Research Article

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Phylogeographic patterns in attached and free-living marine macroalga *Fucus vesiculosus* (Fucaceae, Phaeophyceae) in the Baltic Sea

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Abstract: Sequencing of a mitochondrial intergenic spacer and 23S subunit was used to investigate the phylogeographic patterns in *Fucus vesiculosus*. Samples originated from 21 sites spanning six subbasins of the Baltic Sea. We identify a putative ancestral mitochondrial haplotype that entered the Baltic Sea from the Atlantic, colonising extensively throughout the species' distribution. The dominance of this haplotype is seen in the low overall haplotype diversity (H_d = 0.29). Moreover, there is indication of few spatially aggregated patterns in the deeper demographic time scales (F_{ct} = 0.040; F_{st} = 0.049). Tajima's D (–0.685, p-value 0.297) and Fu's F_S (0.267, p-value 0.591) showed no significant signals of extreme demographic changes. The Baltic Sea free-living *Fucus* is confirmed as *F. vesiculosus* or a closely related species. Haplotype diversities are comparable between forms (attached H_d = 0.306; free-living H_d = 0.268). The relatively short temporal scale for colonisation alongside low variance in the *Fucus* mitochondrial genome results in a rather panmictic structure across the Baltic Sea. Our data suggest that the mitochondrial intergenic spacer and 23S poorly describe the evolutionary dynamics of *Fucus* spp. in such a young, postglacial environment, yet this concatenated-barcode advances our understanding of the colonisation dynamics of *F. vesiculosus* over deeper demographic timescales.

Keywords: barcode sequencing; bladderwrack; colonisation; genetic diversity; mitochondrial DNA.

1 Introduction

Patterns of genomic variation help describe species colonisation scenarios (Rius and Turon 2020). As a young postglacial environment, the Baltic Sea has a relatively recent colonisation history. The young age and marginal conditions result in low biodiversity, having approximately 10 times fewer species compared to the neighbouring North Sea (Elmgren and Hill 1997; Johannesson et al. 2011), with most populations inside the Baltic Sea being less genetically variable than their counterparts in the North Sea and adjacent areas (Johannesson and André 2006). Relatively few species provide the foundation of the coastal ecosystem. *Fucus* is one of the few genera of canopy forming, perennial macroalgae within the Baltic Sea, with *Fucus vesiculosus* having the most widespread distribution of the three native *Fucus* species (*F. radicans*, *F. serratus*, and *F. vesiculosus*). The species entered the Baltic Sea c. 4000–8000 years ago (Russell 1985). Two forms of this species are thought to occur, as either epilithic or benthopleustophytic (hereby referred to as attached and free-living). The forms can be found both sympatrically and allopatrically. Free-living forms display various morphotypes, yet all establish stable populations with unattached thalli lying singly or forming entangled mats on any substratum type. Generally found in more sheltered areas, they are common throughout the Baltic Sea (Luther 1981; Meyer et al. 2019; Svedelius 1901). Despite the widespread distribution, the current taxonomic status of the Baltic

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Sea free-living form is tentative due to the limitations of traditional morphological classification.

The Baltic Sea has had a tempestuous past, having only reached its current brackish state once the connections to the Atlantic re-opened c. 8000 years ago (Björck 1995). As a marine species, *F. vesiculosus* entered the Baltic Sea through these connections, colonising first the southern parts of the Baltic Sea in a west to east direction (Ardehed et al. 2016; Björck 1995; Rößler et al. 2011). The northern Baltic Sea was later colonised following a coastal pathway along the south eastern region (Zillén et al. 2008). Colonisation of new areas is generally characterised by recurrent founder effects and genetic bottlenecks; resulting in decreased genetic variation but also potentially in distinctive allele frequency patterns. Thus, the establishment of new *F. vesiculosus* populations within the Baltic Sea has presumably left distinct colonisation characteristics compared to the ancestral Atlantic population.

Populations of *F. vesiculosus* have also experienced rapid declines during the 1970s and 1980s in coastal zones of Estonia (Kukk and Martin 1992; Martin 2000), Finland (Haahtela 1984; Kangas et al. 1982; Rönnberg 1984; Rönnberg et al. 1985), Germany (Vogt and Schramm 1991), Lithuania (Olenin and Klovaité 1998), Poland (Plinski and Florczyk 1984), and Sweden (Engkvist et al. 2000; Kautsky et al. 1986; Lindvall 1984; Nilsson et al. 2004; Rosemarin et al. 1986). Population recovery has not occurred over much of the species distribution, including in some parts of Finland (Snickars et al. 2014), Germany (Fürhaupter et al. 2008), and Sweden (Nilsson et al. 2004). Moreover, in some areas of the Archipelago Sea, a continued decline of *F. vesiculosus* has been reported (Vahteri and Vuorinen 2016). Conversely, local recovery or expansions have occurred in other parts of Sweden (Engkvist et al. 2002; Eriksson et al. 1998; Nilsson et al. 2004) and Finland (Kangas and Niemi 1985; Rönnberg et al. 1985).

Accordingly, the Baltic Sea *F. vesiculosus* population has undergone both recent and historic demographic changes that may dramatically affect the genetic structure and diversity of the population. However, the effects of these demographic changes when considering both forms concurrently are poorly understood. This study investigated the genetic structure and demographic history of both forms of *F. vesiculosus* within the Baltic Sea using mitochondrial markers. There is no universally accepted DNA barcoding marker for brown algae, with barcodes often selected depending on the genus studied (Bartolo et al. 2020). Consequently the most informative marker currently available for *Fucus*, the mitochondrial (mtDNA) intergenic spacer (IGS), was selected alongside the lower resolution mtDNA 23S subunit region (Coyer et al. 2006a). The study aimed to identify phylogeographic patterns within the Baltic population, particularly those relating to the intraspecific relationships between forms. We predicted that in the deeper demographic timescale both forms would be well connected and originate from the same genetic source.

2 Materials and methods

2.1 Study location and sample collection

Fucus vesiculosus thalli were sampled during 2017–2018 from 21 sites spanning six subbasins (Table 1). All sites were in close proximity to the shore in relatively sheltered inlets, often connected to a larger channel with greater water movement. These sheltered locations were often associated with common reed *Phragmites australis* beds. Free-living populations were either lying individually or forming dense mats on the bottom substratum or on occasion entwined with *P. australis*. Attached populations were adhered to bedrock, stones or pebbles. At each site several 5 cm pieces of thalli from each form were randomly sampled by SCUBA diving, snorkelling or wading. In allopatric sites only one form was sampled. Attached samples were taken from hard-bottom substrata, whereas free-living samples were from both soft and hard bottom substrata. Sampling depth varied per site within the interval of 0.5–4 m. At sympatric sites collection of samples occurred at the most similar depth possible. All sample sites were known to consist solely of *F. vesiculosus*, as defined by the recorded distribution of *F. vesiculosus*, *F. radicans*, and *F. serratus*. Additional morphological identification of thalli from both forms was used to provide further confirmation of an absence of *F. radicans*. All samples were cleaned of epiphytes and stored in silica gel. To ensure all thalli were unique individuals, DNA microsatellite genotyping was performed to allow for the identification and removal of clonal lineages (Preston et al. 2022).

2.2 DNA extraction and sequencing

Genomic DNA was extracted using the NucleoSpin® plant II DNA extraction kit (Machery-Nagel, 740,770.250) from approximately 1 g of dried apical tips. Extraction followed the standard kit protocol and PL1 buffer for cell lysis with a single protocol alteration of an extended incubation of >30 min within PL1. The mtDNA IGS and 23S were targeted using primers from Coyer et al. (2006a). Additional nuclear and organelle markers (plastid-encoded intergenic RuBisCO spacer and internal transcribed spacer region of the nuclear ribosomal DNA [nrDNA-ITS]) were screened, but both provided low sequence variance or poor sequencing reads (Supplementary Table S1). The conclusion to not utilise the nrDNA-ITS is supported by previous studies that note the marker was poor in resolving relationships between closely related *Fucus* species (Coyer et al. 2006a; Laughinghouse et al. 2015; Serrão et al. 1999). First stage PCR reactions (12.5 µl total volume) consisted of 6.25 µl of AccuStart™ II PCR ToughMix® (Quantabio, 95,142), 0.625 µl of each primer (10 µM), 2.5 µl of diluted DNA and 2.5 µl of mQH_2O. Reactions were run on a Veriti 96-Well (Applied Biosystems™) thermal cycler with an initial denaturing step at 94 °C for 3 min, 30 cycles of denaturing at 94 °C for

Table 1: Location, sampling period and number of samples of *Fucus vesiculosus* from the Baltic Sea used in the study.

Site code	Subbasin	Region	Locality	Site location[a] (decimal degrees)	Year	Site type	No. of thalli sequenced	
							Free-living	Attached
AS	Northern Baltic Proper	Askö	Sweden	58.81731 17.46569	2017	Mixed	3	3
AS	Northern Baltic Proper	Askö	Sweden	58.82953 17.61564	2017	Mixed	3	3
AS	Northern Baltic Proper	Askö	Sweden	58.806 17.51478	2017	Mixed	2	3
AS	Northern Baltic Proper	Askö	Sweden	58.89456 17.62786	2017	Mixed	3	3
AS	Northern Baltic Proper	Askö	Sweden	58.93689 17.60736	2017	Mixed	3	3
AS	Northern Baltic Proper	Askö	Sweden	58.90914 17.66022	2017	Mixed	3	3
HS	Arkona Basin	Hiddensee	Germany	54.58202 13.11155	2018	Mixed	3	3
HS	Arkona Basin	Hiddensee	Germany	54.60418 13.14341	2018	Attached	–	3
OL	Gulf of Bothnia	Olkiluoto	Finland	61.236 21.42139	2017	Free-living	2	–
SA	Gulf of Riga	Saaremaa	Estonia	58.37155 22.97865	2018	Free-living	3	–
SA	Gulf of Riga	Saaremaa	Estonia	58.21857 22.50418	2018	Free-living	3	–
SE	Archipelago Sea	Seili	Finland	60.23086 21.95522	2018	Mixed	3	2
SE	Archipelago Sea	Seili	Finland	60.23871 21.94003	2018	Mixed	1	3
TZ	Gulf of Finland	Tvärminne	Finland	59.90994 23.38147	2017	Mixed	3	3
TZ	Gulf of Finland	Tvärminne	Finland	59.90469 23.37603	2017	Mixed	3	3
TZ	Gulf of Finland	Tvärminne	Finland	59.89903 23.3915	2017	Mixed	3	3
TZ	Gulf of Finland	Tvärminne	Finland	59.89328 23.38222	2017	Mixed	3	3
TZ	Gulf of Finland	Tvärminne	Finland	59.85006 23.24636	2018	Mixed	3	3
TZ	Gulf of Finland	Tvärminne	Finland	59.81163 23.20649	2017	Attached	–	3
TZ	Gulf of Finland	Tvärminne	Finland	59.839 23.24913	2017	Attached	–	3
TZ	Gulf of Finland	Tvärminne	Finland	59.81239 23.10628	2017	Attached	–	3

[a]Values listed as latitude (1st) and longitude (2nd).

30 s, annealing at 51 °C for 30 s and extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. Amplified products were checked for quality using gel electrophoresis and subsequently purified using ExoSAP-IT™ (Applied Biosystems™, 78,200.200.UL) following the standard kit protocol using 2 µl of amplified product. Purified PCR products were diluted to approximately 10 ng of template DNA per reaction. Second stage sequencing PCR reactions (10 µl total volume) consisted of 0.25 µl of BigDye™ Terminator v1.1 (Applied Biosystems™, 4,337,450), 2 µl of BigDye™ Terminator v1.1 & v3.1 5X Sequencing Buffer (Applied Biosystems™, 4,336,697), 1.3 µl forward or reverse primer [10 µM], 1 µl of diluted PCR product, and 5.45 µl of mQH_2O. PCR reactions were run with an initial denaturing step at 96 °C for 1 min, 30 cycles of denaturing at 96 °C for 30 s, annealing at 50 °C for 15 s and extension at 60 °C for 4 min, and a final extension at 72 °C for 10 min. Sequencing amplification products were purified using Cleanseq® Dye-Terminator Removal Kit (Agencourt®, A29151) following the standard kit protocol with a final elution of 40 µl in 0.1 mM EDTA, pH 8.0, RNase-free buffer (Invitrogen™, AM9912).

Final reactions were analysed on an ABI 3730 DNA analyser (Applied Biosystems™) at the Molecular Ecology and Systematics Laboratory, University of Helsinki.

Due to noisy signalling in the sequencing chromatograms, cloning of PCR-amplified fragments was employed to provide readable sequences. Five mtDNA IGS and 16 mtDNA 23S sequences were obtained in this manner. Cloning was performed using the TOPO™ TA Cloning™ Kit for Sequencing with pCR™4-TOPO® TA vector (Invitrogen; 450,030) and DH5α *Escherichia coli* competent cells. Ligation was performed using the standard kit protocol and approximately 20 ng of fresh PCR product. Transformation was performed by heat shock, then competent cells were spread onto ampicillin LB plates and incubated overnight. Clones were checked for inserts using blue-white screening. Colony PCR provided secondary screening for inserts and was performed on recombinant clones using the commercially available sequencing primer M13. Colony PCR reactions (12.5 µl total reaction) consisted of 6.25 µl OneTaq® Hot Start 2X Master Mix with Standard Buffer (New England Biolabs, M0484S), 0.5 µl of each primer (10 µM), and 5.75 µl of mQH_2O. Single recombinant clones inoculated each PCR mix, with multiple colony PCRs being performed per plate. PCR reactions were run with an initial denaturing step at 94 °C for 5 min, 35 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 40 s and extension at 68 °C for 45 s, and a final extension at 68 °C for 5 min. Reactions with inserts of appropriate size were then processed identically to non-clone reactions, with the exception of M13 Forward primers being used in the sequencing of the cloned PCR.

2.3 Data analysis

A total of 100 individual thalli were sequenced either through traditional PCR or cloning for each marker (see Supplementary Table S2 for number of thalli analysed). Each of the 100 individual thalli had representative sequences for both markers. Forward and reverse sequences were aligned by ClustalW and edited with MEGA X (Kumar et al. 2018) and by eye. As all cloned sequences were obtained using the M13 forward primer, multiple forward sequences were checked for congruence per cloned sample. Unique sequences were deposited to GenBank under accession numbers MZ711443–MZ711447 (IGS) and MZ779027–MZ779028 (23S). Trimmed mtDNA IGS and 23S sequences were combined to form a concatenated-barcode sequence of 866 bp for each sample. These sequences were used within all haplotype analyses.

BLASTN searches were used to obtain additional sequences from the National Center for Biotechnology Information (NCBI) (Altschul et al. 1990) (Supplementary Table S3). For factorial analysis, mtDNA IGS sequences for *F. vesiculosus* and related *Fucus* spp. were obtained, totalling 194 sequences. Factorial analysis was performed on DARwin v. 6.0.021 (Perrier and Jacquemoud-Collet 2006), using the Kimura dissimilarity method with a pairwise deletion threshold of 80% and 2000 bootstrapping. A second BLASTN search was used to obtain shorter sequences from the most polymorphic region (c. 210 bp) (pr-IGS) of the longer IGS for *F. vesiculosus* (Coyer et al. 2011a, 2006a). Of the sequences returned only those with geographic identifiers were selected due to the discrepancies in the reporting of haplotype frequencies into the repository. All *F. vesiculosus* mtDNA IGS sequences collected from both BLASTN searches were trimmed to 207 bp and repeat haplotypes from the same geographic area were removed. Repeat sequences per geographic area were defined by the country of origin, or in the case of USA, Canada and France, the state/province/region, that had been defined within the original study's sampling model. A haplotype network was created using the default haplotype network settings of the R package Pegas: Population and Evolutionary Genetics Analysis System (Paradis 2010).

Genetic differentiation (F_{ST}), nucleotide diversity (π), haplotype diversity (H_d) and the average number of nucleotide differences (K) were calculated using DnaSP v6 (Rozas et al. 2017). The genetic structure of the Baltic Sea *F. vesiculosus* populations was assessed with several Analysis of Molecular Variance (AMOVAs), which estimate the amount of variation explained by each hierarchical level, and uses covariance components to estimate fixation indices based on haplotype frequencies associated with different levels. AMOVAs and associated F_{ST} values were estimated using Arlequin 3.5.2.2 (Excoffier and Lischer 2010) with p-values generated using 1000 permutations. Two *a priori* hypotheses were used within AMOVA analyses. In hypothesis I, populations were first grouped by form and then by populations at each subbasin. This assumes a structuring of groups by form, and then that populations in each subbasin possess secondary structuring within each form. In hypothesis II, populations were first grouped by subbasin and then by form within these geographic groups. This assumes a primary structure formed by geographic location and a secondary structure of up to two different forms in each subbasin. Both hypothesises were modified *a posteriori* to exclude the attached site at Seili. AMOVAs were performed for the whole population and for each form or subbasin (Askö, Seili, Tvärminne) separately.

Additionally, relative frequencies of haplotypes and two tests for neutrality; Tajima's D (Tajima 1989) and Fu's F_S (Fu 1997) were implemented in Arlequin 3.5.2.2 (Excoffier and Lischer 2010). Tests for neutrality had p-values generated using 1000 permutations. A haplotype analysis map was drawn using PopART (Leigh and Bryant 2015) followed by manual editing. Relationships between haplotypes were estimated using default haplotype network settings on R package Pegas: Population and Evolutionary Genetics Analysis System (Paradis 2010). Rarefaction analysis to determine haplotype richness was performed using iNEXT Online (Chao et al. 2016) with bootstrapping of 1000 and a confidence interval of 0.95%.

3 Results

3.1 Resolution of intra- and interspecific phylogenetic relationships

Factorial analysis of mtDNA IGS haplotype sequences of several *Fucus* species (Figure 1) identifies three clusters: clusters A and C represent the previously recorded species complexes of 1: *F. vesiculosus*, *F. spiralis*, *F. vesiculosus* var. *spiralis*, *F. cottonii*, *F. virsoides*, and *F. guiryi*; and 2: *F. gardneri*, *F. distichus*, and *F. evanescens*, respectively, whereas cluster B represents a single species, *F. ceranoides*, that had previously been resolved within species complex 1 (Coyer et al. 2006a; Serrão et al. 1999) although clear demarcation of *F. ceranoides* from all other species within

complex 1 is supported by Neiva et al., (2010). The five Baltic Sea IGS sequences obtained in this study fall within cluster A, identifying them as *F. vesiculosus* or closely related species within the species complex.

3.2 Intraspecific diversity

Low nucleotide diversity was observed within the whole population (π = 0.001, average pairwise F_{ST} = 0.002). The F_{st} value demonstrated a rather genetically homogenous structure across the majority of the *F. vesiculosus* distribution. There was discrepancy in the levels of genetic variation between nucleotide and haplotype diversities within the populations (Table 2). The intraspecific genetic diversity within the Baltic Sea population therefore is for the most part a manifest of the number and composition of haplotypes within the population rather than that of nucleotide differences. A total of six haplotypes were identified in the concatenated-data, with the number of haplotypes varying from one to five in individual sites. The most common haplotype (H-I) was found in all populations, whilst the forms shared two haplotypes (H-I, H-VI). The overall haplotype diversity was relatively low, but varied widely among populations (H_d scale = 0–0.56, Table 2). The attached populations showed a greater haplotype diversity, containing all but one of the haplotypes, whilst the free-living populations consisted of three haplotypes and a lower haplotype diversity (Table 2).

Haplotype networks illustrate the relationships among DNA sequences between haplotype and population. Within the network the most common haplotype (H-I), present in all populations, forms the central node (Figure 2A). Two groups of less common haplotypes radiated from the central haplotype, one with two smaller nodes and the other with a doubleton and two singleton haplotypes. The first group represents 12% (n = 12) of the sampled individuals, whilst the second 4% (n = 4). These groups are not well resolved as H-IV and H-V are also connected to H-VI by only two mutational differences.

Table 2: Haplotype analysis using concatenated-barcode mtDNA intergenic spacer (IGS) and 23S for the whole Baltic sea population of *Fucus vesiculosus*, by form and by subbasin.

	n	*h*	*c-h*	H_d	*K*
Whole population	100	6	I, II, III, IV, V, VI	0.286	1.223
Attached	53	5	I, II, IV, V, VI	0.306	1.771
Free-living	47	3	I, III, VI	0.268	0.601
AS	35	5	I, II, III, V, VI	0.402	2.326
AS A	18	3	I, II, V	0.307	3.320
AS F	17	3	I, III, VI	0.485	1.103
HS	9	1	I	0.000	0.000
HS A	6	1	I	0.000	0.000
HS F	3	1	I	0.000	0.000
OL	2	1	I	0.000	0.000
SA	6	2	I, VI	0.533	1.067
SE	9	2	I, VI	0.556	1.111
SE A	5	2	I, VI	0.400	0.800
SE F	4	1	I	0.000	0.000
TZ	39	3	I, IV, VI	0.101	0.513
TZ A	24	3	I, IV, VI	0.163	0.833
TZ F	15	1	I	0.000	0.000

IGS, mtDNA intergenic spacer; 23S, mtDNA 23S; *n*, number of samples; *h*, number of haplotypes; *c-h*, mtDNA haplotype identification; H_d, haplotype diversity; *K*, average number of nucleotide differences. Site abbreviations: AS, Askö; HS, Hiddensee; OL, Olkiluoto; SA, Saaremaa; SE, Seili; TZ, Tvärminne. Form abbreviations: A, attached; F, free-living.

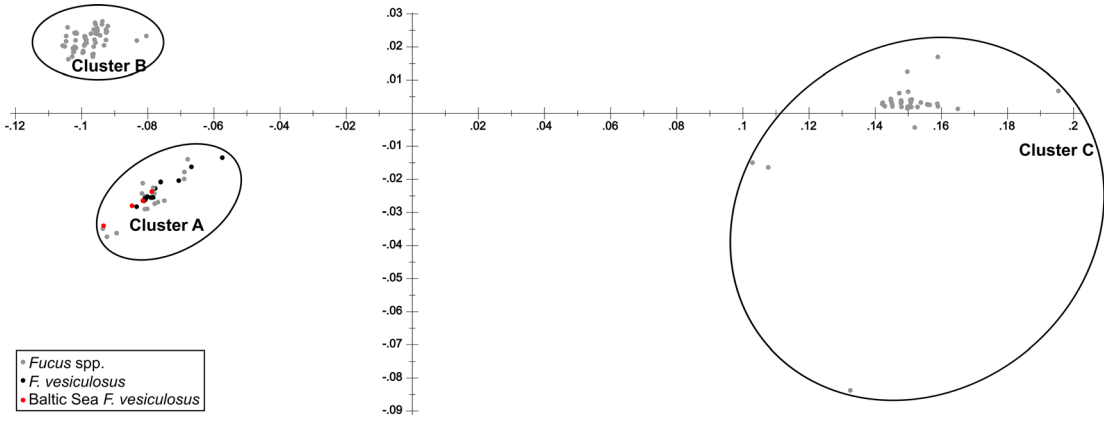

Figure 1: Factorial analysis using mtDNA intergenic spacer (IGS) sequences for several *Fucus* species. Black, red and grey points represent *Fucus vesiculosus*, Baltic sea *F. vesiculosus*, and all other *Fucus* species, respectively. Species within cluster A: *F. vesiculosus*, *F. spiralis*, *F. vesiculosus* var. *spiralis*, *F. cottonii*, *F. virsoides*, *F. guiryi*; cluster B: *F. ceranoides*; cluster C: *F. gardneri*, *F. distichus*, *F. evanescens*.

Sequence divergence is generally low. The network does not show distinct groups relating to site. As predicted by coalescent theory the ancestral haplotypes will be those that are most frequently sampled and will likely have a larger number of connections or descendant haplotypes. Consequently, the common haplotype (H-I) is the putative ancestral sequence within the Baltic Sea, from which the other haplotypes arose.

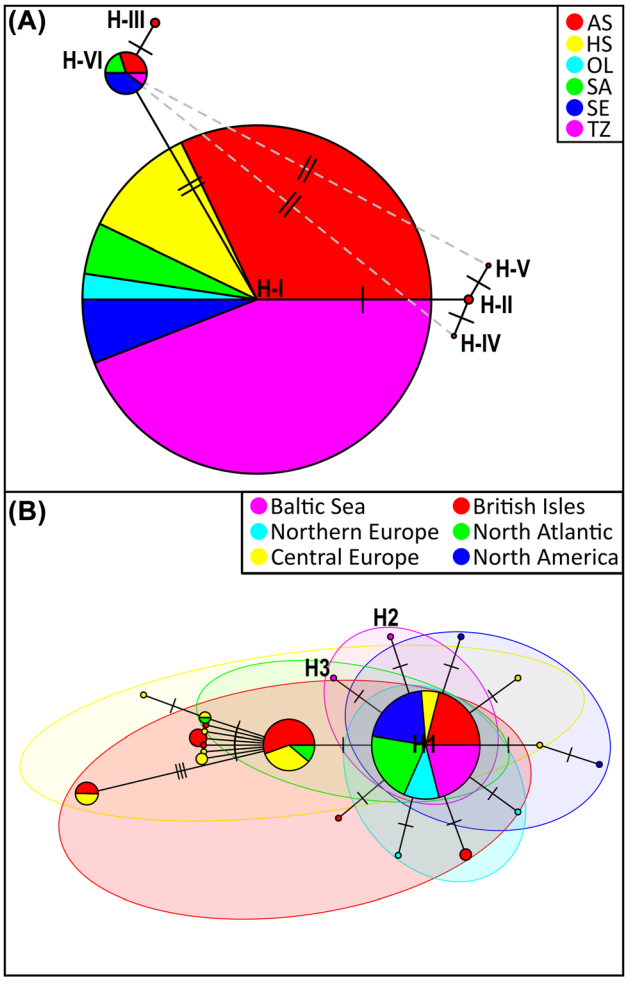

Figure 2: Haplotype network analysis of *Fucus vesiculosus* using (A) concatenated-barcode mtDNA intergenic spacer (IGS) and 23S, and (B) mtDNA polymorphic region-intergenic spacer (pr-IGS). Circle size is proportional to the haplotype frequency. A branch represents a genetic distance and hash marks represent a single mutation. Branches drawn in broken grey lines represent genetic distances between non-linked haplotypes [not shown in (B)]. All undrawn branches between non-linked haplotypes within B equal <2 mutations. Segment colouration indicates the geographic origin of each haplotype whilst ellipses represent the coverage of each geographic region within the network. Site abbreviations: As, Askö; HS, Hiddensee; OL, Olkiluoto; SA, Saaremaa; SE, Seili; TZ, Tvärminne.

The global network of mtDNA pr-IGS haplotypes identified three haplotypes within the Baltic Sea (Figure 2B). Concatenated-barcode haplotypes H-I, H-IV, H-V, and H-VI were included within pr-IGS H1, and concatenated-barcode haplotypes H-II and H-III were represented by pr-IGS H2 and H3, respectively. Globally 21 haplotypes were observed. Mutations between all global sequences equate to a maximum of three. Haplotype H1 forms a large, central cluster as the most abundant and widespread haplotype for the species. As this haplotype corresponds with the Baltic Sea putative ancestral haplotype this indicates that the ancestral haplotype of the Baltic Sea originated from outside the Baltic Sea. Two unique pr-IGS haplotypes spanning from the central node by a single mutation further supports this by demonstrating the formation of new haplotypes within the Baltic Sea itself.

3.3 Population structure and connectivity

The six haplotypes were not equally represented throughout the population (Figure 3). The putative ancestral haplotype (H-I) was dominant in all populations except in the attached Seili population. The second most common haplotype (H-VI) was observed in four populations.

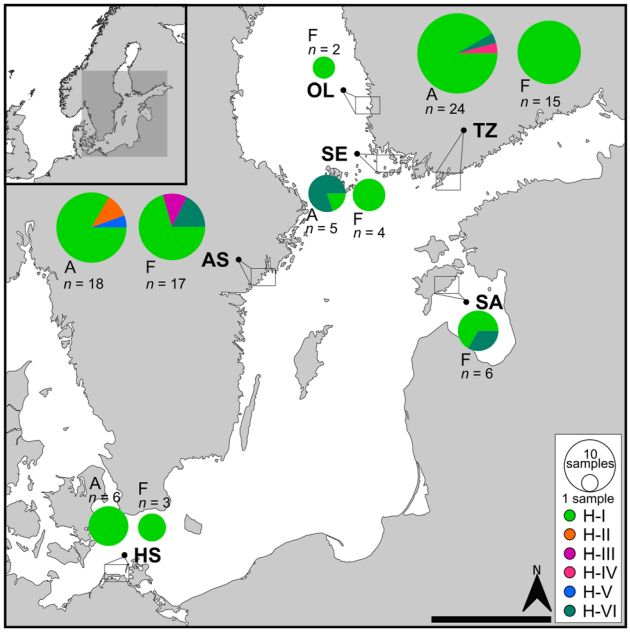

Figure 3: Distribution of concatenated-barcode mtDNA intergenic spacer (IGS) and 23S haplotypes found within the Baltic sea *Fucus vesiculosus* population. Circle size is proportional to sample size (*n*) and form is indicated as A (attached) or F (free-living). Site abbreviations: As, Askö; HS, Hiddensee; OL, Olkiluoto; SA, Saaremaa; SE, Seili; TZ, Tvärminne. Scale: 200 km.

All other haplotypes were only observed in a single population, representing a small fraction of the observed haplotypes. One of the four attached and four of the six free-living populations were monomorphic. Despite the close proximity of the attached and free-living forms at each site, haplotype composition and frequency was dissimilar for all sympatric populations except at Hiddensee where both forms were monomorphic.

Sample size had a marked effect on the number of haplotypes observed within a site. The two sites, Tvärminne and Askö, with the greatest sample numbers (n = 53 and 47, respectively) had the greatest number of observed haplotypes, demonstrating that greater sampling effort resulted in higher numbers of rare sequences. Accordingly, we tested how representative the sampling was using rarefaction analysis on the whole population and separately for each site (Supplementary Figure S1A). The rarefaction analysis indicated that haplotype count was well represented in the whole Baltic Sea and in all individual populations except at Tvärminne (Supplementary Figure S1B). Thus, there is indication that sampling effort does not markedly affect common haplotype representation, rather only that of rare haplotypes.

Spatial structure was first tested using two *a priori* hypotheses concerning the hierarchical organization of the data (Table 3). According to hypothesis I, there was no genetic differentiation between the forms, but there was significant genetic structuring within the two forms (Table 3). When the forms were analysed separately, genetic differentiation was significantly larger than zero among the attached populations, and much smaller and not significantly larger than zero among the free-living populations (Table 3). The attached sample from Seili showed much larger pairwise F_{ST} values (Supplementary Table S4) than

Table 3: Hierarchical AMOVAs testing two hypotheses, grouped by (I) form or by (II) subbasin.

Samples	Source of variance	*Df*	*SS*	% Of variance	Fixation indices	p-value
Hypothesis I						
Attached–Free-living	Populations among groups	1	0.056	−9.02	F_{CT} = −0.090	0.870^{NS}
	Among populations within groups	8	3.612	27.05	F_{ST} = 0.180	0.000^{c}
	Within populations	90	10.502	81.97	F_{SC} = 0.248	0.000^{c}
Attached	Among populations	3	2.657	38.29	F_{ST} = 0.383	0.000^{c}
	Within populations	49	5.286	61.71		
Free-living	Among populations	5	0.955	6.73	F_{ST} = 0.067	0.167^{NS}
	Within populations	41	5.216	93.27		
Hypothesis I: Seili attached omitted						
Attached–Free-living	Populations among groups	1	0.163	−0.65	F_{CT} = −0.007	0.431^{NS}
	Among populations within groups	7	1.135	4.65	F_{ST} = 0.040	0.146^{NS}
	Within populations	86	9.702	96.00	F_{SC} = 0.046	0.164^{NS}
Attached	Among populations	2	0.181	−0.67	F_{ST} = −0.007	0.455^{NS}
	Within populations	45	4.486	100.67		
Hypothesis II						
AS–HS–OL–SA–SE–TZ	Populations among groups	5	1.863	0.64	F_{CT} = 0.006	0.347^{NS}
	Among populations within groups	4	1.805	20.40	F_{ST} = 0.210	0.001^{b}
	Within populations	90	10.502	78.95	F_{SC} = 0.205	0.023^{a}
AS	Among populations	1	0.335	3.87	F_{ST} = 0.039	0.198^{NS}
	Within populations	33	6.493	96.13		
SE	Among populations	1	1.422	72.03	F_{ST} = 0.720	0.044^{a}
	Within populations	7	0.800	27.97		
TZ	Among populations	1	0.048	−0.28	F_{ST} = −0.003	-0.541^{NS}
	Within populations	37	1.875	100.28		
Hypothesis II: Seili attached omitted						
AS–HS–OL–SA–SE–TZ	Populations among groups	5	0.915	4.00	F_{CT} = 0.040	0.413^{NS}
	Among populations within groups	3	0.383	0.94	F_{ST} = 0.049	0.128^{NS}
	Within populations	86	9.702	95.05	F_{SC} = 0.001	0.170^{NS}

Associated p-values calculated by 1000 permutations (p-value significance level: NS > 0.05, ^{a}≤0.05, ^{b}≤0.01, ^{c}≤0.001). *df*, degrees of freedom; *SS*, sum of squares. Site abbreviations: AS, Askö; HS, Hiddensee; OL, Olkiluoto; SA, Saaremaa; SE, Seili; TZ, Tvärminne.

any other population. Therefore, hypothesis I was modified *a posteriori* by removing this sample from the data. Consequently, the significant genetic structure among populations within the forms disappeared, as was also mirrored within the attached population analysis. Thus, the significant genetic structuring observed in our *a priori* hypothesis I was a result of high genetic divergence at a single site. Using hypothesis II, there was no genetic differentiation among subbasins, but there was significant genetic structuring within the subbasins (Table 3). The calculation of fixation indices was not possible for monomorphic sites (KU, OL, HS). Comparisons of form types at both Tvärminne and Askö indicate that genetic differentiation is low between the forms at these locations. As the attached and free-living populations at Seili were highly differentiated (F_{ST} = 0.720*), the significant genetic structuring was caused by the high levels of genetic differentiation at this one site. As with hypothesis I, once the attached population at Seili was omitted from the analysis, the genetic differentiation between and within populations became non-significant (Table 3).

Significant genetic differentiation was observed between the attached Seili population and the attached populations at Tvärminne, Askö, and Hiddensee, and also the free-living populations at Tvärminne, Askö, and Seili (Supplementary Table S4). The F_{ST} values were all >0.3, indicating high levels of differentiation. Free-living populations at Tvärminne and Askö were also significantly genetically differentiated (Supplementary Table S4), though to a lesser extent. No genetic differentiation was observed between any other populations.

3.4 Intraspecific divergence and demographic history

Excluding mutations associated with indels, haplotypes had 1–3 substitutions from the putative ancestral haplotype (Figure 2A). Assuming that *F. vesiculosus* entered the postglacial Baltic Sea at the earliest 8000 years ago (Ardehed et al. 2016) and a generation time of 4 years (Lüning 1985), calculations indicate a substitution rate of 2.3×10^{-6} per generation and a sequence change of 0.5% since earliest colonisation. When considering the whole population or each form separately both Tajima's D and Fu's F_S neutrality tests show no significant signals of recent bottlenecks or population expansions (Table 4). Thus, the null hypothesis of neutrality cannot be rejected and extreme demographic changes, i.e. population expansion or retraction, cannot be verified.

Table 4: Results of Tajima's D and Fu's F_S neutrality tests for the whole population of *Fucus vesiculosus* and each form, including associated p-values calculated by 1000 permutations.

	n	Tajima's D	p-value	Fu's F_S	p-value
Whole population	100	−0.685	0.297	0.267	0.591
Attached	53	−0.736	0.272	1.712	0.810
Free-living	47	−0.241	0.430	0.738	0.639

n, number of samples.

4 Discussion

In this work, we show that the attached and free-living forms of the Baltic Sea *F. vesiculosus* share multiple haplotypes and that both forms belong to the same gene pool. Consequently, we provide no evidence to discredit the previous morphological taxonomic classifications, and thus Baltic Sea free-living *Fucus* should continue to be considered *F. vesiculosus*. Our data reveals an overall low level of nucleotide and haplotype diversity in the mitochondrial genome. Few discernible patterns relating to recent population declines were observed. Mitochondrial haplotypes unique to the Baltic Sea were also found. The distribution of mitochondrial diversity defines a largely panmictic (gene-flow without barriers) population structure in the deeper demographic timescales. Genetic differentiation between sympatric populations of both attached and free-living forms was usually weak confirming that the two forms are drawn from the same genetic source at the majority of sites. We suggest that Baltic Sea populations were historically well connected, with the post-glacial colonisation process resulting in little differentiation within the mitochondrial genome between populations.

4.1 Genetic diversity and population structure

Low nucleotide diversity in the mitochondrial genome of the Baltic *F. vesiculosus* (π = 0.001) corresponds well with the low nucleotide diversity in other *Fucus* species [e.g. *F. ceranoides* (π = 0.02–2.1; Neiva et al. 2010), *F. distichus* (π = 0–0.01; Coyer et al. 2011b, 2006a), *F. serratus* (π = 0–0.09; Hoarau et al. 2007), *F. spiralis* (π = 0–0.008; Coyer et al. 2011a) and North Atlantic *F. vesiculosus* (π = 0–0.005; Coyer et al. 2011a)]. Conversely, our results show considerably lower overall haplotype diversity in the Baltic Sea *F. vesiculosus* (overall H_d = 0.29) compared to the higher haplotype diversities found in

F. distichus (maximum H_d = 0.8; Coyer et al. 2006a), *F. serratus* (maximum H_d = 0.7; Hoarau et al. 2007), and in North-Atlantic *F. vesiculosus* (maximum H_d = 0.6; Coyer et al. 2011a). However, haplotype diversities varied greatly in all these studies, including our own, ranging from zero to the highest possible value. In addition, within-population haplotype diversities in our study (H_d scale = 0.1–0.56) are comparable to several North Atlantic *F. vesiculosus* populations (H_d scale = 0.12–0.6; Coyer et al. 2011a). The observed low overall haplotype diversity is partially a result of the fixation of a single haplotype within edge populations in the north and south, whilst populations in the central regions of the Baltic Sea presented higher levels of genetic diversity. Haplotype diversity also varied according to form. Attached populations generally had higher haplotype diversities (H_d scale = 0–0.4) compared to free-living sympatric populations, which were usually monomorphic. The only exception was Askö, where haplotype diversities for both forms were relatively high, with the haplotype diversity in the free-living population being greater than in the attached population.

The lower overall genetic diversity in the Baltic Sea *F. vesiculosus* population may represent the past colonisation history, as has been suggested in several species that have colonised the Baltic Sea either in historical times (Johannesson and André 2006) or have been recently introduced (Cristescu et al. 2001). Decreasing genetic diversity following colonisation events has been shown in other *Fucus* species. Populations of *F. distichus* demonstrate a clear gradient of decreasing haplotype diversity along their proposed colonisation pathway (Coyer et al. 2006a) whilst North Atlantic populations of *F. serratus* (Hoarau et al. 2007), *F. spiralis*, and *F. vesiculosus* (Coyer et al. 2011a) display low diversity as a consequence of recolonisation from refugia after the last glacial maximum. Furthermore, the ubiquity of a single mtDNA IGS haplotype in North American *F. vesiculosus* populations is also hypothesised to be the result of recent colonisation from Europe (Muhlin and Brawley 2009). Thus, we suggest that the low overall genetic diversity observed within the Baltic Sea is a product of past colonisation from the Atlantic after the present-day Baltic Sea was formed.

Our results show that *F. vesiculosus* populations in the Baltic Sea are well connected over deeper demographic timescales suggesting a rather panmictic population structure with sporadic localised areas of limited gene flow. Recent population bottlenecks could cause the observed low genetic diversity, but neutrality tests provide no significant indication of past declines, thus it appears the patterns relate to the relatively recent colonisation. Population structure is defined by low variation among subbasins (F_{ct} = 0.040) and among populations within subbasins (F_{st} = 0.049), indicating few spatially aggregated patterns in the deeper demographic time scales. Although potential shallow signatures of fine-scale spatial genetic structuring within more recent demographic history can also be seen due to the discrepancies in variation explained by the F_{CT} and F_{ST} values.

Several previous studies of *F. vesiculosus* within the Baltic Sea demonstrate recent isolating processes. *Fucus vesiculosus* has been found to be spatially differentiated at small scales along the Swedish east coast (Pereyra et al. 2009; Tatarenkov et al. 2007) and along the western coast of Finland (Rinne et al. 2018), but also at a larger spatial scale throughout the Baltic Sea (Ardehed et al. 2016). This corroborates the shallow signatures of isolating processes within our data, whilst not invalidating the high connectivity observed within deeper demographic timescales.

It is important to consider that the marker's genetic system and mutation frequency affects the resolution in the data and the likelihood to unveil potential genetic structuring at different demographic timescales. All aforementioned studies used DNA microsatellite genotyping compared to the mitochondrial sequencing used in this study. DNA microsatellites have high mutation rates predisposing them to describing contemporary dynamics whilst the lower mutation rates in mitochondrial genomes favour historic dynamics (Sanchez et al. 2020; Waples 1998). Thus, rather than finding conflicting evidence, the lack of congruence between our results and the previous studies may also be due to the lack of resolution for recent demographic processes in our study. The microsatellite genotyping of an expanded version of this sequencing dataset supports the validity of this assumption (Preston et al. 2022).

The dominance of a single putative ancestral haplotype may be a result of the dispersal capabilities of *Fucus* thalli. Dispersal in the form of rafting is suggested to be an important means of gene flow in *Fucus* spp. in the Baltic Sea (Pereyra et al. 2013; Tatarenkov et al. 2007). Floating *Fucus* rafts have been observed at densities ranging from a few to almost 80 items per km^2 in the Northern Baltic Proper and can move hundreds of kilometres whilst remaining physiologically viable (Rothäusler et al. 2020, 2015). Unfortunately, the differences in the rafting potential of thalli from each form is currently poorly understood, thus any assumptions of the rafting capabilities due to form alone would be

erroneous. Nevertheless, high rafting potential may be a key component of the observed homogenised population structure.

4.2 Resolution of the free-living form

Stable populations of free-living *Fucus* within the Baltic Sea are assumed to originate from attached *F. vesiculosus* populations, whereby detached pieces of thalli from the main attached thallus accumulate in still locations and persist over time (Bauch 1954; Den Hartog 1959; Häyrén 1949; Luther 1981; Svedelius 1901). In this study, genetic techniques were used to corroborate this notion by showing that the most common haplotypes found in the free-living form of *F. vesiculosus* group with specimens within and outside the Baltic Sea. Unattached *Fucus* are also common outside the Baltic Sea, with several unattached *Fucus* populations being associated with attached *F. spiralis* (Mathieson et al. 2006), *F. vesiculosus* (Coyer et al. 2006b), and *F. distichus*/*F. gardneri* (Kucera and Saunders 2008; Neiva et al. 2012; Serrão et al. 2006) using molecular characteristics, and *F. spiralis* and *F. vesiculosus* (Baker and Bohling 1916; Cotton 1912; Mathieson and Dawes 2001) using morphological characteristics. Our results support these previous studies indicating that unattached populations belong to the same genetic entity as the local attached populations. However the sharing of haplotypes could also be a result of high levels of gene flow through sexual reproduction. Sexual reproduction is thought to be absent or at least rare in the life cycle of free-living *Fucus* within the Baltic Sea, either due to the absence (Svedelius 1901) or sterility (Bauch 1954; Häyrén 1949) of receptacles. However, receptacle-rich specimens have been found recently in Sweden (S. Qvarford, pers. comm.) and Finland (R. Preston, pers. comm.). Further research is required to determine the viability of the free-living form before connectivity via sexual reproduction can be rejected.

At two of the three sympatric sites (Tvärminne, Seili), the free-living populations were monomorphic despite the attached populations having multiple haplotypes. If the hypothesis of a local origin is correct, this would suggest that the free-living population is only a subsample of the local attached population. However, at the third site (Askö) the haplotype diversity of the free-living population was greater than that of the attached population. Both Askö populations had two private haplotypes and the two forms were relatively strongly differentiated (F_{ST} = 0.04). This suggests that the free-living population in Askö is not solely drawn from the local attached population but has also received immigrants.

4.3 Colonisation and demographic history of *F. vesiculosus* in the Baltic Sea

The distribution of mitochondrial haplotypes in the Baltic Sea and the Atlantic Ocean suggests that a single haplotype colonised the Baltic from the Atlantic, and became increasingly geographically and reproductively isolated from the parental populations. Current unique mitochondrial diversity in the Baltic Sea compared to the founding North Atlantic populations can then be explained by novel mutations, and subsequent directional selection and genetic drift. The findings of a single mtDNA PR-IGS haplotype corresponding with our putative ancestral haplotype along the Scandinavian coastline (Coyer et al. 2011a) as well as the analogous demographic characteristics of another recently colonized *F. vesiculosus* population in North America (Muhlin and Brawley 2009) supports this assumption. Hence, the observed level of genetic variation within the Baltic Sea appears a result of recent divergence, rather than post-colonisation reduction of variation within the Baltic Sea population.

Despite the well-documented population declines and subsequent recovery of attached *F. vesiculosus* within the Baltic Sea, the data showed no solid indications of population bottlenecks or recent population expansion. Recovery is often localised, for example within the S and SW Finnish archipelagos, particularly around the Hanko peninsula (Kangas and Niemi 1985; Rinne and Salovius-Laurén 2020; von Wachenfeldt et al. 1986) and our sampling sites at Seili (Rönnberg et al. 1985). However, many other sites are yet to recover (Rinne and Salovius-Laurén 2020; Snickars et al. 2014; Vahteri and Vuorinen 2016). It is therefore possible that declines and recovery may not have been extreme enough, having only a limited effect on the genetic diversity, and thus the bottleneck signal could not be detected. Furthermore, the limited genetic variation among our samples effectively homogenises the population.

Mitochondrial barcode sequencing lacks the resolution to adequately describe the evolutionary dynamics or recent demographic changes of *Fucus* spp. in the young, postglacial Baltic Sea. However, the markers accurately describe the colonisation of a single haplotype from the Atlantic, and gives an indication of post-colonisation divergence within the sea itself.

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Data availability: DNA sequences: Genbank accessions MZ711443-MZ711447 (IGS) and MZ779027-MZ779028 (23S). Figshare: full concatenated and separate IGS and 23S datasets available at DOI 10.6084/m9.figshare.17049041.

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