Patterns of evolution in a young species, the Baltic seaweed *Fucus radicans*

Doctoral Thesis

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To my beloved family and friends who
have supported me to the fullest
throughout these roller coaster years

Abstract

To be able to interpret patterns of biodiversity it is important to understand the processes by which new species evolve and how closely related species remain reproductively isolated and ecologically differentiated. This thesis centers on the evolution of the two brown algae *Fucus radicans* and *Fucus vesiculosus* in the Baltic Sea, where *Fucus radicans* very recently diverged from *F. vesiculosus*, and where both species have aquired the unique character of asexual recruitment forming clones. Speciation is one of the most fundamental processes in biology, yet poorly understod. It is generally argued to build on a slow accumulation of differences between populations living in allopatry, but the formation of *F. radicans* most likely occurred without geographic separation and provides a unique opportunity to study the differentiation and evolution of a new species following a recent, rapid and sympatric speciation event. We investigated the spatial patterns of clonality in *F. radicans*, to understand how clones affect the potential for sexual reproduction, recombination and local adaptation. Results showed that rare sexual events, occasional long-distance migration and somatic mutations of large clones contribute new genotypic variation in populations of *F. radicans*. We further analysed the genetic structure of *Fucus* from areas not earlier investigated along the Finnish and Russian coasts of the Baltic Sea, comparing the results with previous data from Bothnian Sea and Estonia. The results indicated a genetic structure strongly affected by the geographic isolation of populations, but in most cases separating the two taxa at a local scale. In addition, we found some cases of strong genetic asymmetries with populations having fewer rare alleles than others, either as a consequence of directional gene flow or founder effects during colonization. Interestingly, in contrast to earlier findings from the Bothnian Sea, sexual reproduction remained important in the lowest salinities (<3‰) in eastern Gulf of Finland. Nevertheless, in *F. radicans* a few old clones have dominated much of the species' distribution over extensive periods of time suggesting that the distribution of genotypes cannot solely be ascribed to natural selection. Using a spatially explicit model, we tested a neutral scenario of colonization of a new environment by a species with the potential of both sexual and asexual reproduction. We found that a pattern with spatially and temporally dominant clones mixed with areas of sexual reproduction is quite unlikley during and thousands of generations after colonization of a new environment even so sexual reproduction eventually takes over. One hypothesis connected to the speciation process is that herbivores may have had different co-evolutionary effects on the two species. We assessed differences in inducible herbivory defense using an ecological experiment complemented by gene expression analysis. We found that *F. vesiculosus* produced deterrent chemicals faster than *F. radicans*, and that patterns of gene expression were different between the species under grazing by isopods. The evolutionary changes observed in transcriptional regulation of grazing resistance in these two macro algae has likely evolved under differential selection, with *F. vesiculosus* mainly distributed in areas of strong grazing pressure, while *F. radicans* is not found in such areas. My investigations have contributed with further understanding of the Baltic Sea *Fucus* system and its intriguingly rapid and recent speciation. In current times, climate change threatens Baltic Sea species and biodiversity, and increased knowledge of processes generating and maintaining biodiversity in this ecosystem seems particularly important and needed.

Key words*:* brown algae, *Fucus radicans*, sympatric speciation, clonality, somatic mutations, microsatellites, reproductive barriers, asymmetric migration, transcriptomic differences

Populärvetenskaplig sammanfattning *Swedish summary*

För att kunna tolka mönster vad gäller arter och biodiversitet är det viktigt att ha kunskaper om de processer som leder till att nya arter bildas, och hur nära besläktade arter upprätthåller olikheter och barriärer. Denna avhandling behandlar den evolution som nyligen ägt rum hos två brunalger smaltång (*Fucus radicans*) och blåstång (*Fucus vesuculosus*), i Östersjön. Smaltången beskrevs nyligen som en egen art separerad från sin nära släkting blåstången. Smaltång finns bara i Österjön och artbildningen har sannolikt skett i Östersjön efter sista istiden. Detta ger oss ett unikt tillfälle att studera evolution hos två arter som har separerat trots att de inte har levt åtskilda från varandra. Dessa är viktiga exempel som kan bidra till ökad förståelse för hur nya arter och ny biodiversitet uppkommer. Unikt för båda arterna i Östersjön är att de här kan föröka sig med kloning. För att förstå hur klonalitet påverkar möjligheten till sexuell förökning och lokal anpassning hos arten undersökte vi den rumliga utbredningen av olika kloner hos smaltång. Det visade sig att bara ett fåtal sexuella händelser, spridning över långa distanser samt mutationer bidrar till att upprätthålla den genetisk variationen som idag finns i populationer av smaltång, och att arten i stora delar av sitt utbredningsområde domineras av ett fåtal stora kloner. Vidare analyserade vi bestånd av *Fucus* från finska och ryska kusten av Östersjön som tidigare inte studerats och jämförde med data från bestånd av båda arterna från norra delen av Östersjön och från Estland. Resultaten visar att den övergripande genetiska strukturen hos båda arterna är starkt påverkad av geografisk isolering, men i de flesta fall kunde arterna ändå särskiljas på en lokal skala. Vidare såg vi att vissa populationer hade färre ovanliga genvarianter än andra, antingen orsakades detta av genflöde i en viss riktning, eller av genetiska förluster i samband med etablering av nya bestånd. Oväntat var att sexuell förökning var hög i Finska viken, trots den låga salthalten (<3‰), vilket motsäger resultat från Bottenviken där asexuell förökning är vanligast i låga salthalter. Att ett fåtal kloner hos smaltång har motstått miljöförändringar och dominerar mycket av artens utbredningsområde och har gjort så under lång tid antyder att mönstret i fördelningen av kloner inte endast beror på naturlig selektion. Vi använde datormodellering för att testa om ett mönster likt detta kan uppstå av ren slump. Våra simuleringar visade att vid kolonisering av ett nytt område kan ett fåtal stora kloner få fotfäste och spridas före andra genetiska individer. Dessa kloner kan förbli dominanta under tusentals generation efter etableringen. Idag saknas kunskap om arternas ekologiska skillnader. Något som kan ha bidragit till arternas olikheter är deras tolerans mot betning. Vi testade om det fanns skillnader i arternas kemiska försvar mot betning genom att mäta skillnader i genuttryck under betning. Resultaten visade att blåstång producerar anti-betningkemikalier fortare än smaltång. Våra resultat visade också att det finns en skillnad i genuttryck mellan de två arterna under betning. De evolutionära olikheter i denna reglering av betningsförsvar hos dessa två algarter har troligen utvecklats under olika selektionstryck, eftersom blåstången existerar i samvaro med betare, medan smaltången lever i områden utan betare. Mina studier har bidragit till ökad kunskap om *Fucus* i Östersjön och hur den har utvecklats i samband med en osedvanligt snabb artbildningsprocess. Eftersom klimatförändringar hotar biodiversiteten i Östersjön är det viktigt att öka förståelsen om processer som genererar och upprätthåller biodiversitet.

List of papers

This thesis is based on the following papers, which will be referred to in the text by the Roman numerals below:

Paper I:

Ardehed A, Johansson D, Schagerström E, Kautsky L, Johannesson K, Pereyra R. *(manuscript, submitted)*. Complex spatial clonal structure in a macroalgae with both sexual and asexual recruitment - the Baltic Sea *Fucus radicans*.

Paper II:

Ardehed A, Johansson D, Sundqvist L, Schagerström E, Zagrodzka Z, Kovaltchouk N, Kautsky L, Johannesson K, Pereyra R. *(manuscript)*. Genetic characterization of west Finland and Gulf of Finland *Fucus* and their relationship to *Fucus vesiculosus* and *F. radicans*

Paper III:

Rafajlovic M, Kleinhans D, Gulliksson C, Fries J, Johansson D, Ardehed A, Sundqvist L, Pereyra R, Mehlig B, Jonsson P R, Johannesson K. *(manuscript, submitted)*. The formation and persistence of dominant clones in facultative asexual species.

Paper IV:

Ardehed A, Kruse I, Weinberger F, John U, Johannesson K, Pereyra R. *(manuscript)*. Grazing induces gene expression differences between sympatric populations of the recently formed Baltic Sea endemic seaweed *Fucus radicans* and its sister species, *F. vesiculosus*.

Innehållsförteckning

Introduction

Speciation and divergence mechanisms

Speciation is the evolutionary process by which new biological species arise. This process is fundamental to biology and in focus for intense research and debate among evolutionary biologists ever since Darwin published his classical work "On the Origin of Species" in 1859. The biological species concept defines species as reproductively isolated units where, somewhat simplified, individuals of a species should exchange genes while individuals of different species should not (Mayr 1963). Consequently, current gene flow is a key parameter to understand the relationship among closely related taxa. New species may arise in several ways (e.g. Rieseberg *et al.,* 2003; Coyne & Orr, 2004). Using a simplified approach, speciation can be divided in allopatric speciation where geographical isolation is the key to divergence, and sympatric speciation, where speciation occurs without external isolating barriers between diverging populations. However, despite earlier doubts and discussions (e.g. Mayr 1963; Felsenstein 1981) it has become increasingly apparent that geographic isolation is not necessarily required for speciation (Butlin 2010; Abbott *et al.,* 2013; Seehausen *et al.,* 2014). Instead, it seems as if a variety of different speciation mechanisms are available, for example, speciation through hybridization with or without chromosomal duplication (Rieseberg and Willis, 2007; Mallet 2007), speciation driven by ecological mechanisms (Schluter 2009), and speciation in parallel (Schluter and Nagel, 1995; Johannesson 2001). In most cases, the origin of species is a slow process, but polyploid or hybrid speciation can occur in one or a few generations (Butlin 2010). Other modes of speciation require progressive build-up of reproductive isolation (Butlin 2010). The available data are limited but the duration of this process appears to vary widely; from 4,000 years in the explosive radiation of cichlid species flocks, to 1 million years in allopatric *Drosophila* species pairs (reviewed by Coyne and Orr, 2004), and 0.01 to 5 million years in birds alone (Price 2007).

Up until now, the general view of speciation was that reproductive isolation evolves during long periods of allopatry. This view is now contrasted with findings that characters affecting reproductive isolation can diverge rapidly in sympatry as a result of natural selection (e.g. Butlin *et al.,* 2014). The sympatric speciation theory is more recent (Maynard Smith 1966) and suggests that under certain circumstances speciation can occur in populations without the presence of separating barriers, typically in recently colonized or isolated areas that offer a variety of new niches (Schliewen *et al.,* 2001). It is also suggested that sympatric speciation occurs more rapidly than does allopatric speciation, as the ecological drivers important to the former are more rapid than stochastic drivers important to the latter (Bush and Smith, 1998; McCune and Lovejoy, 1998; Johannesson 2001). An important mechanism in plants but also known in some animals, is speciation by polyploidy, where characters can change within relatively short time by chromosome multiplication. Polyploidy is also suggested to be one of the predominant modes of sympatric speciation in plants due to large effects on gene regulation (Otto and Whitton, 2000).

Reproductive barriers are often in focus in speciation research since isolating barriers between groups are central to the Biological Species Concept (Mayr 1942), and thus the evolution of

these are believed to be at the very core of species formation. In the early stages of reproductive isolation, gene flow is likely to be reduced at just a few loci, scattered around the genome, which contribute directly to local adaptation, mate choice, sexual conflict or genetic incompatibility between diverging populations. Many studies assume ongoing gene flow between species, even though stochastic variation due to recent coalescence times and incomplete lineage sorting can lead to low divergence and high heterogeneity in a similar way, particularly when they are combined with selection (Noor and Bennett, 2009; Hahn *et al.,* 2012; Seehausen *et al.,* 2014). What has been coined "allelic surfing" is a stochastic mechanism by which allele frequency differences may be a consequence of stochastic effects at the very margin of an expanding population (Excoffier and Ray, 2008; Excoffier *et al.,* 2009). Along the edge of a species' distribution groups of individuals may be very small and genetic drift a prevailing factor. Rapid expansion of these populations as the colonization wave proceeds, may establish genetic differences among local groups generated by drift. This may cause genetic structures among parts of the species' distribution that are unrelated to gene flow (or selection) (Excoffier *et al.,* 2009).

Gene flow within species may be hampered by geographic isolation and almost ceases when populations are separated by distances that considerably exceed dispersal ranges of individuals (Wright 1943). In closely related taxa with poor dispersal this may lead to situations in which genetic differentiation between distant populations of the same taxa is similar to differentiation between sibling taxa due to genetic isolation by distance within each species and incomplete lineage sorting between species. This is, for example, the case in marine snails of the genus *Littorina* in which populations of one species, *L. saxatilis*, from Spain and Sweden are as genetically different as are populations of this species and the closely related species *L. arcana* (Panova *et al.,* 2014).

Several studies suggest sympatric speciation. A few examples are: differentiating to host plant adaptation in flies (Feder *et al.,* 1988), adaptation to different patterns for sexual selection in African cichlids (Schliewen *et al.,* 2001), adaptive niche separation in salmons (Lu and Bernatchez, 1999), and an example in *Littorina* species of parallel evolution of local adaptation and reproductive isolation (Butlin *et al.,* 2014). New approaches for gathering large amounts of genomic data in non-model organisms have begun to produce intriguing and partly unexpected insights and clarity into the genetics of speciation. The population genomics approach can be used to identify regions of reduced gene flow by detecting loci with greater differentiation than expected from the average across many loci. In plants, however some of these adaptive forces do not seem applicable due to the sessile life strategies in plants, which inhibit active choosing among patchy micro niches and are excluding the impact from active mating.

Groups of closely related taxa provide important case studies for understanding species formation and formation of new biodiversity. Among brown macroalgae, the species *Fucus radicans* provides a unique opportunity to study a rapid speciation event that took place recently inside the Baltic Sea. What makes this case special is that the speciation event potentially occurred in sympatry (the two species currently live in close overlap in the same habitat, and with largely overlapping geographic distributions (Forslund *et al.,* 2012), and also that the speciation event seems to have taken place very recently (<6000 y).

The study organisms and their natural habitat

Macro algae of the genus *Fucus* (Phaeophyceae) are a group of large, perennial brown seaweeds, common in temperate and arctic waters on the northern hemisphere. Since the *Fucus* provides habitat and shelter for many organisms (Hagerman 1966; Kautsky *et al.,* 1992; Christie *et al.,* 2009; Dijkstra *et al.,* 2011) they can be considered foundation species (e.g. Korpinen *et al.,* 2010; Dijkstra *et al.,* 2011). Foundation species are species that give structure and increase the complexity of a habitat, as well as, providing shelter and protection from both abiotic and biotic factors to a wide variety of other marine organisms (Dayton 1972; Roff and Zacharias, 2011).

The two brown algae *Fucus radicans* and *Fucus vesiculosus* are important perennial macroalgal species in the Baltic Sea, and they are both essential foundation species for tidal and subtidal rocky bottom ecological communities of the eastern and northern parts of the Baltic Sea, *F. vesiculosus* being widespread through the whole Baltic.

Fucus vesiculosus established in the Baltic area around 6000 years ago, shortly after the formation of this marginal marine environment. This alga has a large range of tolerance to different environmental factors such as temperature, exposure, and salinity (Lüning 1990; Bäck *et al.,* 1992; Chapman 1995; Nygård and Dring, 2008). A smaller morph of what was earlier believed to be a dwarf form of *F. vesiculosus* is found in the northern parts of the Baltic Sea, assumed to be smaller due to stress from very low salinity (Wærn 1952; Ruuskanen and Bäck, 1999). However, by using a combination of morphological and genetic analyses, it was later shown that this morph was in fact a separate species (Bergström *et al.,* 2005), and was named *Fucus radicans* Bergström & Kautsky (**Fig. 1**).
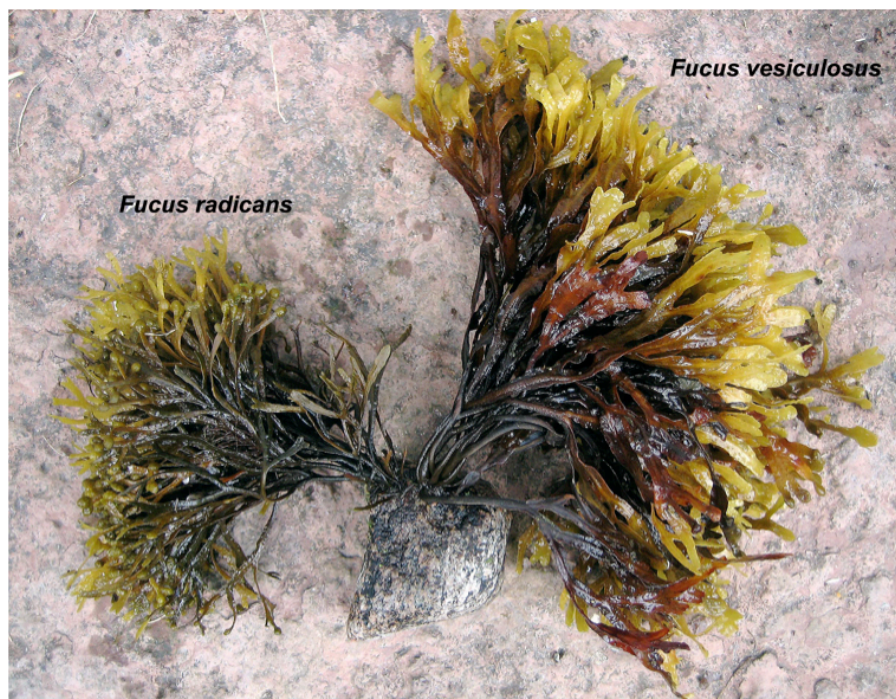

Fig 1. The two *Fucus* species that are the focus of this thesis. To the right *is F. vesiculosus*, to the left is *F. radicans*, which is endemic to the Baltic Sea. *F. radicans* differs from *F. vesiculosus* morphologically in having a more shrubbery appearance, being smaller in size and slimmer in the dimensions. It also lacks the

vesicles often present in *F. vesiculosus*. They may live in strict sympatry, as obvious from the picture. **Photo:** Lena Kautsky

It seems as this species is endemic to the Baltic Sea, and formed from *F. vesiculosus* through presumably sympatric speciation inside the Baltic area only a few thousand years ago (Pereyra *et al.,* 2009). Both algae are found on hard bottom substratum. *Fucus vesiculosus* is common throughout the Baltic Sea, while *F. radicans* is common in the northern parts of Baltic, and have also been found in two Estonian sites (Forslund *et al.,* 2012). Thus, the two species lives in sympatry and have overlapping distributions in the Baltic Sea.

In many areas of the Baltic Sea both *F. radicans* and *F. vesiculosus* live sympatrically (even attached to the same rock as in **Fig. 1**) and studies using microsatellite markers have indicated that there is reproductive isolation between them (Bergström *et al.,* 2005; Pereyra *et al.*, 2009). Notably, there is yet no clearly discernable pattern of different use of the habitat when living closely together. Nevertheless, experimental studies have unveiled differences between the species in both physiological and ecological traits (e.g. Råberg *et al.,* 2007; Gylle *et al.,* 2010; Gunnarsson and Berglund, 2012; Schagerström *et al.,* 2014).

Both species are dioecous (male and female reproductive organs on separate individuals) and they reproduce sexually by synchronized release of gamets to the surrounding waters from late spring to autumn, with peaks at calm conditions and full moon (Serrão *et al.,* 1996). Gametes (particularly eggs) have negative buoyancy, thus the potential for spread is mainly local within a few meters from the mother plant (Serrão *et al.,* 1997), somewhat restricting gene flow (Tatarenkov *et al.,* 2007). Somewhat surprisingly, it has been found that both species can reproduce asexually (Tatarenkov *et al.,* 2005), which is rare or absent in fucoids outside the Baltic. Earlier studies of *F. radicans* have shown that a few large clones dominate large parts of the species' distribution, with, in particular, one female clone dominating large parts of the Swedish coast and extending its distribution to northern Finland (Johannesson *et al.,* 2011). Populations of *F. radicans* in Estonia are mainly sexually reproductive (Bergström *et al.,* 2005; Johannesson *et al.,* 2011). The adventitious branches that grow from the thallus are the main source of vegetative reproduction in both *F. radicans* and *F. vesiculosus*, forming rhizoids and reattaching to a surface (Tatarenkov *et al.,* 2005). Importantly, asexually recruited individuals produce gametes and are also sexually functional (Lena Kautsky, pers. comm.). Even so the two species are reproductive isolated (Pereyra *et al.,* 2009), genetic interactions through past or present hybridization and introgression seems possible, as zygotes forms readily from crossbreeding in the laboratory (Forslund & Kautsky, 2013).

The Baltic Sea poses an extreme marginal environment for many organisms and has a salinity gradient from 20ppm down to almost freshwater (2 psu in the north) (Bernes 2005). It is the largest brackish water body in the world and formed only around 8500 years ago (Zillén *et al.,* 2008). Most marine species that inhabits the Baltic Sea colonised it during the more saline Littorina period, 6000-4500 years ago. As the area has gone through a successive transformation of the environment towards freshwater conditions, the marine organisms in the area have since then been going through an intensive adaptation, with some species being extinct sometime after their invasion (Johannesson & André, 2006). The brackish environment today generally presents a challenging environment and due to the low salinity many species in the Baltic Sea functions at or close to their physiological limits (e.g Westerbom *et al.,* 2002; Bergström *et al.,* 2003) and many populations are genetically differentiated from and have a lower genetic diversity than populations outside the Baltic Sea (Johannesson and André, 2006). In combination with the short history of the Baltic Sea under

present conditions (Voipio 1981; Björck 1995; Winsor *et al.,* 2001) this is probably an explanation to why the Baltic Sea is species poor with a combination of marine and freshwater species (Remane and Schlieper, 1971; Snoeijs 1999).

The recent origin of the Baltc Sea, and the fucoids within this system provides unique opportunities to study rapid evolution and speciation, and also how (if) asexual reproduction interacts with this process.

Given the expected decrease in salinity and increase in temperature due to anthropogenically induced environmental changes (Meier 2006), there is an urgent need to understand the potential for rapid adaptation in Baltic Sea organisms in general. The situation of largely asexual reproduction in Baltic Sea *Fucus* hinders the formation of new genotypes, and may consequently lead towards long-term restriction in adaptation capabilities. This could possibly make the clonal populations of the endemic *F. radicans*, and also *F. vesiculosus,* vulnerable to long-term changes in the environment (Spielman *et al.,* 2004). In the time being, climate change threatens species and biodiversity, so increased knowledge of processes generating and maintaining biodiversity is important and needed.

Aims of this thesis

The overall objectives of the thesis was to understand fundamental components of the postglacial evolution of *Fucus vesiculosus* and *F. radicans* in the Baltic Sea, with particular focus on the new and endemic species, *F. radicans*. By combining genomic and microsatellite data with spatially explicit modelling, and covering an extended area of the *Fucus* distribution compared to earlier studies, I aimed to increase our understanding of the evolution of this intriguing system of two recently split species in the Baltic Sea. In particular, I focused on the effects of clonality on spatial genetic structure in *F. radicans*, on both a local scale and a larger geographical scale to explore the evolutionary effects of asexual reproduction as a dominating reproductive strategy in at least part of this species' distribution (**Paper I**). I furthermore characterized the population genetic structure of earlier un-investigated *Fucus* populations from "new" parts of the Baltic Sea (Gulf of Finland and Western parts of the Finnish coast) to assess these populations' genetic relationships to earlier described populations of Baltic *F. radicans* and *F. vesiculosus* (**Paper II**). To test the possibility of non-selection as an important evolutionary force, we investigated whether the pattern of distribution of clones observed in *F. radicans* can be explained without invoking selection among clones. Furthermore, we here aimed to explore how dispersal dynamics, with local dispersal and occasional long-range dispersal, may contribute to observed patterns (**Paper III**). Finally, using a global transcriptomic approach we investigated the molecular basis of inducable herbivory defense in the two fucoid species and to gain a first insight into what ecological mechanisms stimulated the species divergence, and/or maintain their present reproductive isolation (**Paper IV**).

In the following text I describe the content of this thesis in more detail.

Patterns of evolution in the young seaweed *Fucus radicans*

Geographic patterns of clonal distribution in a young marine species

Clonality is a common life-history strategy in a wide variety of organisms, like plants, protists and aquatic algae. The result of clonality is genetically identical offspring, where existing genotypes are "copied" and conserved, but single-locus differences can be contributed also by somatic mutations (Arnaud-Haond *et al.,* 2007). In sessile or sedentary species with both sexual and asexual reproduction, the distribution of female and male clones affects the potential for sexual reproduction, recombination and local adaptation. Asexual reproduction, in particular, influences the number and the spatial distribution of reproductive units, and thus interferes with local patterns of dispersal, which in turn affects the species' potential to evolve (Johannesson *et al.* 2011).

Asexual reproduction is suggested to be an evolutionary successful strategy in marginal environments, such as the Baltic Sea, where individuals with otherwise uncommon phenotypes, are relatively more common and more successful than in other places. Clonal reproduction is also common along the margins of species' distributions, and it has been suggested that this is a consequence of species hybridization, high physiological stress in marginal environments favouring certain genotypes ("frozen niche hypothesis"), or high costs of sexual reproduction in these habitats (Vrijenhoek, 1984; Silvertown, 2008; Bengtsson 2009; Vrijenhoek & Parker, 2009). It has also been suggested that asexual reproduction has initial advantages during colonization of new areas where allee effects may impede sexual reproduction (Baker, 1967; Hörandl *et al.,* 2008).

In plants and macroalgae, new vegetative individuals (*ramets*) are produced from the original parental individual. Together they are part of the same genetic unit (the *genet*), making the primary outcome of clonal growth an increase in the size of the genet (Vallejo-Marin *et al.,* 2010). In macroalgae, thalli (individuals) formed by cloning are the result of adventitious branches getting loose and reattach to the substrate at some distance from the parental thallus. Thus in macroalgae, ramets of a clone (a genet) are free living from each other, in contrast to clones of terrestrial plants or seagrasses that are often connected to the parental plant by roots or rhizomes (Hämmerli & Reusch, 2002; Xue-Hua *et al.,* 2006; Zipperle *et al.,* 2011).

There are two main spatial strategies of vegetative recruitment: (*i*) the Phalanx strategy, where aggregated structures with genetically identical ramets are clumped together, forming discrete clones in which ramets of other genets are more or less excluded. The second one is (*ii*) the Guerrilla strategy, in which genets are intermingled through a more efficient spread of ramets, resulting in clones being spatially mixed and less discrete (Lovett-Doust, 1981; Alberto *et al.,* 2005; Ruggiero *et al.,* 2005; Vallejo-Marin *et al.,* 2010). In plants where new ramets form while still connected to the parental plant, the ramets cannot disperse easily and this often result in high levels of clonal aggregation (Reusch *et al.,* 1999; 2010; Zipperle *et al.,* 2011). In contrast, marine macroalgae and some aquatic plants that drop off vegetative fragments, which may be transported at least short distances by the aquatic medium, are likely to show less aggregation of single clones (Vallejo-Marin *et al.,* 2010).

The young algae *F. radicans* is capable of sexual reproduction, but all thalli may also form new attached thalli asexually by dropping off vegetative propagules, and in some areas the species reproduces almost exclusively using this mechanism. **Paper I** of this thesis investigated how importantly a clonal strategy that dominate in some areas affects the local spatial genetic structure (SGS) of *F. radicans* in these areas The aim was to study if reproduction was influenced by the spatial genetic structure, that is, if at small spatial scales the distribution of clones of different sex affected the opportunities for sexual reproduction (forming new genotypes from recombination of existing ones) of individual plants, and thus the species' potential to evolve local adaptation (Reusch *et al.,* 1999; Epperson, 2001; Charpentier, 2002; Vekemans & Hardy, 2004; Ruggiero *et al.,* 2005; Pereyra *et al.,* 2013).

By using genetic information from nine microsatellite loci (see details for procedure in **Paper I, II** and Johannesson *et al.,* 2011) we estimated genotypic diversity and described mapped clones (genotypes) of *F. radicans* and their spatial distribution over macrogeographic (the species' distribution in northern and eastern Baltic, using 16 sampled sites) and microgeographic (<100 m, using three of the 16 sites) scales. Our prediction was that in contrast to seagrasses that show a Phalanx distribution of clones (Hämmerli & Reusch, 2002; Zipperle *et al.,* 2011), *F. radicans* would have a more intermingled configuration of genotypes and sexes, due to mixing of fragments from several parental thalli, and this should promote sexual recruitment and hence genotype diversity of populations.

We reconstructed the genetic relationships of clones and their macrogeographic distribution over all 16 sites included in the study. Practically, we used information from the nine microsatellite loci to identify multi locus genotypes (MLGs). Genotypes that differed by only 1-2 mutations, and if sex was scored, were of the same sex, were assumed to be of the same multi clonal lineage (MLL) (Arnaud-Haond *et al.,* 2007). Results show that sexual recruitment was predominant in southern and eastern areas, while in northern and southernmost areas asexual recruitment dominated (**Fig. 2 a-b**). In the north, however, clones of both sexes were locally intermingled at several sites but sexual recruitment was nevertheless low, maybe due to low salinity, but could also be the result of eggs and sperm produced not being fully vital and useful anymore, due to the effective spread by asexual reproduction. In western sites, a strong female bias may contribute to low sexual recruitment, even so other factors may also be important. The two largest clones, one female (blue) and one male (green), were distributed over 100-550 km of coast (**Fig. 2 a-b**). The distribution of the blue female was in many ways exceptional; this clone being common in 12 of 16 sites. Also the large male clonal lineage (green) was also extensively distributed and present in 8 of 16 sites. Notably, the genotype of the yellow clonal lineage suggested this is an offspring of a cross between the blue female and the green male.

In addition, in each local population, small and local clones were found that differed by 1-2 mutations from the large clones (**Fig. 2 c**). This suggests the large clones have been around for a long time and evolved new genotypes by somatic mutations.
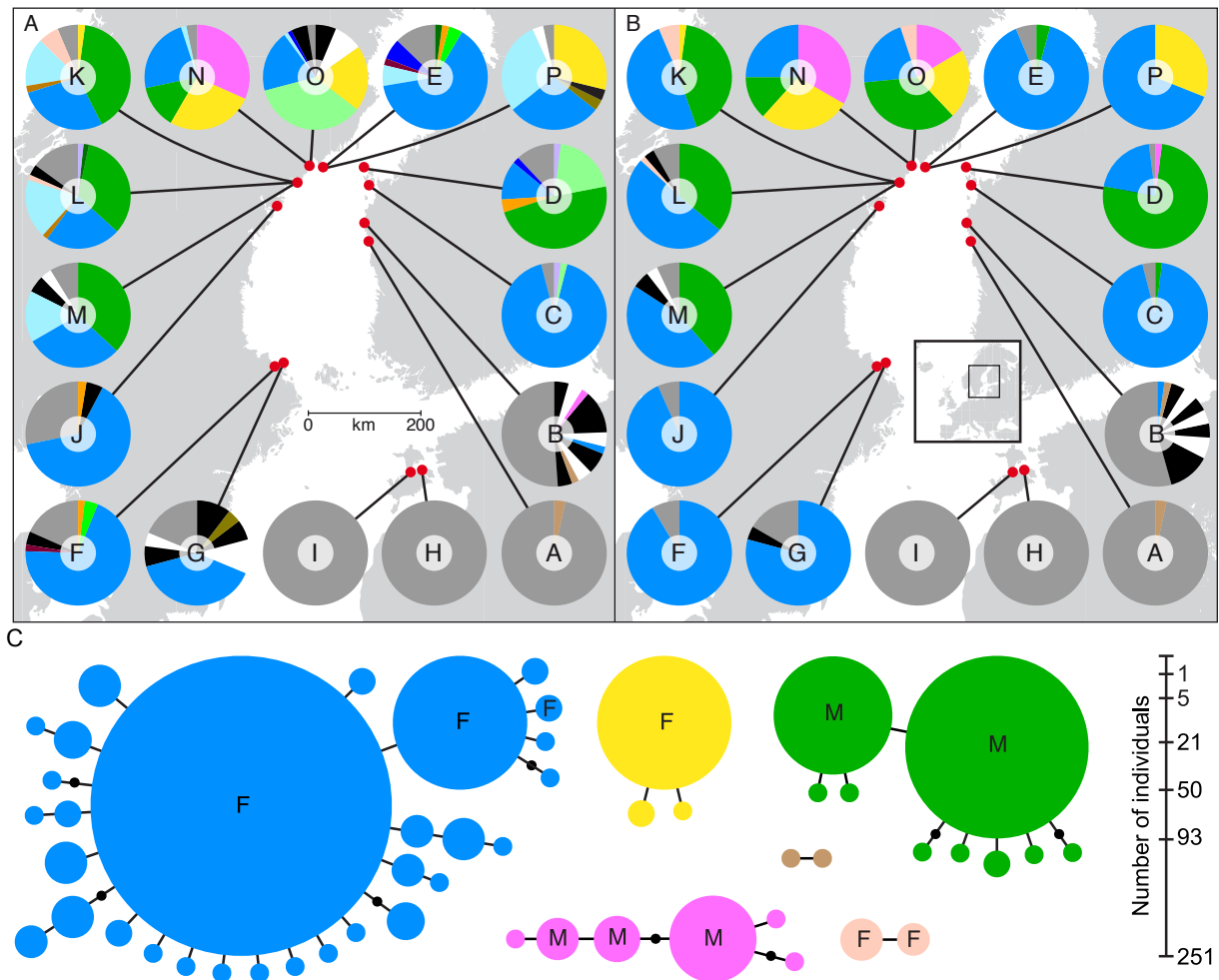
Fig. 2 a-c. Macrogeographic distribution of singletons (grey), local clones (black and white), and widespread clones (different colours) in the Baltic Sea *(a)*. In *(b)* MLGs that differ with 1-2 mutations were merged into multilocus lineages (MLLs). (For details on sample sizes and positions see Table 1 in **Paper 1.**) *(c)* Network of MLGs and identification of MLLs. Each circle represents a separate MLG, and each colour indicates a separate MLL (same colours as in Fig. 1 a-b). Circle size is proportional to numbers of thalli, and letters indicate that sex was confirmed (F for female, M for male). MLGs are clustered into MLL by joining those MLGs that differ by 1-2 mutations.

Results from analyses of local spatial structure (see details in Fig. 1 of **Paper I**) shows that overall, pairs of ramets, or pairs of genets, that were genetically similar were not more spatially associated in the populations than other pairs. In conclusion, the overall microgeographic genetic structure of *F. radicans* was in all three locally studied populations, characterized by a high level of intermingling of ramets (a Guerrilla strategy) of a few dominant clones with additional scattered thalli of small clones. It was also obvious from the results that several clones had extensive distributional ranges. With respect to gender distribution, two of the three populations were made up by dominant clones of different sexes, while in the third population, the two dominant clones were both females, and no male was found.

Understanding the clonal patterns in this species provides a basis for understanding the dynamics of *F. radicans* populations in relation to their habitats. A heterogeneous genetic structure over macrogeographic scale, such as described here for *Fucus radicans*, is commonly found also in terrestrial and aquatic plants with both sexual and asexual

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reproduction (Eckert, 2002; Hörandl *et al.,* 2008), but have to our knowledge not earlier been shown in any other species of macroalgae than *F. radicans*. Such a structure may include variable and strongly skewed sex ratios (Eckert, 2002), which we also observed in *F. radicans*.

Most asexual populations of plants have only local clones (Eriksson, 1993, Reusch & Boström, 2010), but in invasive species, the same clones may be widely distributed and the pattern we observe in north and west populations of *F. radicans* is similar to patterns found in populations of species that have recently invaded a new area (Eckert, 2002; Kliber & Eckert, 2005). This may suggest that the establishment in the north and west of *F. radicans* was recent (compared to the establishment in the south and east) and that the widespread clones are comparably young. However, the many local mutant clones deriving from the large female clone and established in all of the sites where the female clone is dominant, strongly suggests that the female clone is old and has been present in these sites over a long period of time. Indeed, it seems likely that to reach such a large size and establish over such a large geographic area, this clone originated early in the history of this young species, that is, some few thousand years ago (Pereyra *et al.,* 2013). A possible hypothesis is that the dominating clones of *F. radicans* have superficial phenotypes that resist both the marginal environment and temporal and spatial variation ("the frozen nisch hypothesis", Vrijenhoek, 1984). With strong selection among clones, it is unexpected, however, that the same few clones would have the highest fitness in local populations throughout large parts of the species' distribution and through long periods of time. Hence, after all, the question remains if natural selection is at all important in explaining the macrogeographic genetic structure observed. An alternative explanation is that the geographic distribution of sexual and asexual populations and the dominance of a few widespread clones is an effect of an asexual wave in the initial phase of colonization of a new area, and the pattern established by such a wave being around for extensive numbers of generations (see **Paper III**).

The Guerrilla strategy efficiently mixed clones of both sex in northern populations of *F. radicans*, but despite this, there is considerably less sexual activity than in fully sexual areas. Earlier studies have shown that low salinity restricts successful fertilisation due to, for example, lysis of the egg cell or polyspermy (Serrão *et al.,* 1999), and this may contribute to the low sexual recruitment in the northernmost Swedish and Finnish sites where salinity is lower (3.5-4.3 PSU, however, see also Paper II) than in the sites with sexual dominance in southernmost sites Finland and Estonia (5.2-5.8 PSU, Johannesson *et al.*, 2011). However, Swedish thalli of *F. radicans* produce more adventitious branches and fewer receptacles than thalli of *F. radicans* from sexual populations in Estonia (Forslund & Kautsky, 2013). Thus, although the old female clone is still sexually active, this clone seems to allocate more energy to asexual reproduction than do thalli from a fully sexual Estonian population, and perhaps it is not a low salinity but the strong femaled-biased sex ratio that impedes sexual recruitment in the southern Swedish sites.

The complex structure of clones of *F. radicans* in the Baltic Sea seems not only a consequence of selection of the most fit phenotypes as old clones have successfully established in various local environments and have survived through centuries of climate variation. Rather it seems as if stochastic processes have been heavily involved. Rare sexual events, occasional long-distance migration and somatic mutations contribute new genotypic variation in northern and western populations, nevertheless the same few clones have resisted environmental changes and have dominated much of the species' distribution over extensive

periods of time. This suggests that the distribution of clones of *F. radicans* cannot solely be ascribed to natural selection.

Population genetic structure of a recently evolved species in a marginal habitat

The evolutionary relationships between closely related species, like *F. radicans* and *F. vesiculosus*, may be complex when both geographic isolation and a close common ancestry influence the genetic differences that have evolved. Nevertheless, groups of closely related taxa provide significant case studies for understanding species formation as in recently formed species less of the patterns of divergence observed are caused by accumulation of differences after the actual speciation event.

Applying the biological species concept (Mayr 1963), one may use current gene flow is a key parameter to understand the relationship among closely related taxa. Somewhat simplified, individuals of a species should exchange genes, while individuals of different species should not, and consequently, genetic differences between samples of putative species suggest a barrier to gene flow. Gene flow within species is, however, also impeded by geographic isolation among populations within species, and genetic differences within species can be caused by isolation by distance. Finally, two recently split taxa will share a lot of their genetic characteristics due to a common ancestry (incomplete lineage sorting).

In the Baltic Sea, the two brown algae *F. vesiculosus* and the endemic *F. radicans* are extremely closely related due to a very recent (<6000 y) split, consequently they share a large part of the variation in sequenced genes and are indistinguisable from sequences of commonly used genetic markers (Bergström *et al.,* 2005). Also, in the fast mutating microsatellite alleles, they share almost all alleles but differ only in frequencies of alleles (Tatarenkov *et al.,* 2005; Pereyra *et al.,* 2009). In addition, each species is strongly genetically structured due to isolation by distance within each species (see **Paper I** and Tatarenkov *et al.,* 2007).

The two taxa live in true sympatry in many areas with well-mixed stands even on a scale of < 1m^2, Therefore cross-fertilization, if possible, is not prevented by physical isolation of gametes. In these fully sympatric locations it is possible to assess the degree of current gene flow and assess their species status, following the biological species concept. This has been done, and in earlier studies reproductive isolation between the two species was evident from differences in allele frequencies of analysed microsatellite loci (e.g. Bergström *et al.,* 2005; Pereyra *et al.,* 2009; Pereyra *et al.* 2013). However, the taxonomic relationship between the two *Fucus* species has earlier been investigated in the northern part of the Baltic Sea, and, to some extent, around the Estonian island Saaremaa (Johannesson *et al.,* 2011; Forslund *et al.,* 2012), while no genetic studies have been performed on *Fucus* from western Finland and the Finnish and Russian coasts of Gulf of Finland.

In this study we analysed the population genetic structure of 12 *Fucus* populations along the Finnish and Russian coasts of the Baltic Sea using the same 9 microsatellite markers as in earlier studies (see details for procedure in **Paper I, II** and Johannesson *et al.,* 2011). The

results were compared with data from 17 earlier analysed populations of *F. vesiculosus* and *F. radicans* in the Bothnian Sea, and around the island of Saareema in Estonia (**Fig. 3**). The individuals from the 12 new populations were sampled and labelled as *"F. vesiculosus-like"* or *"F. radicans-like"* based on morphology (mainly thallus width but also overall appearance), and thereafter genotyped. Individuals of four samples, all from the Finnish coast of the Gulf of Finland, did not clearly divide into "*vesiculosus*-like" and "*radicans*-like" morphotypes, and were left untagged (populations X, Y, V, W of **Fig. 3**). As there is a salinity gradient from the centre of the Baltic Sea and into the Gulf of Finland that parallels the salinity decrease from the Baltic Sea centre and northward, we were able to look for parallel trends in, for example, degree of asexual reproduction. In an earlier study, a significant trend of increasing asexual recruitment with decreasing salinity from the Baltic Proper to the Bothnian Sea has been observed in *F. radicans* while the was no significant trend in *F. vesiculosus* (Johannesson *et al.,* 2011). It has been suggested that increased asexuality may be explained by increasing problems with polyspermy in low salinities (Serrão *et al.,* 1999). A competing hypothesis, however, is that asexual recruitment and spread of clones is favoured during establishment of facultative asexuals in a new area (Baker's law), and that due to this effect asexual recruitment may remain dominating for thousands of generations in some areas (see **Paper III**).

We found a complex genetic structure both affected by geographic isolation and by the two taxa largely sharing a common ancestral gene pool. Numbers of MLGs (genets) compared to number of ramets (thalli) varied greatly among populations (see Table 1 in **Paper II** for details). Likewise, the number of globally unique MLGs ("singletons") varied greatly on a geographic scale and seemingly independent of the variation in salinity (**Fig. 3**). For example, while populations in the northern part of the Sea of Bothnia with very low salinity were highly clonal, this was not the case in the populations living in similarly low salinities in eastern Gulf of Finland, some of which were instead highly sexual (**Fig. 3**).

We used a PCA analysis to display the relative position of the 29 populations in a multidimensional genetic space (**Fig. 4**). This genetic map unveiled some intriguing patterns. Firstly, there was a strong geographic component in the total variation. In most cases, populations from the same region (colour on left side of circles) and of the same species ended up close to each other. Thus, for example, the most northern samples of *F. radicans* (purple/green circles) all appeared close on the genetic map (**Fig. 4**). Similarly, all samples of *F. vesiculosus* from western Finland (yellow/red) grouped nicely. This geographic component of variation was also very obvious in the Gulf of Finland, with the four samples of unknown morphotype forming one group, and the two Russian samples forming another group. Also the three Estonian samples formed a rather tight group, despite both species being sampled in this area.
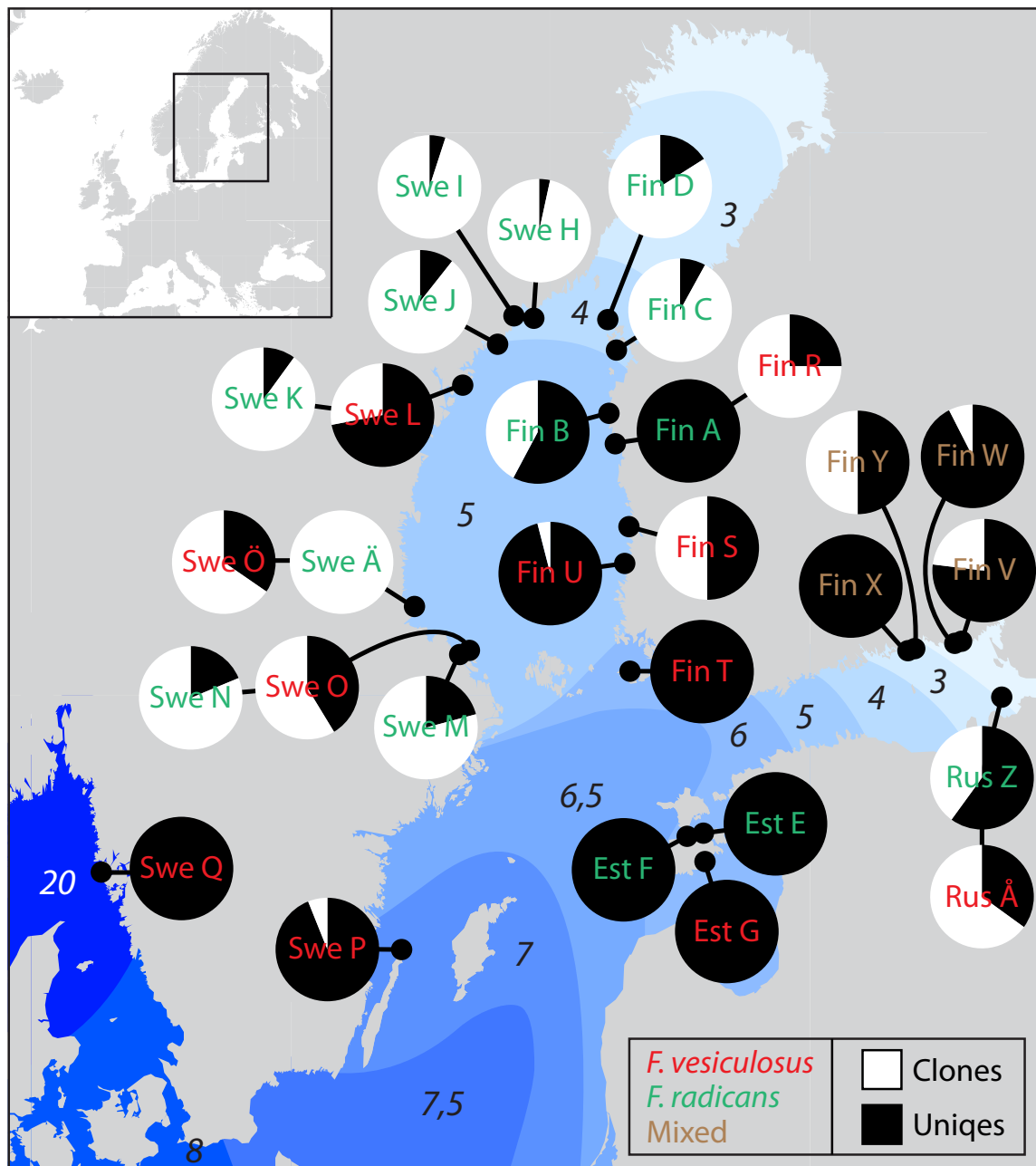
Fig. 3. Geographic map of the sampled 29 localities of *Fucus* in the Baltic Sea. Included is information on frequency of unique singletons (black in circles) vs. clones (white in circles) per population, in relation to salinity in that region. Abbreviations within circles denote population. Colour of abbreviation denotes species belonging. The brown abbreviations indicate populations that display mixed morphs with unclear species belonging.

Secondly, there was an appearant species division overlaying the geographic pattern when the genetic similarity of populations of the two different species was sampled in the same area. Thus, in most cases these pairs of populations were clearly genetically separated (pairs connected by broken lines in **Fig. 4**). The difference between the two Russian samples (Pop Z and Å) was, however, small compared to the geographic component of separation to other samples, and a similar trend was observed in the Estonian samples where, in fact, the different species were also sampled in two separate areas.

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Somewhat, surprisingly, the samples of *F. vesiculosus* from the Baltic Proper (Pop P) and the entrance of the Baltic Sea (Pop Q) were, despite being separated by a large geographic distance, notably similar.

Due to the strong geographic component of genetic separation, it was not possible to infer the taxonomic status of the four samples from the Finnish part of the Gulf of Finland that were recognized as being of mixed morphologies (Pop V, W, X, Y), despite a strong morphological variation including both typical *F. radicans* and typical *F. vesiculosus* morphologies (Pereyra unpublished results) they appeared closely related to each other. Most surprisingly, the appeared genetically more closely related to the distant Estonian samples (G, E, F) than to the nearby Russian samples (Z, Å).
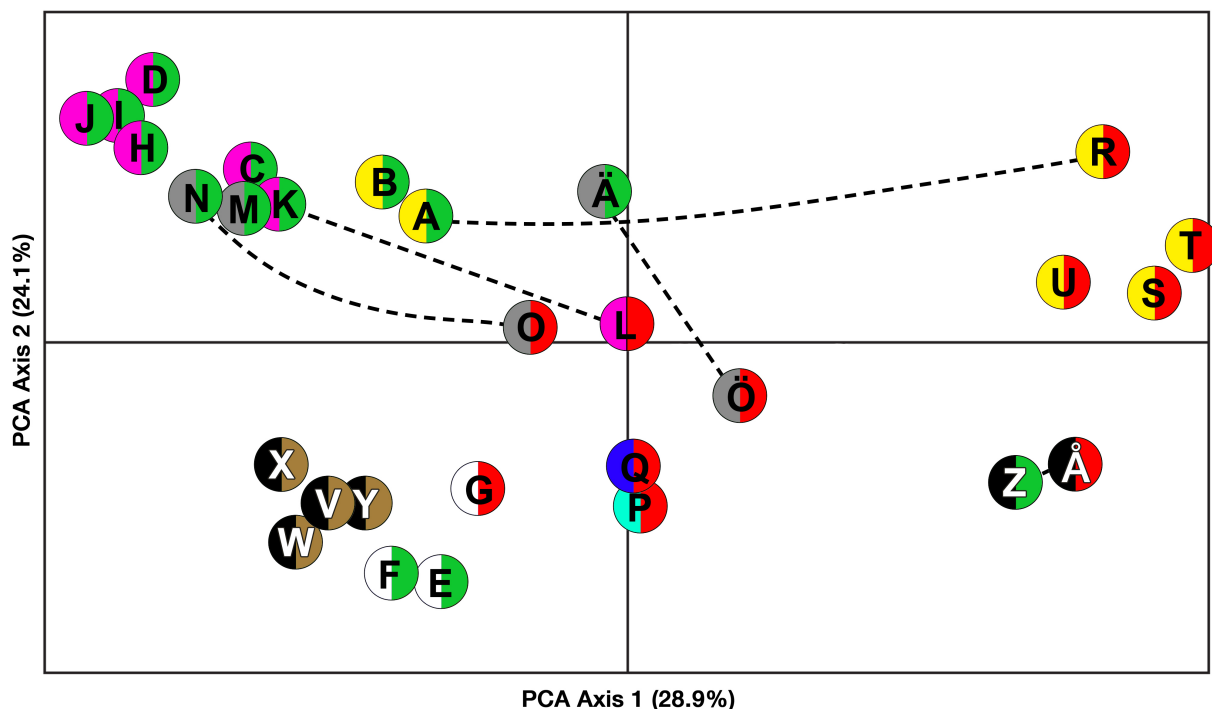

Fig. 4. Principal component analysis (PCA) describing the relationship of the 29 *Fucus* localities in the Baltic Sea on genet level with 10 000 randomizations. Circles with letters inside denote population. Right side of circle indicates species belonging, and left side of circle indicates what region the population is from (**purple** = North Bothnian Sea; **grey** = Swedish East coast; **white** = Gulf of Riga; **black** = Gulf of Finland; **light blue** = Baltic Proper; **dark blue** = North Sea; **yellow** = West Finland). Dotted lines connect populations that were sampled at the same locality, but display genetic differentiation. The overall $F_{ST} = 0.19267$; $p = 0.0001$.

We looked for genetic asymmetries in three different groups of samples: (1) All the *F. vesiculosus* populations from the Baltic Proper plus the population from the Baltic entrance (see Fig. 4 a in **Paper II**). 2) All Gulf of Finland populations including the Estonian populations (see Fig. 4 b in **Paper II**). 3) All *F. radicans* populations from the Bothnian Sea (**Fig. 5**). In the first comparison we found a clear asymmetry with fewer private alleles in the Baltic Proper than in the Baltic Sea entrance. This either indicated a gene flow out of the Baltic Sea, or that the Baltic populations had lost rare alleles present during colonization with these alleles still present in Baltic Sea entrance. We found no clear asymmetries among the

populations inside the Gulf of Finland, although a few populations appeared as richer in private alleles than others (c.f. T, X with G and Z, and see Fig. 4 a-c in **Paper II**). In the comparison of the *F. radicans* populations of the Bothnian Sea we found two populations, A and B, with more private alleles than the others. Thus these two populations were either sink populations assuming the asymmetries being a consequence of current gene flow, or these populations represented the original pool of genetic variation in the area, with several of the other populations (e.g. I, J, D) having lost genetic variation, and in particular, rare alleles during colonization.

Thus, these analyses of population assymetries indicate a system with, in some cases, strongly skewed distributions of private alleles, and there may be alternative explanations to this pattern, at least in some areas (**Fig. 5,** see also Fig. 4 a and 4 b in **Paper II**). As the main Baltic Sea water circulation is counter-clockwise (Leppäranta and Myrberg, 2009), and surface water flow out of the Baltic Sea, this suggests that there may be a dispersal of *F. vesiculosus* out of the Baltic Sea, predominantly from the Swedish coast of the Baltic Sea. This pattern is, possibly, supported by these analyses, which showed an asymmetry in the gene flow in the direction from Swedish Baltic Sea sites of *F. vesiculosus* to the North Sea site. However, as the asymmetry analysis takes into account the distribution of unique alleles, and infers gene flow in the direction to the population with most unique alleles, there is an alternative explanation. If the Baltic Sea populations of *F. vesiculosus* lost all but the most common alleles during the colonization of the Baltic (due to genetic drift in small founder groups) North Sea populations will have more rare alleles than the Baltic Sea populations. Such a bias will generate the same asymmetry as a uni-directional gene flow from Baltic to North Sea populations.

Perhaps the most interesting finding of this study was the complex genetic structure between and within both species, in which, in particular, geographic isolation of populations seemed to play a major role, in addition to species delimitations. Part of the complexity of the genetic structure in the Baltic *Fucus* is likely caused by the mixed sexual and asexual reproduction, that allows for similar genotypes (clonal lineages, see **Paper I**) to establish over vast geographic areas, and similar to what is sometimes found in both terrestrial and aquatic plants (Eckert 2002; Hörandl *et al.,* 2008). Furthermore, while the morphological characterization based on the original descriptions of *F. radicans* in most of the investigated sites matched a subdivision into two genetic entities - and hence supporting the presence of two different taxa, this was not true in four Finnish populations in the Gulf of Finland. This is a very interesting finding that needs to be further investigated, using additional samples from the area and more comprehensive molecular markers.
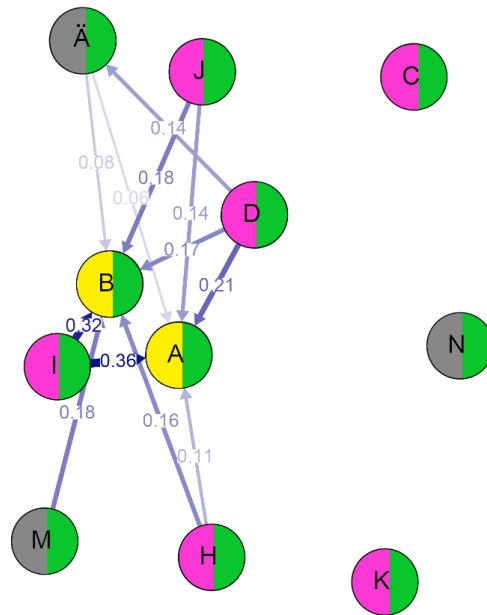
Fig. 5. Results from analyses of migration patterns in a subset of *F. radicans* populations from the Bothnian Sea. Arrows between populations show which direction gene flow is going. Numbers by arrows show the significant asymmetric migration value. Circles with letters inside denote population. Green color (right side of circle) denotes *F. radicans,* and colors for regions (left side of circle) correspond to colours in **Fig. 4**.

A neutral model explaining clonal spread in a facultative asexual species

Discontinuously distributed and widely dominant clones are more frequently found in aquatic species colonising new (e. g. postglacial) areas (Ting and Geller, 2000; Eckert *et al.,* 2003; Kliber and Eckert, 2005; Tatarenkov *et al.,* 2005; Darling *et al.* 2009; Pettay *et al.,* 2009; Koenders *et al.,* 2012) than in terrestrial plant species (Carino and Daehler, 1999; Paun *et al.,* 2006; Wilk *et al.,* 2009), and in species that reproduce both sexually and asexually, clones are often more common in young or marginal areas, just like in *F. radicans*. A number of hypotheses based on selection have been proposed to explain the dominance of asexual reproduction in marginal areas. For example, asexual reproduction has been suggested to favour maintenance of generally or specifically successful genotypes (Parker 1977; Peck *et al.,* 1998; Stenberg *et al.,* 2003; Kearney 2005; Hörandl 2009) or to be insusceptible to inbreeding (Haag and Ebert, 2005; Pujol *et al.,* 2009). By invoking selection for asexual reproduction, however, these hypotheses suggest that a spatial separation of clones and sexual recruits implies a permanent loss of sexual reproduction in marginal areas (Eckert 2002), although in a majority of cases, clones have preserved sexual reproductive organs (Green and Noakes, 1995; Johnson *et al.,* 2010) as do *F. radicans*. This leads to the question if the clonal patterns observed arise as a consequence of selection resulting in a permanent loss of sexual reproduction in marginal areas. Or alternatively, can dominance of asexual reproduction in marginal areas arise and persist for relatively long periods of time as a consequence of demographic processes during the expansion of a facultative asexual species into a new habitat? In **Paper III** we investigate under which conditions dispersal dynamics can

contribute to such a pattern in facultative asexual species expanding into a new area, using *F. radicans* as an empirical example for testing the model. Both short-range and long-range dispersal dynamics are incorporated to understand the consequences of reproductive-assurance arguments (Baker 1955; Tomlinson 1966; Pannell and Barrett, 1998) on emerging patterns. The approach of this study was to formulate and investigate a model for a species colonising a new habitat in which each individual may reproduce both sexually and asexually. We did not involve adaptation to the conditions of the environment, that is, genetic differences among individuals were assumed to be neutral (see details of modelling in the Materials and Methods-section in **Paper III**). In this respect our approach offered an alternative to existing models for establishments in marginal environments (Peck *et al.,* 1998; Hörandl 2009).

The potential for long-distance dispersal was found to be a key component, whether or not selection among clones or random events are crucial in structuring the geographic pattern of clones and unique genotypes in *F. radicans*. With long-distance dispersal, a clone present in one area is able to spread and establish in a distant area, and a unisexual population may turn into a bisexual population and initiate sexual reproduction. In *F. radicans,* gametes, zygotes, adventitious branches and thalli all have negative bouyancy (Serrão *et al.,* 1996; D. Johansson, pers. obs.). However, there are some indirect observations showing long-distance dispersal is possible. A single large thallus of an attached *F. radicans* was found 50 km northeast of the nearest population at the border of the species' distribution (Länsstyrelsen, 2008), and a single drifted (fully vital) thallus was found in a shore 18 km north of the same populations (R. Pereyra, pers. obs.). From results in **Paper I**, we also found single thalli that were genetically assigned to populations from distant sites, rather than to the population in which they were found.

Comparing the patterns obtained under three different values of clonal birth rate all else being equal (**Fig. 6**), we found that under the lowest rate of clonal reproduction tested (sexual reproduction being 100 times more successful than asexual reproduction), populations recruited sexually were dominated both in space and time (**Fig. 6 a, d**). Under higher rates of clonal reproduction (sexual reproduction being only X10 or X2 as succesful as asexual reproduction in Fig. 3b and 3e, respectively), however, large single-sex colonies were established. In these latter cases, we observed three phases of spatial distribution of sexes. The first phase occurred during colonisation and was characterised by an expansion of single sex-colonies consisting of single clones. This colonization was faster for higher rates of clonal reproduction (c.f. **Fig. 6 b-c**). While sexual reproduction was locally impossible in the single-sex areas established, sexual reproduction spread progressively from the centre of the expansion ("a sexual wave"). The speed of this wave was slow when asexual reproduction was more successful (**Fig. 6 b-c**). The second phase was initiated when the habitat was fully occupied. Following the sexual wave, the large single sex colonies formed during the first phase of the colonisation decreases as the region where sexual reproduction is possible expands. When the sexual wave moves more slowly (i. e. when asexual reproduction is relatively successful) the clonal colonies persists for ten to hundred of thousands of years (**Fig. 6 e-f**). The third phase started when the dominant clonal colonies finally disappeared. In this phase the overall frequency of asexual individuals fluctuated around a constant value.

We also analysed what would happen if the species had a capacity for occasional long-range dispersal an we found that this will increase the speed of colonisation (**Fig. 7**). In addition, aided by long-range dispersal, a clone could spread and become dominate in multiple (distant) areas (**Fig. 7 b-c).** Finally, occasional long-range dispersal also resulted in the formation of more than two dominant clones, as additional clones (to the two original ones) formed during

the colonization phase by a long range transport of an individual originating in a sexually recruiting deme. With long-range dispersal, the persistence time and the size of individual clonal colonies were on average smaller than with only local dispersal, and this effect was stronger when long- range dispersal were more frequent (**Fig. 7**).
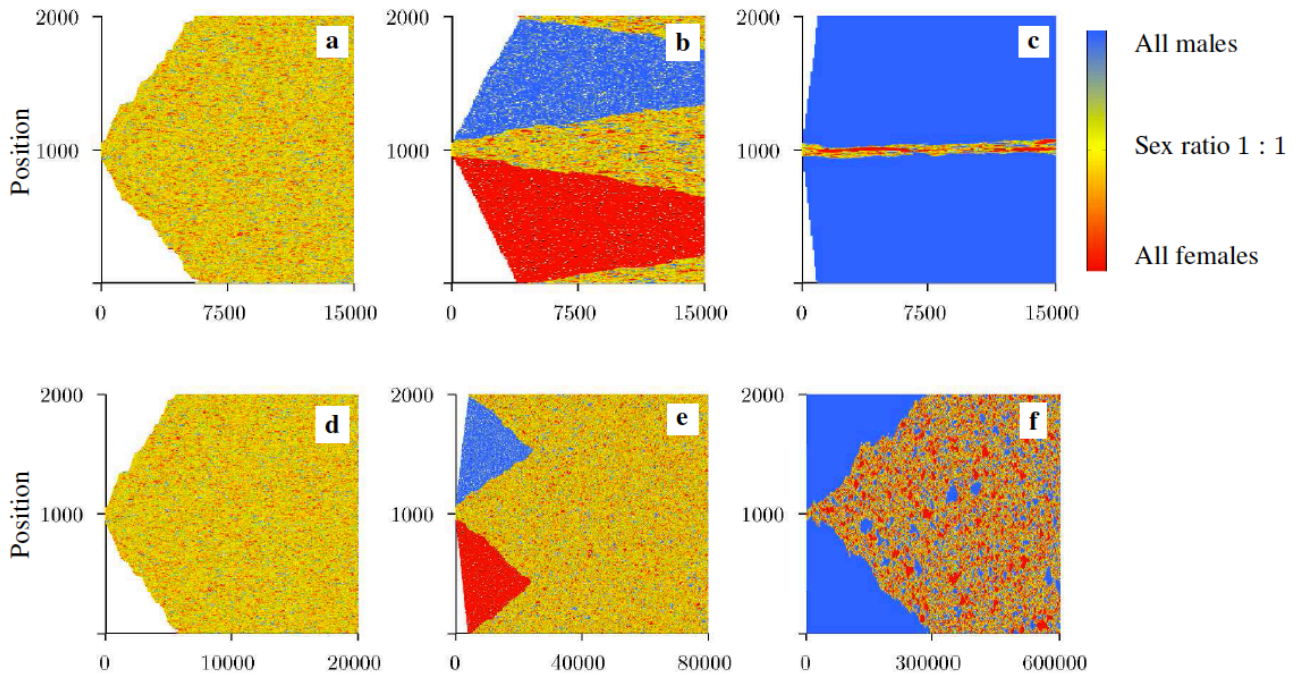

Fig. 6 a-f The importance of the rate of clonal reproduction *c*. Panels *a-c*: space-time patterns of local sex ratios (pools of 8 neighbouring patches) obtained from single stochastic realisations of the model with $c = 0.02$ in *a)*, $c = 0.2$ in *b)*, and $c = 1$ in *c)*. Empty patches are coloured white. Panels *d-f*: same as in a-c, respectively, but during longer times (more generations). In this figure only short-range dispersal is taken into account.
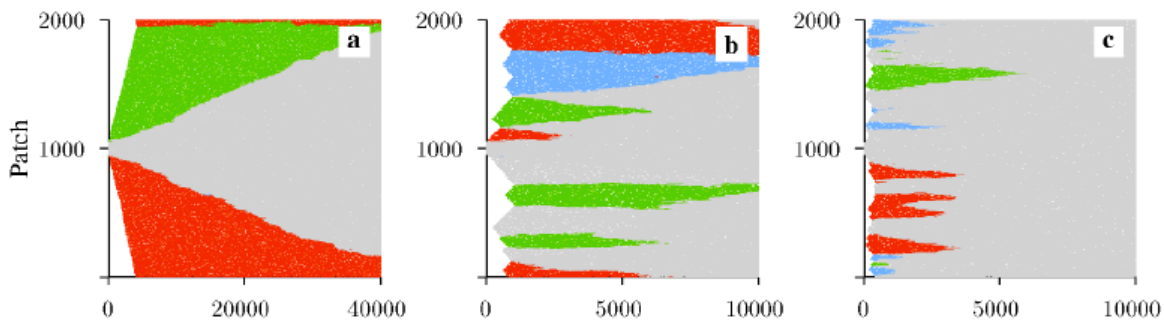

Fig 7. Dominant clones. Panels *a-c* show space-time patterns of three largest clonal colonies (coloured green, red and blue). Genotypes differing from the three largest clones are coloured grey, and empty patches are coloured white. The values of the long-range dispersal rate are: $r = 0$ in a, and d, $r = 10^{-4}$ in b, and e, and $r = 10^{-3}$ in c, and f. The per-individual rate of production of sexual propagules is fixed to two.

The results of the stochastic model were compared with the geographic patterns observed in *F. radicans* to test of the model predictions were consistent with pattern of distribution of clines in this species. As described already, the individuals of *F. radicans* in the northern and western part of the species' distribution produce both gametes and asexual fragments

(Tatarenkov *et al.,* 2005), while local populations in the south eastern part of the species' distribution (Estonia) are almost exclusively sexually recruited (Pereyra *et al.,* 2009). The latter area has also been suggested to be the native area of the species, from which further colonization of the northern parts of the Baltic Sea took place (Pereyra *et al.,* 2013). Comparing the large scale distribution of genotypes in *F. radicans*, with that predicted by the model, there is a striking resemblance. While some areas (Estonia and western Finland) have dominance of fully sexual populations, the northern and western parts of the species' distribution are dominated by a handful of large clones (**Fig. 2**). This pattern is consistent with the patterns predicted from our model with occasional long-range dispersal: a highly uneven distribution of clones, a few dominant large clones widely and discontinuously spread over the species distribution, but also high genotypic diversity and sexual activity in parts of the distribution.

Results showed that in strongly perennial species without the capacity for long-range dispersal, the dominance of asexual recruitment arises even when the rate of successful clonal production is only a few percent of the rate of gamete production (see Fig. S1 in **Paper III**). However, in annual species with limited dispersal capabilities, a clonal dominance arises only when the rate of clonal production is almost at a similar level as, or higher than the rate of production of successful gametes (see Fig. S2 in **Paper III**). Thus, our results suggest that perennial species are more likely to persist than annual species, and the clonal patterns emerging during colonisation are supported by perenniality.

Our model results thus show that in expanding perennial species with no long-range dispersal, dominant clonal colonies establish and persist for up to tens of thousands of reproductive seasons, even when individuals have a ten times higher capacity for sexual as compared to asexual reproduction. In expanding annual species, however, this occurs when the individual capacity for clonal reproduction is approximately equal to that for sexual reproduction. Furthermore, we show that occasional long-range dispersal facilitates colonisation of new habitats and, in the short run, promotes the spatial separation of sexual and clonal recruits, as suggested by Baker (1955). In the long run, however, occasional long-range dispersal facilitates the expansion of sexual recruits and promotes erosion of the dominant clonal colonies formed during colonisation.

Gaining insight into mechanisms of divergence and reproductive isolation

Adjustment of gene expression has a fundamental role in adaptation to long-term environmental changes, and hence variation in gene expression is an important component of population and species evolution (Lopez-Maury *et al.,* 2008). It is well documented how terrestrial plants respond in complex ways to the combination of stress and herbivory (e.g. Herms and Mattson, 1992; Pennings and Paul, 1992; Mole 1994; Dicke *et al.,* 2003; Chen 2008). However, much less is known about similar responses of seaweeds, and especially species differences in the molecular mechanisms underlying the chemical defense in macroalgae (Dethier *et al.,* 2005). The brown algae *F. vesiculosus* and *F. radicans* have been shown to have significant genotypic differences between them (Bergström *et al.,* 2005; Pereyra *et al.,* 2009) but additional information on transcriptomic

differences represents many more loci and may identify functionally important genes, which may have central roles in the evolution of ecological differences affecting reproductive isolation between the species.

Brown algae are important primary producers (Schmidt *et al.*, 2011) and often get exposed to herbivory from organisms like isopods and gastropods (Hagerman, 1966). The effect of grazing leads to physiological stress in the algae, and since grazed individuals are rarely killed, this stress can be sustained over long time periods (Dethier *et al.*, 2005). Under selection from grazing, chemical defense against herbivore attacks has evolved in many species of algae, and brown seaweeds contain secondary metabolites that may deter feeding by herbivores (Steinberg, 1988; Pavia and Toth, 2000) by making the algae less palatable and sometimes even toxic to grazers (Pennings and Paul, 1992; Augner 1995; Forslund *et al.*, 2012). The production of secondary metabolites in brown algae is costly (Van Alstyne, 1988; Pavia *et al.*, 1999; Strauss *et al.*, 2002), and it is presumably advantageous for the algae to respond to grazing only when herbivores are present and actively grazing. This type of inducible defense is common in brown algae (e.g. Ragan and Glombitza, 1986; Van Alstyne 1988; Lvessalo 1989; Van Alstyne and Paul, 1990) and major regulation occurs at both transcriptional and post-transcriptional level, which is decisive to adaptation in changing environments.

We hypothesise that there is potential for local adaptation to herbivores in *F. vesiculosus* from the Swedish West coast compared to Baltic *F. vesiculosus* and *F. radicans*, and that this may be connected to the ecological separation (and hereby reproductive isolation) of *F. vesiculosus* and *F. radicans*. Isopods of the genus *Idotea* are important grazers of macroalgae in the Baltic Sea, and in particular in the southern Baltic Sea several species are frequent (Leidenberger *et al.*, 2012) overlapping in distribution with *F. vesiculosus* but to a lesser degree with *F. radicans* that has its main distribution in the northern part of the Baltic Sea (Forslund *et al.,* 2012). Furthermore, although grazing experiments show that the isopods have a preference for *Fucus* species over other macroalgae (Jormalainen *et al.*, 2001; Forslund *et al.*, 2012), they prefer *F. radicans* to *F. vesiculosus* (Forslund *et al.,* 2012; Gunnarsson and Berglund, 2012). In addition, *F. radicans* seems to have lower levels of phlorotannins (that helps to resist grazing) than *F. vesiculosus* (Forslund *et al.*, 2012).

With the development of functional genomic techniques, the evolution of gene expression differences following a recent speciation event may be investigated (Chelaifa *et al.*, 2010). We used a microarray developed earlier for *F. vesiculosus* to provide a good basis for mapping contigs differentially expressed between the two closely related *Fucus* species by so called heterologous hybridization. By using transcriptomic analysis we addressed the following specific questions:

1) How is gene expression altered by herbivore grazing and are there differences in the transcriptome profiles of *F. vesiculosus* and *F. radicans*? And how many and which genes are involved in the response to isopod grazing?
2) What are the differences in gene expression between both species? And how many and which genes (by functional annotation) are different between both species?
3) How can expression differences be interpreted in relation to evolutionary history of the two species and to their environments?

These questions are of high relevance and important to study in order to gain a deeper understanding of the speciation event, but our study was just a first step to unravel the molecular, genetic, and evolutionary causes involved.

Two similar and fully replicated herbivore grazing experiments were performed, and we used a combined approach to induce and test the defense reaction in the two algae species: the experiment potentially inducing defense in the algae by direct grazing of isopods ('induction experiment') was followed by a feeding assays with isopods (*I. granulosa*), testing palatability differences between potentially induced and control algal tissues ('feeding assay'). The induction experiment followed four individuals of each of the two species of *Fucus* (each individual split in two replicates) over three time points, both for control and treatment for *F. radicans* and *F. vesiculosus*. Using microarray technology, grazing-induced genes that were differentially expressed between ungrazed control tissue and grazed tissue in *F. radicans* and *F. vesiculosus* were identified within and between species, and between three given time points (see details of procedure in **Paper IV**).

The feeding preference assay showed differences in consumption between *F. vesiculosus* and *F. radicans* tissue when grazers were given the choice between reconstituted food made of control thalli and previously grazed pieces (see Fig 1 in **Paper IV**). Algal material previously grazed for 3 days was preferred over the control tissue from *F. radicans* whereas the opposite happened in *F. vesiculosus*, where control tissue was preferred over previously grazed tissue during this time. However, the consumption pattern switches for algae grazed for 7 days. These results suggest that *F. vesiculosus* produces deterrent chemicals against grazing faster than *F. radicans* over the time-period of 3-7 days, and that the *Fucus* thalli were indeed different with respect to timing.

The microarray analyses demonstrated differential gene expression in grazed tissue of both *F. radicans* and *F. vesiculosus,* compared to the expression patterns of the same genes in non-grazed control thalli. In total, 292 genes were differentially expressed in *F. radicans* across all time points, and 240 genes were differentially expressed in *F. vesiculosus* across all time points (**Fig. 8**). In *F. radicans*, the total number of up- and down regulated genes was highest after 1 day of grazing, and in comparison, the number of responsive genes in *F. vesiculosus* was highest after 3 days of grazing (**Fig. 8**). In total, more genes were differentially expressed in *F. radicans* than in *F. vesiculosus* during our experiment. However, for each time point, the number of responsive genes for each species was different (**Fig 8**). For example, 1 hour after grazing was initiated, *F. radicans* showed more genes that were both up- and down-regulated than *F. vesiculosus,* and none of these genes were shared between species. Also after 1 day of grazing *F. radicans* showed the highest number of responsive genes, and very few genes were further shared between both species. However, after 3 days of grazing, the number of responsive genes in *F. vesiculosus* had increased and was larger than in *F. radicans*. This suggests that *F. radicans* and *F. vesiculosus* may display differences in the timing of their gene regulation linked to their induced responses to grazing.

The largest expression changes among the up-regulated genes (illustrated as the highest values of fold change, FC) in *F. radicans* were after 1 day of grazing, whereas largest changes occurred in *F. vesiculosus* after 3 days of grazing (**Fig. 9**). Among the down-regulated genes, the highest differential expression in *F. vesiculosus* was also after 3 days whereas the down-regulation differences in *F. radicans* were less evident when comparing the three time points.
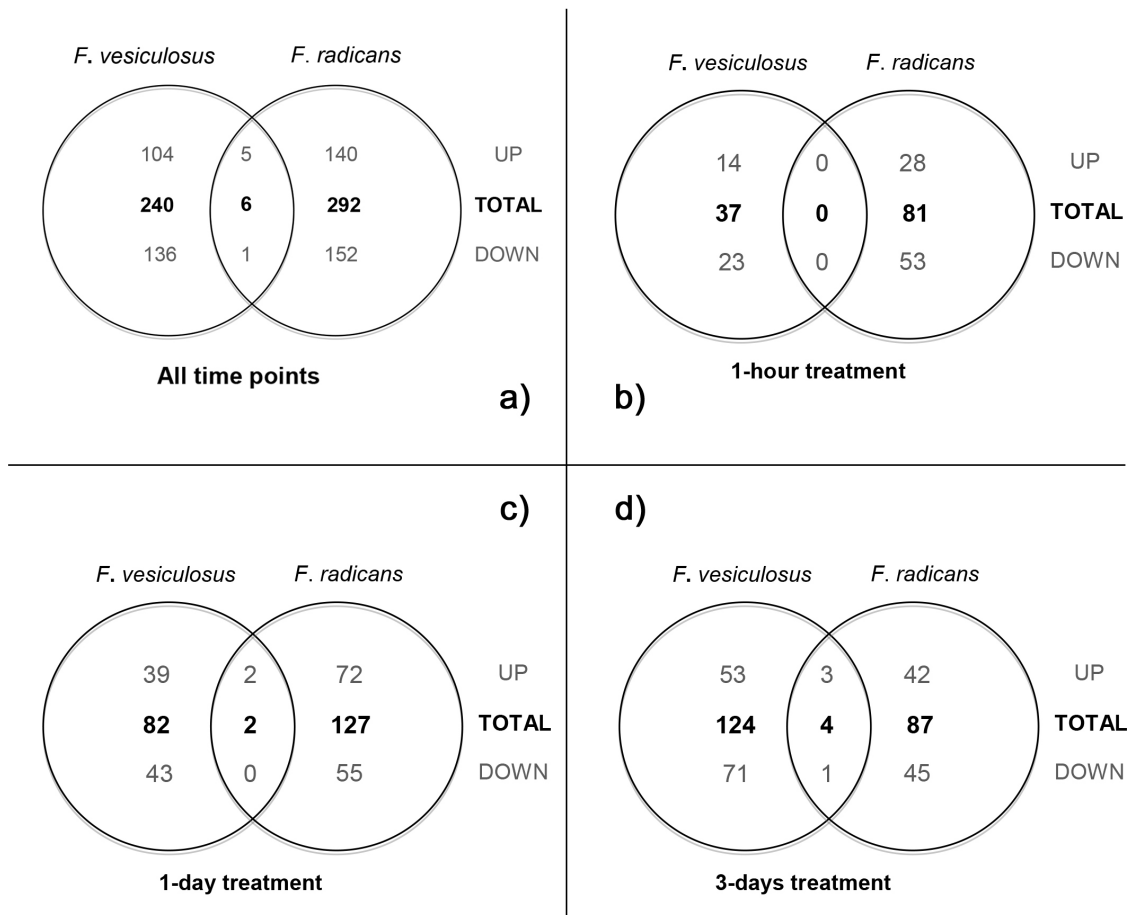
Fig. 8. Venn diagrams of number of responsive genes between species, in total (a) and for each time point (b - 1h, c - 1 day and d - 3 days). Regulated transcripts are shown in total (black numbers), and as up- and down-regulated genes (grey numbers). Numbers of responsive genes unique to each species, and shared between species are indicated.

We further explored the possibility that despite finding less number of genes expressed in one species at a particular time point (**Fig. 8**), the expression would be higher (expressed as higher fold-changes) compared to the other species. Our results showed that this was not the case after 1-hour and 1-day treatment, where *F. vesiculosus* showed less expressed genes than *F. radicans* (see Fig. 4 in **Paper IV**). At these time points, both up- and down-regulated genes showed higher fold changes in *F. radicans* than in *F. vesiculosus*. However, our hypothesis of less genes but those then being highly expressed was true after 3 days, where *F. radicans* showed less up-regulated genes than *F. vesiculosus* but the expression magnitude was on average higher in *F. radicans*.

Lastly, we hypothesised whether lower expression in *F. vesiculosus* was associated with higher constitutive expression in *F. vesiculosus*. In total, 292 genes appeared significantly expressed in *F. radicans* across all time points in the experiment. From these, 83 genes (28%) appeared to have higher constitutive expression in *F. vesiculosus* compared to *F. radicans* across all time points while the remaining genes showed smaller expression changes in *F. vesiculosus* compared to *F. radicans* and may be considered potentially stress-indicator genes (see Fig. 5 in **Paper IV**).
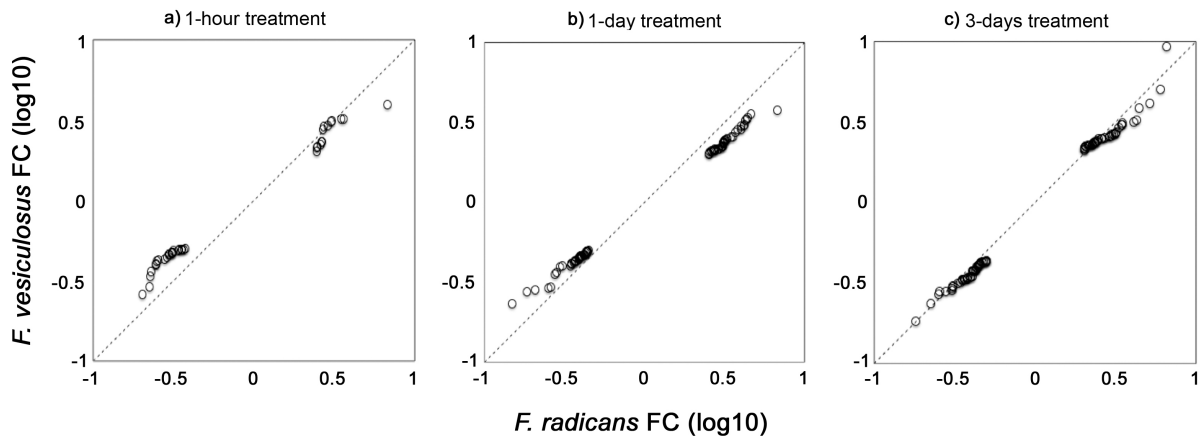
Fig. 9. Scatter plots analysing fold changes (FC) of gene expression between genes from the different time points (1 h, 1 day and 3 days) in pair-wise comparisons within each species (*F. vesiculosus* and *F. radicans*). Upregulated genes are above 0, and downregulated genes are below 0.

To be able to give information about how many and which genes are different between the two species, a generalised classification was performed for all significantly regulated genes of the different time points under grazing treatment. The number of genes and their process they are classified in are listed in Table 1 in **Paper IV**, where they are divided into function per species and per time point. Up- and downregulation of genes involved in defense, signalling/sensing, intracellular trafficking, and energy response was found in the grazed seaweed pieces of the two macro algae.

Our results suggest that this species induces resistance to grazing at slower rate than *F. vesiculosus*, as we found no effect after 3 days, but resistance was induced after 7 days. A slower response in *F. radicans*, compared to *F. vesiculosus* makes sense if the former is under less selective pressure from grazers for an efficient response. The slower response may also let grazers simply graze away *F. radicans* where the grazers are present.

In this study, we found support for many genes involved in anti-grazing defense that may be persistently expressed in *F. vesiculosus*. That is, among the nearly 300 genes that were differentially expressed during grazing in *F. radicans*, more than a quarter (83 genes) seem to be part of a constitutive defense in *F. vesiculosus*. In addition, we found potential stress-indicator genes for which *F. vesiculosus* showed smaller expression changes than did *F. radicans*. These genes showing smaller expression change may be related to more general stress responses that both species are subject to, and therefore any higher constitution may not confer any advantage to *F. vesiculosus*. As higher constitutive expression may prepare an individual for frequently encountered stress, this suggests that *F. vesiculosus* may be more resistant to herbivore grazing since it is frequently subjected to this pressure, and therefore experience overall lower physiological stress than *F. radicans* when exposed to isopods.

This study suggests a certain level of temporal variability in induced anti-herbivory traits of *F. vesiculosus* and its close relative *F. radicans*, and reveals increased expression of genes that may have a general stress- or grazing response. Unfortunately, the genes which showed the highest up- or down-regulation after 1 hour, 1 day and 3 days of grazing (up to ~7-fold change in expression in *F. radicans*) remained with unknown function. Due to the fact that some of these unknown genes show the highest response to grazing, these genes may be considered as defense-related, however, it is not possible to draw any definite conclusions

about their involvement in herbivore resistance. However, the genes that were observed to be regulated at the highest level are certainly interesting candidates to look into further, to be able to find key genes that are involved in the anti-herbivory response in *F. radicans*, as well as *in F. vesiculosus*.

One assumption of ecological microarray studies is that expression levels of genes are subject to evolutionary pressure, and that intertaxa differences in expression are due to adaptation to different environments (Kammenga *et al.,* 2007). Since *Idotea spp.* are major herbivores of *Fucus* in the Baltic Sea and cause significant biomass losses of tha algae (Rohde and Wahl 2008) one might expect inducible defenses of the prey species to be evolutionarily attuned to the activity patterns of this herbivore. We conclude that the evolutionary changes that we observe in transcriptional regulation of grazing resistance has evolved under differential selection, with *F. vesiculosus* mainly distributed in areas of strong grazing pressure, while *F. radicans* not found in such areas. This is consistent with earlier studies showing gene expression differences being adaptive (e.g. Rockman *et al.,* 2006; Tirosch *et al.,* 2006; Odom *et al.,* 2007).

Conclusions and future perspectives

The results from **Paper I** in this thesis have further strengthened the picture of profound asexual reproduction in *F. radicans* in the northern and western distribution of the species, and added a few more widespread and presumably old clones found in the northern part of the distribution. Two clones, in particular, are very widespread and considered to be of old age. Also, both clones and sexes appeared to be intermingled at local scale, giving good opportunities for sexual reproduction. However, despite of this intermingled pattern, asexual reproduction dominates in *F. radicans*. We suggest two hypotheses explaining the frequent occurrence of asexual reproduction in *F. radicans*. The first is that sexual reproduction is selected against in low salinity due to polyspermy and subsequent failure of embryo development, like earlier believed (Serrão *et al.,* 1999). This hypothesis is, however, not supported by the observations of high sexual reproduction in the lowest salinities (<3‰) in eastern Gulf of Finland. The second hypothesis makes the assumption that in a species with both sexual and asexual reproduction, and even if the former is favoured over the latter over long-evolutionary times, asexual recruitment can be predominant during colonization of a new area, due to the much higher potential of asexual reproduction in the low density front of an expanding species, due to the problem of sexual individuals to find mates (Baker, 1967; McCarthy, 1997). This is also supported from modelling results in **Paper III**, and some areas might initially have been colonized by a few clones, but has later, due to gene flow from other populations, received more individuals that increased the potential for sexual reproduction, as predicted by the neutral model (**Paper III**). As a next step, an analysis of the spatial genetic structure of clones also in *F. vesiculosus* may give further insight to evaluate these two hypotheses.

The results from **Paper II** overall indicate a complex genetic structure strongly affected by the geographic isolation of populations of *Fucus* in the Baltic Sea, where additional stochastic mechanisms may contribute to further complicate the picture, but in most cases the two taxa

separate when analysed at a local scale. It also seems, as dispersal and gene flow are less important than other local factors at larger geographic scales in the both species. Part of the complexity of the genetic structure in the Baltic *Fucus* is likely caused by the mixed sexual and asexual reproduction, that allows for similar genotypes (clonal lineages, see **Paper I**) to establish over vast geographic areas, and similar to what is sometimes found in both terrestrial and aquatic plants (Eckert 2002; Hörandl *et al.,* 2008). A concept named "allelic surfing" which is a mechanism by which allele frequency differences may be established and succeequently spread as a consequence of stochastic effects at the very margin of an expanding population (Excoffier and Ray, 2008; Excoffier *et al.,* 2009). Indeed, this mechanism is very similar to the stochastic effects that may explain the complex distribution of sexual and asexual populations in *F. radicans* (**Paper III**). Given that each species has a complex population genetic structure, and that the two species are extremely recently separated, it comes as no surprise that the separation of *F. radicans* and *F. vesiculosus* is sometimes vague. It is not unusual to find closely related species that are very similar and overlap in morphological space, and possibly hybridize in some areas while not in others (Haas *et al.,* 2009; Mikhailova *et al.,* 2009; Hermansen *et al.,* 2011). It is also expected in very recently diverged species that a large amount of the genetic variation will be shared due to incomplete lineage sorting. With such minor species distinctions, it would not be surprising to find the two species hybridize in some of their sympatric areas, in particular, since hybridization is commonly found among closely related species of *Fucus* (Coyer *et al.,* 2002).

We here show that one way to explore the evolutionary history of the divergence, the speciation mechanisms and also the processes of local adaptation is to take advantage of transcriptomics approaches (**Paper IV**). By exploring the transcriptome profiles under grazing treatments, we gained further insight into the ecological of divergence and, indirectly, the maintenance of reproductive isolation between the two *Fucus* species, and as the evolutionary changes that we observe in transcriptional regulation of grazing resistance in these two macro algae has likely evolved under differential selection, with *F. vesiculosus* mainly distributed in areas of strong grazing pressure, while *F. radicans* is not found in such areas and this could, together with other factors, influence the distributional range of *F. radicans*. There was difficulties to identify candidate genes for defense induction due to the limited annotation success, in particular since both of these brown algae are non-model organisms without whole-genome sequencing. However, the genes that were observed to be regulated at the highest level are certainly interesting candidates to look into further, to be able to find key genes that are involved in the anti-herbivory response in *F. radicans*, as well as *in F. vesiculosus*.

To further elucidate the complex structure of the two *Fucus* species in the Baltic Sea I suggest using extensive numbers of genome-wide markers, complemented with intensive sampling, experimental crossbreeding and modelling the role of genetic drift. Such ambitious investigations will likely be rewarding in gaining further understanding of the Baltic Sea *Fucus* system and its intriguingly rapid and recent speciation.

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