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DIVERGENCE PATTERNS IN *FUCUS* SEAWEEDS (PHAEOPHYCEAE) IN THE NORTHERN BALTIC SEA AND IN THE TJONGSPOLLEN AREA

Tesi di Laurea in:
Habitat marini: struttura, processi e mapping

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To my mother who gave me the passion for the sea,
to my father who has been my point of reference for all my life.

A mia madre che mi ha trasmesso l'amore per il mare,
a mio padre che per tutta la vita è stato il mio punto di riferimento.

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1. INTRODUCTION

1.1 Speciation and divergence mechanisms

Speciation is the evolutionary mechanism by which new and distinct biological species arise. Since 1859 when Darwin published his iconic work “On the Origin of species”, this process has been underlined fundamental in biology.

Species, as defined by the biological species concept, are groups of individuals considered reproductively isolated units. Individuals of the same species exchange genes, this not happen considering different species individuals (Mayr 1963).

Biogeographically, speciation can be classified in three modes: allopatric speciation, where the divergence cause is the geographical isolation; sympatric speciation, where the divergence is within a freely interbreeding population without any physicial barriers; and parapatric speciation where there are no specific extrinsic barriers to gene flow. The population is continuous, but nonetheless, it does not mate randomly. Individuals are more likely to mate with their geographic neighbors than with individuals in different parts of the population's range. In this mode, divergence may happen because of reduced gene flow within the population and varying selection pressures across the population's range.

Many processes have been underlined as a speciation mechanisms, such as speciation through hybridization, chromosomal rearrangements (Rieseberg and Willis, 2007), speciation as a consequence of ecological mechanisms, where in some cases ecological divergence is a proxy for the presence of divergent selection (Schluter 2009, Nosil et al., 2008) and speciation in parallel (Johannesson 2001). In most cases, the process of speciation usually takes long time.

The recent theory of sympatric speciation suggests that under certain circumstances speciation can occur in populations in absence of separating barriers, especially in recently formed or isolated areas that offer a variety of new ecological niches (Schliewen *et al.*, 2001). Therefore, sympatric speciation often occurs more rapidly than allopatric speciation.

Divergence and differentiation can vary during speciation and it can be seen in different stages (Fig 1.1.1).

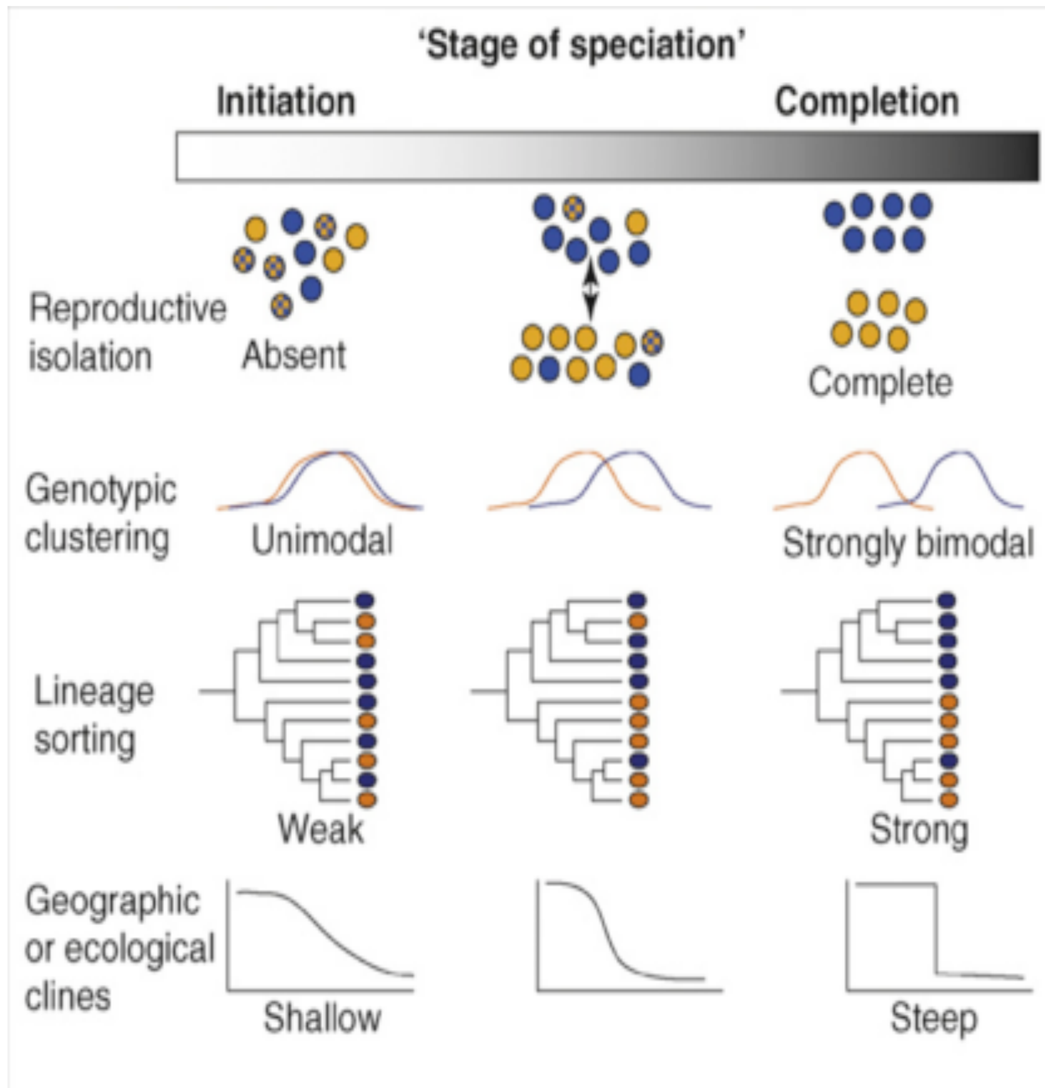

Figure 1.1.1: (Scheme from Nosil et al., 2008).The continuous nature of divergence during speciation. Different levels of speciation in natural populations from absence of divergence to complete reproductive isolation. The distribution of gene frequencies in individuals from two different populations can vary from unimodal to bimodal genotypic clustering, that is reflected in lineage sorting and geographic or ecological clines.

However, the continuous nature of divergence during a speciation process is challenging to disentangle in natural populations and the dynamics of differentiation are still a matter of debate among evolutionary biologists.

Groups of closely related taxa constitute important case studies to understand species and new biodiversity formation. However, it is important to assess the divergence among them at different organismal levels and from an integrative perspective (Fig. 1.1.2).

For this purpose, this study used the brown seaweeds genus *Fucus* as a model to study speciation, as they may constitute a good opportunity to study divergence at different stages.

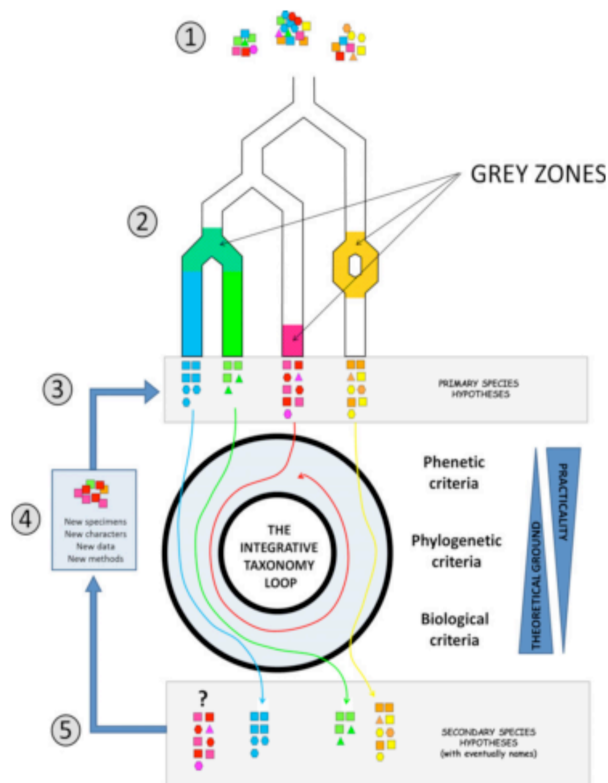

Figure 1.1.2 : (Pante et al., 2015), species hypothesis based on the approach of integrative taxonomy

1.2 Genus *Fucus*

The brown macroalgae genus *Fucus* (Phaeophyta) is a group of perennial brown seaweeds (Fig 1.2.1) common in temperate and arctic coastal areas (White, 2004). These macroalgae are found in habitats ranging from the rocky intertidal to brackish salt marshes on the northern hemisphere (Bergström *et al*., 2005).

Fucus seaweeds are considered key ecosystem-structuring species (Chapman 1995, Tatarenkov *et al*., 2007), that means they give structure and increase the complexity of the habitat, providing refuge and protection for other marine organisms from abiotic and biotic factors (Dayton, 1972; Roff and Zacharias, 2011).

The brown colour of these algae, as well as the other *Phaeophyceae*, is a result from the presence of xanthophyll pigment: fucoxanthin, which masks the other typical algal pigments as Chlorophyll *a* and *c*, β-carotene and the other xanthophylls.

The genus *Fucus* typically show strong morphological and physiological variation (Knight and Parke, 1950; Burrows and Lodge, 1951; Pèrez-Ruzafa *et al*., 1993; Malm *et al*., 2001; Ruuskanen and Bäck, 2002), showing puzzling taxonomic designations with significant intraspecific variability throughout its geographic range (Coyer *et al*., 2006), and for this it is believed that the group has large phenotypic plasticity (Scott *et al*., 2001). However, in some cases, this variability may be genetically determined (Pearson *et al*., 2000).

For these reasons this genus offers the opportunity to investigate divergence due to local adaptation, as well as, speciation mechanisms.

Figure 1.2.1: Example of brown seaweeds genus *Fucus* in intertidal habitat

1.3 The Baltic Sea and Tjongspollen, two unique ecosystems

The Baltic Sea (Fig. 1.3.1), currently a large postglacial brackish water body, might be considered a unique and marginal (geographically and ecologically) ecosystem, with a surface salinity ranging from 1 to 2 psu (practical salinity unit) in its inner parts up to 25 psu with predominantly atidal conditions (HELCOM, 1996).

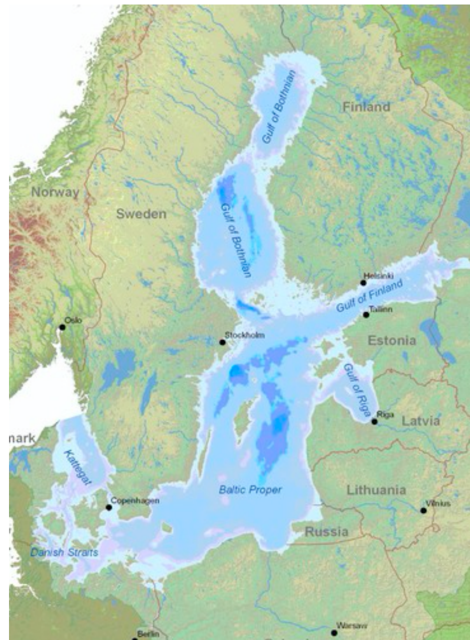

Figure 1.3.1: The Baltic Sea (Bergström *et al*., 2005)

As its documented postglacial history showed, 11,000 years ago the Baltic Sea was subjected a globally increased sea level and a regional isostatic rebound after the retreat of the last continental ice sheet (Ignatius *et al*., 1981).

After the period of freshwater, the Baltic Sea was colonized by biome coming from the Atlantic Ocean as the entrance was wider than today and the salinity of the Baltic Sea was around 25 psu (Snoeijs, 1999).

After this, the salinity progressively neared current levels, with a consequent rapid adaptive challenge for the marine biota and with the extinction or retreat of those species that were unable to adapt to new environmental conditions (Russel, 1985).

Today the Baltic Sea hosts both marine and fresh water taxa with a low total number of species, especially in its inner areas.

It is possible to observe that the progressive salinity decreasing, from the entrance of the Baltic Sea to its inner part (Bergstrom *et al*., 2005), is accompanied by a reduction in the number of species comparing approximately 840 species of marine macrofauna (Kautsky and Kautsky, 2000), 220 marine macroalgae (Snoejiis, 1999) at the entrance with 80 and 60 species in its inner parts.

More detailed studies showed many kinds of adaptation to the brackish water conditions including physiological adaptations (Rietema, 1991, 1993, 1995; Kristiansen *et al*., 1994; Düwel, 2001).

Moreover, Baltic Sea populations may show marked genetic differences with North Sea ones (Väinöla and Hvilsom, 1991; Luttikhuizen *et al*., 2003; Olsen *et al*., 2004; Johannesson & André, 2006), some probably due separate evolutionary lineages (Väinöla, 2003). In fact, isolated populations that inhabit marginal environments tend to be more genetically differentiated than either central population or isolated populations that not live in extreme environmental conditions (Bouza *et al*., 1999).

Another pheripheral environment is Tjongspollen, a sheltered pool located in the southwest coast of Norway (Fig. 1.3.2).

Figure 1.3.2: Tjongspollen area (Norway)

The surrounding area is almost uninhabited and the pool is therefore hardly influenced by anthropogenic activity. This sheltered area is connected to the adjacent

sea through a narrow channel, characterized by specific hydrographic conditions that result particularly in warm waters during summer and low salinity level. The benthic algal flora of the pool show a large number of rare species (e.g. *Chondria dasyphylla* and *Codium vermilara*) believed to have arisen due to the marginal environmental conditions of this area

Directional selection is a strong promoter of speciation, even in the presence of gene flow (Rieseberg *et al*., 2002; Korol *et al*., 2006; Doebeli *et al*., 2003). In more detail, environmental stress along gradients is a potential source of new species (Pereyra *et al*., 2009).

As evident from recent evolutionary events, *Fucus* seaweeds have been subjected to phenotypic and genetic radiation (Leclerc *et al*., 1998; Serrão *et al*., 1999b).

1.4 Morphological traits in *Fucu*s

Fucus seaweeds are morphologically characterised by a perennial thallus with irregular or disc-shaped holdfast or with hapter and the erect portion of the thallus is dichotomous. Air vesicles appear in pairs in some species, and in some instances, considerable variation is observed in the fronds, characterised by large or narrowed branches, completely flat or undulating, more or less branched. In the apices of the final branching are located conceptacles embedded in receptacles. However, while some morphological characters may be species-diagnostic to certain extent some overlapping may complicate the species identification in the field.

Three formally described *Fucus* species occur in the Baltic Sea while six have been reported in Tjongspollen area. *Fucus vesiculosus* and *F. serratus* occur in both regions while *F. radicans* is the third occurring species in the Baltic. Otherwise in Tjongspollen, *F. ceranoides*, *F. distichus*, *F. evanescens* and *F. spiralis* also occur. Perhaps the most distinctive species morphologically are *F. evanescens*, without bladders and inconspicuous midrib, *Fucus serratus* with broad and highly serrated blades and *Fucus distichus* also with inconspicuous midrib but shows distinctive enclosed cavities in the cortex. By contrast, *F. ceranoides*, *F. spiralis*, *F. radicans* and *Fucus vesiculosus* have distinctive midrib, which makes their differentiation

difficult. *Fucus vesiculosus* posses air bladders almost across its whole distribution helping differentiate this species from *F. ceranoides* and *F. spiralis*. However, it lacks these air vesicles in the Baltic and becomes narrower, challenging its morphological differentiation from *F. radicans*. *Fucus radicans* does not have bladders and it is considered a miniaturised form of *F. vesiculosus*. However, individuals of both taxa with overlapping sizes may be difficult to identify to species level.

Despite the morphological similarity among *Fucus* species, they may differ at other organismal level physiological and ecological traits (Gylle *et al*., 2010; Gurnnarsson and Berglund, 2012; Schagerström *et al*., 2014).

For example, *Fucus radicans* and *F. vesiculosus* are dioecus and they reproduce sexually by synchronized release of gametes to the surrounding waters, in a temporal window from late spring to autumn (Serrão *et al*., 1996). However, it has been found that both species can reproduce asexually (Tatarenkov *et al*., 2005), this feature being unique in fucoids. Earlier studies involving *F. radicans* have shown the dominance of one female clone in large part of the Swedish coast and in northern Finland (Johannesson *et al*. 2011). In contrast, Estonian populations mainly display sexual reproduction.

Figure 1.4.1: Two species of Fucus; Left is *F.radicans* and right *F.vesiculosus*. Ph: Lena Kautsky.

Despite the fact that both taxa appeared with limited gene flow between them (Pereyra *et al*., 2009), their potential for hybridization cannot be ruled out as they

show no prezygotic or early postzygotic reproductive barriers (Forslund and Kautsky, 2013).

1.5 Role of phlorotannins in brown seaweeds

Brown macroalgae contain secondary polyphenolic metabolities called phlorotannins (Pavia *et al.*, 1999). Phlorotannins are polymers of phloroglucinol (1,3-5 trihydroxybenzene) (Ragan and Glombitza, 1986) and among different tannins, this type are the most relevant ones synthesised in different taxa (Connan *et al.*, 2004).

The different roles of phlorotannins are still under investigation. However, many studies have shown the evidence that abiotic factors such as salinity range, nutrients and solar irradiance might play an important role in the phlorotannins content, regulation and production.

In particular, there is evidence that phlorotannins are involved as protection against pathogen and UV radiation induced-damage avoiding photodestruction of algal compounds (Pavia *et al.*, 1997; Targett and Arnold, 1998;) and they can also act as grazing deterrents.

It has been demonstrated, UV radiation can be an important factor to explain intraspecific variation in phlorotannins content of marine brown seaweeds (Pavia *et al.*, 1997). This has been shown in the brown alga *Ascophyllum nodosum* exposed to UV-irradiance shock with an increase of 30% in the mean phlorotannin concentration (Pavia *et al.*, 1997).

At an intraspecific level a common explanation of different contents of phlorotannin is the Carbon/Nutrient (C/N) ratio (Bryan *et al.*, 1983). It is predicted that the production of polyphenolic and other C-based metabolites is determined by the relative supply of carbon (through photosynthesis) and essential nutrients. Accordingly, high levels of light with low levels of nutrients should be followed by an excess of carbon, which can be used for production of carbon-based chemical defence as polyphenolics (Pavia *et al.*, 1997).

Baltic Sea *Fucus* present comparatively larger amounts of phlorotannins around 8-14% of dry weight (mg of DW) than 2-10% common in marine populations from Skagerrack and the North Sea (Kubanek *et al*., 2004).

Grazing by herbivores is one of the stressors for many plant and algal species. The loss of biomass by grazing constitutes a continual physiological stress and under grazer selection pressure, chemical defence has evolved in many species of seaweeds as well as terrestrial plants.

Brown seaweeds of the genus *Fucus* are continuously exposed to herbivory from gastropods and isopods (Hagerman, 1966). Through constitutive and inducible chemical defense, algal tissue becomes less palatable and even toxic for grazers (Forslund *et al*., 2012).

However, the production of phlorotannins as secondary metabolities is costly in terms of energy for algae, so it is presumably advantageous for the seaweeds to produce grazing defenses, only when herbivores are present and actively grazing, therefore they are known as inducible defenses (Van Alstyne and Paul, 1990).

The most dominant grazer of *Fucus* is the isopod Idotea, and in particular *I. baltica*, common in the southern Baltic Sea (Leidenberger *et al*., 2012). There is extensive evidence of the preference of Idotea for *Fucus* species (Jormalainen *et al*., 2001; Forslund *et al*., 2012).

However, some species may be more palatable than others and thus, the grazing pressure may limit the distribution of some algae (Forslund et al., 2012). Therefore, the herbivory pressure may contribute to the divergence among species and/or populations in levels of phlorotannin production against grazing.

1.6 Aim of the study

The wide distribution of *Fucus* macroalgae along environmental gradients as well as the exposure to biotic interactions with grazers may be strong promoters of divergence at different organismal levels.

In this general framework its possible to hypothesise that adaptation to a shifting environment will result in migration, mortality or local adapation of the species.

Genetic variation provides the necessary “fuel” for populations and species to evolve and adapt in changing environments (Hoffman *et al*., 2011; Lohbeck and Reusch, 2012), and may be reflected by physiological/morphological variations in the organisms.

Therefore, the aim of the following study is to describe and understand the divergence patterns in *Fucus* species from marginal areas, based on phenetic, phylogenetic and biological taxonomical criteria (Pante *et al*., 2015) characterised by algal morphology, allele frequencies of five microsatellite loci and levels of phlorotannins.

Considering that different marginal ecosystems are characterised by different species (Johannesson and Andrè 2006), this study will use a large number of individuals from Baltic Sea locations as well as a number of *Fucus* seaweeds from another brackish area outside the Baltic, namely Tjongspollen, located in the west coast of Norway, in order to better understand the species dynamics in *Fucus* seaweeds.

In short the main questions that this work will address are: What is the magnitude of divergence in each of the three different characters of the integrative taxonomy (morphology, genetics and biochemistry)?

Is there any correspondence between morphological, genetical and chemical traits in *Fucus* seaweeds?

Is it the same divergence type and magnitude in each trait?

2. MATERIALS AND METHODS

2.1 Sampling and study area

Individuals from sympatric populations of *F. radicans* and *F.vesiculosus,* commonly found at distance < 1, were collected in August 2014 from 10 localities in the Gulf of Bothnia along Swedish and Finnish coasts (Fig. 2.1.1).

Figure 2.1.1: Sampling area.

We also considered another peripheral area, namely Tjongspollen (Norway), characterised by different *Fucus* species but nonetheless useful to assess the extent of divergence among taxa in other brackish water areas.

Sampling was done at 0.5-3 m depth in approximate circular areas of 20m diameter, picking individuals separated by at least 1m to avoid the risk of picking individuals of the same clone.

Four localities were sampled in Sweden: Barsta, Djursten, Järnäs and Kuggören; six localities from Finland: Björneborg, Hällkalla Rauma, Sälskäret, Södra Vallgrund and Storskäret (Tab. 2.1). Finally, Norwegian *Fucus* specimens were collected from Tjongspollen at low and high salinity levels (Fig. 2.1.2).

Figure 2.1.2: Sampling area of Tjongspollen (Norway). The sheltered area is characterised by low salinity level, outside that area the salinity level is higher

Table 2.1: Sampling data

Location	Country	Label	Sample Size	Latitude	Longitude
Barsta	Sweden	BAR	140	62°51'39.60"N	18°24'7.44"E
Björneborg	Finland	BJO	43	61°28'48.00"N	21°20'56.57"E
Djursten	Sweden	DJU	95	60°22'7.32"N	18°24'4.14"E
Hällkalla	Finland	HAL	50	63°18'26.54"N	21° 5'22.62"E
Järnäs	Sweden	JAR	70	63°26'8.03"N	19°39'58.61"E
Kuggören	Sweden	KUG	140	61°41'55.62"N	17°30'59.16"E
Rauma	Finland	RAU	30	61° 8'36.52"N	21°18'17.81"E
Sälskäret	Finland	SAL	30	62°20'0.99"N	21°12'55.45"E
Södra (Val)	Finland	SOD (val)	80	63°20'13.00"N	21°22'55.00"E
Storskäret	Finland	STO	81	62°28'28.43"N	21° 8'8.93"E
Tjongspollen	Norway	TJO	72	59°40'57.45"N	5°14'40.49"E

2.2 Morphometric Analysis

Two individual measurements were used to determine morphological traits (Bergström *et al* 2005; Pereyra *et al*., 2009; Pereyra *et al*., 2013):

- frond width in the youngest dichotomy measured at three separate branches.
- distance between dichotomies, measured from the oldest dichotomy.

Three measuments were taken for each morphological variable. These variables were previously used to discriminate morphologically *F. radicans* and *F.vesiculosus* (Bergström *et al*., 2005). The measurement of morphological variables was performed for each individual sampled for each sampling spatial unit. The classification has been done (Fig. 2.2.1) with the program of image analysis ImageJ64 (Schneider *et al*., 2012) to estimate quantitatively the morphometric identification variables, to identify potentially misclassified individuals and the presence of hybrids in the populations analysed.

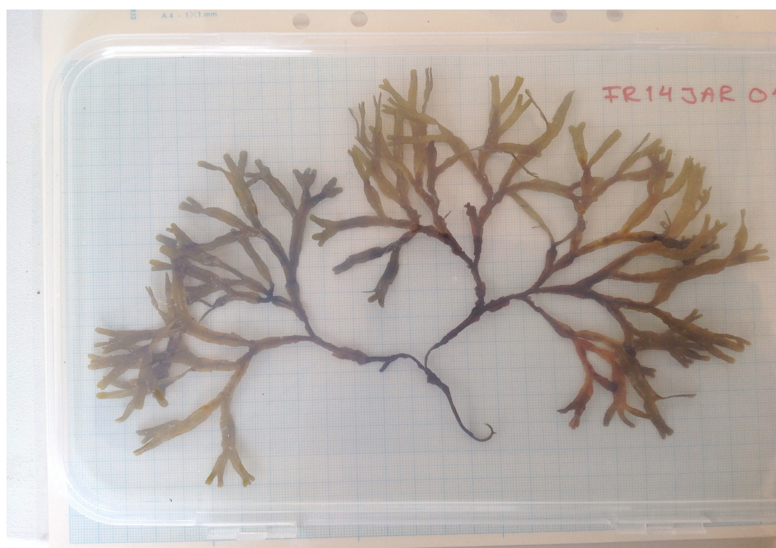

Figure 2.2.1: example of picture of Fucus radicans from Jarnas analysed with image analysis program imageJ-

2.3 DNA extraction, PCR and Genotyping

DNA was extracted from dried algal tissue, adding 500 µl of 2% CTAB Extraction buffer, 17.5 µl of 0.1 M DIECA, 5 µl of 2-Mercaptoethanol, 10 µl RNase A solution (10mg/ml) and the mixture was incubated for one hour at 65 °C. After that DNA was extracted using the NucleoSpin Plant II Kit following the manufacturer's instructions of Macherey-Nagel and the Zymo DNA Clean & Concentrator for the cleaning up and DNA purification following the manufacturer's instructions of Zymo research (Zymo Research Corporation, Irvine, CA, U.S.A) (full protocol Appendix A).

Individuals were genotyped at five polymorphic microsatellite loci L20, L38, L58, L85, L94 (Engel et al. 2003). Polymerase Chain Reaction (PCR) was performed in 10 µl reactions containing 0.5 µl DNA template and 9.5 µl containing 0.54 µl BSA 0.5 µg/ µl, 1.07 µl buffer 10x, 0.21 µl dNTPS 10 mM, 0.43 µl MgCl 50 mM, 0.54 each Forward and Reverse Primer 10 µM, 0.06 µl Platinum Taq polymerase for all loci except L58 for which 0.11 µl RBC Taq was used (Appendix B).

DNA products were visualised in 2% agarose gel (electrophoresis process) to verify the successful amplification. Alleles sizes were resolved and scored using Beckmann Coulter automated capillary sequencer (Tab. 2.2) and CeqMan 8000 software (Beckman-Coulter Inc., Fullerton, CA, USA) (Pereyra et.al., 2013).

Table 2.2: Dye and load informations for genetic analysis

locus	load µl
L20 (black)	**1.5**
L38 (green)	**1.3**
L58 (green)	**0.9**
L85 (black)	**1.5**
L94 (blue)	**0.6**

A ca. 350 bp region of the mitochondrial 23S subunit (Oudot-Le Secq *et al*., 2005) was used to assess the depth of divergence among Fucus individuals from Tjongspollen. PCR reactions were carried out in 43 ul volumes containing 1-3 ng of DNA template, 1 uM of each forward and reverse primer, 2 mM of dNTPs, 0.85 mM MgCl2 and 0.2 units of Platinum Taq (Invitrogen). PCR profile consisted of 94ºC, 2 min; followed by 94ºC, 30 s, 54ºC, 30 s, and 72ºC, 40 s for 40 cycles; and a final extension at 72ºC for 5 min. Sequencing was performed by Macrogen Co. (Seoul, South Korea).

2.4 Phlorotannins extraction

The chemical analyses consisted in the quantification of phlorotannins content for 20 individuals for each location (with the exception of Tjongspollen were all individuals were chemically analysed).

For phlorotannins extraction, 5-8 mg of dried tissue was ground to fine powder. For each sample, 1.5 ml aqueus acetone (60%) was added, a solvent that would extract polar coumpounds, such as phlorotannins.

Crude extracts were subsequently centrifuged, the acetone was evaporated in vacuum (rotary evaporator) and the remaining portion of water solution was filtered, with 20 µm filters, to remove any lipophilic material and diluted with distilled water.

Concentration of phlorotannins was quantified using the Folin-Ciolcateu (F-C) method (Folin and Ciolcateu, 1927). This method consists on an oxidation/reduction reaction, adding sodium carbonate (Na_2CO_3, 20g/100 ml) to the polyphenolic extract.

The reduced reagents produce a blue coloured solution that has been measured spectrophotometrically at wavelength of (λ_{max}) 725 nm.

A standard curve for phloroglucinol was used (CAS no. 6099-90-7) to calculate the concentration in each extract.

The percentage of phlorotannin in dry tissue was calculated from concentrations values. (full protocol Appendix E)

2.5 Statistical analysis

GENECLONE 2.0 (Arnaud and Belkhir, 2007a) was used to estimate the extent of clonality in each locality by determining the probability of occurrence of repeated genotypes at random. . Those repeated genotypes identified as clones were removed from the subsequent genetic analysis of differentiation.

Program STRUCTURE 2.2 was used to infer the presence of distinct genetic clusters, assigning individuals to different groups to identify hybrids, putative migrants and individuals with admixed ancestry.

Bayesian approach is used by this program assuming a model in which there are K number of genetic cluster, each of which is characterized by a set of different allele frequencies at each locus and each individual in the sample are probabilistically assigned to clusters (Pritchard *et.al*., 2000a; Falush *et al*., 2003a-2007).

The model used by this program assumes Hardy-Weinberg and linkage equilibrium (Pritchard *et al*., 2000b) and therefore the need to remove the repeated genotypes, as clonality violates these assumptions.

Factorial component analysis (FCA) of GENETIX 4.05 program (Belkhir, *et al*., 2004), PCA (Principal Component Analysis) and classical MDS (Multi Dimensional Scaling) from the statistical program R (R Core Team, 2014), (Appendix F) were used to determine if there is a concordance between the two morphometric variables, the allele frequences of five microsatellites loci analysed and the chemical content of Phlorotannins.

Finnaly FastTree implemented in Geneious 9.0 has been used to compute a maximum likelihood tree to estimate the phylogenetic relationships for Tjongspollen individuals. The program uses Jukes Cantor model (Jukes and Cantor, 1969) of nucleotide evolution that produces a maximum likelihood estimate of the number of nucleotide substitutions between two sequences, and Shimodaira and Hasegawa test (Shimodaira and Hasegawa, 1999) to compute node support values.

3. RESULTS

Results from three levels (morphometry, genetics and chemistry) of analysis were elaborated to observe if there is a real correspondence between morphological, genetical and chemical traits in our investigation object, the brown seaweeds genus *Fucus*, considering geographical distances and differentiation between species.

First, 881 individuals of wich 445 from Sweden, 364 from Finland and 72 from Norway were analyzed morphometrically. Results were performed trying to characterize different groups of individuals considering two morphological variables: thallus length and distance between dichotomies.

Second, genetics data (MGLs of five microsatellites) were analysed to determine the genetic differentiation level. DNA sequences of 768 total individuals were compared to a reference sequences to determine the genetic make up (genotype) of all *Fucus* individuals analysed.

Third, chemical data, including Phlorotannins content in terms of percentage of dry weight of tissue, were analysed using 250 individuals.

3.1 Morphometry

The distribution of data points in Figure 3.1.1 shows a discernable segregation of samples from Järnäs and Djürsten that are situated in diametrically opposed in the plot. In the middle of the plot with a partial overlap are located the samples from Barsta and Kuggoren, the former particularly aggregated with Djursten whilst the latter toward Järnäs.

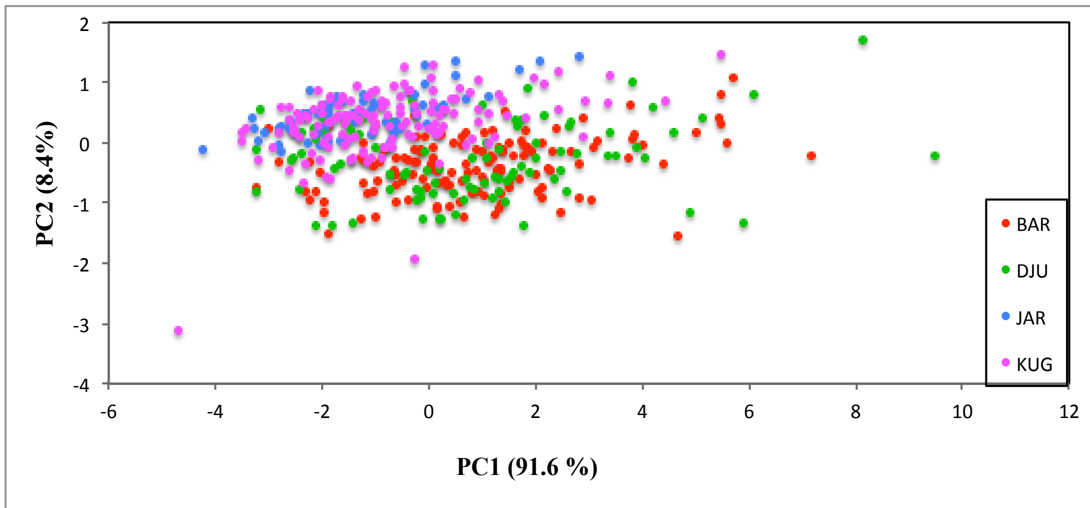

Figure 3.1.1: Result of Principal Component analysis based on morphometric variables from Barsta, Düjrsten, Järnäs and Kuggören localities. The two axis (PC1 and PC2) are first and second principal component that explain the percentage of morphometric variability.

Results from the Finnish localities show a complete overlap of morphological traits among individuals and therefore, it is not possible to distinguish any (Fig. 3.1.2).

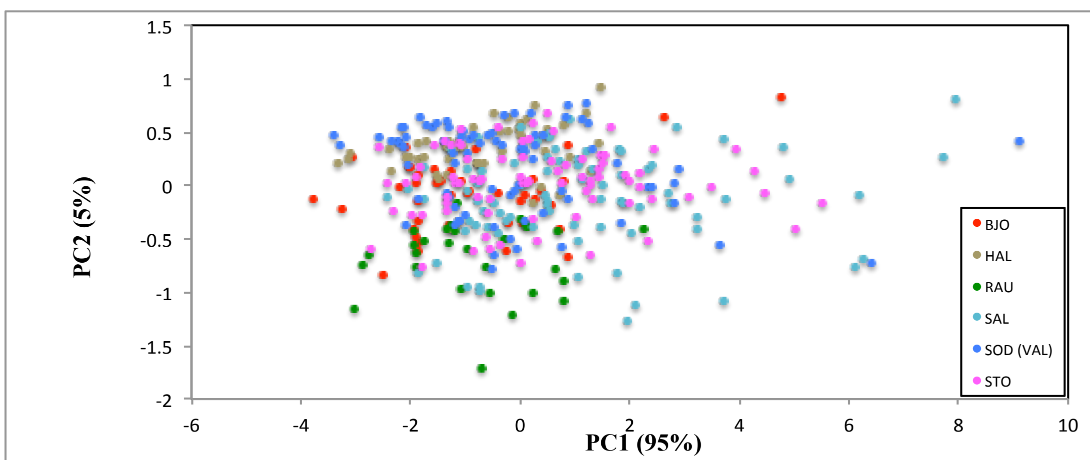

Figure 3.1.2: Result of Principal Component analysis based on morphometric variables from Finland localities. The two axis (PC1 and PC2) are first and second principal component that explain the percentage of morphometric variability.

The morphological differentiation among Tjongspollen samples is clearer than the Finnish and Swedish individuals. Although the data points appear scattered, the aggregation of taxa according to the field identification is evident from the plot with rather limited morphological overlap (Fig. 3.1.3).

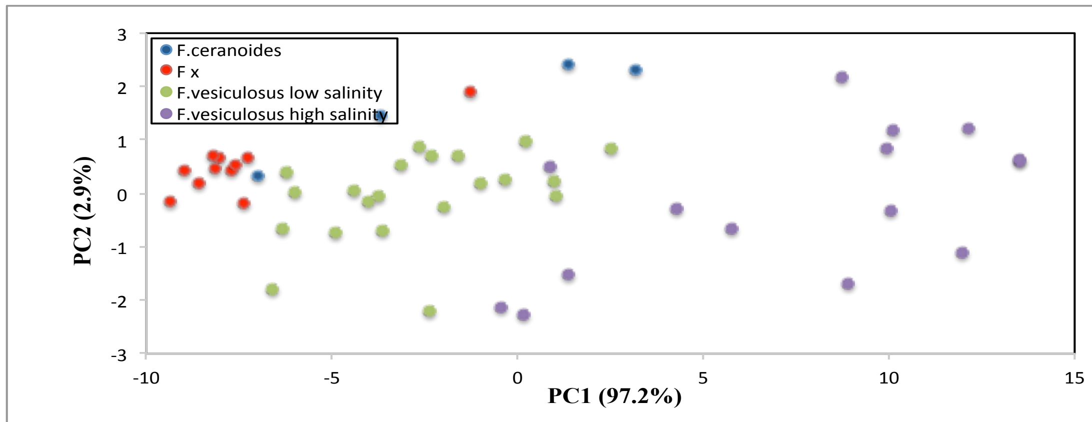

Figure 3.1.3: Result of Principal Component analysis based on morphometric variables from Norway (Tjongspollen), different colours are associated in relation of the species characterization considering morphometric variables. The two axis (PC1 and PC2) are first and second principal component that explain the percentage of morphometric variability.

3.2 Genetic results and populations structure

Genetics results considering all individuals from each locality revealed different levels of diversity.

Hällkalla, Järnäs, Södra (Val), where higher levels of clonality (96-100% of clonal individuals) are identified.

The lowest levels of clonality has been identified in Barsta and Björneborg.

In Fact, Shannon index values adapted for genotypic biodiversity (H^{ll}) show that these locations are characterised by higher levels of genotypic diversity with corresponding values of Eveness index (V^{l} H^{ll}) higher than locations where there is a dominance of clonal individuals (Tab. 3.1).

Table 3.1: Estimates of genetic variability at 5 microsatellites loci in *Fucus* populations. Values shown include all copies of the same genotype (clones).

Location	Country	Label	*n*	C	H^{II}	$V^{I}H^{II}$	C %
Barsta	Sweden	BAR	140	38	4.71	0.98	27.14%
Björneborg	Finland	BJO	43	10	3.6	0.99	23.25%
Djursten	Sweden	DJU	95	91	1.95	0.76	95.79%
Hällkalla	Finland	HAL	50	49	0.71	0.51	98.%
Järnäs	Sweden	JAR	70	70	0.41	0.59	100%
Kuggören	Sweden	KUG	140	63	3.54	0.79	45%
Rauma	Finland	RAU	30	13	3.06	0.97	43.33%
Sälskäret	Finland	SAL	50	41	2.27	0.80	82%
Södra (Val)	Finland	SOD (val)	50	48	0.74	0.41	96%
Storskäret	Finland	STO	51	19	3.53	0.96	37.25%
Tjongspollen	Norway	TJO	50	9	3.76	0.98	18%

n number of samples **C** clonal individuals $\mathbf{H^{II}}$ Shannon index $\mathbf{V^{II}H^{II}}$ Evenness C% percentage of clonality

Results from FCA based on allele frequencies, without any repeated genotype (clones) showed discernable aggregation by geographic locality within each region in Sweden (Fig. 3.2.1) and also among putative taxa from Norway as identified in the field.

By contrast, considering finnish locations (Fig. 3.2.2) it is not possible to distinguish between localities and is possible to evidence an overlap with an exclusion of some sample from Sälskäret that appear separate from the main group.

Finally for set of samples from Norway, in Figure 3.2.3 It is easier to observe three distinct genetics patterns showing one cloud represented by *F.ceranoides*, one by *Fucus vesiculosus* with a single set of points including samples collected in high and low salinity environment and another one for *Fucus* X (X= species not identified).

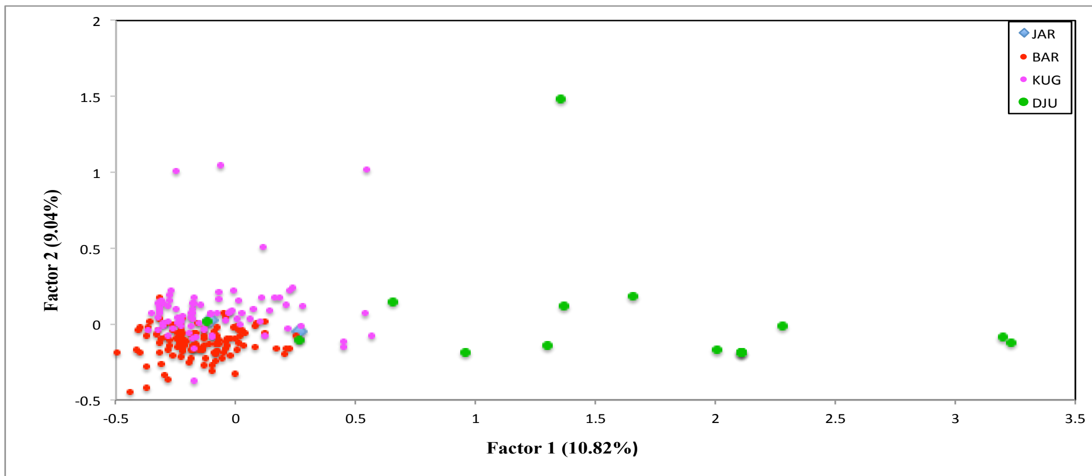

Figure 3.2.1: Factorial correspondence analysis based on allele frequencies from five microsatellite loci of samples from Sweden (withouth clones).

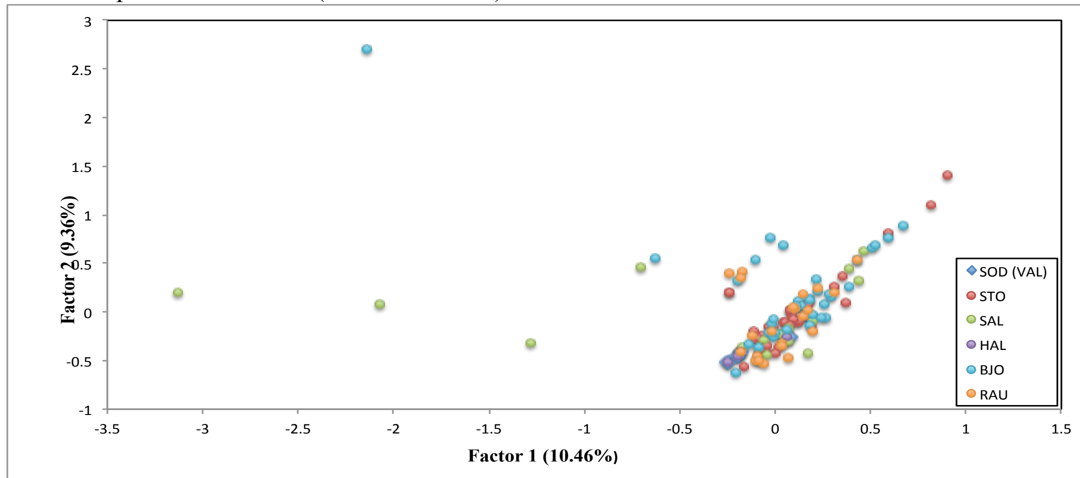

Figure 3.2.2: Factorial correspondence analysis based on allele frequencies from five microsatellite loci of samples from Finland (withouth clones).

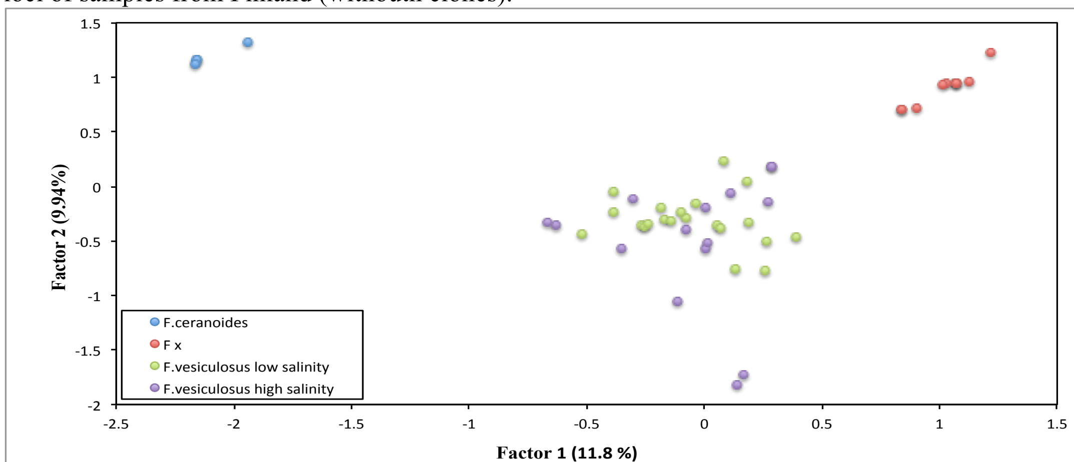

Figure 3.2.3: Factorial correspondence analysis based on allele frequencies from five microsatellite loci of samples from Norway.

Results from Bayesian genetic clustering analysis (Fig. 3.2.4) showed a clear separation among Swedish localities. Within Barsta and Kuggören, individuals belonged to two different genetic entities. Another overlap is possible to be observed between Kuggoren and Järnäs while there is a separation between Djursten and Järnäs .

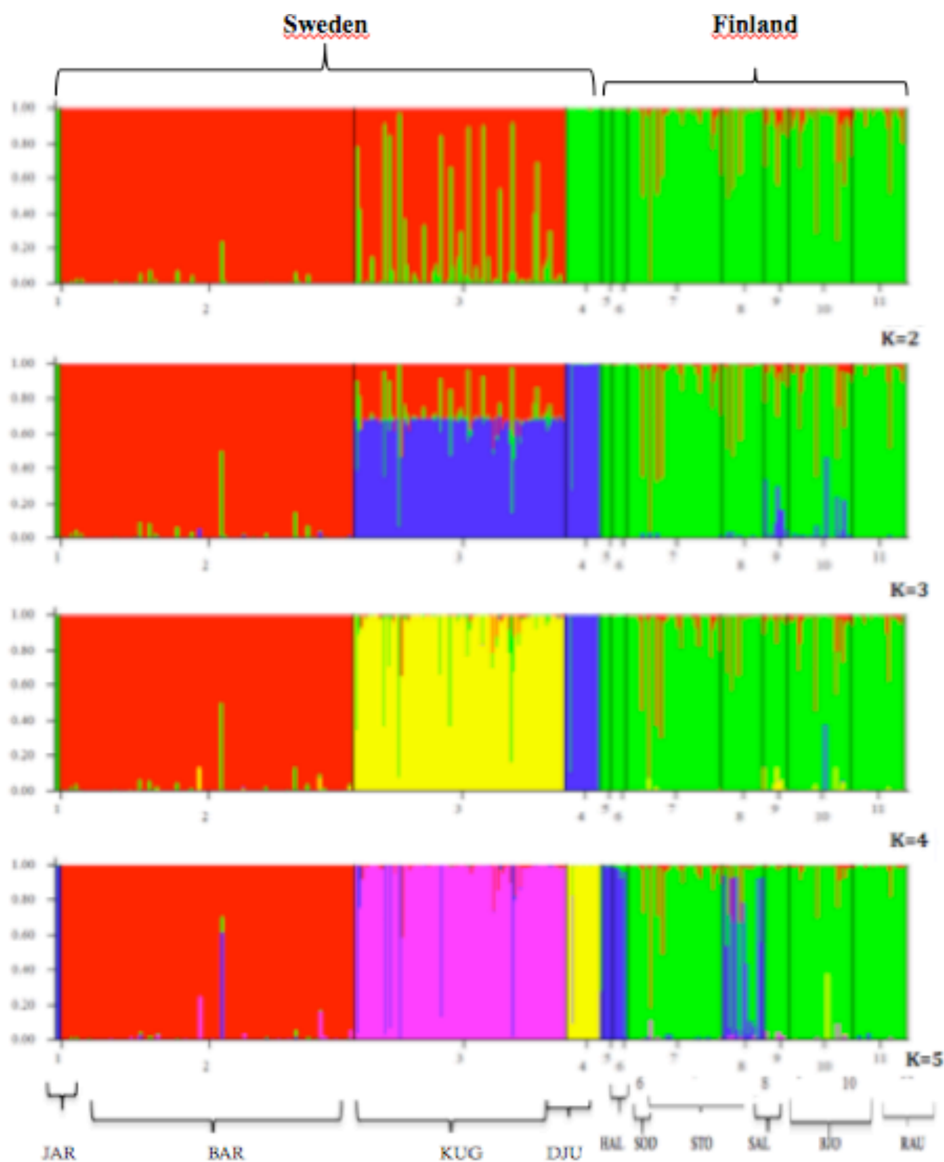

Figure 3.2.4: Bayesian assignment analysis based on genotypes data of five microsatellite loci (without considering clones) of *Fucus* from ten Swedish and finnish locations.. Each vertical bar represents one individual. Fraction of colour in an individual represents its estimated membership to a certain genetic group. Black lines separate different localities. Sampling sites are labelled below the figure. K is a priori user-defined number of gene pools.

Genetic separation is showed between swedish and finnish locations, while the first are also separated each other (Fig. 3.2.1), the same uniformity showed by FCA (Fig. 3.2.2) is present also with Bayesian analysis considering finnish populations, where there is a dominance of one genetic entities putatively *F.radicans*, with the exception of some individuals that may be *F.vesiculosus* or hybrids between the two species.

In Tjonspollen, bayesian analysis (Fig. 3.2.5) revealed a clear genetic separation among putative taxa and ecomorphs (i.e. *F. vesiculosus* high salinity and low salinity). This analysis identifies a cohesive gentic identity that comprises all individuals from a previously unidentified morphology. Given this large genetic differentiation and in order to test the evolutionary depth of this divergence, further insight was gained from sequencing analysis.

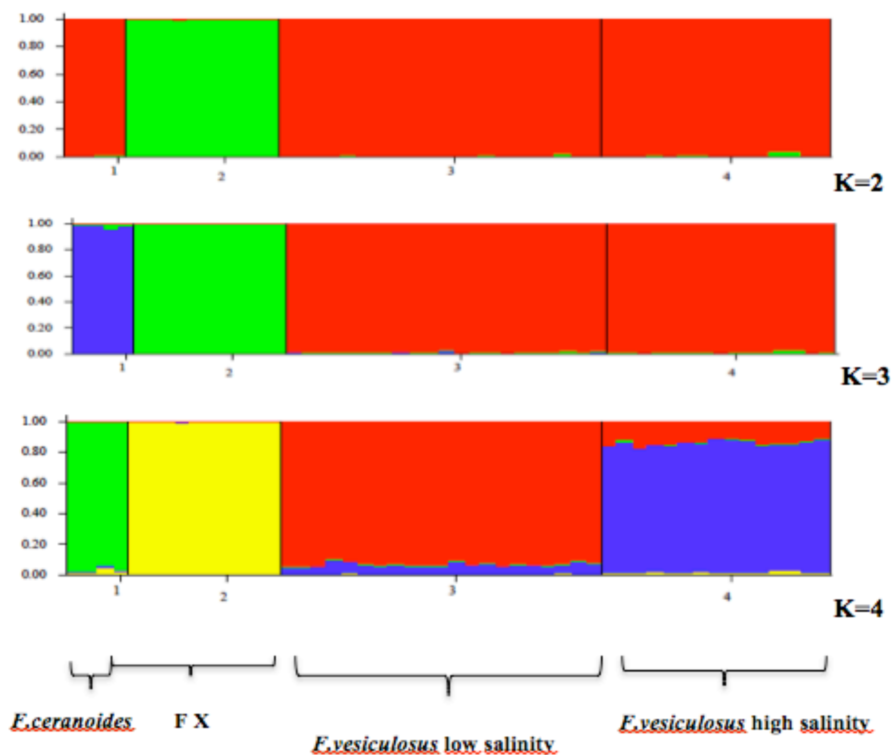

Figure 3.2.5: Bayesian assignment analysis based on genotypes data of five microsatellite loci (withouth considering clones) of *Fucus* from Tjongspollen. Each vertical bar represents one individual. Fraction of colour in an individual represents its estimated membership to a certain genetic group. Black lines separate different species. Sampling species of *Fucus* are labelled below the figure. K is a priori user-defined number of gene pools.

Figure 3.2.6, not only confirms the results from microsatellites but also unveils a closer genetic affinity of Fucus X with *F.serratus* than with *F.vesiculosus* or *F.ceranoides*.

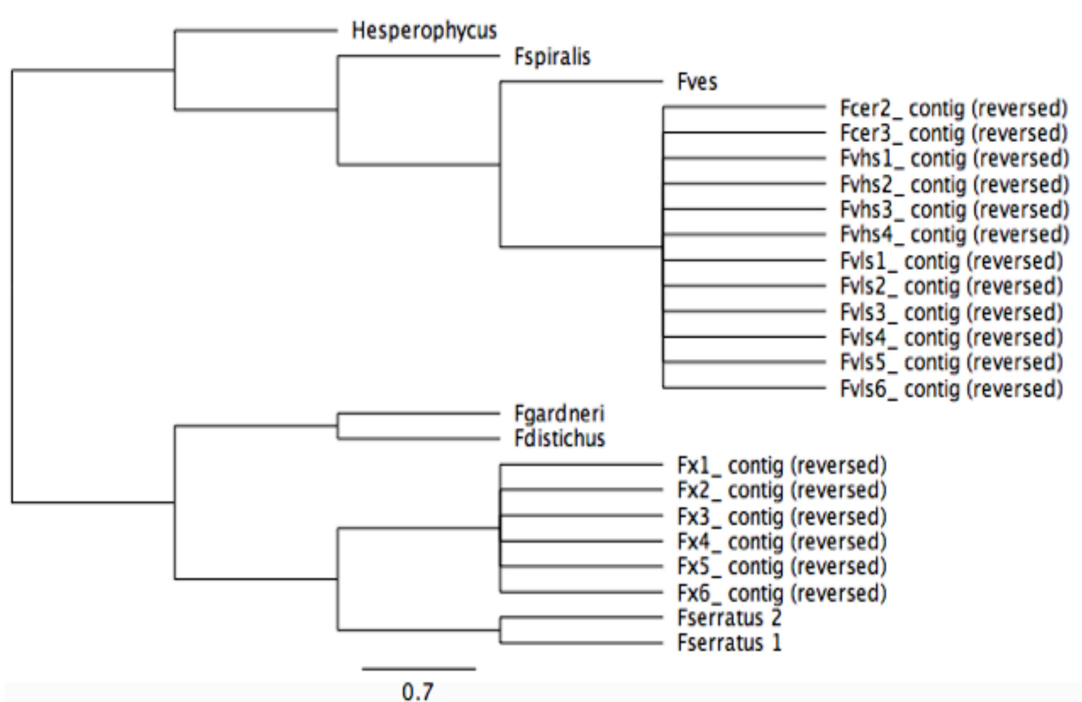

Figure 3.2.6: Phylogenetic tree showing evolutionary relation, based on genetical distances between *Fucus* species (Jukes and Cantor, 1969; Shimodaira and Hasegawa, 1999).

3.3 Phlorotannins content in algal tissue

The multidimensional scaling plots based on percentage of phlorotannin content in dry weight of algal tissue show no discernable pattern among populations from any of the Baltic localities. (Figures: 3.3.1, 3.3.2, 3.3.3, 3.3.4, 3.3.5,).

Phlorotonnanins concentration range 8-14% of dry weight in all samples but the differences within the range are usually considered normal in natural populations and not diagnostic.

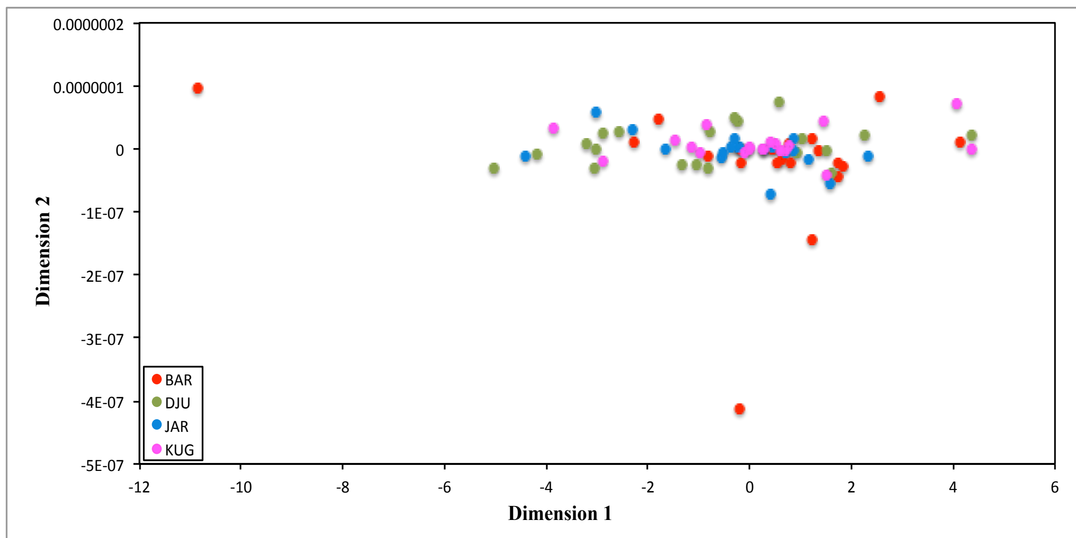

Figure 3.3.1: Phlorotanin content in *Fucus* from Swedish localities. Multidimensional scaling (MDS), based on distances matrices. Plot performed on percentage content of phlorotannin in dry weight of the algal tissue.

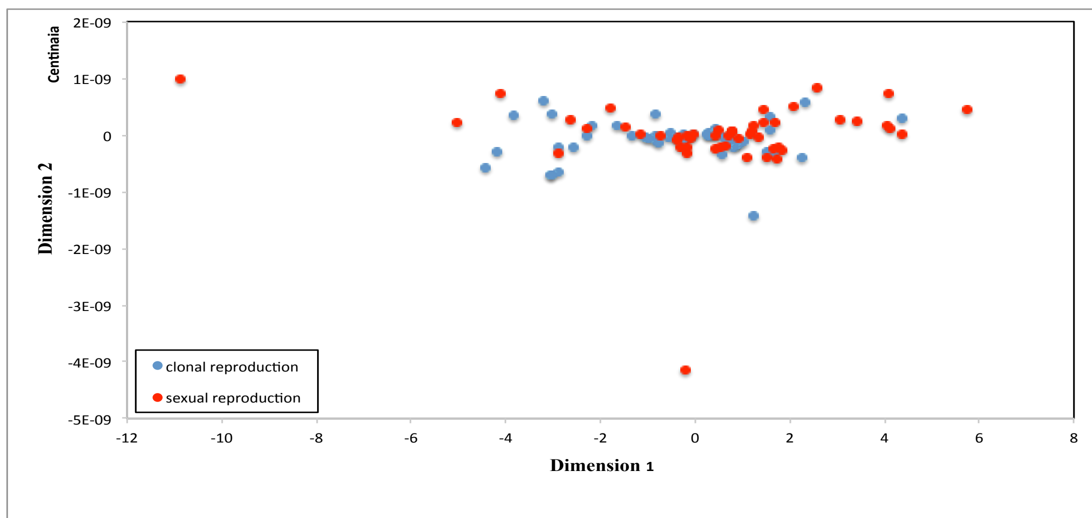

Figure 3.3.2: Phlorotannin content in Swedish *Fucus* individuals with different reproductive strategies (sexual vs clonal) without any putative species subdivision. Multidimensional scaling (MDS), based on distances matrices. Plot performed on percentage content of phlorotannin in dry weight of the algal tissue. .

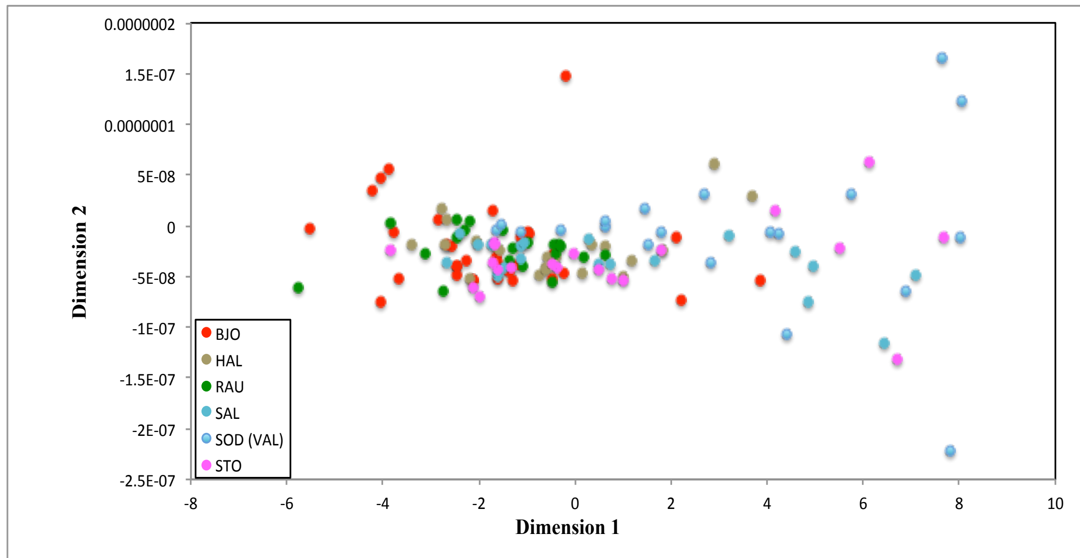

Figure 3.3.3: Phlorotanin content in Fucus from Finnish localities. Multidimensional scaling (MDS), based on distances matrices. Plot performed on percentage content of phlorotannin in dry weight of the algal tissue.

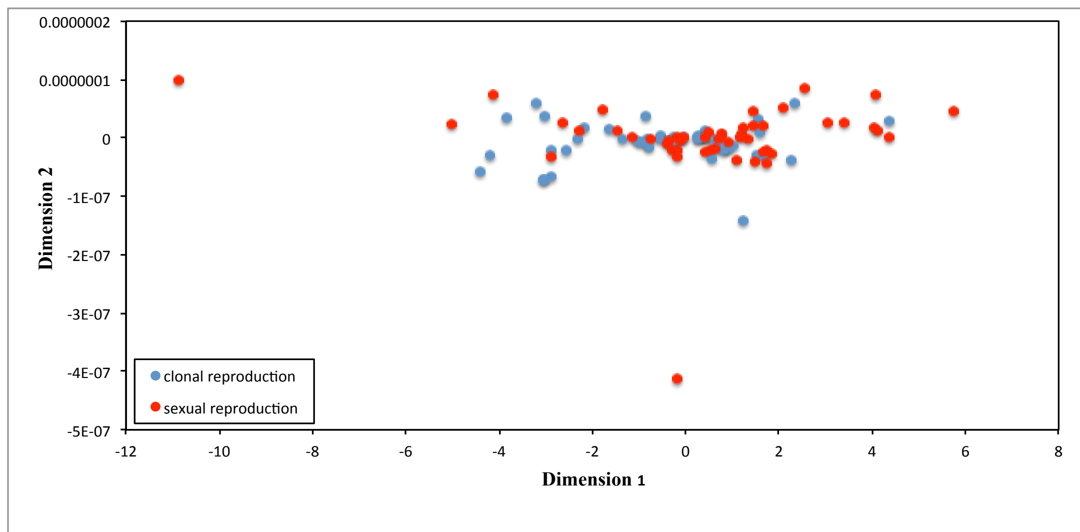

Figure 3.3.4: Phlorotannin content in Finnish *Fucus* individuals with different reproductive strategies (sexual vs clonal) without any putative species subdivision. Multidimensional scaling (MDS), based on distances matrices. Plot performed on percentage content of phlorotannin in dry weight of the algal tissue.

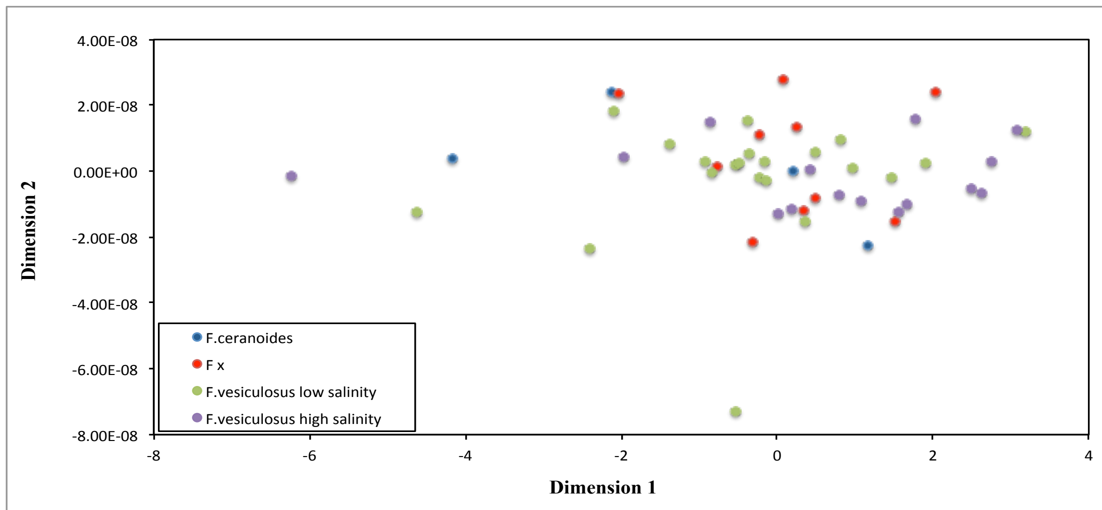

Figure 3.3.5: Phlorotanin content in *Fucus* from Tjongspollen, Norway. Multidimensional scaling (MDS), based on distances matrices. Plot performed on percentage content of phlorotannin in dry weight of the algal tissue.

4. DISCUSSIONS AND CONCLUSIONS

The objective of this study was to assess the degree of divergence of *Fucus* seaweeds from the Baltic Sea and Tjonspollen in Norway at three biological levels:

- Morphology
- Genetics
- Chemistry

The purpose of this assessment was to determine whether any biotic or abiotic processes promote the local adaptation of particular traits and above all, if they promote divergent ecotypes at any organismal level.

The results from this study showed divergence at morphological and genetic levels to certain extent but complete lack of divergence at biochemical level (i.e. constitutive phlorotannin production) in the Baltic Sea or Norway.

Morphological divergence was clearly evident in Tjongspollen (Norway) among putative taxa as they were identified in the field and this divergence corresponds with their neutral genetic divergence.

In the Baltic, there are some distinguishable patterns in the morphology of the Swedish individuals according to locality to certain extent but not among putative taxa within localities. Likewise, these morphological patterns have genetic correspondence among localities but not within each locality.

At the biochemical level, measured by the phlorotannin contents there were neither evidence of divergence in Norway or the Baltic Sea nor any discernable aggregation pattern among or within localities.

4.1 Morphology and genetics

Morphological analysis from Swedish localities showed discernable patterns by locality that could be explained by geographical distance separating them to a certain extent. Individuals from Järnäs and Djursten are at the two extreme localities (northern and southern-most of this study) and show no overlap in morphological traits.

It is known that the environment may have a large influence in the morphological traits in fucoid algae (Knight and Parke, 1950; Burrows and Lodge, 1951; Pèrez-Ruzafa *et al*., 1993; Malm *et al*., 2001; Scott *et al*. 2001; Ruuskanen and Bäck, 2002; Coyer *et al*. 2006;) and the distance among localities may translate into differences in environmental conditions.

Thus, as geographic distances become larger, the more different the environment may become the more the morphology diverges.

The results support this hypothesis, as it is evident when plotting the morphology of individuals belonging to the two localities mediating the extremes (Barsta and Kuggören).

There is larger morphological overlap among individuals from contiguous localities than the more geographically distant. Nonetheless, considerably reduced gene flow and significant environmental differences are needed among localities for this hypothesis to hold as it is predicted by ecological divergence theory (Fig. 1.1.1). The steeper the cline is, the stronger the reproductive isolation might become.

The reproductive biology of *Fucus* also contributes to geographic reproductive isolation as gametes sink immediately after release, with limited dispersal capacity (Serrão *et al*., 1996), largely depending on strong water movements (currents, tides, upwelling) to disperse. In addition, *Fucus* vegetative reproduction in the Baltic effectively isolates individuals from gene flow, further contributing to the reduced gene flow.

In contrast, *Fucus* individuals from the Finnish coasts show no discernable pattern in morphological traits and instead, show large overlap among all the localities. This suggests either, homogeneous environmental conditions across this area have influenced their morphology or that other extrinsic or intrinsic factors influence more the morphological traits than the environment in this region.

In terms of correspondence with the genetic data, the morphological results show some congruence with the genetic subdivision to support the hypothesis of divergence due to different environmental conditions. However, the genetic results also show further genetic subdivision within some localities both in Finland and

Sweden that is not evident from the morphology (Fig. 3.2.4). This, of course, may not come as surprise if the environmental or ecological clines are not steep enough to create divergence at both organismal levels and since microsatellites are putatively neutral they are not expected to be associated with the morphological traits. By first identifying the repeated genotypes from each locality (Tab. 3.1), it was possible to infer together with previous studies which individuals correspond to *F. radicans* and *F. vesiculosus*. Johannesson et al. (2011) reported high incidence of clonality in *F. radicans* and much lower in *F. vesiculosus*. In addition, these authors reported the presence of at least two conspicuously dominating genotypes of *F. radicans* covering large geographical areas that are also present in this study (Fig. 3.2.4); blue bars in Järnäs, Hällkalla, South Vallgrund and Sällskaret).

Therefore, it can be concluded that while it is possible to find genetic divergence in *Fucus* from the Baltic Sea, in the absence of diagnostic morphological characters or when morphological traits are continuous, it is essential to use complementary information to distinguish between putative taxa such as reference individuals with diagnostic genetic and/or morphological traits to compare with.

A completely different scenario unveiled regarding Norwegian *Fucus*, there is a strong divergence at morphological and genetic level among co-occurring individuals.

That is, there is no obvious environmental cline as a potential divergence mechanism, suggesting that either historical or ecological mechanisms of divergence have or are acting in this area. Both morphological and genetic analysis show marked cohesiveness of *F. ceranoides*, *F. vesiculosus* from inside and outside Tjongspollen and also those to date formally undescribed individuals. The latter in fact, appeared largely differentiated and they may well warrant a new species status.

Regarding *Fucus vesiculosus* from low and high salinity (inside and outside Tjongspollen pool, respectively), the genetic separation is noticeable between them, which highlights the strong environmental influence both in morphology and genetically, effectively drifting apart.

4.2 Phlorotannins content

The phlorotannin range observed in this study in Baltic and Norwegian seaweeds is between 10-14%.

This is in line with previous studies (Kubanek *et al*., 2004) that underlined the higher phlorotannins content in the brackish water ecosystems such as the Baltic area and Tjongspollen pool with high levels of stress factors, like low salinity range and high seasonal temperature fluctuation, for local populations so that they have evolved varous physiological adapations (Rietema, 1991, 1993, 1995; Kristiansen *et al*., 1994; Düwel 2001; (Heggøy 2001).

As shown before, phlorotannin levels in the brown algae are significantly higher in Spring than in Autumn as a consequence of nitrogen availability (Chapman and Craigie ,1978; Hanisak 1983; Steinberg et al., 1994), and light availability (Pavia et al., 1997).

Nevertheless, despite the concordance with the expected salinity ranges according to the environmental conditions, the chemical analysis showed no differentiation between different groups. This evidence conflicts with previous studies regarding phlorotannins content as chemical defense against herbivores in which it has been shown a strong preference of the isopod Idotea balthica towards F. radicans in the presence of F. vesiculosus (Forslund *et al*., 2012; Ardehed et al., not yet published).

However, this might be explained by the fact that the present study analysed constitutive levels of phlorotannins and previous observed differences may have been inducible

Moreover, *I. baltica*, underlined as a potential driver of phlorotannin production as inducible defence (Forslund *et al*., 2012), is largely absent in the Swedish coasts of the Gulf of Bothnia so levels of phlorotannin might be depending mostly by environmental conditions.

4.3 Conclusions and future perspectives

In conclusion our study, based on phenetic, phylogenetic and biological taxonomical criteria, represent a favourable start point to understand the divergence patterns in *Fucus* species from marginal areas.

Nonetheless, the fact that there is strong divergence in morphology and genetics among several putative taxa without any obvious physical barriers or clines needs further investigation to unveil the mechanisms of divergence.

Our future perspectives include to investigate deeper about distribution and adaptative genetic variation among species that might probably be better explained by next-generation DNA sequencing (NGS) (Allendorf et al. 2010; Funk et al. 2012).

Moreover, our chemical results represent an interesting subject to carry out future studies about phlorotannins production in *Fucus* seaweeds, and the mechanisms of phenotypic plasticity due to local adaptation in marginal ecosystems.

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APPENDIX A

Extraction protocol for RAD-seq of *Fucus*

I. EXTRACTION STEP

1. Place a small piece of algal tissue (10-15 mg; ~2.0 x 0.7cm) in a microcentrifuge 1.5ml tube along with two small stainless steel balls bearing (5mm) and close the lid. Place it into a milling instrument (MixerMill MM 301, Retsch) and pulverized the tissue for 1.5 min at a frequency of $30s^{-1}$. (*See note in appendix to wash bearing balls)

2. Suspend the ground material in 1mL of 100% acetone for 10 min in the rotor.

3. Centrifuge samples at 11000 rpm for 1 min, pour out the acetone and discard it.

4. Repeat steps 2 and 3 and air-dry samples for 5-10 min (Avoid overdrying).

5. Add 500 µl of 2% CTAB Extraction buffer to each tube. Use a pipette tip to scrape the tissue pellet off the tube wall (See Appendix).

6. Add 17.5 µl of 0.1M DIECA, 5 µl of 2-Mercaptoethanol under fumehood, 10 µl RNase A solution (10mg/ml), vortex vigorously and incubate the mixture at 65°C for 1 hour. Vortex the mixture every 10 min. (For 30 samples, one may prepare in advance 16 mL of CTAB buffer, 560 µl DIECA, 320 µl RNase A and 160 µl 2-Mercaptoethanol).

7. From this point, we use the NucleoSpin Plant II Kit from Macherey-Nagel. Preheat Nuclease-free ddH_2O to 65°C for final elutions (55 µl per extraction).

8. (It is highly recommended that the following step is done in batches of 10 or 15 samples). Place a NucleoSpin Filter (violet ring) into a new collection tube (2ml) and load the crude lysate onto the column using wide-bore filtered tips. Centrifuge for 2 min at 11,000 rpm, collect the clear flow-through and discard the Filter. If not all liquid has passed the filter, repeat the centrifugation step. If a pellet is visible in the flow-through, transfer the clear supernatant to a new 1.5ml tube using wide-bore pipette tips. Alternatively, place the NucleoSpin Filter in a new 1.5ml tube and pass the pre-cleared supernatant through the Filter once more to remove solid particles completely.

9. Add 450 µl Buffer PC and mix thoroughly by gentle inversion a few times.

10. Place a NucleoSpin Plant II Column (green ring) into a new Collection tube (2ml) and load a maximum of 700 µl of the sample. Centrifuge for 1 min at 11,000 rpm and discard the flow-through.

11. Add 400 µl Buffer PW1 to the NucleoSpin Plant II Column. Centrifuge for 1 min at 11,000 rpm and discard the flow-through.

12. Add 700 µl Buffer PW2 to the NucleSpin Plant II Column. Centrifuge for 1 min at 11,000 rpm and discard the flow-through.

13. Add another 200 µl Buffer PW2 to the NucleSpin Plant II Column. Centrifuge for 2 min at 11,000 rpm and discard the flow-through.

14. Place the NucleoSpin Plant II Column into a new 1.5ml tube. Pipette 50 µl of ddH_2O (preheated 65°C) onto the membrane. Incubate the NucleoSpin Plant II Column for 5 min at 65°C. Centrifuge for 1 min at 11,000g to elute DNA.

II. CLEANING-UP STEP

15. From this point, we use the Zymo DNA Clean & Concentrator. Preheat Nuclease-free ddH_2O to 65°C for final elutions (65 µl per extraction).

16. Add 100 µl of DNA Binding Buffer to each DNA sample. Mix 1 sec in vortex.

17. Transfer mixture to a Zymo-Spin column in a collection tube.

18. Centrifuge for 30 seconds at 11500 rpm. Discard the flow-through.

19. Add 200 ul of DNA Wash Buffer to the column. Centrifuge for 30 seconds at 11500 rpm and discard the flow-through.

20. Repeat the wash step.

21. Place the Zymo-Spin Column into a new 1.5ml tube. Pipette 35 µl of ddH_2O (preheated 65°C) onto the membrane. Incubate the column for 5 min at 65°C. Centrifuge for 30 seconds at 11500 rpm to elute DNA.

III. QUALITY-CHECK STEP

22. Run 5 µl of each extraction in a 1% agarose gel with a 20 kb ladder at 50 Volts for 1.5 hours. While running the gel, measure DNA concentration in Nanodrop. For further steps, avoid using samples with values less than (260/280 < 1.8) and (260/230 < 1.2). In addition, only samples that appear in the agarose gel with little or no degradation and a high molecular band must be used further.

23. Check REAL DNA concentration with 1 µl in Qubit and keep the rest at 4°C for further concentration. Only samples with Qubit concentrations above 10ng/ul will be useful.

IV. DNA CONCENTRATION ADJUSTMENT STEP

Note: the following steps can be substituted for simple evaporation in speed vac to resuspend in the needed volume for a final concentration of 50ng/µl in 1x TE buffer (use excel sheet "concentrate dna" to calculate final elution volume for each sample in last step).

24. Adjust the concentration of monovalent cations in the sample by adding 1/10th volume of 3 M sodium acetate to the sample. For 50 µl of DNA, add 5 µl of 3M NaAOC.

25. Add 1 volume of isopropanol and mix gently. If there is sufficient DNA in the sample, you will see a white precipitate form very rapidly.

26. Recover the DNA by centrifugation for 20 min at 4°C max speed. Carefully pour out isopropanol to avoid disturbing the pellet.

27. Add 2 volumes of 70% ethanol to remove residual salt, centrifuge at max. speed for 5 minutes. Carefully pour out ethanol to avoid disturbing the pellet.

28. Evaporate off residual ethanol (max 15 min.) and resuspend the DNA pellet in the needed volume of 1x TE Buffer for a final concentration of 50ng/µl.

Additional information

Bearing balls for grinding can be reused by rinsing them in 10% bleach solution and rinse well with ddH_2O before using them again. Tissue can be silica-dried but must be less than 3-month old or fresh to avoid degraded DNA. If fresh, grinding should be also done with the aide of liquid nitrogen by placing the tube with the tissue into the nitrogen for a 30s, grind it quickly.

Extraction buffer

2% CTAB, 3% Polyvinylpirrolidone (PVP-40 or 0.1% PVPP-"PVP-cross linked"), 3.5mM diethyldithiocarbamic acid (DIECA), 1.4 M NaCl, 20mM EDTA pH 8.0, 100mM Tris-HCl pH 8.0, 1% mercaptoethanol (PVPP- DIECA, complexors of tannins and polyphenols and mercaptoethanol, reductant):

2 g	CTAB
10 ml	Tris-HCl 1M pH 8.0
4 ml	EDTA 0.5 M
28 ml	NaCl 5M or 8.1 g NaCl
3 g	PVP-40 (MW: 40,000)

Important note on the pvp
PVP-40 is soluble in water and may be added in larger amounts. It works much better when there are larger amounts of tissue >10mg. In contrast, a cross linked version of this chemical called PVPP Polyvinylpolypirrolidone (also appears as "PVP-cross linked") is NOT soluble in water but it's known to have strong binding properties with tannins and polyphenols but it is not so useful with large amounts of tissue >10mg since the silica filters get easily clogged. The latter may be also used in the buffer but instead in 0.1% (100mg in 100ml) but still will not dissolve. It will just need to be stirred every time before use.

Add ddH2O to make 100 ml. Heat with stirring to dissolve CTAB (be careful not to boil over). AUTOCLAVE

Stock solutions
1 M Tris, pH 8.0: for 1 L
121.1 g Tris
700 ml ddH2O
Dissolve tris and bring to 900 ml.
pH to 8.0 with concentrated HCl (will need ~50ml)
Bring to 1 L.

0.5 M EDTA pH 8.0: for 1 L
186.12 g of EDTA
750 ml ddH2O
Add about 20 g of NaOH pellets
Slowly add more NaOH until pH is 8.0, EDTA will not dissolve until the pH is near 8.0.

5 M NaCl: for 1 L
292.2 g of NaCl
700 ml ddH2O
Dissolve and bring to 1 L.

1.1 M Diethyldithiocarbamic acid (DIECA): for 100 ml
2.253 g of DIECA
70 ml ddH2O
Dissolve and bring to 100 ml

APPENDIX B

Master mix for PCR amplification for each locus.

L20	Fucus:L20Ta53	
Batch	PLATE 8	
	n. of samples	88
MasterMix	x1	93
BSA 0,5 ug/ul	0.54	49.82
buffer 10x	1.07	99.64
dNTPs 10 mM each	0.21	19.93
MgCl2 50 mM	0.43	39.86
Primer F 10 uM	0.54	49.82
Primer R 10 uM	0.54	49.82
Taq (platinum)	0.06	5.30
H20	6.12	569.31
		883.50
Aliquotes	9.50	9.50
DNA templ	0.50	
	10.00	

L58	Fucus:L38L58	
Batch	PLATE 8	
	n. of samples	88
MasterMix	x1	93
BSA 0,5 ug/ul	0.54	49.82
buffer 10x	1.07	99.64
dNTPs 10 mM each	0.21	19.93
MgCl2 50 mM	0.32	29.85
Primer F 10 uM	0.54	49.82
Primer R 10 uM	0.54	49.82
Taq (RBC)	0.11	10.63
H20	6.17	573.98
		883.50
Aliquotes	9.50	9.50
DNA templ	0.50	
	10.00	

L94	Fucus:L94TA57	
Batch	PLATE 8	
	n. of samples	88
MasterMix	x1	93
BSA 0,5 ug/ul	0.54	49.82
buffer 10x	1.07	99.64
dNTPs 10 mM each	0.21	19.93
MgCl2 50 mM	0.35	32.55
Primer F 10 uM	0.54	49.82
Primer R 10 uM	0.54	49.82
Taq (platinum)	0.06	5.30
H20	6.20	576.61
		883.50
Aliquotes	9.50	9.50
DNA templ	0.50	
	10.00	

L38	Fucus:L38L58Ext_61	
Batch	PLATE 8	
	n. of samples	88
MasterMix	x1	93
BSA 0,5 ug/ul	0.54	49.82
buffer 10x	1.07	99.64
dNTPs 10 mM each	0.21	19.93
MgCl2 50 mM	0.30	27.90
Primer F 10 uM	0.54	49.82
Primer R 10 uM	0.54	49.82
Taq (platinum)	0.06	5.30
H20	6.25	581.26
		883.50
Aliquotes	9.50	9.50
DNA templ	0.50	
	10.00	

L85	Fucus:L85TA61	
Batch	PLATE 8	
	n. of samples	88
MasterMix	x1	93
BSA 0,5 ug/ul	0.54	49.82
buffer 10x	1.07	99.64
dNTPs 10 mM each	0.21	19.93
MgCl2 50 mM	0.64	59.80
Primer F 10 uM	0.54	49.82
Primer R 10 uM	0.54	49.82
Taq (platinum)	0.06	5.30
H20	5.91	549.36
		883.50
Aliquotes	9.50	9.50
DNA templ	0.50	
	10.00	

APPENDIX C

Thermocycling regimes for PCR amplification

L20

STEP	TEMPERATURE	TIME
Initial Denaturation	95°C	2 min
34 cycles	95°C 53°C 72°C	40 sec 40 sec 30 sec
Final Extention	72°C	30 min
Hold	4°C	

L38

STEP	TEMPERATURE	TIME
Initial Denaturation	95°C	2 min
34 cycles	95°C 58°C 72°C	40 sec 40 sec 30 sec
Final Extention	72°C	2 min
Hold	4°C	

L58

STEP	TEMPERATURE	TIME
Initial Denaturation	95°C	2 min
34 cycles	95°C 58°C 72°C	40 sec 40 sec 30 sec
Final Extention	72°C	2 min
Hold	4°C	

L85

STEP	TEMPERATURE	TIME
Initial Denaturation	94°C	2 min
34 cycles	94°C 61°C 72°C	30 sec 30 sec 30 sec
Final Extention	72°C	2 min
Hold	4°C	

L94

STEP	TEMPERATURE	TIME
Initial Denaturation	95°C	2 min
34 cycles	95°C 58°C 72°C	40 sec 40 sec 30 sec
Final Extention	72°C	3 min
Hold	4°C	

APPENDIX D

How to run microsatellites on Beckman Coulter CEQ 8000

By Anna-Karin Ring Updated 2013-09-05

General guidelines:

Hand in the risk assessment before you perform your first operation.
Always use lab coat and gloves in the post PCR room.
Always throw away or change gloves before you leave the post PCR room.

Book your run on the booking sheet in the post PCR room. If you are planning to run several runs tell the lab responsible so we will not run out of chemicals.

Fill the information needed about your run in the Beckman log book, and if you experience any problem during the run or after analyzing your results make a note about it in the book, as well as discussing it with the lab responsible.

If you want more information about the machine or the software you can find manuals in the DNA- lab. For info on analyzing the results see the manual "STR locus tags and allele list generation".

Before you start:
Prepare a excel spread sheet with your sample configuration (a "sample set up") and save it on a memory stick (.xls format!)

Put SLS and size standard in fridge to defrost.

Starting and preparing the computer:
Turn the Beckman machine and the computer on (no password).

Create a folder where your data will be stored (preferably within a folder with your name on the Beckman computer's "My documents"), where you save all your data.

Open the program CEQ system
From main menu open **Database**
(the database in bold is the active one where all data from the run will be stored)
Make a new project for each run (ex "AK 20100329 COD ST set1"). You create a new project within the database by right click on the active database and choose **new project.**

Open **Setup** from main menu – this will create a new sample plate – view by subject ID (tick this box)– copy paste your sample set up from your excel file into the sheet of the setup program. (If you do like this sample name and subject ID will have the

same designation. You can also choose to have a different sample name and subject ID, in that case make two set ups in excel and fill sample name and subject ID separately. See Beckman instruction book for more information, or ask.)

Mark your sample set up, choose **Edit -> Auto-fill method name -> All sample sets Choose method -> Frag-3 (for size standard 400) or Frag-1 (for size standard 600)**

NB! If your not running a whole plate you have to mark only the sample sets you are going to run, (*only whole rows*): tick **Selected samples sets only** instead of **All sample sets.**

Mark your sample set up and choose **Analysis-> Export data**
Choose your folder where the data will be stored (on the desktop) -> save as type: CEQ (".cq") (for microsatellite data). Don't keep too much run data in your folder as it uses a lot of space in the computer. Clear the folder regularly.

Save the set up file
File -> save as -> Database: "20100325" (i.e. the active database), -> project name: "AK 20100329 COD ST set1"
On the right hand plate saved appears. Do not close this window.

Installing the capillary array:
Fill the **wetting tray with ddH2O** and put the lid on (no bubbles!). Bring the box with the **capillary array** in use from the fridge.

From main menu open **Run -> Direct control** (shows a display of the machine on the screen)
Place the marker on the picture of the capillary, right click -> choose **release manifold plug**

A small window will appear: add the serial number of the capillary array (if not stated) and tick **Install capillary array**
Fill the box "Nbr of runs": (stated on the capillary box)
Days on instrument: put 0

The machine will tell you when it is ok to start with the installation of the capillaries.

Open the front of the machine, unscrew the two covers and take the **manifold plug** out by pulling the red handle towards you. Put the manifold plug inside at the front of the machine.
Carefully take the covers off the capillary array. Keep them steady by keeping them at the table whilst pulling the covers, slowly and straight, off. Always handle the capillary array as instructed!

Put the capillaries into the machine, carefully. Put the covers of the machine back on and put the wetting tray into the machine as soon as you have the capillaries installed. *You can never keep the capillary array in the machine without a filled wetting tray.*

Preparing the buffer plate:
Fill the wells of the buffer plate with buffer solution, it is kept in the fridge.

Gel cartridges: are stored in the fridge. From the **direct control** display click the gel cartridge and choose install gel. Push the lower door of the machine and take away the yellow dummy and put the cartridge there instead. Put the cap on the yellow dummy and keep it in the machine, in the front.

Preparing the chemicals:
Count how much of the two chemicals SLS and size standard you need (those are kept in the freezer) NB! SLS is sensitive to air and the size standard is sensitive to light.
SLS: 34.55 uL/sample (well)
Size standard: 0.55 uL/sample (well)

For one whole plate add 1 105 uL of SLS in each of three eppendorf tubes and add 17,6 ul of size standard in each tube. Mix.

SLS is stored in eppendorf tubes or in original packaging in the freezer. The bigger original tubes with SLS can be put in the fridge approx. 1 h before you need it and it will be defrosted and ready to use.
Spin down eppendorf tubes before opening.

If you are using a new SLS bottle, measure the volume you need for your sample plate and put it in eppendorf tubes. The remaining SLS has to be put in eppendorf tubes as well (as it is air sensitive) and mark the tubes with content and date incl. year (*very important*) and put in the freezer for the next user.

Size standard 400 is used for runs with fragment shorter than 400 bp. Spin down the defrosted size standard before opening.

Preparing your PCR products:
Take a **Beckman sample plate** and put it onto a cooling block.

Transfer the PCR products to the Beckman sample plate. The standard recommended by Beckman is:
2.8 ul of the black pcr product (D2)
1.4 ul of the green pcr product (D3)
0.8 ul of the blue pcr product (D4)

But you can modify this according to your own primers and how well they amplify, and as well to your own set of colors (for example running 4 loci in one run). Recommended maximum volume of PCR product is 5 ul in total.

Completing the Beckman sample plate:
Use the dispenser pipette to dispense the SLS+size standard solution in the Beckman sample plate, 35 ul/sample.

Put a drop of mineral oil on top of every sample (prevents evaporation)

Check for air bubbles in the wells. Spin down the plate in the centrifuge.

Observe that you cannot run less than one whole row, so if you have less than 8 samples in a row you still need to add SLS and buffer for the whole row.

Starting the run:
Go back to your sample setup and choose **Run sample plate**
Choose who's the operator "AK" and choose your project: "-------"

From the window that appears **Load plates ->** (check that everything is ready) **-> start**
Open the machine, put your sample plate in the tray in the back and press slightly to put it in place (watch out for the capillaries!)
Put the buffer plate in the front tray and put the white lid on, with the "lip" facing you!! Close the door of the machine and choose **Load -> START**

To do after the run is completed:
The run will last for number of rows-1 h = 12 rows, approx. 11 hours.

IF THE MACHINE IS NOT TO BE USED SOON AGAIN
When the run is complete and as soon as possible and if the machine is not to be used soon again: **Direct control -> release capillary -> install manifold plug** and follow the instructions on the screen to take the capillaries out. Clean the wetting tray.
Release the gel cartridge and put the yellow dummy into the machine from **Direct control-> release gel cartridge-> install plug.** Follow the instructions on the screen.

Used sample and buffer plate are disposed off in dangerous waste box (put in plastic bag) - or if you have not used a whole plate keep in a plastic bag marked with date and your name to be used later.

Turn the computer and machine off. Remember to fill in the data about your run in the Beckman log after analyzing you result.

IF THE MACHINE IS TO BE USED SOON AGAIN

If the capillaries are installed, a wetting tray must be present at all times, the water must be changed after the run is completed.

When the run is complete and as soon as possible, from **Direct control -> change wetting tray** –take your plates out of the machine and clean and fill the wetting tray with new distilled water.
From **Direct control -> release gel cartridge -> install plug.** Follow the instructions on the screen.

Used sample and bufferplate are disposed off in dangerous waste box (put in plastic bag) - or if you have not used a whole plate keep in a plastic bag marked with date and your name to be used later.

Remember to fill in the data about your run in the Beckman log after analyzing you result.

APPENDIX E

Determination of total phlorotannins in *Fucus* seaweeds

Preparation of seaweeds samples

1. Homogenize the freeze-dried sample with a mortar and pestel.
2. Extract in 60-vol% acetone or methanol for 1 hour on a shaking table. Note the exact weight of the sample. For *Fucus* we used between 5-8 mg sample in 1.5 ml acetone. Transfer the sample to a marked 1.5 ml Eppendorf tube.
3. Centrifuge at the highest speed in a microcentrifuge for 2 min and transfer 1 ml of the supernatant to a new marked Eppendorf tube (1.5ml).
4. Evaporate the acetone in vacuo (takes about 1 h in the rotary evaporator). Make sure that the samples do not dry out. Add dH_2O if the samples are left for a longer period.
5. Filter (20 µm) the samples in order to remove precipitated lipophilic material. Make sure to filter the whole sample. Rinse the tube several times with dH_2O.
6. Dilute to 10 ml with dH_2O.

Folin-Ciolcateus analysis

1. Add 7 ml dH_2O and 1 ml of the diluted sample to a test tube.
2. Add 0.5 ml of Folin-Ciolcateus reagent and mix thoroughly.
3. Add 1.5 ml of sodium carbonate (Na_2CO_3, 20g/100ml), mix and note the time of addition.
4. Prepare a blnk with dH_2O instead of sample every 20 samples.
5. Measure the absorbance a 725nm after 2 hours incubation.
6. Calculate the concentration in the samples using the standard curve.

Standard curve

1. Make a dluition series with phloroglucinol (0, 10, 20…..100 µg/ml)
2. Make a Folin-Ciolcateus analysis and measure the absorbance a 725 nm. The relationship between the concentration and absorbance should be linear.

Note

1. Phlorotannins are sensible to light and high temperature (>40°C).
2. Your measurements should preferably be in the middle of the standard curve. The above given amounts and volumes are based on analyses with *Fucus* material. *Ascophyllum* and *Laminaria* usually have lower phlorotannin concetrations, so you may have to adjust the diluition of your sample.

APPENDIX F

R Instruction

1. Principal Component Analysis

```
dati=read.csv(file itinere)                    # read file
attach(dati)
library(ggbiplot)                              #open package
pca=princomp(dati[,3:4],center=T,scale=T)  # principal component analysis
```

2. Metrical Multidimensional Scaling (mMDS)

```
dati=read.csv(file itinere)                    # read file
attach(dati)
library(lattice)                               # open package
d=dist(dati[,1]
fit=cmdscale(d,eig=TRUE, k=2)                  # MDS
scores=cbind(x,y)                              # take scores for the plot
```

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