Molecular and Morphological Studies of Norwegian Eelgrass Populations

Eli Johansen

Master of Science Thesis

Integrative Systematics of Plants and Fungi, Natural History Museum and

Department of Bioscience and, University of Oslo.

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Abstract

According to the Norwegian Flora (Elven et al. 2022), we have three accepted species of eelgrass in Norway: *Zostera marina* L. (eelgrass, ålegras), *Z. noltii* Hornem. (dwarf eelgrass, dverg- ålegras), and *Z. angustifolia* (Hornem.) Reichenbach (narrow-leaved eelgrass, smalt ålegras). Other studies only support two species: *Zostera marina* (eelgrass, ålegras) and *Z. noltii* Hornem. (dwarf eelgrass, dverg- ålegras), and regards *Z. angustifolia* as a subspecies of *Z. marina* (den Hartog 1972, Borum et al. 2004, Larkum et al. 2006). This study aims to investigate how many *Zostera* species are present in Norway and whether the morphologically species delimitation is confirmed by the molecular analyses, and which names should be applied to these taxa. Target capture sequencing analysis was applied to resolve the genetic delimitations. The morphological analyses were statistically significant with respect to differences between the three taxa in several characters: leaf width, apexes, number of veins, sclerenchymatic tissue, flowers, and seeds. *Zostera angustifolia* have a higher percentage of specimens with long, tough strands of sclerenchymatic tissue, while none of the *Z. noltii* specimens had visible fibers. The molecular analyses only supported two species; *Zostera noltii* and *Z. marina.* The *Zostera* specimens examined were collected from eelgrass beds during fieldwork in Oslofjorden, including Bliksekilen in Vestfold and Kurefjorden in Østfold, where all three taxa co-occur.

Keywords: eelgrass, dwarf eelgrass, narrow-leaved eelgrass, target capture analysis.

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1 Introduction

1.1 The genus *Zostera*

Zostera L., eelgrass, is a marine vascular plant genus in Zosteraceae in the monocot order Alismatales. *Zostera* is distributed in the Pacific and Atlantic Oceans, around the British Isles, on the coast of Denmark, Sweden, and Norway. Eelgrass grows in shallow waters, forms extensive meadows, and stabilizes the sediments. It also attenuates wave energy, improves water quality and clarity by directly trapping suspended particles, nutrient uptake, and retaining organic matter (Duarte, 2002; Spalding et al., 2003). Eelgrass meadows form the basis of many coastal food webs and provide habitat to various organisms. Previous studies have found eelgrass to have a high degree of morphological plasticity that allows it to survive and adapt to environmental changes (Short and Wyllie-Echeverria, 1996). Most of the species in the genus are perennial and monoecious. The leaves are attached to a sub-surface thick, horizontally growing rhizome with fine roots (Phillips & Menez, 1988; Borum & Greve, 2004) (Figure 1). The recently formed internodes are light green, while older segments turn yellow or brown (Greve & Binzer, 2004). *Zostera* reproduces both asexually, through vegetative clonal growth, and sexually, through the production of flowers and seeds (Phillips & Menez, 1988; Darnell et al., 2015). Geographic variation in the reproductive strategy of *Z. marina* on the Pacific coast of North America is reflected in differences in flowering frequency, seed production, and the effects of salinity on germination.

1.2 Genus delimitation

In Tomlinson and Posluzny's (2001) taxonomic revision of *Zosteraceae,* the genus *Zostera* was split into four genera, namly *Zostera, Nanozostera, Phyllospadix* and *Heterozostera*. They suggested an elevation of *Zosterella* from subgeneric to generic rank, *Nanozostera*. *Zostera noltii* was assigned to this genus, while *Z. marina* as the type-species, remained in *Zostera*. Molecular and morphological data indicated that *Phyllospadix* represents the most divergent taxon, followed by *Zostera*, while *Heterozostera* and *Nanozostera* are closely related sister-clades. This splitting of the genus has not been taken up by other authors and are today regarded as synonyms of *Zostera* by most others (POWO 2023, WFO 2023).

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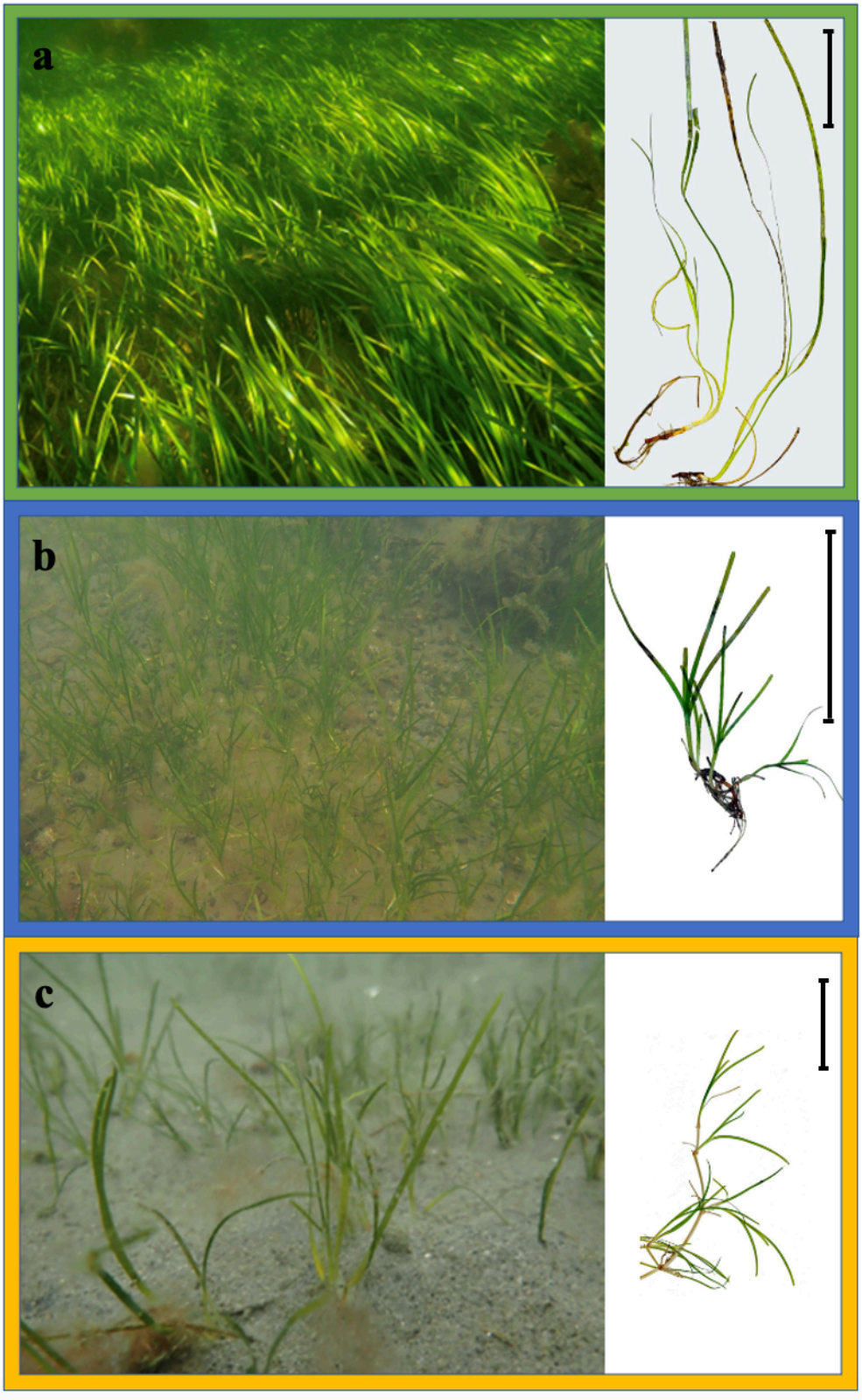

Figure 1. Habitat and specimens of **(a)** *Zostera marina*, **(b)** *Z. angustifolia*, and **(c)** *Z. noltii.* Photo: Eli Rinde (habitat) and Birna Rørslett (specimens). 10 cm scales. Photos: Habitat: Eli Rinde, specimen: Birna Rørslett.

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1.3 Species delimitation

According to the Norwegian Flora (Elven et al., 2022), we have three accepted species of eelgrass in Norway: *Zostera marina* L. (eelgrass, ålegras), *Z. noltii* Hornem. (dwarf eelgrass, dverg- ålegras), and *Z. angustifolia* (Hornem.) Reichenbach (narrow-leaved eelgrass, smalt ålegras) (**Figure 1**). By many authors, *Zostera angustifolia* is considered to be a separate species based on its distinct morphological characteristics, eg. Tutin et al. (1976), Czerepanov (1995), Coyer et al. (2013a), Rørslett & Mjelde (2021b), and Elven et al. (2022). However, Den Hartog, in his "Sea-grasses of the world" (1970), did not agree with this view and reduced *Z. angustifolia* to a synonym under *Z. noltii*. Later, his investigations at the Copenhagen herbarium led Den Hartog (1972) to conclude that *Z. angustifolia* represents a narrow-leaved form of *Z. marina*, consistent with Hornemann's proposal in 1816. This view is also shared by many other authors, eg. Markgraf (1972), Markgraf & Zoller (1981), Stace (1997), Borum et al. (2004), Olsen et al. (2013). Hence, there are some disagreements in the status of both *Z. noltii* and *Z. angustifolia*.

Zostera marina. Carl Linnaeus described *Z. marina* in 1753 based on a type from the Baltic Sea (Figure 1a). This species is the most common seagrass in the northern hemisphere (Linnaeus, 1753; Phillips & Menez, 1988; Boström et al., 2003; Borum et al., 2004) (Figure 2a). *Zostera marina* is adapted to relatively cold habitats with temperatures ranging between -1°C in winter and approximately 25°C in summer (Greve & Binzer, 2004). Studies have shown that *Z. marina* has high phenotypic plasticity that allows this species to occupy different habitats and environmental conditions (Backman, 2011). They form beds on soft sediments at intermediate depths (Figure 1a). *Zostera marina* is a perennial seagrass, usually distributed in the lower intertidal and subtidal relatively sheltered area (Park et al., 2016), and therefore has little exposure to stressors such as desiccation, high irradiance, and waves (Jacobs et al., 1984; Madden et al., 1993). *Zostera marina* is commonly found along the coast of Norway, although its occurrences become sparse towards the northernmost regions. The occurrences in Finnmark represent the northernmost occurrence globally (Jørgensen & Bekkby, 2013) (Figure 2a).

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Zostera angustifolia. *Zostera angustifolia* was described by Hornemann as *Zostera marina* var*. angustifolia* in 1816 based on a type from Denmark as *Zostera marina ß [var.] angustifolia* (Hornemann, 1816) (Figure 1b). Reichenbach (1845) erected *Z. angustifolia* in 1845 to species level closely related to *Z. marina*. Tutin (1936) described the same taxon in 1936 as *Z. hornemanniana*. This name was later synonymized by Michael D. Guiry (algabase.org retrieved: 12.01.2023). *Zostera angustifolia* is distributed in the temperate biome of the northern hemisphere (Figure 2b), with its native range in North and Northeastern Europe and the Russian Far East (POWO, 2023). Generally, the distribution of *Z. angustifolia* is poorly known, as the species often co-occurs with *Z. marina. Zostera angustifolia* grows scattered on sheltered tidal mudflats in estuaries and coastal lagoons, usually in more shallow and more turbid water than *Z. marina*. It is usually found on mud or muddy sands between the half-tide and low-tide marks (Stewart et al., 1994). It often occurs in mixed beds with *Z. marina* and *Z. noltii*, predominating in waterlogged depressions between the free-draining hummocks dominated by *Z. noltii* (Davison & Hughes, 1998). *Zostera angustifolia* is exposed to diverse stressors, such as desiccation and changes in salinity, pH, or oxygen concentration (Hily et al., 2003). *Zostera angustifolia* has been recorded along the Norwegian coast, predominantly exhibiting a similar distribution pattern to *Z. marina* (Rørslett & Mjelde, 2021b) (Figure 2b).

Zostera noltii. *Zostera noltii* was described by Hornemann in 1832 based on a type from the North Sea or the Baltic Sea (not specified in the original description) (Figure 1c). Hornemann initially named the species *Zostera noltii* as a tribute to Professor Nolte. However, there have been disagreements regarding the spelling, sometimes referred to as *Zostera noltei* in the literature. The Norwegian flora and the International Plant Names Index (IPNI) use the spelling *Zostera noltei.* However, Hornemann (1832) first used the name *Zostera noltii*, which is considered the correct name according to Plants of the World Online (POWO 2023) and World Flora Online (WFO). *Zostera noltii* (Figure 2c) thrives in cold habitats in the north but prefers higher temperatures than *Z. marina* (Greve et al., 2004). The absence of *Z. noltii* in the Northern/Arctic part of Europe might be due to a higher temperature required for flowering than that of *Z. marina. Zostera noltii* forms dense beds in the muddy sand of intertidal areas, where *Z. marina* is sparse due to its lower tolerance to desiccation (Borum

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& Greve, 2004) (Figure 1c). In Norway, the seagrass species *Z. noltii* is categorized as highly threatened (EN) because of its limited and fragmented distribution, and the ongoing decline in its occurrence and habitat quality, as stated by Solstad et al. (2022). *Zostera noltii* is a priority species under the Natural Diversity Act (Ministry of Climate and Environment, 2015), and an action plan has been established to preserve it, as documented in Lundberg (2009) and Environment Directorate (2014). *Zostera noltii* is distributed on both sides of Oslofjorden, in several areas around its estuaries, and at locations along the southern coast up to Jæren and Hardanger (Figure 2c). The occurrences of *Z. noltii* in Norway are the northernmost known worldwide (Lundberg, 2013).

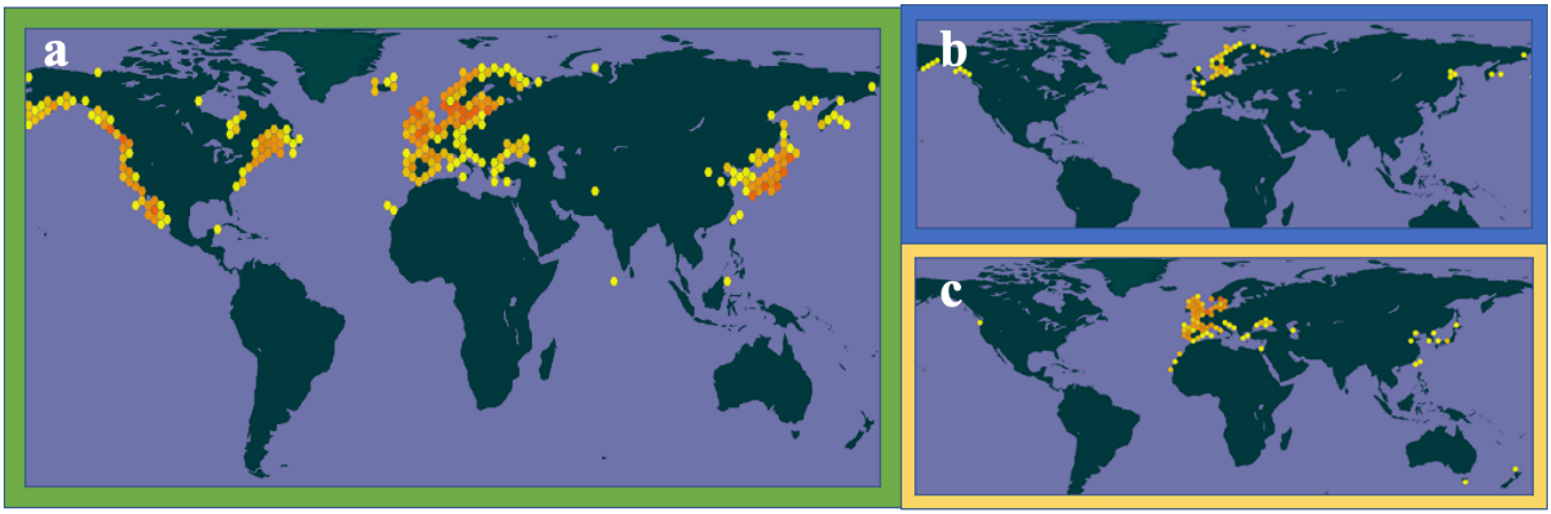

Figure 2. Global distribution of **(a)** *Zostera marina,* **(b)** *Zostera angustifolia*, and **(c)** *Zostera noltii.* Source: gbif.org (retrieved on February 23rd, 2023).

1.4 Phenotypic plasticity and morphology

Zostera marina has shown remarkable phenotypic plasticity in response to various environmental factors such as light, temperature, salinity, and nutrient availability. Olesen & Sand-Jensen (1993) found that plants grown under low light conditions had longer, narrower leaves with higher chlorophyll content, while those grown under high light conditions had shorter, wider leaves with lower chlorophyll content. Short (1987) found that plants grown under high nutrient conditions had higher shoot density, leaf length, and leaf width than those grown under low nutrient conditions. Li et al. (2023) found that plants

exposed to high water motion had shorter and thicker leaves, higher shoot density, and shorter internode length than those grown under low water motion conditions. Nejrup & Pedersen (2008) found that plants grown under low saline conditions had a reduced shoot biomass compared to those grown under high salinity conditions. These studies demonstrate the remarkable ability of *Z. marina* to modify its morphology and physiology in response to different environmental factors, which allows it to thrive in a wide range of marine habitats and might explain the species' worldwide distribution.

1.4.1 Leaf morphology

The leaves of *Zostera angustifolia* (1-3 mm) and *Z. noltii* (<1.5 mm) are described to be narrower than those of *Z. marina* (≥ 4 mm) (Figure 1) (Rørslett & Mjelde, 2021a, b and c). *Zostera marina* has mucronate leaf apex, while *Z. noltii* and *Z. angustifolia* have rounded but emarginate apexes as the plants matures (Figure 3). The number of parallel leaf veins are assumed to differ between the three species, *Z. marina* has between 5 and 11, *Z. angustifolia* between 3 and 5 and *Z. noltii* has normaly 3 (Rørslett & Mjelde, 2021b) (Figure 4a). The leaves also have fiber bundles, these can be seen when the leaves are pulled apart, as long, tough strands of sclerenchymatic tissue emerge (Figure 4b). Mjelde and Rørslett (2021b) observed that *Z. marina* has fewer and shorter strands than *Z. angustifolia*.

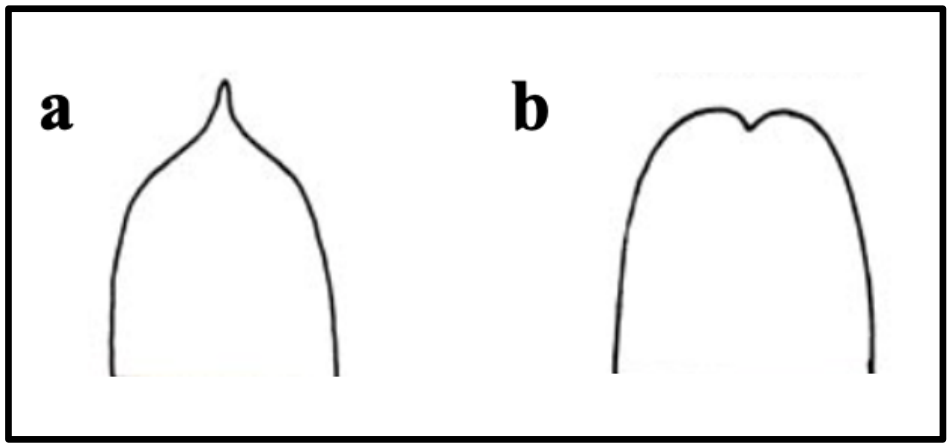

Figure 3. Leaf apex; **(a)** emarginate and **(b)** mucronate.

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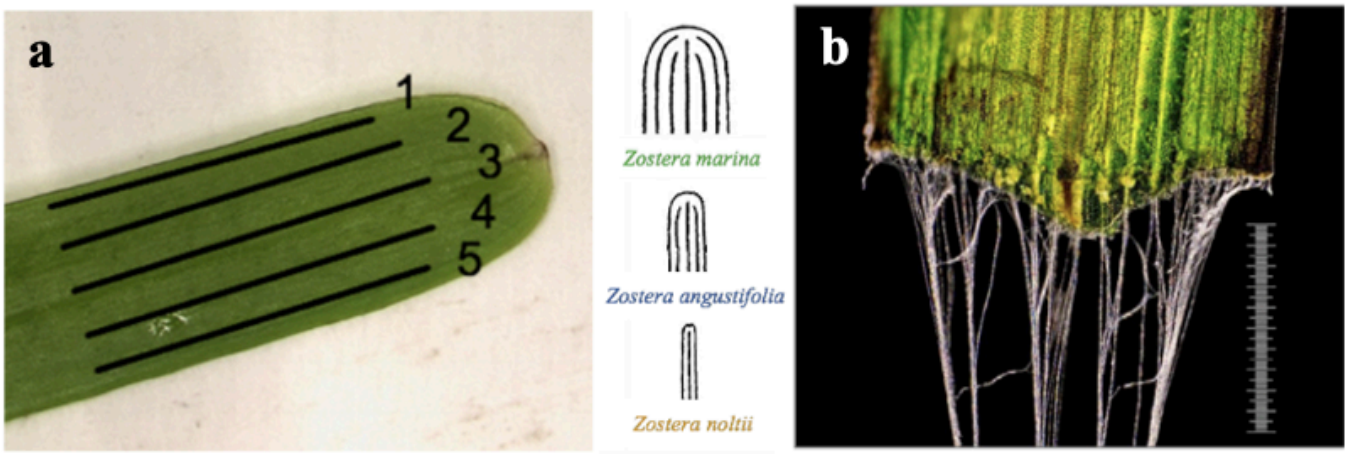

Figure 4. (a) Parallel veins of the three species, *Z. marina* (5-11), *Z. angustifolia* (3-5) and *Z. noltii* (3). **(b)** Tough strands of sclerenchymatic tissue, 1 mm scale with 1/100 mm divisions. Photo: **(a)** Eli Johansen and **(b)** Birna Rørslett.

1.4.2 Reproduction structures

The reproductive structures of *Zostera* specimen consist of a flattened spadix with flowers on one side, enclosed within a spathe (Figure 5c). Male and female flowers are found on the same individual and are small and greenish and partly hidden in pockets within the leaf sheaths (Figure 5b). Flowering can be observed from early spring to fall. The shoots change morphology during flowering to produce more leaf bundles separated by long, thin stem segments (Borum et al., 2004). The male flowers release pollen from the tip of a thick, cupped anther in long strands and produce several thousand grains per square meter (Christensen et al., 2004; Borum et al., 2004) (Figure 5e). The female flowers capture floating pollen threads and bend down against the blade to produce fruit containing a seed (Figure 5d). *Zostera noltii* has the smallest seeds (2-3 mm) with red-brown color, while *Z. marina* (4- 4.5 mm) and *Z. angustifolia* (3-3.5 mm) have larger yellow-orange or light brown seeds with stripes (Rørslett & Mjelde, 2021 a, b, c). Seasonal reduction in salinities enhance seed germination, and there is a higher incidence of flowering (Phillips, 1983).

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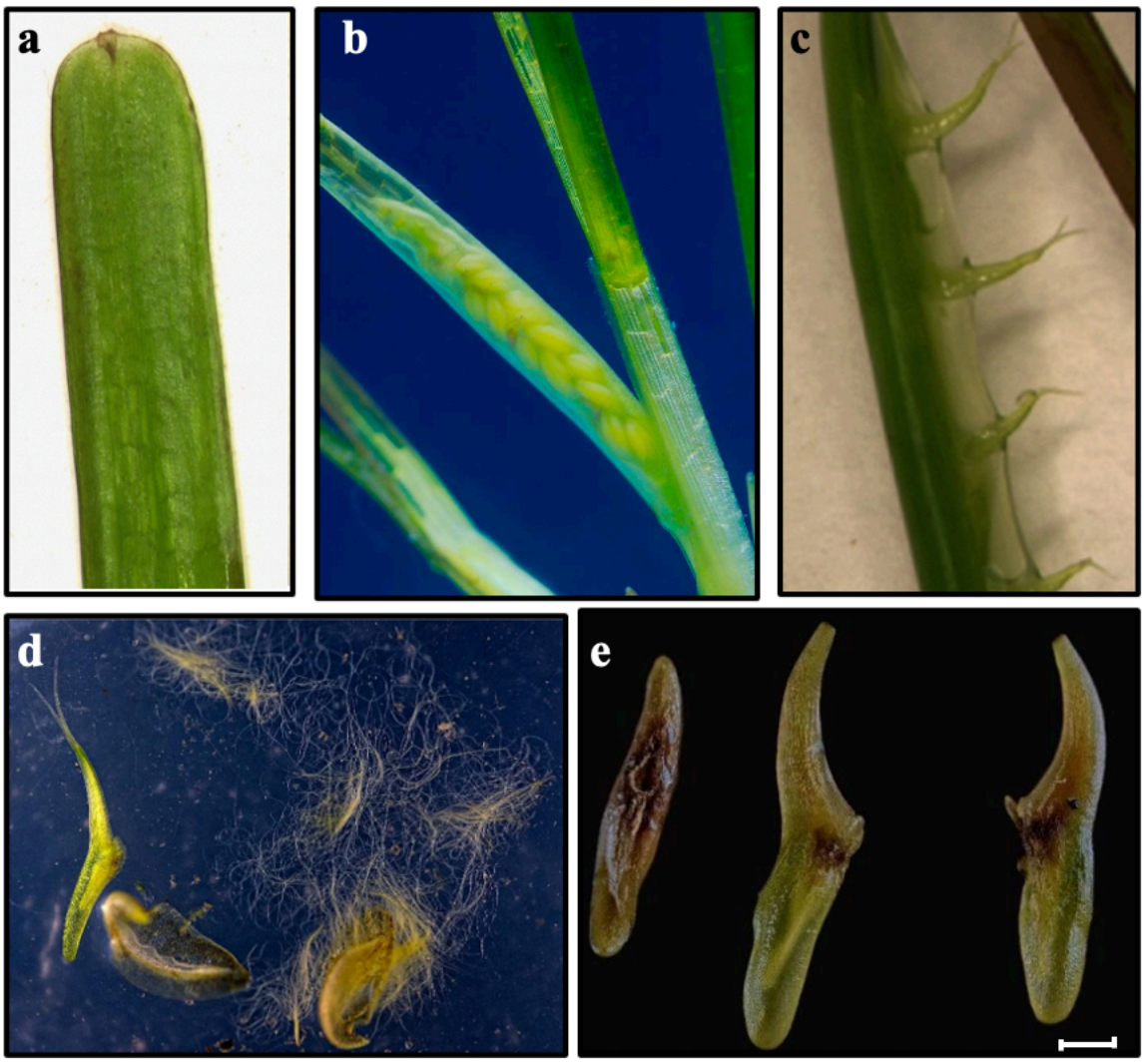

Figure 5. (a) *Zostera marina* leaf apex. **(b)** Fertile *Z. angustifolia* shoot with flowers enclosed within a spathe. **(c)** Flattened spadix with *Z. angustifolia* flowers. (**d)** Female *Z. angustifolia* flower with fruit node, stigma with two spreading scar lobes and anthers that release masses of thread-shaped pollen grains. **(e)** Male *Z. marina* flower with anther (left) and two fertilized female flowers (right). Scale 1 mm. Photos: (**a** and **c)** Eli Johansen and (**b**, **d** and **e**) Birna Rørslett.

1.5 Genetic diversity and hybridization

Several studies have investigated the genetic diversity within the genus, specifically focusing on *Z. marina* and *Z. angustifolia*. J. Brenchley, in personal communication cited in Davison and Hughes (1998), suggested that *Z. marina* and *Z. angustifolia* may represent variants of a single species. Coyer et al. (2013) conducted a global study using four genetic loci to

compare the 'wide-leaved' *Zostera marina* var*. angustifolia* from three different locations. They concluded that these specimens were indistinguishable from *Z. marina*, further supporting the idea that *Z. angustifolia* is not a distinct species but rather conspecific with *Z. marina*. Olsen et al. (2013) focused on Norwegian fjord populations and used microsatellite loci to examine the genetic differentiation between *Z. marina* and what they denoted the '*angustifolia*' morphotype. Neither this study found any significant genetic distinction between the two taxa. These studies collectively highlight the genetic similarities between *Z. marina* and *Z. angustifolia*, providing evidence that supports the hypothesis of *Z. angustifolia* being a morphological form or ecotype within the broader *Z. marina* species. Olsen et al. (2014) found evidence of hybridization between *Z. marina* and related species *(Zostera pacifica*) based on the genetic markers analysed. This suggests that hybridization events occur in natural populations, leading to genetic exchange between different seagrass species. Additionally, researchers have observed multiple paternity lineages within *Z. marina*, indicating that individual plants can be fertilized by multiple males (Reusch, 2000). Molecular studies have not provided supporting evidence for hybridization between *Z. marina* and *Z. noltii*. However, they provide insights into the reproductive strategies and population dynamics of *Z. marina*, which are important considerations for understanding the evolutionary processes and conservation implications of this seagrass species.
The previous studies used microsatellite loci (Olsen et al., 2013) and multiple genetic loci (Coyer, 2013) did not find any significant genetic distinction between *Z. marina* and *Z. angustifolia*. It is possible that the previous methods were not sensitive enough to discriminate between the closely related *Z. marina* and *Z. angustifolia*. Therefore, the present study will utilize the highly sensitive target capture analysis to shed more light on the species delimitation of the Norwegian *Zostera* taxa. The chosen method is not designed for hybrid identification. Instead, it targets 353 nuclear angiosperm genes to explore the phylogenetic relationships within *Zostera*.

1.7 Research aim

The aim of this study is to conclude on the number of *Zostera* species present in Norway and which names to apply to these taxa. In this project, I will also investigate whether the morphological species delimitations are confirmed by the target sequencing analysis.

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2 Material and methods

2. 1 Field Sampling

One hundred and eight *Zostera* specimens were collected from eelgrass beds during fieldwork in Oslofjorden, including Bliksekilen Vestfold and Kurefjorden in Østfold, in collaboration with NIVA, on 1/7/21 and 1/9/21 (Figure 6). The collection sites were selected to areas where all three taxa's morphological variations were known to co-occur. Eelgrass beds were accessed by snorkeling from land. Species were identified based on morphological characters at the site of collection (overall size, leaf width, shape of stem). Leaf samples were gathered from the same rhizome to ensure they represent the same populations and placed in 1-liter containers containing seawater and stored in a cooler with freezing elements under transportation and later stored in a refrigerator (4°C).

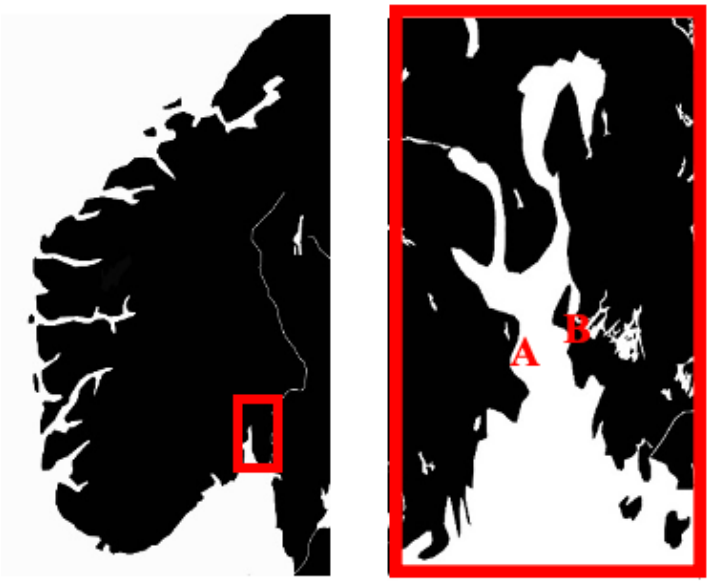

Figure 6. Map of the fieldwork sites in Oslofjorden, **(A)** Bliksekilen, Vestfold (59.325457° N, 10.500504° E) and **(B)** Kurefjorden, Østfold (59.293327° N, 10.741410° E). Source: gbif.org (retrieved on May 25th, 2023).

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2. 2 Morphology

2.2.1 Laboratory work

All specimens (108 in total) were photographed, pressed as herbarium vouchers, and deposited at the Oslo herbarium (O). From each specimen, a total of 1cm x 1cm leaf tissue were cut using a scalpel and placed in 25 ml containers with lids and silica crystals for dehydration and conservation of DNA. The morphology of the leaves and the fertile parts were investigated and photographed under a dissecting microscope (Infinity, Lumenera) with a camera (Nikon SMZ1270). The leaf's width and apex were recorded for each individual (**Figure 3**). Leaves from the fall samples were ripped and pulled apart to detect whether long, tough strands of sclerenchymatic tissue emerged (Davies et al 2007). Leaf apexes were bleached with chlorine; then dyed using Lactophenol Cotton Blue (Sigma-Aldrich) to visualize the leaf veins and strands of strong tissue before counting the number of veins in a dissecting microscope (Infinity, Lumenera). The color of mature seeds was documented, and their length was measured.

2.2.2 Statistical Analyses

To investigate the differences in mean leaf width between the *Zostera species*, I used one-way ANOVA. The residuals were visually inspected to assess the assumption of homogeneity of variance, and a histogram was used to verify normal distribution. The pairwise differences in leaf width between species were assessed using Welch's t-test with the option 'Assuming Unequal Variances' selected, as the standard error varied among the three taxa. The null hypothesis was that there was no significant difference in leaf width between the three plant species (i.e the ANOVA), and the two species compared (t-test). The ANOVA and t-test were performed using Microsoft Excel version 16.73. Due to limited availability of mature seeds, and a substantial variation in seed size, it was not possible to get a valid statistical analysis of possible differences in seed size for the three Z. species.

2.3 Molecular analyses

2.3.1 DNA extraction

DNA tissue from four *Zostera marina*, five *Z. angustifolia*, and three *Z. noltii* individuals was extracted from the silica-dried leaves collected during the fieldwork. DNA was extracted at the DNA laboratory at the Natural History Museum, UiO, using the E.Z.N.A.® SP Plant Mini Kit (Omega Bio-Tek, Atlanta, USA) following the manufacturer's recommended protocol. The total volume of the extracted and eluted DNA was 100 µL per sample. Two µL of the eluted double-stranded DNA were used for quantification by Qubit® 2.0 fluorometer using the dsDNA HS assay kit (Life Technologies, Carlsbad, CA, USA), high sensitivity, to measure the concentration of DNA in each sample following the protocol provided by Mardis & McCombie (2017). Two µL of the eluted double-stranded DNA was used for a NanoDrop One (Thermo Fisher Scientific, USA) spectrophotometry test to determine the degree of contamination in the extracted samples, following the protocol provided by García-Alegría et al. (2020). Three µL of the total DNA was used for gel electrophoresis to check the DNA fragments' size, according to the protocol provided by the manufacturer, using the FastRuler Low Range DNA ladder.

2.3.2 DNA yield

The DNA concentration was calculated for all samples using qubit. The DNA concentration from each sample was calculated through the specified DNA yield (>10 ng/µL for degraded samples). Duplicated samples were pulled, and afterwards transferred to a plate, for drying using a vacuum centrifuge (DNA120 SpeedVac, ThermoSavant) according to the protocol provided by Baker et al. (2022). The samples were dried for seven hours and prepared for shipping covered with Adhesive Sealing Sheet. Library prep, target capture and Illumina sequencing were performed by Arbor Biosciences (MI, USA). The samples were divided in two groups of six samples for target capture reactions using the Angiosperm353 probe kit (Johnson et al., 2019). The enriched libraries were sequenced on an Illumina NovaSeq 6000 S4, using 150 base pair (bp) paired end reads and to a depth of 1 Gbp per sample.

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2.3.3 Read mapping, exon extraction, and removal of paralogous gene copies

The analyses was conducted using the System for Automated Geoscientific Analyses (SAGA) computer (Conrad et al., 2015), and a pipeline designed at the Natural History Museum, UiO, that handles target capture data obtained through the Angiosperms353 bait kit. The raw data file were unzipped and quality-checked using FastQC version 0.11.9 to identify the data quality of the samples downstream analysis (Wingett & Andrews, 2018). Trimmomatic version 0.39 (Bolger et al., 2014) was used to remove adapters and low-quality bases from the reads using the following parameters: LEADING:30, TRAILING:30, SLIDING WINDOW:4:30, and MINLEN:36. The MINLENGTH setting, with the score set to 36, was used to remove shorter reads that might not have been positioned uniquely against hybrid sequences. The cleaned reads were quality-checked using FastQC. Angiosperm353 exons were recovered from the trimmed paired read and were mapped to the mega353 target file (McLay et al., 2021) to assemble the clean reads into sequences using the HybPiper pipeline version 1.3.1. (Johnson et al., 2016). The mega353 target file is available on GitHub (https://github.com/chrisjackson-pellicle/NewTargets). The trimmed reads were mapped to the target sequences using BWA version 0.7.17 (McLay et al., 2021). The reads were distributed to separate directories and assembled *de novo* using SPAdes version 3.15.3 (Bankevich et al., 2012). Extraction of exons for each target region of the samples was done using Exonerate (Slater & Birney, 2005) to assign the contigs to the appropriate target file. The HybPiper stats script was used to evaluate the success of the assembly, including the number of genes recovered compared to the 353 target genes. The HybPiper recovery heatmap was used to compare samples in an image viewer. The Paralog script (Johnson et al., 2016) was used to identify and evaluate paralogs which are genes arising from duplication events within the genome. All paralogous genes were excluded from the further analysis.

2.4.2 Sequence concatenation

As outgroups in the phylogenetic analyses, Angiosperm353 exons (protein-coding regions), for *Potamogeton wrightii* Morong and *Phyllospadix iwatensis* Makino were downloaded from the Kew Tree of Life database (Baker et al., 2022). *Potamogeton wrightii* and

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Phyllospadix iwatensis were chosen as outgroups as they are established sister groups to *Zostera* based on Kew's Tree of Life phylogeny of *Zostera* (Baker et al. 2022). Angiosperm353 exons of *Zostera marina* from the Kew Tree of Life Explorer were included in the ingroup. The outgroups were concatenated with the ingroup using AMAS (Borowiec, 2016). The ingroups and the outgroups were aligned using MAFFT version 7, using the default settings (Katoh & Standley, 2013), and trimmed with ClipKIT (Steenwyk et al., 2020). The aligned trimmed sequences were concatenated, and alignment statistics were calculated using AMAS (Steenwyk et al., 2020).

2.4.3 Phylogenetic reconstruction

Each trimmed alignment was used to generate an unrooted gene tree with IQ-TREE version 2.1.3 (Minh et al., 2020). The ultrafast ModelFinder option was used to estimate the best-fit nucleotide substitution model for the alignments (Kalyaanamoorthy et al., 2017), and gene trees were inferred using maximum likelihood. UFBoot (Hoang et al., 2018) was used to assess branch support and to generate 1000 bootstrap replicates for each gene tree. The gene trees from IQ-TREE version 2.1.3 (Minh et al., 2020) were combined and used to construct a species tree in ASTRAL-III version 5.7.8 (Zhang et al., 2018) using the multi-species coalescent model (Sayyari & Mirarab, 2016). Branch support was scored using local posterior probability measuring the support of a quadripartition (quartet frequencies) (Sayyari & Mirarab, 2018). One multi-specie coalescent specie tree was based on 262 nuclear regions present in the 15 samples (100% gene coverage). A second phylogenetic tree was constructed using the remaining 342 nuclear regions after the exclusion of paralogous sequences. The generated species tree was plotted in FigTree version 1.4.4 (Rambaut, 2014) and iTol (Letunic and Bork, 2021). The tree was rooted in FigTree, using *Potamogeton wrightii* and *Phyllospadix iwatensis*was.

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3 Results

3.1 Leaf morphology

3.1.1 Width

The average leaf width and standard error were highest for *Z. marina* (3.6± 0.99 mm), followed by *Z. angustifolia* (2.6 ± 0.53 mm) and *Z. noltii* (1.3 ± 0.3 mm) (Figure 6). The One-way ANOVA indicated a significant difference in leaf width among the three plant species, *Z. marina*, *Z. angustifolia*, and *Z. noltii* ($F(2, 83) = 82.85$, $p < 0.05$). Based on these results, the null hypothesis that there is no significant difference in leaf width between the three plant species can be rejected. The significant p-value and high F-value indicate that there is a significant difference in leaf width among the three species. The pairwise t-test showed a significant difference in leaf width between *Z. marina* and *Z. angustifolia* ($t(47) = 4.80$, $p < 0.05$, two-tailed). The mean leaf width of *Z. marina* was significantly larger than that of *Z. angustifolia*. Furthermore, the t-test confirmed significant differences in leaf width between *Z. marina* and *Z. angustifolia* compared to *Z. noltii*.

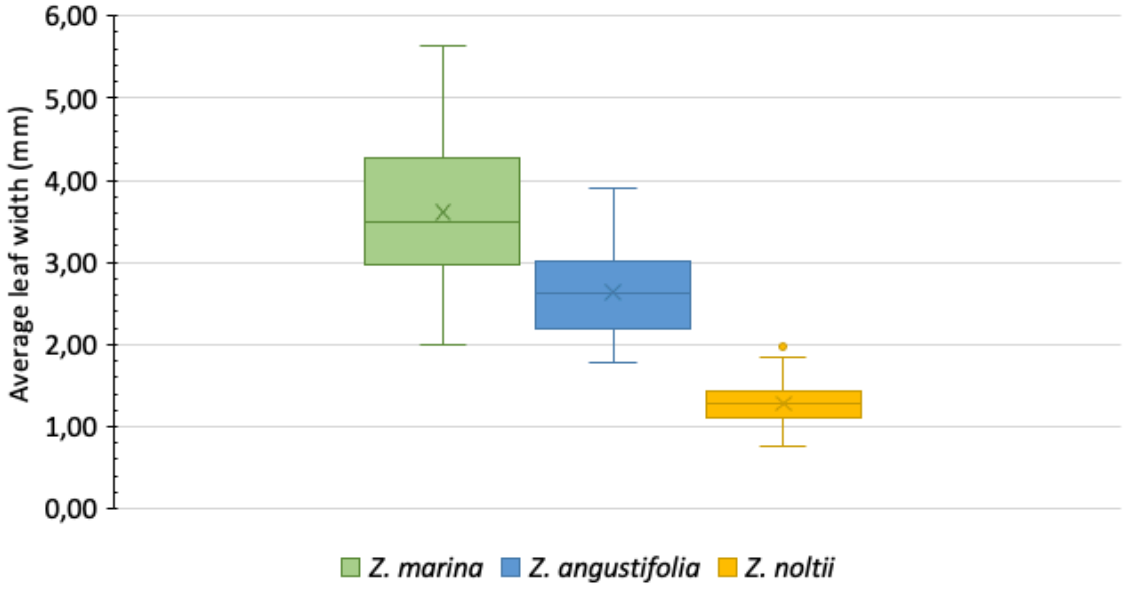

Figure 7. Average leaf width of *Z. marina* (3.61± 0.99 mm), *Z. angustifolia* (2.64 ± 0.53 mm), and *Z. noltii* (1.29 ± 0.3 mm) measured with a caliper. Error bars indicate standard error.

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3.1.2 Leaf apex

The leaf apexes of *Z. angustifolia* were emarginate, mucronate, or rounded. *Zostera noltii* had filiform leaf shape, with emarginate, truncate or unevenly rounded leaf apexes. The total percent of emarginate leaf apexes was lowest for *Z. marina* (31,4%), then higher for *Z. angustifolia* (48,4%) and highest for *Z. noltii* (94,1%) (Figure 7). The percentage of emarginate leaf apexes was higher in the fall than in the spring for all taxa. The frequency of emarginate leaf apexes increased from spring to fall for each of the taxa. The increase was from 5.9% to 58.8% for *Z. marina*, from 0% to 58.8% for *Z. angustifolia*, and from 56.6% to 75% for *Z. noltii*. The largest increase in frequency was observed for *Z. angustifolia*.

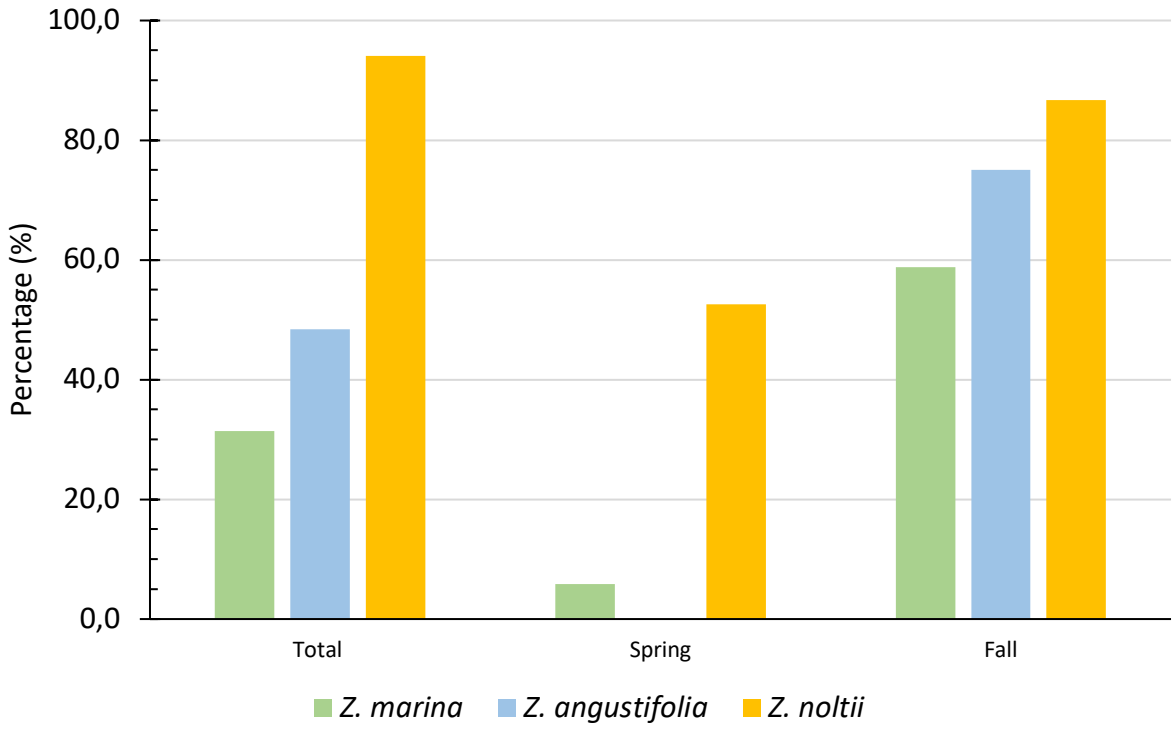

Figure 8. Percentage of *Z. marina*, *Z. angustifolia*, and *Z. noltii* with emarginate leaf apex.

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3.1.3 Veins

The number of veins was recorded for 12 *Z. marina*, 15 *Z. angustifolia*, and 12 *Z. noltii* individuals. The average number of veins varied among the studied taxa, with *Z. marina* exhibiting the highest mean value (5±0), followed by *Z. angustifolia* (4±1) and *Z. noltii* (3±0). (Figure 9). The veins of *Z. marina* merge at the leaf apex, and the mid vein splits into two at the very end of the leaf apex (Figure 9a). The side veins of *Z. noltii* merge with the midrib before the leaf apex (Figure 9c).

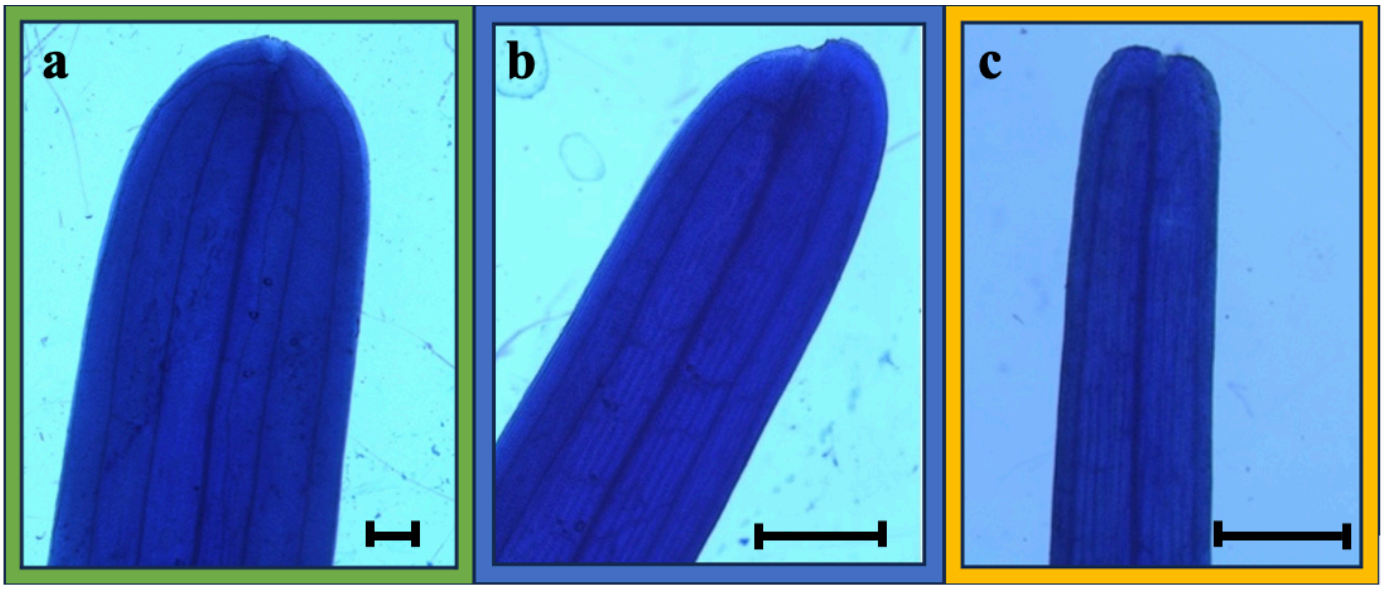

Figure 9. Example of the number of parallel veins found in the three taxa **(a)** *Zostera marina* (five veins), **(b)** *Zostera angustifolia* (five veins), **(c)** and *Zostera noltii* (three veins). 1 mm scale.

3.1.4 Cellulose fiber test

In the fall 55,6% of *Z. marina* and 85,7% *Z. angustifolia* collected had long, tough strands of sclerenchymatic tissue emerging when broken apart (Figure 10). None of the *Z. noltii* specimens had visible fibers.

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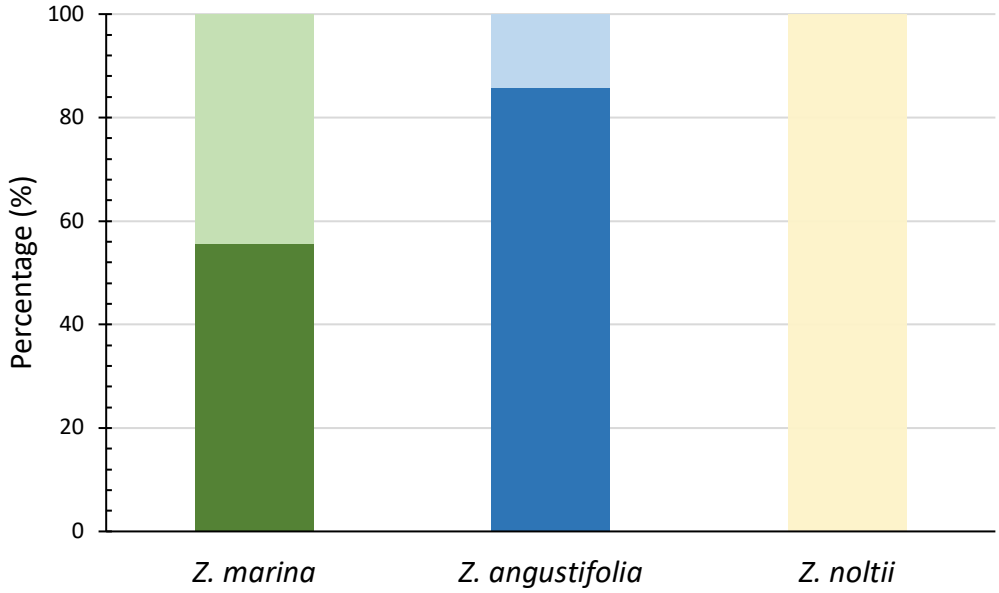

Figure 10. The cellulose fiber test scores presented as percentages of *Z. marina*, *Z. angustifolia*, and *Z. noltii* with long tough strands of sclerenchymatic tissue. Dark shade indicates individuals with visible fibers when pulling leaves apart, and light shade indicates those not fibrous.

3.1.5 Flowers and seeds

The flowers of *Z. marina* and *Z. angustifolia* were light green, and *Z. noltii's* flowers were red (Figure 11a, b, c). *Zostera marina* has larger seeds than *Z. angustifolia* and *Z. noltii* (Table 1). The *Z. marina* seeds had a green-brown color with stripes (Figure 11 1e). The *Z. angustifolia* seeds were dark brown, light yellow-brown, and light green with stripes (Figure 11 2e). The *Z. noltii* seeds were dark brown and green-brown without stripes (Figure 11 3e).

Table 1. Seed morphology of *Zostera marina*, *Z. angustifolia*, and *Z. noltii*.

	Z. marina	*Z. angustifolia*	*Z. noltii*
Seed length (mm)	3,45 ± 0,07	3,03 ± 0,3	2,05 ±0,07
Seed Color	Green-brown	Dark brown, light yellow-brown, and light green	Dark brown and green-brown
Seed Pattern	Stripes	Stripes	No stripes

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Figure 11. Reproduction morphology variations in **(1)** *Zostera marina*, **(2)** *Zostera angustifolia*, **(3)** *Zostera noltii.* **(a)** Flattened spadix with flowers on one side, enclosed within a spathe, **(b)** unfertilized female flowers, **(c)** fertilized female flowers and fruits, **(d)** Seeds and fruits, **(e)** Seeds. Scale: 1 mm. Photo: Eli Johansen.

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3.2 Molecular Analyses

3.2.1 Gene recovery, read depth and alignment

A total of 70.7 million reads was obtained for the 12 samples sequenced for this study, 54% of which were successfully mapped to the 353 angiosperms protein-coding loci. The number of genes recovered ranged from 310 to 325, and the percentage of the reads on target ranged from 42 to 62%. There were 16 paralogous loci flagged by HybPiper (Figure 12). Sequences were obtained for 342 nuclear regions, as different samples obtained sequences from different loci. After alignment and trimming, including the two outgroup taxa and *Z. marina* from the Kew Tree of Life Explorer, the gene alignment lengths ranged between 96 to 3503 base pairs. The number of parsimony informative sites was 19,806, where 72,5% was present in the 15 samples.

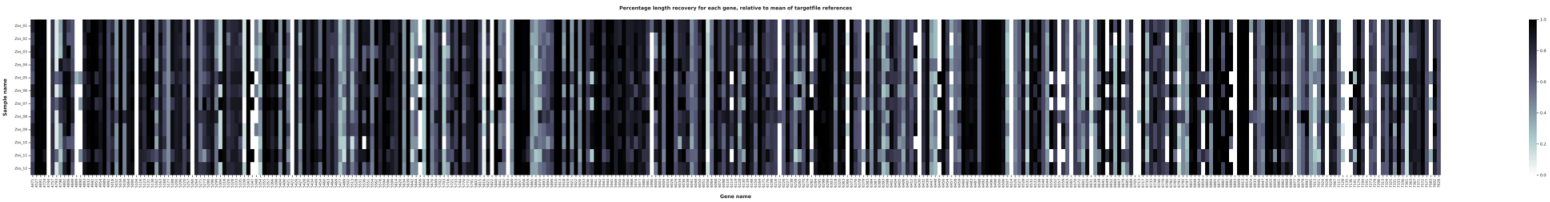

Figure 12. Heatmap depicting the recovery of *Zostera* samples and genes. The y-axis represents each sample, while the x-axis represents each gene. The color gradient indicates the extent of recovery, with darker shades indicating better recovery of both samples and genes.

3.2.2 Multispecies coalescent species tree

The final phylogenetic analysis was based on 15 specimens, 12 obtained in the sequenced target capture samples and three downloaded from the Kew Tree of Life Explorer (Baker et al., 2022) (Appendix 2). One multi-specie coalescent specie tree was based on 262 nuclear regions present in the 15 samples (100% gene coverage) (Figure 13). The second multi-specie coalescent specie tree was based on 342 nuclear regions (Appendix 1). The trees obtained from ASTRAL-III (Figure 13 and Appendix 1) had a final normalized quartet score of 0.68, which is considered to be strong support (Sayyari & Mirarab, 2016).

The phylogenetic tree splits into two sister clades. *Zostera noltii* diverges into a genetically distinct sister clade with high local posterior probability (LPP=1). Located in the sister clade,

Z. marina, is nor monophyletic as it is nested with *Z. angustifolia* with low posterior probability. *Zostera angustifolia* comes out as polytomy. The first *Z. marina* sample that diverges is from the Kew database with a high posterior probability value. The phylogenetic tree does not support the three species hypothesis (Figure 13).

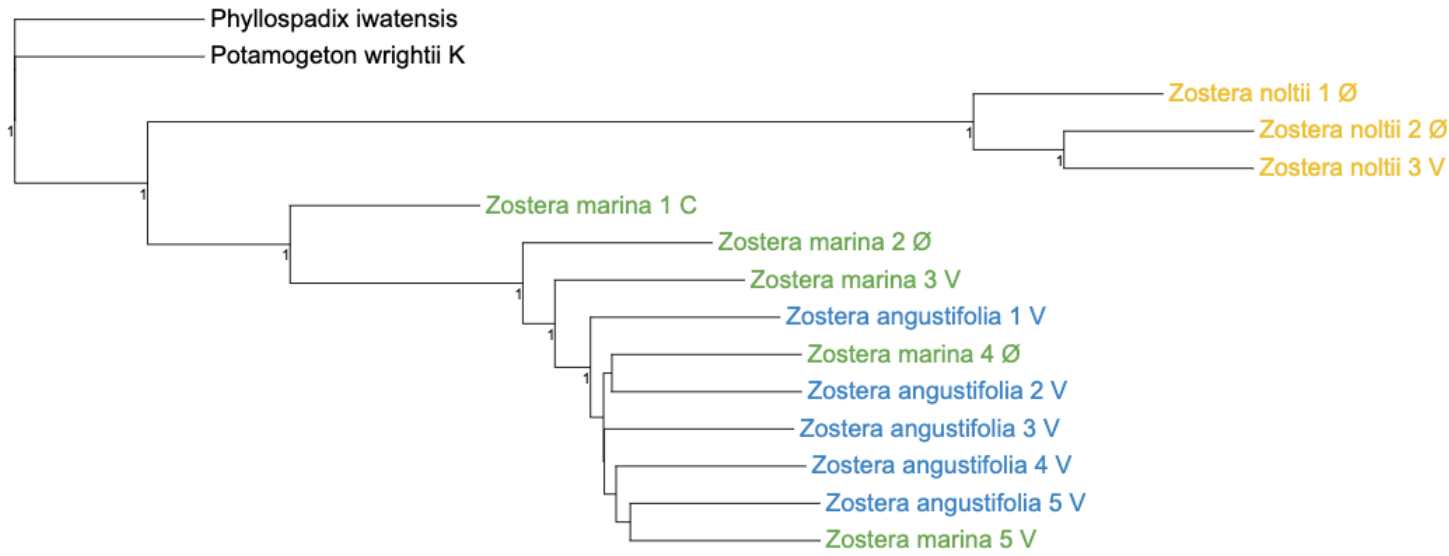

Figure 13. Multispecies coalescent tree inferred using ASTRAL-III with 100% gene coverage using 262 nuclear regions of the ingroup and outgroup taxa. The branch support values are local posterior probabilities (LPP) that were collapsed below LPP = 0.9. *Potamogeton wrightii* and *Phyllospadex iwatensis* are outgroups. C= Malibu, Southern California, USA. V= Vestfold, Bliksekilen, Norway. Ø= Østfold, Kurefjorden, Norway. X= Xiaoshi Island, Yellow Sea, China.

4 Discussion

This study found distinct morphological variations among *Zostera marina*, *Z. angustifolia*, and *Z. noltii*, which were not reflected in the molecular analyses. The morphology of the three taxa differs with respect to leaf width, apexes, number of veins, sclerenchymatic tissue, flowers and seeds. This study found a significant difference in leaf width among the three taxa (ANOVA) and *Z. marina* and *Z. angustifolia* specifically (t-test) (Figure 7). *Zostera noltii* had the highest total percentage of individuals with emarginate leaf apexes, then *Z. angustifolia,* and lastly *Z. marina* (Figure 8). The mucronate leaf apex was the dominant

form in the spring and emarginated in the fall for *Z. marina* and *Z. angustifolia*, and *Z. angustifolia* displayed the highest increase from spring to fall. Emarginate leaf apex was the dominant form for *Z. noltii* throughout the season. All the *Z. marina* specimens had five veins, *Z. noltii* had three, while the *Z. angustifolia* ranged between 3 and 5 (Figure 9). It was a notable difference between *Z. angustifolia* and *Z. marina*, with *Z. angustifolia* having a higher percentage of specimens with long, tough strands of sclerenchymatic tissue (Figure 10).

In shallow waters, where plants are exposed to strong waves and mechanical forces, it is advantageous for leaves to be flexible to bend with water currents. The presence of fibers in the leaves can help with this. None of the *Z. noltii* specimens had visible fibers, which may indicate that this species has a different adaptation strategy. In contrast, *Z. marina* had fewer and shorter strands of sclerenchymatic tissue, suggesting that it may be less well-adapted to these conditions. These findings align with the observations made by Rørslett and Mjelde (2021 a, b, c). The flowers of *Z. marina* and *Z. angustifolia* were light green, and *Z. noltii's* flowers were red (Figure 11b). The size differed among the three taxa, *Z. marina* having the largest seeds, then *Z. angustifolia*, and lastly *Z. marina* (Table 1). The *Z. marina* and *Z. angustifolia* seeds had stripes, while *Z. noltii* did not have stripes (Figure 10e). *Z. angustifolia* is consistent with what den Hartog (1970) defined as "ecotype" previously known as *Z. marina* var. *angustifolia*. This ecotype is characterized by smaller size and distinct growth strategies compared to the common *Z. marina* ecotype (den Hartog, 1972; Olsen et al., 2013; Talbot et al., 2016).

The molecular analyses, based on 262 (Figure 13) and 324 (Appendix 1) nuclear regions, clearly show that the *Zostera noltii* accessions resolves as a genetically distinct clade with high support (LPP= 1). The *Z. marina* and *Z. angustifolia* accessions forms a monophyletic clade, with high support (LPP= 1). This clade does not have much internal structure, however the Californian accession of *Z. marina* resolves as sister to the rest of the clade with Norwegian accession only (LPP= 1). *Zostera noltii* is clearly separated from the other taxa, which indicates that there are pre- or postzygotic fertilization barriers. Hence, this

analysis can clearly reject the hypothesis put forward by den Hartog (1970) that *Z. angustifolia* is a brackish water form of *Z. noltii.* The present study (Figure 13, Appendix 1) supports the suggestion that *Z. angustifolia* is a narrow-leaved form of *Z. marina*, like several other studies (den Hartog, 1972; Markgraf, 1972; Markgraf & Zoller, 1981; Stace, 1997, Borum et al., 2004).

On the other hand, the morphological analyses demonstrate a high variation within the complex. This high phenotypic plasticity has led to identification of as many as 13 subspecific names for the taxa (POWO, 2023). Earlier studies have reported that abiotic factors play a crucial role in shaping the morphology and physiology of *Z. marina*, which allows it to thrive in a wide range of marine habitats and this might explain the species' worldwide distribution. Light conditions (Olesen & Sand-Jensen, 1993), nutrient availability (Short, 1987), water motion (Li et al., 2023), and salinity (Nejrup & Pedersen, 2008) are factors known to affect the morphology of *Zostera*. Because of this plasticity we specifically selected two areas where all three taxa occur, to minimize environmental differences. *Zostera noltii* was sampled at an approximate depth of 0.5 meters*. Zostera marina* and *Z. angustifolia* were found growing in mixed beds. However, measurements of water depth and precise exposure to currents and waves were not recorded in this study.

The molecular analyses of individuals of the three taxa do not support the species delimitation based on morphology. This might have several reasons. A main limitation of the study is that the sampling approach may have influenced the results, as the collection of individuals might have been biased towards the broader, larger ones for *Z. marina* and the smaller, narrower ones for *Z. angustifolia*. As leaf width is an important character for *Z. angustifolia*, this is difficult to circumvent. This potential bias have implications for the interpretation of the morphological analyses.

Furthermore, during the fall few sterile shoots were observed in the samples, especially for *Z. angustifolia.* This might be due to the degradation of plant material by solar radiation, transportation, and storage (Vähätalo et al., 1998). This could have influenced the obtained results of leaf width (Figure 7) and number of veins (Figure 9), as fertile shoots

might have been mistaken for sterile shoots, which are known to be narrower (Evans, 1985). The mistake could also have affected the number of veins assigned to each specimen, as the number of veins might be correlates with leaf width.

The form of the leaf apex of vegetative shoots was assigned to each specimen as mucronate or emarginate (Figure 8). However, the two forms were found on leaves at the same shoot. The mucronate leaf apex was the dominant form in the spring and emarginated in the fall for all three taxa. Indicating that juvenile and intermediate vegetative leaves have the emarginat form while adult leaves have the mucronate form, making this character less useful in species diagnostics. However, almost all *Z. noltii* specimens (94%) had emarginate leaf apexes throughout the season. These findings are consistent with the description of the plants by Rørslett & Mjelde (2021a), which suggests that some of the taxa become emarginate with maturity. The present study suggests that emarginate leaf apexes is a sign of structural damage from mechanical stress, varying with taxa and season.

Compared with the study by Rørslett & Mjelde (2021a), the length of the *Z. marina* seeds was shorter in the present study (Table 1). The smaller seed size in the present study might be due to lack of maturity of the seeds. Additionally, only a few selected seeds were available for analysis. In future studies, a larger sample size would be necessary to support the present observations. Despite these overall differences, the variation in seed measurements had a large standard error, making it challenging to analyse and compare the different taxa. The small sample size result in nonsignificant differences. However, the test had too low power to be able to identify differences in seed size. A larger sample size is needed to make a more robust test and conclusion. Additionally, there was no guarantee that the seeds were fully ripe, further complicating the analyses.

Due to potential limitations in the present study, it is still possible that there are three distinct species present. Factors such as hybridization, introgression, and genetic mixing could have influenced the observed pattern. Hybridization can introduce challenges in species identification, making it difficult to differentiate between them based solely on the analyzed data. Given their overlapping habitat and non-discriminatory water pollination method, it is expected that introgression and mixing could contribute to the observed

patterns, including genetic exchange and the blending of traits among species. It is more typical to see genetic mixing in populations with sexual reproduction rather than a sharp divide, which would be expected with vegetative reproduction (Ruckelshaus, 1996). These processes may introduce complexities in species delimitation and emphasize the need for further investigations considering hybridization and genetic exchange to obtain a more comprehensive understanding of species diversity within this system.

Coyer et al. (2013) suggested that the large species, *Z. marina*, and the small species, *Z. noltii,* could potentially crossbreed, resulting in hybrids. However, there is no present evidence to suggest that this is occurring. If crossbreeding occurs, the offspring should either be sterile or establish itself as an allopolyploidy with double chromosomes, but in this case, they all have the same chromosome number 2n = 12 (Elven et al 2022). Hybridization at the same ploidy level is very rare among plants (Nowak et al 2020).

Previous studies have used microsatellite loci (Olsen et al., 2013) and multiple genetic loci (Coyer et al., 2013) and did not find any significant genetic distinction between *Z. marina* and *Z. angustifolia.* One interpretation of the application of such methods is that they are not sensitive enough to discriminate between the closely related *Z. marina* and *Z. angustifolia.* Therefore, in the present study a method (target capture analysis) that should be both sensitive and powerful including more than 300 nuclear regions (Appendix 1) was chosen. This method is clearly sensitive enough to discriminate on species level, as *Z. noltii* is forming a well-supported monophyletic clade. However, *Z. marina* and *Z. angustifolia* did not form monophyletic clades. This means that these two taxa are more closely related to each other than to *Z. noltii*. One could claim that this analysis is not sensitive enough to separate between *Z. marina* and *Z. angustifolia*. However, as the accessions of the two potential taxa are intermingled, and e.g *Z. marina* 4 and 5 are more closely related to *Z. angusitifolia*, than they are to *Z. marina* 1,2, and 3. This indicates that the applied method is adequate to investigate the main research question; how many *Zostera* species do we have in Norway?

Eli Johansen
University of Oslo
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5 Conclusions

The molecular analyses support two species; *Zostera noltii* and the *Z. angustifolia*/*Z. marina* complex. As *Z. marina* is a Linnaeus name from 1753, it has priority over Hornemann's *Z. angustifolia* from 1816, and *Z. marina* is subsequently the valid name.

6 Future research

The present study does not support the delimitation of *Zostera angustifolia* at the subspecific level; however, another analysis including accessions from a wider geographical area could possibly conclude differently.

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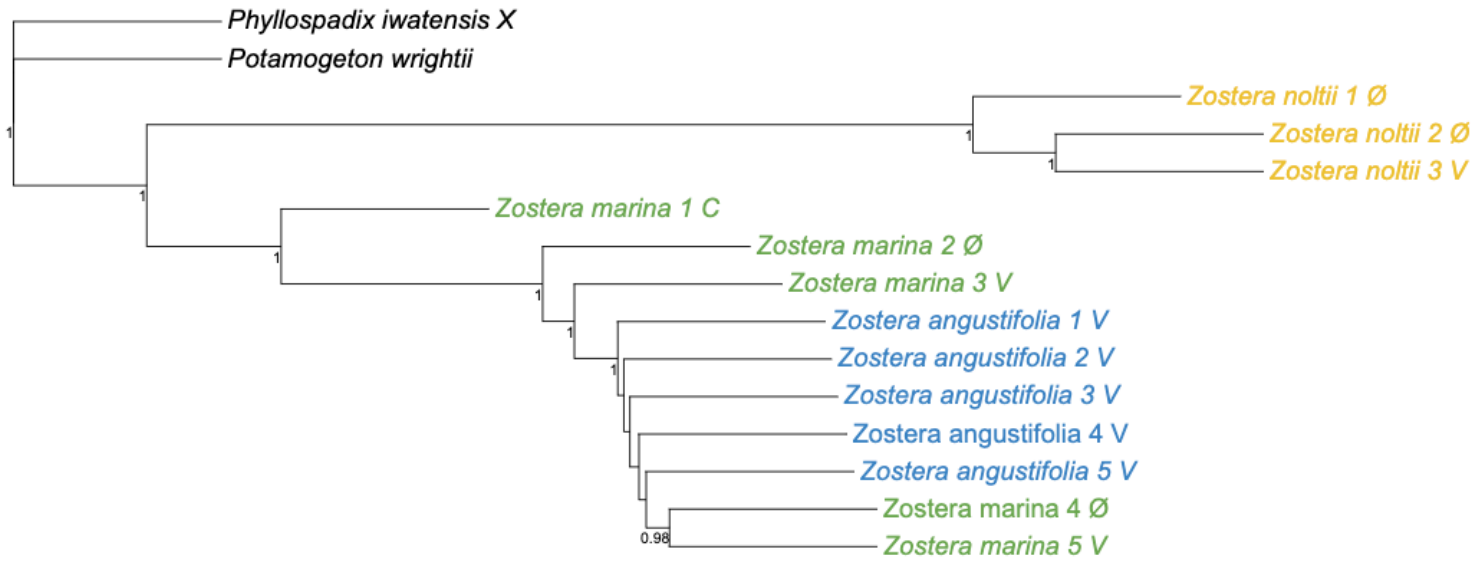
Appendix



Appendix 1. Multispecies coalescent tree inferred using ASTRAL-III based on 342 nuclear regions of the ingroup and outgroup taxa. The branch support values are local posterior probabilities (LPP) that were collapsed below LPP = 0.9.. The tree is rooted by *Potamogeton wrightii* and *Phyllospadex iwatensis*. C= Malibu, Southern California, USA. V= Vestfold. Ø= Østfold, Kurefjorden, Norway. X= Xiaoshi Island, Yellow Sea, China.

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Appendix 2. List with voucher information (taxon name, voucher information, herbarium and origin) of specimens used in the morphological and phylogenetic analysis. Species from the genera *Phyllospadix iwatensis* and *Potamogeton wrightii* are serving as outgroups in the multispecies coalescent tree and were downloaded from the Kew Tree of Life Explorer database. Herb. = voucher holding herbarium. Abbreviations: O= Oslo Botanical garden. K= Kew Royal botanical garden. n/a = not available. Number after taxon names refers to individuals in the multispecies coalescent trees. E. Johansen et al, refers to the following persons: H. Christie, S. Fredriksen, W. Eikrem, B. Rørslett, E. Rinde, C. Sletten.

Taxon name	Voucher Specimen (Herb)	Analysis	Location
Zostera marina (1)	Savolainen, V. 98/3 (K)	DNA	Malibu, Southern California, USA
Z.marina (2)	E. Johansen et al., 7. (O)	DNA, Morphology	Kurefjorden, Østfold, Norway
Z. marina (3)	E. Johansen, 89. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	DNA, Morphology	Horten, Vestfold, Norway
Z. marina (4)	Johansen, E51, E. Rinde. (O)	DNA, Morphology	Horten, Vestfold, Norway
Z, marina (5)	E. Johansen et al., 28. (O)	DNA, Morphology	Horten, Vestfold, Norway
Zostera angustifolia (1)	E. Johansen, 70. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	DNA, Morphology	Kurefjorden, Østfold, Norway
Z. angustifolia (2)	E. Johansen et al., 36. (O)	DNA, Morphology	Horten, Vestfold, Norway
Z. angustifolia (3)	E. Johansen et al., 34. (O)	DNA, Morphology	Horten, Vestfold, Norway
Z. angustifolia (4)	E. Johansen, 103. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	DNA, Morphology	Horten, Vestfold, Norway
Z. angustifolia (5)	E. Johansen, 67. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	DNA, Morphology	Kurefjorden, Østfold, Norway
Zostera noltii (1)	E. Johansen, 79. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	DNA, Morphology	Kurefjorden, Østfold, Norway
Z. noltii (2)	E. Johansen, 78. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	DNA, Morphology	Kurefjorden, Østfold, Norway
Z. noltii (3)	E. Johansen et al., 42. (O)	DNA, Morphology	Horten, Vestfold, Norway
Phyllospadix iwatensis	n/a	DNA	Xiaoshi Island, Yellow Sea, China
Potamogeton wrightii	Maurin, O. 4353 (K)	DNA	n/a
Z. marina	E. Johansen et al. , 1. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. marina	E. Johansen et al. , 2. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. marina	E. Johansen et al., E3. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. marina	E. Johansen et al., E4. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. marina	E. Johansen et al. , 5. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. marina	E. Johansen et al., 6. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. angustifolia	E. Johansen et al. , 8. (O)	Morphology	Kurefjorden, Østfold, Norway

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Taxon name	Voucher Specimen (Herb)	Analysis	Location
Z. angustifolia	E. Johansen et al. , 9. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. angustifolia	E. Johansen et al. , 10. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. angustifolia	E. Johansen et al., 11. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. angustifolia	E. Johansen et al. , 12. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. angustifolia	E. Johansen et al., 13. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. angustifolia	E. Johansen et al., 14. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. angustifolia	E. Johansen et al., 15. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. angustifolia	E. Johansen et al., 16. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. noltii	E. Johansen et al., 17. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. noltii	E. Johansen et al., 18. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. noltii	E. Johansen et al., 19. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. noltii	E. Johansen et al., 20. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. noltii	E. Johansen et al., 21. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. noltii	E. Johansen et al., 22. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. noltii	E. Johansen et al., 23. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. noltii	E. Johansen et al., 24. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. noltii	E. Johansen et al., 25. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. noltii	E. Johansen et al., 26. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. marina	E. Johansen et al., 27. (O)	Morphology	Horten, Vestfold, Norway
Z. marina	E. Johansen et al., 29. (O)	Morphology	Horten, Vestfold, Norway
Z. marina	E. Johansen et al., 30. (O)	Morphology	Horten, Vestfold, Norway
Z. marina	E. Johansen et al., 31. (O)	Morphology	Horten, Vestfold, Norway
Z. angustifolia	E. Johansen et al., 32. (O)	Morphology	Horten, Vestfold, Norway
Z. angustifolia	E. Johansen et al., 33. (O)	Morphology	Horten, Vestfold, Norway
Z. angustifolia	E. Johansen et al., 35. (O)	Morphology	Horten, Vestfold, Norway
Z. noltii	E. Johansen et al., 39. (O)	Morphology	Horten, Vestfold, Norway
Z. noltii	E. Johansen et al., 40. (O)	Morphology	Horten, Vestfold, Norway
Z. noltii	E. Johansen et al., 41. (O)	Morphology	Horten, Vestfold, Norway
Z. noltii	E. Johansen et al., 43. (O)	Morphology	Horten, Vestfold, Norway
Z. noltii	E. Johansen et al., 44. (O)	Morphology	Horten, Vestfold, Norway
Z. noltii	E. Johansen et al., 45. (O)	Morphology	Horten, Vestfold, Norway
Z. noltii	E. Johansen et al., 46. (O)	Morphology	Horten, Vestfold, Norway
Z. marina	Johansen, 47, E. Rinde. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. marina	Johansen, 48, E. Rinde. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. marina	Johansen, 49, E. Rinde. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. marina	Johansen, 50, E. Rinde. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. noltii	Johansen, 52, E. Rinde. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. noltii	Johansen, 53, E. Rinde. (O)	Morphology	Kurefjorden, Østfold, Norway

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Taxon name	Voucher Specimen (Herb)	Analysis	Location
Z. marina	E. Johansen, 54. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. marina	E. Johansen, 55. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. marina	E. Johansen, 56. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. marina	E. Johansen, 57. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. marina	E. Johansen, 58. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. marina	E. Johansen, 59. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. marina	E. Johansen, 60. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. marina	E. Johansen, 61. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. angustifolia	E. Johansen, 62. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. angustifolia	E. Johansen, 63. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. angustifolia	E. Johansen, 64. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. angustifolia	E. Johansen, 65. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. angustifolia	E. Johansen, 66. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. angustifolia	E. Johansen, 68. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. angustifolia	E. Johansen, 72. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. marina	E. Johansen, 73. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. noltii	E. Johansen, 75. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. noltii	E. Johansen, 76. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. noltii	E. Johansen, 77. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. noltii	E. Johansen, 80. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. noltii	E. Johansen, 81. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway

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Taxon name	Voucher Specimen (Herb)	Analysis	Location
Z. noltii	E. Johansen, 82. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. noltii	E. Johansen, 83. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. noltii	E. Johansen, 84. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. noltii	E. Johansen, 85. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. noltii	E. Johansen, 86. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Kurefjorden, Østfold, Norway
Z. marina	E. Johansen, 87. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Horten, Vestfold, Norway
Z. marina	E. Johansen, 88. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Horten, Vestfold, Norway
Z. marina	E. Johansen, 90. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Horten, Vestfold Kommune, Norway
Z. marina	E. Johansen, 91. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Horten, Vestfold Kommune, Norway
Z. marina	E. Johansen, 92. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Horten, Vestfold Kommune, Norway
Z. marina	E. Johansen, 93. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Horten, Vestfold Kommune, Norway
Z. marina	E. Johansen, 94. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Horten, Vestfold Kommune, Norway
Z. marina	E. Johansen, 95. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Horten, Vestfold Kommune, Norway
Z. angustifolia	E. Johansen, 96. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Horten, Vestfold Kommune, Norway
Z. angustifolia	E. Johansen, 97. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Horten, Vestfold Kommune, Norway
Z. angustifolia	E. Johansen, 98. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Horten, Vestfold Kommune, Norway
Z. angustifolia	E. Johansen, 99. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Horten, Vestfold Kommune, Norway
Z. angustifolia	E. Johansen, 100. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Horten, Vestfold Kommune, Norway
Z. angustifolia	E. Johansen, 101. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Horten, Vestfold Kommune, Norway
Z. angustifolia	E. Johansen, 102. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Horten, Vestfold Kommune, Norway
Z. angustifolia	E. Johansen, 104. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Horten, Vestfold Kommune, Norway

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Z. angustifolia	E. Johansen, 105. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Horten, Vestfold Kommune, Norway
Z. noltii	E. Johansen, 106. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Horten, Vestfold Kommune, Norway
Z. noltii	E. Johansen, 107. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Horten, Vestfold Kommune, Norway
Z. noltii	E. Johansen, 108. H. Christie, B. Rørslett, E. Rinde, C. Sletten. (O)	Morphology	Horten, Vestfold Kommune, Norway

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Appendix 3. Hybpiper statistics file summarises target enrichment and gene recovery efficiency for the sequenced *Zostera* samples. These include the number of reads, number of reads on target, percent reads on target, number of genes with reads, number of genes with contigs, number of genes with sequences, number of genes with sequences > 25% of the target length, number of genes with sequences > 50% of the target length, number of genes with sequences > 75% of the target length, number of genes with sequences > 150% of the target length, and the number of genes with paralog warnings.

	Number of Reads	Reads Mapped	Pct On Target	Genes Mapped	Genes With Contigs	Genes With Seqs	GenesAt 25pct	GenesAt 50pct	GenesAt 75pct	GenesAt 150pct	Paralog Warnings Long	Paralog Warnings Depth	Genes Without Stitched Contigs	Genes With Stitched Contigs	Genes With Stitched Contigs Skipped	Genes With Chimera Warning
Name																
Zos_01	5543126	3220059	58.1	352	339	318	310	259	190	0	2	4	196	122	0	0
Zos_02	5464766	2759895	50.5	353	333	312	302	255	180	0	3	6	187	125	0	0
Zos_03	7288646	4500115	61.7	352	342	319	303	255	172	0	4	6	178	141	0	0
Zos_04	5353262	2902983	54.2	352	331	316	308	264	194	0	1	2	197	119	0	0
Zos_05	5001294	2376840	47.5	352	343	322	314	268	194	0	1	3	197	125	0	0
Zos_06	5448412	3035830	55.7	352	333	315	307	260	190	0	2	3	198	117	0	0
Zos_07	5000662	2312285	46.2	352	342	321	313	264	195	0	1	4	194	127	0	0
Zos_08	7090294	4011063	56.6	352	341	325	316	281	210	0	11	17	188	137	0	0
Zos_09	8765134	4959965	56.6	352	342	320	308	269	196	0	3	4	200	120	0	0
Zos_10	3355942	1924147	57.3	352	334	310	299	255	185	0	2	2	197	113	0	0
Zos_11	3543076	1503742	42.4	352	339	319	309	260	188	0	1	4	198	121	0	0
Zos_12	8815564	4784851	54.3	352	342	316	306	263	191	0	5	8	188	128	0	0