorpha compressa* (Linnaeus) Nees 1820; *Fistularia compressa* (Linnaeus) Greville 1824; *Solenia compressa* (Linnaeus) C.Agardh 1824; *Ilea compressa* (Linnaeus) Gaillon 1828; *Fistularia intestinalis* var. *compressa* (Linnaeus) J.P.Jones & Kingston 1829; *Hydrosolen compressus* (Linnaeus) C.Martius 1833; *Enteronia compressa* (Linnaeus) Chevallier 1836; *Enteromorpha vulgaris* var. *compressa* (Linnaeus) Edmondston 1845; *Ulva enteromorpha* var. *compressa* (linnaeus) Le Jolis 1863; *Enteromorpha intestinalis* var. *compressa* (Linnaeus) Rosenvinge 1893; *Enteromorpha intestinalis* subsp. *compressa* (Linnaeus) M.W.R.N.de Silva & E.M. Burrows 1973	*Enteromorpha compressa* f. *typica* Kjellman; *Enteromorpha linkiana* Greville 1830; *Enteromorpha complanata* Kützing 1845; *Enteromorpha compressa* var. *genuina* Kützing 1845; *Enteromorpha compressa* var. *complanata* (Kützing) Rabenhorst 1868; *Enteromorpha usneoides* Bonnemaison ex J.Agardh 1883; *Enteromorpha compressa* f. *complanata* (Kützing) J.Agardh 1883; *Enteromorpha chlorotica* J.Agardh 1883; *Ulva usneoides* Bonnemaison 1883; *Enteromorpha compressa* var. *bullosa* Schiffner 1938; *Enteromorpha compressa* var. [*torta*] f. *valde-elongata* Schiffner 1938; *Enteromorpha procera* f. *chlorotica* (J.Agardh) V.J.Chapman 1956; *Enteromorpha clathrata* var. *usneoides* (J.Agardh) V.J.Chapman 1956; *Ulva mutabilis* Föyn 1958; *Enteromorpha compressa* var. *usneoides* (Bonnemaison ex. J.Agardh) Bliding 1963	N/A
Ulva curvata (Kützing) De Toni 1889	*Phycoseris curvata* Kützing 1845	*Ulva kuckuckiana* Schmidt	*Phycoseris curvata* Kützing 1845
Ulva fenestrata (Ulva lactuca) Postels & Ruprecht 1840	N/A	*Ulva stipitata* Areschoug 1850; *Ulva lactuca* f. *stipitata* (Areschoug) Kylin 1907	N/A
Ulva flexuosa Wulfen 1803	*Enteromorpha flexuosa* (Wulfen) J.Agardh 1883	*Conferva flexuosa* Roth 1800; *Enteromorpha intestinalis* var. *tubulosa* Kützing 1845; *Enteromorpha tubulosa* (Kützing) Kützing 1856; *Enteromorpha lingulata* J.Agardh 1883; *Enteromorpha compressa* var. *lingulata* (J.Agardh) Hauck 1884; *Enteromorpha prolifera* var. *tubulosa* (Kützing) Batters 1902; *Enteromorpha intestinalis* f. *tubulosa* (Kützing) V.J.Chapman 1937; *Enteromorpha tubulosa* var. *ramosa* Schiffner 1938; *Enteromorpha lingulata* var. *elongata* Schiffner 1938; *Enteromorpha lingulata* f. *genuina* Schiffner 1938; *Enteromorpha tubulosa* var. *vermiculata* Schiffner 1938	N/A

Ulva intestinalis Linnaeus 1753	*Conferva intestinalis* (Linnaeus) Roth 1797; *Tetraspora intestinalis* (Linnaeus) Desvaux 1818; *Scytosiphon intestinalis* (Linnaeus) Lyngbye 1819; *Enteromorpha intestinalis* (Linnaeus) Nees 1820; *Fistularia intestinalis* (Linnaeus) Greville 1824; *Solenia iintestinalis* (linnaeus) C.Agardh 1824; *Ilea intestinalis* (Linnaeus) Leiblein 1827; *Hydrosolen intestinalis* (Linnaeus) C.Martius 1833; *Ulva enteromorpha* var. *intestinalis* (Linnaeus) Hariot 1889; *Ulva bulbosa* var. *intestinalis* (Linnaeus) Hariot 1889; *Enteromorpha compressa* var. *intestinalis* (Linnaeus) Hamel 1931	*Enteromorpha intestinalis* var. *bullosa* Le Jolis; *Enteromorpha intestinalis* f. *genuina* Hauck; *Enteromorpha intestinalis* f. *genuina* Ahlner; *Scytosiphon intestinalis* var. *nematodes* Wallroth 1833; *Enteronia simplex* Chevallier 1836; *Enteromorpha vulgaris* var. *lacustris* Edmondston 1845; *Enteromorpha ntestinalis* f. *maxima* J.Agardh 1883; *Enteromorpha intestinalis* var. *maxima* (J.Agardh) Lily Newton 1931; *Enteromorpha intestinalis* var. *genuina* Schiffner 1938; *Enteromorpha intestinalis* var. *asexualis* Bliding 1963; *Ulva intestinalis* var. *asexualis* (Bliding Taskin 2007	N/A
Ulva kylinii (Bliding) H.S.Hayden, Blomster, Maggs, P.C.Silva, Stanhope & Waaland 2003	*Enteromorpha kylinii* Bliding 1948	N/A	*Enteromorpha kylinii* Bliding 1948
Ulva lactuca Linnaeus 1753	*Phyllona lactuca* (Linnaeus) F.H.Wiggers 1780; *Monostroma lactuca* (Linnaeus) J.Agardh 1883	*Ulva fasciata* Delile 1813; *Ulva lactucaefolia* S.F.Gray 1821; *Ulva lactuca* f. *fasciata* (Delile) Hering 1846; *Phycoseris fasciata* (Delile) Montagne 1856; *Ulva crassa* Kjellman 1877; *Ulva tenera* Kornmann & Sahling 1994	N/A
Ulva laetevirens Areschoug 1854	N/A	*Gemina linzoidea* V.J.Chapman 1952	N/A

Ulva linza Linnaeus 1753	*Solenia linza* (Linnaeus) C.Agardh 1824; *Enteronia linza* (Linnaeus) Chevallier 1836; *Phycoseris linza* (Linnaeus) Kützing 1843; *Enteromorpha linza* (Linnaeus) J.Agardh 1883	*Enteromorpha linza* f. *lanceolata* Unknown authorities; *Enteromorpha linza* var. *angusta* Unknown authoritis; *Ulva linza* var. *lanceolata* Kützing; *Ulva linza* var. *angusta* Kützing; *Ulva lanceolata* Linnaeus 1767; *Ulva intybacea* Lamarck 1779; *Ulva crispata* Bertoloni 1810; *Ulva fasciata* S.F.Gray 1821; *Ulva bertolonii* C.Agardh 1823; *Scytosiphon intestinalis* var. *plynthodes* Wallroth 1833; *Phycoseris crispata* (Bertoloni) Kützing 1843; *Enteromorpha bertolonii* Montagne 1846; *Ulva enteromorpha* f. *crispata* (Bertoloni) Le Jolis 1863; *Ulva enteromorpha* var. *lanceolata* (Linnaeus) Le Jolis 1863; *Enteromorpha bertolonii* var. *lanceolata* (Linnaeus) Grunow 1867; *Enteromorpha lanceolata* var. *crispata* (Bertoloni) Rabenhorst 1868; *Enteromorpha crispata* (Bertoloni) Piccone 1878; *Enteromorpha linza* f. *crispata* (Bertoloni) J.Agardh 1883; *Enteromorpha linza* var. *crispata* (Bertoloni) Hylmö 1916; *Enteromorpha ahlneriana* Bliding 1944; *Enteromorpha bulbosa* var. *japonica* Yoshida et al. 1990	N/A
Ulva pilifera (Kützing) Škaloud & Leliaert 2018	*Enteromorpha pilifera* Kützing 1856*;* *Enteromorpha flexuosa* subsp. p*ilifera* (Kützing) Bliding 1963; *Ulva flexuosa* subsp. *pilifera* (Kützing) M.J.Wynne 2005; *Ulva flexuosa* var. *pilifera* (Kützing) P.Tsarenko 2011	*Enteromorpha intestinalis* var. *capillaris* Kützing 1845; *Enteromorpha prolifera* f. *capillaris* (Kützing) V.J.Chapman 1937	*Enteromorpha pilifera* Kützing 1856
Ulva prolifera O.F.Müller 1778	*Ulva enteromorpha* f. *prolifera* (O.F.Müller) Van Heurck; *Ulva compressa* var. *prolifera* (O.F.Müller) C.Agardh 1823; *Enteromorpha compressa* var. *prolifera* (O.F.Müller) Greville 1830; *Enteromorpha prolifera* (O.F.Müller) J.Agardh 1883	*Enteromorpha salina* Kützing 1845; *Enteromorpha salina* var. *polyclados* Kützing 1845; *Enteromorpha compressa* var. *trichodes* Kützing 1845; *Enteromorpha polyclados* (Kützing) Kützing 1856; *Enteromorpha procera* K.Ahlner 1877; *Enteromorpha prolifera* f. *simplex* K.L.Vinogradova 1974; *Ulva procera* (K.Ahlner) H.S.Hayden, Blomster, Maggs, P.C.Silva, Stanhope & Waaland 2003; *Ulva simplex* (K.L.Vinogradova) H.S.Hayden, Blomster, Maggs, P.C.Silva, Stanhope & Waaland 2003	N/A

Ulva pseudorotundata Cormaci, G.Furnari & Alongi 2014	N/A	*Ulva rotundata* Bliding 1969	N/A
Ulva rigida C.Agardh 1823	*Phycoseris rigida* (Cagardh) Kützing 1843; *Ulva lactuca* var. *rigida* (C.Agardh) Le Jolis 1863	*Phycoseris ulva* Sonder 1845; *Phycoseris gigantea* var. *perforata* Kützing 1849; *Letterstedtia petiolata* J.Agardh 1883; *Ulva thuretii* B.Föyn 1955; *Ulva petiolata* (J.Agardh) Womersley 1956; *Ulva spathulata* Papenfuss 1960; *Ulva scandinavica* Bliding 1969; *Ulva armoricana* P.Dion, B.de.Reviers & G.Coat 1998	N/A
Ulva torta (Mertens) Trevisan 1842	*Bangia torta* (Mertens) C.Agardh; *Prasiola crispa* f. *torta* (Mertens) Brand; *Conferva torta* Mertens 1822; *Schizogonium tortum* (Mertens) Kützing) 1843; *Ilea torta* (Mertens) Trevisan 1845; *Enteromorpha torta* (Mertens) Reinbold 1893	N/A	*Conferva torta* Mertens 1822

5 Discussion

Many, but not all, expected species of the region were detected using *tuf*A barcoding. Introduced species have also been found, as have MOTUs which could not be delimited to species level. As expected, historical estimations of species distributions were not consistent with the results of this study, likely due to widespread misidentifications based on morphological plasticity. The same can be said for the state of European seaweed production.

5.1 Anomalies

A large portion of specimens needed to be re-sequenced due to unusable or low-quality sequences. I believe this was caused by handling issues during PCR or sequencing preparation and does not reflect any genuine problems with amplification of the *tuf*A marker.

A mix-up likely occurred with the aberrant specimens of one tubular *U. laetevirens* and one foliose *U. torta* at some point during collection or processing in the laboratory. The specimens were collected and processed consecutively and came from the same location. Foliose *U. laetevirens* were also collected at the site and mixed morphologies have not been described in other molecular analyses of the species.

5.2 Noteworthy species

5.2.1 Ulva compressa

The mixed morphology of *U. compressa* in response to salinity, where the tubular morph is found in normal salinities and the foliose, drifting morph in low salinities, was mostly confirmed by this study, though some question marks were raised by the occurrence of three foliose specimens collected at two very closely located sites where the salinity was 20,8 and 23,8. The temperature and O_2 levels were not notable at either site, but one of them was described as a green tide. It is possible, but not known, that other environmental stressors, such as high fluctuations in salinity or temperature may have influenced the morphology at the sites. One tubular specimen collected north of Stockholm in 5,4 salinity was DNA barcoded as *U. compressa*. However, since the identification was unreliable at 94,14 %, and half of the chromatogram was of poor quality, it was not included on the distribution map. Based on the extensive sampling of tubular *Ulva* in the Baltic by Leskinen et al. (2004) which found no *U. compressa*, and of tubular and foliose *U. compressa* in a wide range of salinities by Steinhagen et al. (2019d) which only identified tubular specimens at salinities > 17, I consider it most likely to be an incorrect identification, and suggest it is more likely to be the closely related *U. intestinalis*. The foliose morphotype was not detected further into the Baltic than the Danish straits. Whether this reflects its true distribution is not conclusive. Based on previous sequencing of historical vouchers from the Baltic identified as *U. compressa*, which were molecularly identified as *U. intestinalis* and *U. linza* (Steinhagen et al. 2019d), the species likely occurs further east and north in the Baltic than established here.

5.2.2 Ulva laetevirens and U. rigida

U. laetevirens was suggested to be synonymized with *U. rigida* (Steinhagen et al. 2019b) based on the inability to delimit the species using *tuf*A. However, Fort et al. (2020b) demonstrated that they can be separated using *tuf*A and using their suggested reference sequences all specimens in the region were identified as *U. laetevirens*. The species have been shown to co-exist in other parts of Europe (Fort et al. 2020b, Sfriso et al. 2010), but this survey suggests that is not the case in Scandinavia and that *U. laetevirens* is the species present here, contrary to historical records. Fort et al. (2020b) detected *U. rigida* in France and western Ireland but not in the northernmost sampling locations in the Netherlands, where they did find *U. laetevirens*. Combined with the results of this study, this could suggest a northern limit to the distribution of *U. rigida*. Molecular species delimitation should be compared to the morphological characters that Sfriso et al. (2010) described to separate them. As suggested by Fort et al. (2020b), the new findings regarding *U. rigida* and *U. laetevirens* may have nomenclatural effects, as *U. armoricana* and *U. scandinavica*, until now considered synonyms of *U. rigida*, may in fact be synonyms of *U. laetevirens*.

To further add to the confusion, a recent *rbc*L analysis of lectotype material from the southern hemisphere has demonstrated *U. laetevirens* is in fact a synonym of *U. australis* (Hughey et al. 2020). Since the European species these names have been applied to are clearly not conspecific, at least one name must be misapplied. Neither name has priority as the species were described at the same time, but the authors use *U.*

australis to minimize confusion in part due to how the names are applied in public repositories. The future nomenclatural status of what is considered the sister species of *U. rigida* thus remains uncertain.

5.2.3 Ulva intestinalis

In species descriptions and identification keys, *U. intestinalis* is identified in part by its unbranched morphology. Previous molecular studies (e.g. Leskinen et al. 2004) have demonstrated that branching does occur in the species, especially in low salinities. The six branched specimens identified as *U. intestinalis* at five sites in Sweden and Norway were found in salinities ranging from 3,8 to 32. All but one was found in salinity > 20, confirming how unreliable this character is for identification. Of course, in combination with other characters, and with information on the salinity at the collection site, it can still be a useful character (Blomster et al. 1998).

Two Baltic specimens identified as *U. intestinalis* exhibited foliose and monostromatic morphology. Foliose, monostromatic *U. intestinalis* similar to the genus *Monostroma* Thuret has previously been identified on the west coast of Finland under eutrophic conditions using ITS markers (Blomster et al. 2002). They were tide-forming, unlike the specimens in this study, and in culture they only reproduced by cell regeneration into tubular thalli. This may indicate that there is no selection pressure for tide-forming strains of foliose *U. intestinalis*, a mechanism suggested by Fort et al. (2020c) regarding other tide-forming species. As the strains used by Fort et al. were only maintained in vegetative growth before barcoding, the possibility that other strains and species have similar limitations (particularly ones of aberrant morphology) should be investigated.

5.3 Seaweed industry

The most commonly listed species of *Ulva* sold in Europe is *U. lactuca*, which does not occur in northern hemisphere waters. This study confirms the findings of Steinhagen et al. (2019d) that the distribution of *U. fenestrata* is considerably more limited than previously believed and that it is likely in part due to misidentifications of foliose *U. compressa*. It was also found that *U. laetevirens* both overlaps with *U. fenestrata* and occurs in the geographic "gap" (mostly Kattegat) between the detected *U. fenestrata* and foliose *U. compressa*. Thus, I suggest that misidentifications of *U. laetevirens* may also have contributed to the issue from when it was introduced. This needs to be confirmed by sequencing of voucher specimens from the area.

More companies than expected only listed unspecified *Ulva* species. Of the three companies whose material was sequenced, the foliose specimens were identified as *U. laetevirens* in two cases, a species which no company identified by myself or Phyconomy lists. While the DNA barcoding effort of biomass from companies was fairly small, it does confirm that misidentifications occur frequently in the European seaweed industry. According to Fort et al. (2020a), only *U. lactuca* is authorized for food consumption in Europe outside of France, but this does not seem to be accurate as *Ulva* sp. is authorized as a traditional food product (Barbier et al. 2019). However, it is still of great importance to know which species are cultivated, harvested and consumed. If *Ulva* biomass is to be used for high-value products, it needs to be standardized and traceable. For the industry today, it means products are being manufactured and sold with unknown properties and nutritional values. Considering that most of European seaweed production comes from wild harvest, it may also have implications for biodiversity.

5.4 *tuf*A for DNA barcoding, species delimitation and phylogenetic analysis

Using *tuf*A for DNA barcoding was in large part successful but has its limitations. One of them is that *tuf*A, like other organellar markers, is not adequate to identify hybrids, as the specimen will only have the plastid DNA of one parent. Hybrids can and do occur but appear to be rare (Fort et al. 2020b) or less vital than non-hybrids (Xie et al. 2020).

The phylogenetic analysis retrieved a tree which is in overall agreement with previous results using different statistical analyses and genetic markers (Hayden and Waaland 2004, Steinhagen et al. 2019b). Barcoding genes are chosen for species delimitation purposes rather than to reflect evolutionary relationships and rates of change, but a comparison of the topology of trees based on *rbc*L, *tuf*A, complete mitochondrial and chloroplast genomes, and nuclear 45S rRNA showed no large differences in topology for foliose *Ulva* species, but higher bootstrap support for organellar and nuclear trees (Fort et al. 2020b). They also confirmed

that species identification matched between the different methods, and established the investigated species showed low intraspecific and high interspecific organellar variation, confirming they represent true species. However, Fort et al. (2020a) also point out that the same is not necessarily true for all *Ulva* species, and in fact recommend against a DNA barcoding approach for species delimitation of *Ulva*. This is partly because of how commonly *Ulva* sequences are misannotated on NCB – 21 % for foliose species – which was also apparent for some species during this study. However, it is also because several species separate into subgroups. They mention specifically the delimitation of *U. prolifera*, *U. linza*, and *U. procera* (though *U. procera* is currently considered a synonym of *U. prolifera*). The results of the ML analysis in this study retrieved two clear, separate, well-supported clusters for *U. linza* and *U. prolifera*, but species delimitation is not clear (Cui et al. 2018), and the results are dependent on the accuracy of the reference sequences.

According to Fort et al. (2020a), phylogenetic analyses of all NCBI entries for *Ulva* contain 47 accepted species names which separate into around 40 species clusters., or around half of currently accepted species. Some of the remaining species may not turn out to represent different species when molecular methods are used, as has been the case several times previously (e.g. *U. mutabilis* [Steinhagen et al. 2019a], *U. tenera* [Steinhagen et al. 2019b]). However, as this study shows, there are also *Ulva* species which do not have any reference sequences, and thus NCBI does not represent the true diversity of the genus.

5.4.1 Unidentified species

Two clusters which could not be delimited to species level were found in the phylogenetic analysis, one of which may or may not represent more than one species. One possibility is that these clades represent new species, another one that they belong to species without reference sequences. Further species delimitation methods to disentangle species affiliations of the unidentified clusters need to be applied, but there are some potential candidates for the clusters detected in this study. In an ITS/*rbc*L phylogeny (Hiraoka et al. 2017) *U. clathrata* can be found in a similar position as *Ulva* sp. 3–6 in this study. Seeing as *U. clathrata* is one of the species which does not have any *tuf*A reference sequences, I suggest one or more of the MOTUs represent *U. clathrata*. This can be tested by DNA barcoding using *rbc*L. The tubular morphology and the fact that some of the specimens of *Ulva* sp. 4 and 6 were detected in the Baltic, where *U. clathrata* is reported historically, supports this prediction. *Ulva* sp. 1, which is represented by a single sequence, stands out in the phylogram. However, it seems the placement is due to numerous ambiguous base calls, and a visual inspection shows it almost certainly belongs somewhere within the *Ulva* sp. 3–6 cluster. For the other clade, *Ulva* sp. 2, I suggest investigating *U. kylinii* as a possible candidate. *U. kylinii* lacks reference sequences for any marker. It is consistently listed in the literature except in the Baltic flora by Pankow (1990) and described as common in localities near on the northwest coast of Sweden by Kylin (1949). According to Sweden's Virtual Herbarium, historical vouchers, including type material, are available in the botanical collections of Lund University.

5.5 Conclusions and future considerations

Ideally, this study would have included the second marker, *rbc*L, as was originally intended. It would also have been beneficial to include historical vouchers. While inferences regarding previous misidentifications can be made by comparing the results of this survey and historical inventories, they need to be verified by testing historical material. Additionally, expanded sampling would give a more accurate representation of current species distributions. Some such data exists from previous sampling in 2018 and 2019, but it is as yet unpublished and not included in this study.

Of more than 400 scientific names of *Ulva* species, only 89 are currently valid, and half of those are represented on NCBI. Much more work is needed to improve our understanding of *Ulva*, but progress is being made. To move forward, a concerted effort needs to be made on several fronts. Sampling and molecular identification are needed to further elucidate knowledge of current distribution patterns. Sequencing of historical vouchers and type material are needed to clarify taxonomy and species delimitations, generate reference sequences, and understand historical species distributions and how they have changed, for example by introduction of new species. Information in public repositories should ideally be reviewed and corrected. Additionally, recommended reference sequences, as suggested by Fort et al. (2020b) for foliose species, should be identified and/or generated.

Another important factor is to link molecular identification with morphology. We know now that many species descriptions are incorrect or incomplete (e.g. *U. compressa* and *U. intestinalis*), and the same is true

for distribution patterns. The newest identification key for Sweden is the compendium from University of Gothenburg, which was updated in 2010, but it contains errors in both taxonomy and descriptions. Combining molecular and morphological analysis (including sequencing of type material) would provide a greater understanding of natural variability of the species and connect morphological variations with ecological factors. In turn, this would allow us to establish accurate species descriptions and taxonomy which can be used in revised and refined identification keys, including information on their limitations.

The importance of type material has been further high-lighted by the most recent taxonomic revisions by Hughey et al. (2020) mentioned previously, which have demonstrated some results of this study are already outdated. *Ulva* is a very active field of study, and if we are to fully utilize the potential of this diverse genus, the work has just begun.

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Appendix 1

50 shades of green

The diversity of green algae in Scandinavia

Can you name one thing that sushi, plastic, toothpaste, fertilizer, paper, fish food, pharmaceuticals, and biofuel have in common? If your answer is that all of them can be made using green algae, you are correct!

You probably do not give much thought to green algae in your everyday life. Perhaps you enjoy some sushi every now and then, and if you spend time by the coast you will almost certainly have seen them – thin, soft, hollow tubes or large, lettuce-like leaves of different species of the genus *Ulva*. If you have been unlucky, you may have had a visit to the beach ruined by enormous, stinking masses of them. In that form, they are usually called green tides. These are not only a nuisance to beachgoers but can completely suffocate other species living in the ecosystem.

Identifying green algae

Despite the importance of green algae and despite a rich Scandinavian history of studying them, no one knows fort certain which species are present and where. In the past, the only way of describing and identifying species was by their appearance. Looking at a green alga, this can involve examining its shape, texture, and branching patterns. Using a microscope, you can see the size and shape of the cells, the chloroplasts inside the cells, and even study structures inside the chloroplasts. All these characters can be used to identify which species you are looking at. At least that is what we used to think, before we were able to study their DNA.

DNA barcoding

In 2003, a revolution in species identification began when a scientific paper introduced the concept of DNA barcoding. The scientists behind the paper had identified a gene they believed could be used to quickly and easily identify any animal species once a reference sequence of that species existed to compare it to. While it has not been as straight-forward as that, DNA barcoding has changed the way we look at many species – including green algae. Studying their DNA, it was soon understood traditional identification methods are far from reliable. That is why there is a research project in progress investigating *Ulva* species in Scandinavia.

In the lab

This study, which is part of the bigger research project, has used DNA barcoding on over 500 samples of green algae to find out what species they belong to and where along the coasts of Sweden, Denmark, and Norway they can be found. First, DNA was extracted from freeze-dried samples. After that, millions of copies of a specific barcoding gene were made by PCR (polymerase chain reaction). Samples of this amplified gene were sent away for sequencing, and after a few days the DNA sequence were returned. The raw sequences can be directly compared with millions of other sequences in a public database to find the nearest match. Hopefully, the matching sequence is correctly identified and will tell us the species of the sample. Finally, the sequences were used in statistical analyses to find the most likely evolutionary relationship between them – in short, to build a family tree. By including reliable reference sequences in the analysis, the accuracy of the DNA barcoding identification can be validated.

New discoveries

So far, the project has revealed that the distribution and diversity of species differ from historical inventories, which were based on morphological identification. Introduced – and potentially invasive – species have been discovered, and some samples belong to species which are unknown or have at least never been sequenced before.

Future prospects

Because of the many different applications of *Ulva* – as food, biofuel, in medicine and much more – these simple and abundant organisms may hold part of the key to a more sustainable future. But different species have very different properties, so for us to fully utilize their potential, we need to be able to identify them – and that work is just beginning.