PHYLOGEOGRAPHY OF *PHYCODRYS RUBENS* (LINNAEUS) BATTERS FROM MAINLAND NORWAY AND SVALBARD BASED ON NUCLEAR AND MTDNA SEQUENCES AND MICROSATELLITES.

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Abstract:

Phycodrys rubens is a common cold temperate red macroalga in the North Atlantic, a region with a diverse natural history that has shaped the phylogeography for many species. In this study microsatellite loci as well as nuclear and mtDNA sequences were used to investigate the phylogeography and populations genetic diversity of *P. rubens* in the northern North Atlantic. A total of 19 mtDNA cox2-3 intergenic spacer sequences and 37 nrDNA ITS1 sequences were obtained representing 15 locations in Svalbard, Norway and Iceland. In addition 103 individuals from two locations each on Svalbard and outside Bergen were analysed for five microsatellite loci.

Several unique haplotypes were identified among the cox2-3 spacer sequences, and 10 (53%) of the haplotypes were found only once. The analysed ITS1 sequences were less variable, and all ITS1 sequence types were found in more than one population. The geographic distribution of the ITS1 sequences types, with most being shared among geographically widespread populations, suggests a recent common history of populations that are currently widespread. The ITS1 sequences obtained in this study all represented the East Atlantic lineage as identified by van Oppen *et al* (1995). Some of the ITS1 and cox2-3 sequence types and microsatellites genotypes occurred only in mainland Norway or only in Svalbard, indicating isolation of the current populations of *P. rubens*.

From one to four genotypes were identified from each of the five microsatellite loci used, three of the loci had three genotypes, while one locus had four genotypes and the last locus was monomorphic in the tested populations. Three of the tested microsatellite loci gave good resolution and were usable to look at population differences between Svalbard and Bergen. For the microsatellite loci there were differences in genotype frequencies, both between the populations of Svalbard and Bergen, but also between the two populations in each Svalbard or Bergen. For instance two of the genotypes found were only present in one population.

Contents

Introduction:

The Quaternary ice sheets covered large areas of the North Atlantic and caused large-scale shifts in the distribution of organisms (Bennet and Provan 2008). It has been hypothesized that *Phycodrys rubens* (Linnaeus) Batters (1902) survived the late Pleistocene glaciations in ice-free areas in Scotland and Norway (van Oppen *et al* 1995). The natural history of the North Atlantic has influenced the current distribution and genetic diversity of *P. rubens*. The geographical ranges of any species change as the climate alters the environment (Maggs *et al* 2008). Genetic similarities in species individuals could indicate exchange between populations, both resent and old exchanges could lead to geographical distant population having similar genetics diversities. On the other hand genetic differences in species populations could indicate geographical isolations, for example because of past ice cover and changes in water currents. Genetic differences between populations could be enough to recognize some *P. rubens* communities as subspecies (van Oppen *et al* 1995). Paleoecology has shown that in temperate Europe species experienced a cycle of range expansions and contractions (Pielou 1991). Because many species lack fossil record genetic data has been important tools in recent advances in understanding the evolutionary effect of the glaciations (Maggs *et al* 2008).

Evolutionary history of the North-Atlantic macroalgal flora:

The first opening of the Bering Strait is an important geological event, up to c. 120 million years ago (Ma), making dispersal of species from the Pacific Ocean into the Atlantic Ocean possible (Lawver *et al* 1990). The Atlantic Ocean was again cut off from the Pacific Ocean when the Bering Strait closed. The passage opened up for a second time, around late Pliocene (3.5-3.0 Ma years ago), this again gave the possibility that species from the Pacific Ocean might have dispersed into the Arctic and the Atlantic Ocean. The lowering of the Icelandic ridge below sea level in the Miocene (10-15 Ma years ago) is another important geological event in the Atlantic region, making deep-water exchange between the Arctic Ocean and the Atlantic Ocean possible (Kennett 1982). It is this history together with a general cooling of the area in the Miocene, which enhanced a distinct boreal marine province in the Arctic Ocean and the surrounding littoral. This history also started the evolution of a sub-Arctic biota with an Atlantic character (Lindstrom 2002). This was followed by a period of general cooling that lead to the Pleistocene ice age, beginning 1.8 Ma, and ended in the last glacial maximum. The period was characterized by oscillated changes in climate and in the Arctic Ocean freezing.

This also gave a period with repeated glacial and interglacial climate conditions, where the Bering Strait opened and closed at least six times. The diverse history of the region is important in shaping the phylogeography of many species in the northern hemisphere (Bennet and Provan 2008).

Species responses to large-scale climate shifts:

It is clear based on models and pollen records that species reacted individually to the climatic shifts of the Pleistocene, because of individual differences in environmental requirements (Maggs *et al* 2008). Paleoecology has shown that terrestrial species has had a repeated cycle of retraction and expansion during the Pleistocene glaciation (Pielou 1991). Isotherms do to a large extent decide the distribution of marine macro algae, many species are believed to have survived south of the ice-cap. After the Pleistocene glaciation, when the currents changed and the sea warmed, there was a northern expansion of organisms (Maggs *et al* 2008). Species undergoing distribution change is an important effect of large-scale climatic change, and this will influence the genetic variation patterns. As the climate changes species will move from former suitable environment, for instance if sea temperature in an area increases it will not fit the tolerance limits of some species anymore. Distribution changes will probably continue in the future as the marine environment changes (Maggs *et al* 2008). In interglacial periods a warmer climate also reduced the ability for a number of species to survive in the southern part of their range (Hewitt 1996). Expansions from the leading edge are expected to cause bottlenecks for colonizing genomes and this will lead to the loss of genotypes and a tendency towards homozygosity (Maggs *et al* 2008). Recolonized areas may thus show low diversity dominated by a few genotypes (Hewitt 1996). Many marine species have shown large-scale dispersal, and remarkable large-scale structure (Maggs *et al* 2008). However, predicting how individual species will respond to changes in the surrounding environmental conditions is challenging.

Refugia are another important term when discussing the effect of the late Quaternary ice age. Glacial refugia are area were populations are isolated from each other because of the ice-cap, and are often characterized by small effective population size (Bennet and Provan 2008). Isolation into refugia leads to characteristics patterns in the distribution of genetic diversity in species, with high diversity maintained in populations within a refugia and dissimilarity between different refugial populations (Maggs *et al* 2008). Since the effective populations sizes are often small in refugia this will increase genetic drift, and since the populations are isolated there is little genetic inflow (Bennet and Provan 2008). Seven possible LGM (last

glacial maximum) marine glacial refugia have been suggested in the eastern North Atlantic and the Mediterranean Sea (Maggs *et al* 2008); the Canary Islands, the Iberian Peninsula, the Mediterranean Sea, Iceland and the Faroe Islands area, South-West Ireland, Northern Norway and the Western English Channel. The survival and re-colonization of macroalgae in the North Atlantic have been little studied (but see e.g. Gabrielsen *et al* 2002, Provan *et al* 2005, Coyer *et al* 2011). Provan *et al* (2005) used sequencing of the nrDNA ITS, the chloroplast 16S-*trn*I-*trn*A-23S-5S, *rbc*L-*rbc*S, and the mitochondrial *rpl*12-*rps*31 and the cox2–3 spacer to study the population structure of *Palmaria palmata* in the European and North American coasts. Their results pointed to the existence of glacial refugia for *P. palmata* in the English Channel (Hurd Deep) during the late Pleistocene. The refugium would be outside of the range the ice sheet, and would have been lower than the sea level at the time. Modelling reconstruction suggested that the conditions in the late Pleistocene in the English Channel are almost identical to the current conditions on Svalbard (Provan *et al* 2005), where *P.palmata* is currently common. The study also suggested that the species underwent a severe bottleneck during the penultimate glacial maximum, rather than the LGM (Provan *et al* 2005). Another aspect of the responds of northern species to the Quaternary ice ages is the presence of cryptic refugia, which suggest that species could have survived the glacial periods in small-scattered populations. In a sense cryptic refugia are small populations in smaller, less obvious refugia with favorable microclimates in areas that would otherwise have been regarded as inhospitable (Bennet and Provan 2008). Genetic work using microsatellites and mitochondrial markers for *Fucus* spp. (Coyer *et al* 2010) suggested that cryptic refugia might exist for the genus, in Nova Scotia/Newfoundland in Canada and at Andøya in Northern Norway. The data were however not clear and the pattern found might be caused by other reasons (Coyer *et al* 2010).

<u>*Phycodrys rubens*; morphology, habitat and life history:</u>

Phycodrys rubens is a red macro-alga, belonging to the Rhodophyta, the genus is in the sub-family Phycodryoideae of Nitophylloideae (Selivanova and Zhigadlova 2002). *Phycodrys* has about recognized 20 species and the genus has a wide distribution, from temperate to cold waters. *Phycodrys* spp.. are found in the North Pacific Ocean, the Southern Ocean and in the North Atlantic Ocean. *Phycodrys rubens* is one of the most common leafy red-algae in the sub-littoral , growing from l-30 meters, and is the only representative of the genus that occurs in the North Atlantic region. The algae has a wide geographical distribution; growing in Europe, the Arctic and on both sides of the Atlantic. The southern range of the species is not

well studied. *Phycodrys rubens* has a complicated nomenclature history, and this makes the distribution range of the species problematic (Lin and Nelson 2009).).

Phycodrys rubens has irregularly curved blades, with a midrib. It can grow to about 5-15 cm in length and it is most developed in spring (Rueness 1998). Individuals of *P. rubens* are often attached to rocks or shells (Rueness 1998). It is also found as an epiphyte on larger algae such as *Laminaria* spp. and often most of the epiphytic growth on *Laminaria* spp. are composed of *P. rubens* together with some other common species (Whittick 1983). The *Phycodrys* spp.. is perennial and has a lifespan of about four years. New blades are made annually and late in the year the blades disintegrate. The species has an isomorphic *Poolysiphonia*-type life-cycle, with reproductive tissue in the blades. *Phycodrys* spp. can have both sexual and asexual reproduction (Schoschina 1996). This means that in a population it is possible to have individuals which are genetically identical, and therefore have a small effective population size when asexual reproduction is dominant.

There are some morphological characters that can be used to separate the species of *Phycodrys*. These characters include glandular cells, lamina layering, and localizations of tetrasporangia and cystocarps. In order to tell some species apart it is necessary to use several traits, since some species share the same morphology on same aspects but not others. Considering the classification problems within the *Phycodrys* genus, more work on the taxonomy is needed (Selivanova and Zhigadlova 2002). In the *Phycodrys* genus there are also synonyms for the species used in different literature. For *P. rubens* the name *P. crenata* is often used in literature. The use of the name *P. rubens* in the literature is often confusing (Selivanova and Zhigadlova 2002). In addition to the problem with names this species has a very similar morphology as another species; *P. riggi*. However the two species is considered to have different geographical range. *Phycodrys riggi* is found in the Pacific and the Atlantic coast of North America, but there is so far no record of it in Europe (van Oppen *et al* 1995). Historically, in algal research, the mechanisms surrounding the speciation and extinction of seaweeds have been hard to detect. This means that maintenance and distribution mechanisms of genetic diversity in seaweeds have also been less well known (Wattier *et al* 1997). Using molecular methods the phylogeny of *P. rubens* has been reviewed in the past (van Oppen *et al* 1995), there have however not been any studies recently.

Phylogeography:

Phylogeography is the study of genetic lineages by geographic distribution, and is a very useful approach inferring species postglacial history (Taberlet *et al* 1998). Until recently biogeographical studies of organisms with limited fossil records have been difficult (Provan *et a*l 2005). It is often assumed in phylogeography that the populations with the highest diversity are ancestral, and that their locations are the source of diversity to other, less diverse modern populations. However there is a wide range of possible outcomes for isolated populations (Maggs *et al* 2008). Glacial refugia are believed to be characterized by high-frequent central haplotypes, and private haplotypes found in no other location. Recolonized areas on the other hand can show low diversity, if they are populated from one refugia, or high diversity as a result of several populations meeting after isolation (Coyer *et al* 2011). Bottlenecks are also important to consider when using genetic markers in population studies, as they can cause loss of genetic diversity (Maggs *et al* 2008). In addition there are also contact zones, were recolonizing populations meet, where populations may show higher diversity. However, contact zones tend to lack unique haplotypes, which characterizes populations that have been separated for a long period of time, like during the last glacial maximum (Bennet and Provan 2008). The speed of re-colonization could also be important for the genetic diversity found in individual species. Rapid expansions could lead to considerable homozygosity, with derived genomes spread over the colonized area. Slower expansion on the other hand could mean that more of the genotypes would survive, with less genome divergence among populations. However different environmental condition across the distribution range will select for different genomes. The founder effect may also be important for genetic diversities and speciation; if an isolated population recolonizes an area first its haplotypes will most likely be the most common haplotypes in the recolonized area (Hewitt 1996). Because different causes can give similar effects the genetic signature of glacial influence can be hard to detect (Coyer *et al* 2011).

Genetic markers in phylogeography:

A number of genetic markers have been developed for red algae (Saunders and Moore 2013). Many of the markers have been found to show population-level variation, for example the mitochondrial DNA cox2-3 intergenic spacer (cox2-3 spacer), the nuclear ribosomal DNA internal transcribed spacers (ITS1 and ITS2) and the plastidic encoded Rubisco spacer (situated between the Large and Small Rubisco genes; Saunders and Moore 2013). The variability of ITS1 and ITS2 has proven useful to species in resolving closely related taxa, and

also show intraspecific relationships (van Oppen *et al* 1995). As non-coding regions ITS1-ITS2, the cox2-3 and the Rubisco spacer, have less functionally constrains than the coding genes. The cox2-3 spacer has similarly proven useful at intra-and interspecific levels in red algae (Zuccarello *et al* 1999, Gabrielsen *et al* 2002).

Microsatellites are tandem repeat regions in the genome, usually only 1-6 bp long (Selkoe and Toonen 2006). Microsatellites are usually highly polymorphic, with co-dominant inheritance and multiallelic single loci, which is an advantage in when studying population genetics. Multiple genotypes at many loci are often observed when using microsatellites (Wattier *et al* 1997). For population genetics, systematics and molecular ecology the method has been shown to be a valuable tool (Vik *et al* 2012). Microsatellites can be used to determine levels of genetic diversity, and to look at the patterns of genetic diversity and gene flow in and between populations (Selkoe and Toonen 2006). Microsatellites have been developed for a large range of different organisms from mammals and invertebrates to plants (Wattier *et al* 1997). The problem with this method is that there can be a limited number of loci, especially in algae (Selkoe and Toonen 2006). There has been an increase in the use of microsatellites in recent years, which has made microsatellites available for more species (Provan *et al* 2013).

Aim of the study:

The phylogeography of *Phycodrys rubens* was previously studied using nrDNA ITS and rubisco spacer sequence data (van Oppen *et al* 1995). In the study, two lineages of *P. rubens* was identified from the European coast, these lineages were hypothesized to have survived in different refugial populations along the European coast during the LGM (van Oppen *et al* 1995). After the retreat of the Pleistocene ice sheets, *P. rubens* probably migrated northwards establishing populations in recently deglaciated areas. The aim of this study was to extend the study of van Oppen *et al* (1995) to investigate glacial survival and postglacial migration patterns of *P. rubens* along the Norwegian coastline and Svalbard using nrDNA ITS sequences, rubisco spacer sequences and mtDNA cox2-3 spacer sequences. In addition more detailed population genetic variability and differentiation was studied among two populations from Svalbard and two populations from western Norway using microsatellite markers. Potential geographic patterns of the genetic diversity will be explained in relation to the glacial and postglacial history of *P. rubens.*

Material and methods:

Study site and sampling:

Phycodrys rubens was collected from four main sampling sites, two in Svalbard and two outside Bergen, as well as 10 additional sampling sites along the Norwegian coast and one in Iceland (Figure 2.1 and Table 2.1). The sampling locations in Svalbard were at Vestpynten and Dicksonfjorden, both situated in Isfjorden on the western coast of Spitsbergen (site 1 and 2. Figure 2.1). Isfjorden is open-ended and influenced by relatively warm and saline water from the West Spitsbergen Current. The fjord is also highly influenced by melting ice and run-offs from the glaciers (Rasmussen *et al* 2012). The sampling locations outside Bergen were Bukken in Raunefjorden and Vikso at the mouth of Korsfjorden (sites 10 and 11. Figure 2.1)

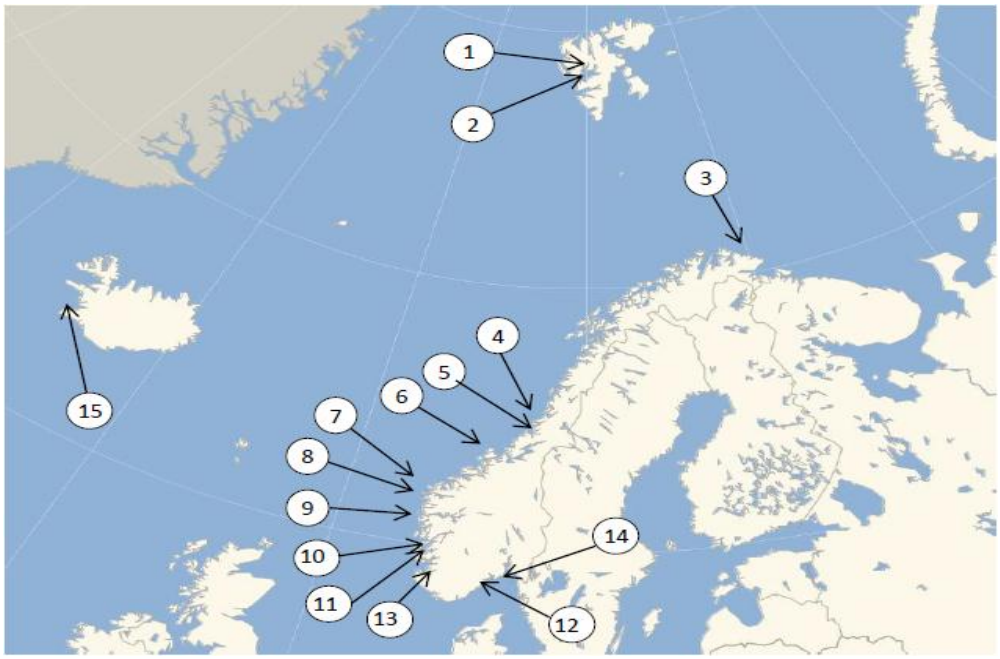

Figure 2.1: Map of sampling sites where material of *P. rubens* was collected.

The Vestpynten sampling site was dominated by small rocks and red algae. The individuals of *P. rubens* collected grew either as epiphytes on *Laminaria digitata* or epilithically. The Dicksonfjorden sampling site was a maerl area with *P. rubens* growing epilithically. A the Vikso sampling site *P. rubens* grew as epiphytes on large kelps, and at the Bukken sampling site *P. rubens* grew epiphytically at a number of different seaweeds species. In addition to these main sampling sites, *P. rubens* was collected populations from along the Norwegian coast as well as Iceland (Table 2.1).

Table 2.1: Overview of localities and collected material; Latitude and longitude are given. Samples DNA extracted are the number of individuals where DNA was extracted. Some of the collected samples were used for microsatellite testing. Samples with good DNA extractions are the samples that produced products after PCR with 28S primers. For some localities information on depth, latitude and longitude was not available.

Location	Region	number	Latitude	Longitude	Depth Sampled	Preservation	Samples collected	Samples DNA Extracted	Samples with good DNAextractions
Dicksenfjorden (Isfjorden)	Longyearbyen, Svalbard	1	78.15,34	15.24,61	5-10m	Silica gel	50	45	38
Vestpynten (Isfjorden)	Longyearbyen, Svalbard	2	78.72,67	15.38,68	4-20m	Silica gel	100	65	50
Berlevåg	Berlevåg, Finnmark	3	-	-	-	Silica gel	30	25	18
Horta	Leka, Nord-Trøndelag	4	65.10,559	11.27,600	6m	Herbarium	6	6	6
Nordøyan	Vikna, Nord-Trøndelag	5	64.50,750	10.33,650	7m	Herbarium	6	6	0
Hitra	Hitra, Sør-Trøndelag	6			-	Herbarium	6	6	6
Erkna	Ålesund, Møre og Romsdal	7	62.33,10	05.57,00	10m	Herbarium	6	6	3
Finnes	Ørsta, Møre og Romsdal	8	-	-	-	Herbarium	30	30	25
Frilsøyene	Fedje, Hordaland	9	60.49,845	05.38,673	10.	Herbarium	6	6	5
Vikso (Korsfjorden)	Bergen, Hordaland	10	60.16,22	05.03,66	5-20m	Silica gel	50	45	39
Bukken (Raunefjorden)	Bergen, Hordaland	11	60.24,20	05.20,33	5-20m	Silica gel	50	35	25
Jomfruland	Kragerø, Telemark	12	58.52,673	09.36,585	3-4m	Herbarium	6	6	6
Jæren	Rogaland	13	58.37,170	05.35,650	12m	Herbarium	6	6	6
Ytre Oslofjorden	Telemark	14	-	-	-	Herbarium	20	15	9
Reykjavik	Seltjarnarnes, Island	15	64.09,309	22. 01,035	-	Silica gel	12	6	6

Thalli of 50-100 *P. rubens* individuals were collected from the four main sampling sites at 5-20 m depth using a triangular dredge. Thalli mostly free from epiphytes were picked at random from the dredges, and individuals growing close together or that were in bad condition were avoided. From each of the sampled individuals, part of the *P. rubens* thallus was dried in silica gel to avoid DNA degradation, and the rest of the thallus was preserved as herbarium specimen (kept at UNIS). The 6-30 individuals of *P. rubens* collected from the remaining sites were sampled by divers or by using a triangular dredge and were either dried on silica gel or preserved as herbarium specimens (Table 2.1).

DNA extraction:

DNA was extracted from 3-55 individuals from each population (Table 2.1) using the DNeasy Plant Mini Kit (QIAGEN, Germany) according to the manufactures protocol. The final step of the protocol was modified to increase the yield of the extracted DNA by; eluting twice in the same AE buffer. After extraction the DNA stocks were stored at -20˚. The quantity and quality of the DNA extraction was checked using a spectrophotometer (NanoDrop 2000 spectrometer, Thermo scientific, Germany) and by visualizing the DNA on a 0.7% agarose gel and as well as by using PCR (see below). Based on the results, samples that showed good DNA quality (limited degraded DNA), were used in the amplifications of the different spacers and microsatellite loci. For all primers used a dilution range (stock, 10x, 100x, 500x, 1000x dilution) was tested to determine which dilution gave the best PCR results with different primers. Based on these results the dilution used for the universal short 28S primers used to

test the DNA (see below) was 10x. ITS1 amplification was a 500x dilution and for the cox2-3 spacer and the Rubisco spacer was a 100x dilution used.

Poolymerase chain reaction:

Poolymerase chain reactions (PCR) were used to test the extracted DNA, using an Eppendorf Ep Gradient PCR cycler (Eppendorf AG, Germany). The PCR reaction was performed in 25 µl volumes containing; 1X Dream Taq buffer, 0.2 mM of each dNTPs, 1 U DreamTaq DNA poolymerase (Thermo Scientific, Germany) and 1 µM of each primers, and amplified a short 28S nrDNA fragment using the primers 28 SF and 28 SR. 2 µl of 10x diluted template DNA was added to the PCR mix. The PCR cycling conditions used were; initial denaturing at 94˚C for 3 min, followed by 30 cycles of 94˚C denaturing for 30 sec, 54˚C annealing for 30 sec, 72˚C elongation for 1 min, and a final extension step at 72˚C for 10 min. The PCR products were visualized on 1-2% agarose gels stained by Gel Red (Biotium Inc, U.S.A). The 28S primers are universally targeted so it is expected that all DNA will show up under standard PCR condition, if the extraction was successful and the DNA has good quality.

Sanger sequencing:

The nrDNA internal transcribed spacer 1 (ITS1), ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco)and cytochrome oxidase subunit 2-3 intergentic spacer (cox2-3 spacer) genes were amplified DNA stocks from two samples from etch localities the, using the gene specific primers (table 2.3). The primers are from the article from Saunders and Moore (2013), previously used on Rhodophyta. The PCR reaction was done in a 25µl volume, with 1X green mix, 0.2 µM of each primer, 1 U Dreasmtaq. However the PCR setup did need optimization, to get as many good PCR products as possible. After the PCR the product was visualized on a 2% gel to see how the PCR had worked

For ITS1 the cycling conditions used were; 94°C for 2 min; 35 cycles of 94°C for 30 s, 50°C annealing for 45 sec, 72°C extension for 2 min; Followed by 72°C final extension for 5 min. For many of the samples this gave good clear PCR bands. However some of the samples needed more optimizing. The annealing temperature was increased to 52˚ for some samples, because extra bands that were sometimes amplified would then largely disappear. Although most samples amplified best at a 500x dilution, some samples would have extra bands that would disappear at a higher temperature. Although most samples amplified best at a 500x dilution, some samples worked better at a 100x dilution or a 1000x dilution. If the PCR

products still represented several bands after these optimizations, the band of correct size was cut out from the agarose gel and cleaned with a gel cleanup kit (Omega Biotec, U.S.A).

The amplification of the cox2-3 and rubisco spacer spacers was done using the similar reaction conditions as for ITS, and the following PCR program (after testing of the cycling conditions initially used for each primer pair); 94°C for 2 min; 5 cycles of 93°Cfor 1 min, 45°C annealing for 1 min, 72°C extension for 1 min; then 30 cycles of 93°C for 30 sec, 55°C annealing for 30 sec, 72°C extension for 30 sec; followed by 72°C final extension. For some of the samples this set-up worked well. To get more usable sequences the PCR was optimized by lowering the annealing temperature to 53°C, producing more samples with usable PCR products. For same samples improved results were obtained using a 500x dilution of the DNA stock instead of a 100x dilution.

Table 2.3: Gene- specific primers used for Sanger sequencing. The primer name refers to the name used in literature. The sequence is the primer sequence from the 5´ to 3´ end and the region refers to were the gene the primers amplify is located. While the size is the expected fragment size with the primers after amplifying, and ann temp represents annealing temperature in the literature. Forward or Reverse represents the direction of the primers, and source article is the study that the primers were obtained from.

Primer name	Region	Sequence	Size	Ann temp	Forward or Reverse	Source article
Rub5	Rubisco	TGTGGACCTCTACAAACAGC	350	55°	F	Zuccarello *et all* 1999
RUB3Por	Rubisco	CCCATAATTCCCAGTA	350	55°	R	Zuccarello *et all* 1999
P1	ITS	GGAAGGAGAAGTCGTAACAAGG	650-1100	50-53°	F	Saunders and Moore 2013
R1s	ITS	TTCAAARATTCGATGAYTC	650-1102	50-53°	R	Saunders and Moore 2013
cox2F	Cox2-3	GTACCWTCTTTDRGRRKDAAATGTGATGC	350-400	45-55°	F	Zuccarello *et al* 1999
cox3R	Cox2-3	GGATCTACWAGATGRAAWGGATGTC	350-400	45-55°	R	Zuccarello *et al* 1999

The PCR products were cleaned using the EZNA Cycle Pure kit (Omega Biotec, U.S.A) following the protocol from the manufactures. The cleaned PCR products were run on 1% agarose gels stained to test the quality and quantity of the cleaned PCR products. A total of 55 PCR products (19 cox2-3 spacer and 36 ITS1) were sequences at GATC Biotech AG (Germany). If the PCR products after cleanup were not strong enough to be sequenced directly, a second PCR (as described earlier for the primes used) was run, with a reduced (15) number of cycles. Alternatively, the samples were concentrated using ETOH precipitation. 5mg/µl LPA, 0.3x NaOAc and 2.5x ETOH was added to the samples, the samples were then vortexed briefly and precipitated at -20°C for 20 minutes supernatant removed. The pellet was washed with 70% ETOH, the supernatant removed and the samples dried in room temperature. Then the samples were resuspended in 10 µl TE solution before Sanger sequencing (at GATC Biotec, Germany).

Microsatellites:

Eight primer sets amplifying poolymorphic di-or trinucletide repeats within *P. rubens* were developed by Ecogenics GmbH (Zurick-Schlieren Switzerland). The extracted DNA used in the PCR amplifications described above did to a large extent not work when using the developed microsatellite primers. The DNA of 92 individuals wasere cleaned using the MoBio soil extraction kit, which enabled amplification using five of the microsatellite loci. Poolymerase chain reactions wereas performed in 10 µl, using 1x PCR buffer, 0.2 mM of each dNTP mix, 0.5 µM of each of the forward (labeled with FAM, NED or VIC) and reverse primers, 1 U HotStarTaq, and 2 µl cleaned genomic DNA. The PCR cycling conditions included an initial denaturation for 15 min at 95˚C followed by 30 cycles of 95˚C for 30 sec, 54-55˚C for 30 sec and 72˚C for 30 sec, followed by an extra 2 min extension at 72˚C.

Table 2.2: Microsatellite markersloci developed for *P. rubens*. Locus represents the name of the individual microsatellite loci developed, F is the forward 5′-3′ primer and R is the reverse 5′-3′ primer. The size range is the size range of the amplified product, and no of alleles are the number of alleles identified in the testing population of P. rubens utilized by Ecogenics when developing the markers.

Locus	Primer sequense 5' - 3'		Size range (bp)	No of allels.
Phyrub_04846	F	CAAACTCAACCTGTCCACGG	211-232	3
	R	GATAATCAACGCGCTCCACC		
Pyrub_05673	F	AATGTTTTTGGGGTCGACGG	196-212	4
	R	AGTTTTGCAATTGTTTCGCAC		
Phyrub_07734	F	CATCCTGCATGCCTTTCACC	111-118	3
	R	CTGCAAGAAGGAACTGACGC		
Phyrub_08003	F	AATATCCAGTCCTGGCTCGC	182-194	2
	R	ACTACTTAACTGCTTTCGAAGAATTG		
Phyrub_08540	F	ATACCGACGTCATCAACCCG	215-234	4
	R	TGCCATGCTATGATGATTGTG		
Phyrub_18475	F	ACAATATGCCGAAAGATGTGC	212-214	2
	R	GTCGTGGAAGACAAAGCAGG		
Phyrub_31074	F	TTCCGAGATACCTGCATGGG	191-194	2
	R	GTGAATTCCAGGTTCGAGCG		
Phyrub_36410	F	TGCGGACATTAGTCTTGGGG	44-87	4
	R	ACGTTAATTCTTCCATCCTTCG		

Data analyses:

The Sanger sequences were analysed using the Geneious program (version 7.0.5). All the sequences were trimmed using the trim end function in the program; the error probability limit was set to 0.05, maximum ambiguities to 10 and maximum length after trim to 700 bp. This was done to remove base hits that were too uncertain. Contigs of each individual were built from the forward and reverse sequences using the De Novo assembly function. The contigs were manually checked and the primers were removed. Blast searches were done using Geneious to check the sequences. The sequences were then assembled using Muscle alignment in Geneious, and then manually checked. Minimum spanning networks (MSN) were drawn for the cox2-3 spacer and ITS1. The MSN were drawn according to the nucleotide differences in the alignment, one substitution means the sequences represented a

different ITS1 sequence type and the change was illustrated with lines. The network with the least amount of changes was selected.

Only five of the eight developed microsatellites loci were optimized for *P. rubens*. The microsatellites were tested on the four main sampling populations; however many of the analysed individual did not give usable results. For the microsatellites the Geneious program was used to view and score the peaks. Microsatellite peak bins were made using the peak bin function, so peaks could be scored. Stutter bands were abundant, these are additional peaks that were mostly smaller than the true fragment, and are usually the results of DNA slippage during PCR. Sometimes it could be hard to see what peak was the original. After the genotypes were scored automatically in Geneious, using predict peak function with a threshold of 200, the genotypes were manually checked. Genotypes were assigned to each individual and population, and the genotype frequencies for etch population were calculated. As *P. rubens* is an isomorph, haplodiplontic species it is not possible to identify the number of alleles accounting for the different homozygote patterns (as the haploid and diploid tissue of *Phycodrys rubens* could not be separated). Thus, further calculations of population genetic parameters could not be performed for the limited dataset available.

Results:

Methodological challenges:

The microsatellites loci developed for *P.rubens* was difficult to amplify. Three of the loci did not work at all, and one of the five remaining loci did not give usable PCR products for more than 11 individuals even with optimization. The microsatellite loci that largely worked did not do so for all of the individuals. This is most likely because of PCR inhibitors in the extracted DNA, which the DNA cleanup and PCR optimizations only partly removed. Problems with PCR amplification of the ITS1, cox 2-3 spacer and spacer regions even after optimization resulted in a reduced number of sequenced individuals compared to the original plan. The test using a dilution series of the DNA showed that highly diluted DNA template gave improved PCR amplifications in most cases, suggesting that PCR inhibitors are present in the DNA templates. However, when DNA was visualized on agarose gels most of the samples apparently had fairly clean DNA bands (for example lane six in Figure 3.1). Most samples had fairly low DNA concentrations (for example lane seven to ten) but still amplified best in

PCR when strongly diluted. The dilution tests showed that cox2-3 spacer and rubisco spacer amplified best using on a 100x dilution. For ITS1 the dilution test showed that samples amplified best bands with a dilution of 500x, so this dilution were used for most samples when amplifying ITS. However, some samples hade clearer bands at a 1000x.

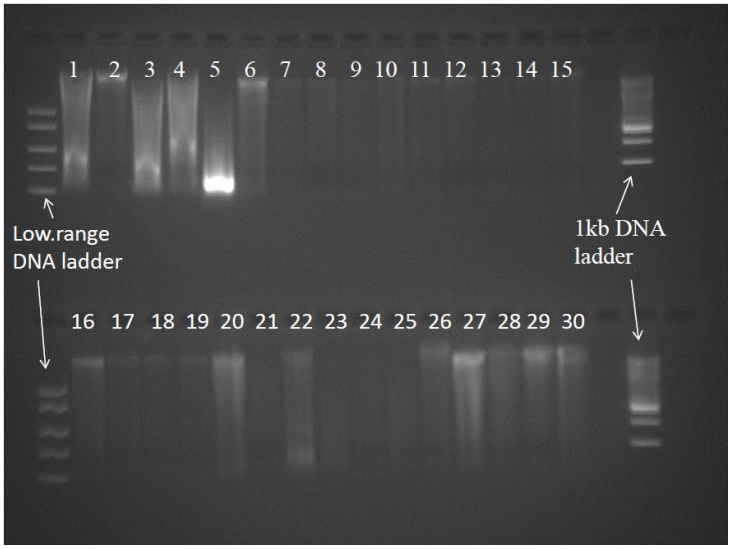

Figure 3.1: Gel picture of DNA, visualized on 1% gel. Five individuals from six of the locations, Bukken (1-5), Vikso (6-10), Finnes (11-15), Oslofjorden (16-20), Jomfroland (21-25) and Horta (26-30). First ladder on the left side is fastruler low range DNA ladder and the right ladder is Generuler 1 kb DNA ladder.

Sequence data:

A total of 19 mtDNA cox2-3 spacer sequences, 37 ITS1 nrDNA sequences and 4 rubisco spacer sequences were obtained from the collected material. The cox2-3 spacer sequences ranged from 303 to 327 bp, ITS1 ranged from 319 to 450 bp and rubisco ranged from 281 to 349 (Table 3.1). For the cox2-3 spacer the first 30 and last 17 nucleotides were not readable for some individuals and were excluded from the alignment. The complete cox2-3 spacer alignment for *P. rubens* had 409 characters; of these 19 were variable sites. Both ITS1 and the cox2-3 spacer showed variations in *P. rubens*. The ITS1 alignment also included previously published *P. rubens* and *P. riggi* (van Oppen *et al* 1995; Goff *et al* 1997), meaning the ITS alignment had a total of 50 sequences. The ITS1 alignment had 746 characters; of these 369 were variable sites. The rubisco spacer alignment had 409 characters; of them 145 were variable site. The rubisco was hard to optimize and the four sequences obtained did not have good quality. Individual B22 from Bukken was excluded from the analyses of both the cox2-3 spacer and the ITS1 sequence results. This is because it was very different from the other sequences in the alignment, including the ITS1 sequence of *P. riggi*, and blast searches (Altschul *et al* 1991) suggested that the B22 individual probably represents a different species

compared to the other samples. This is not unlikely since the Bukken location was collected late in the season, and some of the samples had epiphytes growing on the thallus. The DNA extracted and amplified was most likely from an epiphytic red algae species, and not from a *Phycodrys* spp., although any further identification was not possible because of few available ITS1 and cox2-3 spacer sequences in GenBank.

The 19 individuals sequenced for the cox2-3 spacer represented 10 different mtDNA haplotypes. Haplotype A was the basal haplotype, while the other haplotypes had one or up to 13 nucleotide differences from A. All haplotypes B-G had the same two nucleotide changes separating them from A and H-J. B, C and D haplotypes had the same two one nucleotide substitutions that separated them from the remaining haplotypes. In addition haplotypes B and C had four extra one nucleotide substitutions that D did not have. Haplotype B and C shared many substitutions, but C had five extra nucleotide substitutions separating it from haplotype B. Haplotype E, F and G shared three one nucleotide substitutions. Haplotype F and G both had a one nucleotide substitution that separated them from E. Haplotype D had one unique nucleotide substitutions that separated it from the others. The minimum spanning network drawn reflects the relationship between the different haplotypes (Figure 3.2).

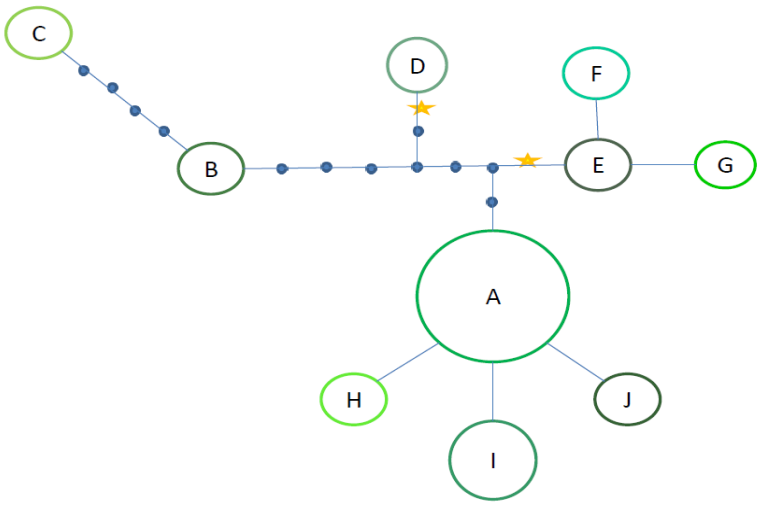

Figure 3.2: Minimum Spanning Network (MSN) based variable nucleotides the Cox2-3 spacer sequences, where lines represent one nucleotide difference and dots potential but undetected haplotypes. The Stars indicates shared nucleotide differences not seen by the lines.

The geographical origin of the cox2-3 spacer haplotypes is shown in Figure 3.3. Haplotypes from both the main mtDNA groups (A and H-J, E-G vs. B-D) was found along the Norwegian coast as well as Svalbard. The most abundant haplotype was A, it was found in four

individuals in Dicksonfjorden, in one individual from Vestpynten and in two individuals from Iceland. E-G and B-D haplotypes was most common on the western coast of Norway and on Iceland. Some of the haplotypes were found on several locations, haplotype A was found on Iceland and both locations on Svalbard (Vestpynten and Dicksonfjorden). While haplotype I was found on Vestpynten and Berlevåg, and haplotype E was found Nordøyan (Trøndelag) and Oslofjorden. The rest of the Haplotypes (B, C, D, F, G, H and K) were each found in one individual at one locality.

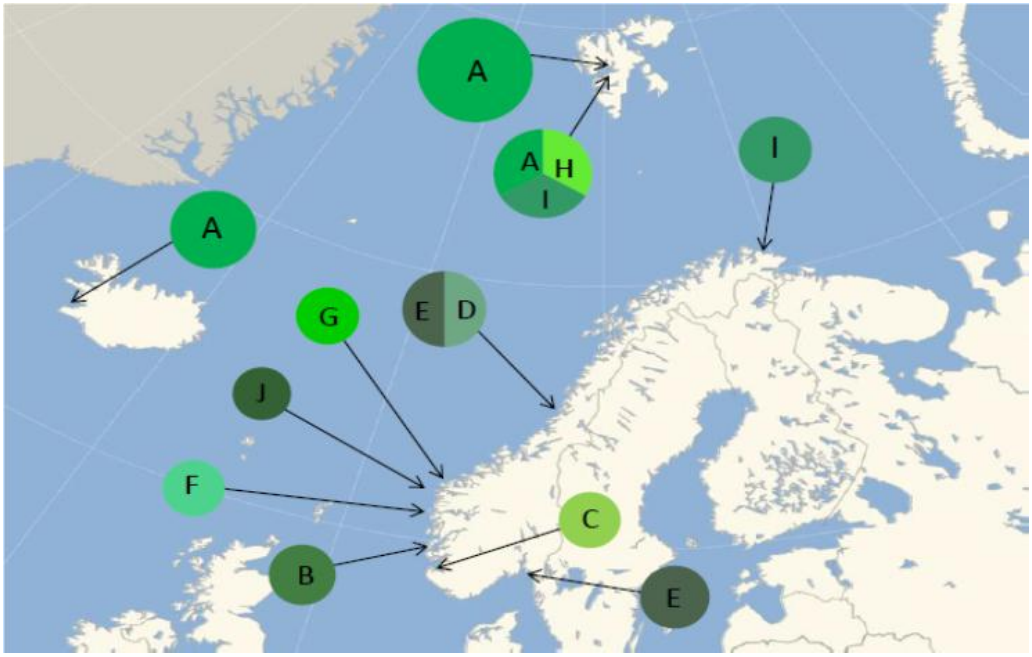

Figure 3.3: Geographic origin of cox2-3 spacer mtDNA haplotypes identified in P. rubens. The letters corresponds to the letters in the MSN. The diagrams show the relationship between the haplotypes frequencies at different locations, for instant the Dickson location has tree individuals with L and one individual with the G haplotype. The larger the diagrams more sequence were identified, for instance the diagram in Iceland is bigger than the F diagram (Erkna) because the Iceland location have two individuals with the A haplotype.

The ITS1 sequence types identified in this study were not as divergent as the cox2-3 spacer haplotypes. The 37 individuals sequenced showed seven different ITS1 sequence types which were identified by six nucleotide substitutions. The basal ITS1 sequence type A was the most common. ITS1 sequence types B, C, D, F and G had one unique nucleotide difference from the A ITS1 sequence type. ITS1 sequence type E shared one nucleotide change from ITS1 sequence type A with D, and had an additional one private nucleotide change. The ITS1 sequence types and their interrelationship are illustrated in the MSN for ITS1 (Figure 3.4). All the ITS1 sequence types found for ITS1 in this study were found in more than one individual.

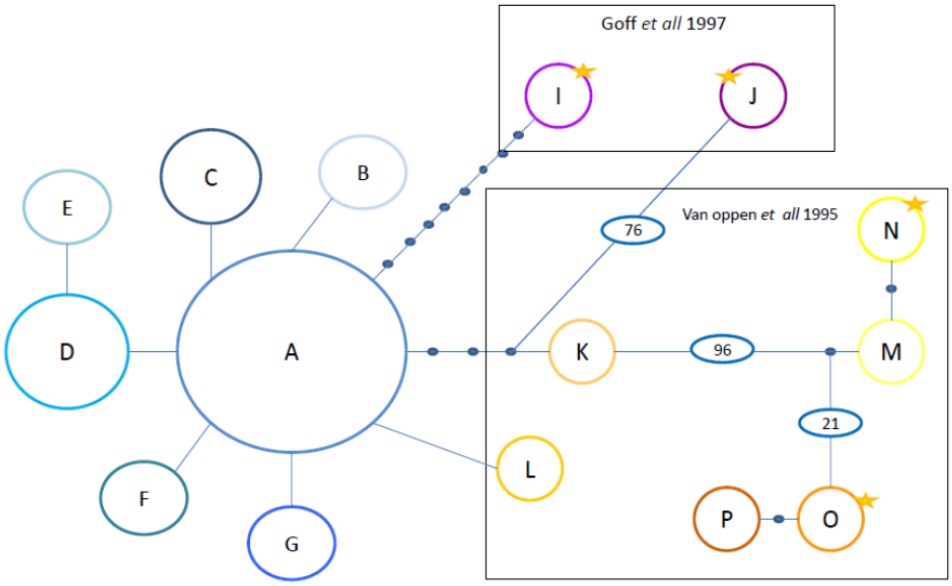

Figure 3.4: The Minimum Spanning Network based connecting the ITS1 sequence types of *P. rubens* found in this study as well as others (van Oppen *et al* 1995, Goff *et al* 1997). Lines represent one nucleotide difference, and dots potential but undetected ITS1 ITS1 sequence types. The Stars indicates shared nucleotide differences not seen by the lines.

As for the cox2-3 spacer the geographical location of the ITS1 sequence types is illustrated on a map (Figure 3.5). According to the map ITS1 sequence type A was found in almost all of the locations, with the exception of Svalbard, Iceland and Horta (Nord-Trøndelag). All of the other ITS1 sequence types were found in more than one location, with the exception of ITS1 sequence type B that was only found in two individuals at Vikso. For instance ITS1 sequence type G was found on Iceland and Horta (Trøndelag), while ITS1 sequence types D and E were found in three locations, on Svalbard and Oslofjorden. ITS1 sequence type F was only found on Svalbard, in one individual at Vestpynten and two individuals at Dicksonfjorden, and is therefore private for Svalbard. And ITS1 sequence type B was only found in two individuals at Vikso and one individual at Jæren location. After A ITS1 sequence type was D the most common, it was found on Svalbard and Jomfruland, and was on these two locations more common than ITS1 sequence type A. ITS1 sequence type C was also commonly found on tree localities (Berlevåg and Møre og Romsdal; both Erkna and Finnes). Thus there were few private ITS1 sequence types in the analysed populations and a shallow MSN of the analysed individuals.

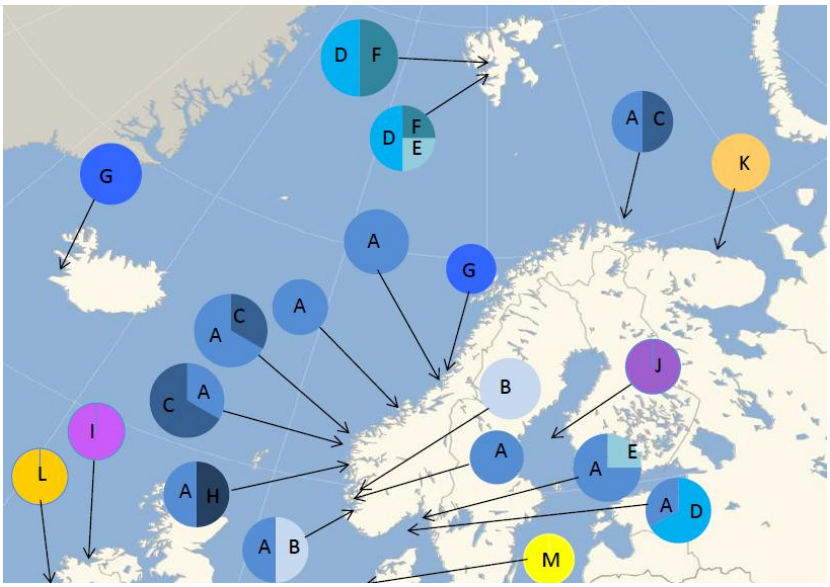

Figure 3.5: Map of geographical origin of ITS1 sequence types of *P. rubens* found in this study as well as others (van Oppen *et al* 1995, Goff *et al* 1997. The letters corresponds to the ITS1 sequence types designation shown also in the MSN. The diagrams show the different found ITS1 sequence types frequencies at different locations. The larger the pie chart the more sequences was obtained from that location.

Available ITS1 sequences from the literature (van Oppen *et al* 1995, Goff *et al* 1997) were included in the ITS1 sequence types network (Figure 3.4). From the MSN it can be seen that the *P. rubens* sequence form Sweden (haplotype J) was very different from the collected *P. rubens* in this study, while the *P. rubens* from Ireland (haplotype I) only differed in 10 nucleotides. Material of *P. rubens* from Ireland and Sweden also shared two nucleotide changes that were unique for these two ITS1 sequence types, illustrated in the figure with the star (Figure 3.4). The Atlantic East sequence from Achill Island (Ireland) and Norris point 1 (ITS1 sequence type L) identified by van Oppen *et al* (1995) only differed with one nucleotide substitution from ITS1 sequence type A, making them part of the same lineage. Also locations Russia and France from the East Atlantic lineage (ITS1 sequence type K) of van Oppen *et al* (1995) were not very different from ITS1 sequence type A, only tree nucleotides. This means that the ITS1 sequence types found in this study (A-G), by Goff *et al* (1997), the sequence from Ireland (ITS1 sequence type I) and the East Atlantic lineage sequences identified by van Oppen *et al* (1995; sequence types K and L, Figure 3.4) had very little differences between them. There was a lot of difference between the East Atlantic lineage and the Baltic sequences identified by van Oppen *et al* (1995; ITS1 sequence type M and N). The East Atlantic lineage was also different from the analysed West Atlantic populations identified van Oppen *et al* (1995; ITS1 sequence type O) and *P. riggi* identified by van Oppen *et al* (1995; ITS1 sequence type P), not shown in Figure 3.5. The differences

indicate that the ITS sequences from populations in the West Atlantic identified by van Oppen *et al* (1995) belong to a different lineage.

Microsatellite markers:

All the loci analysed were trinucleotide repeats. Between 9-23 individuals from each population were scored for two to five of the microsatellite loci. Locus 31074 was homozygote among the 10 samples tested. Loci 7734 and 8540 were heterozygote, both having three genotypes in the analysed material (the heterozygote and both homozygotes). Locus 4846 was also heterozygote, but had four genotypes. While for locus 8003 only few individuals (12) produced readable peaks, this locus was therefore excluded from further analysis.

Based on the number of genotypes found in the different populations (appendix) the frequencies of genotypes for the different loci and populations were calculated (Table 3.1, Figure 3.6). The genotypes were named according to the alleles present, and although it is uncertain how many alleles a homozygote individual had, they were named e.g. 114114 if only allele 114 of locus 7734 was observed. For the locus 7734, the genotypes 114114 and 114117 were completely absent from Vikso, while the genotypes were uncommon in both Bukken and Dicksonfjorden populations. While the Dicksonfjorden and Vestpynten had all three genotypes identified in locus 7734, Vestpynten was the only location where genotype 114114 was more frequent than genotypes 114117 and 117117. For locus 8540 there were no usable genotype sequences for the Bukken population, and only one individual from Dicksonfjorden that worked, so these two populations were not analysed for the 8540 locus. For the other populations, Vikso and Vestpynten, there was a clear difference in locus 8540 genotype frequencies. The Vikso population had only one of the three genotypes; all 18 individuals had only the 214214 genotype. While the Vestpynten population had all the 214214, 214233 and 223233 genotypes; the 233233 genotype had the highest frequency, while 214233 genotype had low frequencies (found in three individual) and the 214214 genotype was almost absent (only found in one individual). The 4846 locus had a genotype 216219 that were only found in one individual at one location (Bukken). The 219219 genotype were also found at its highest frequency at Bukken and had low frequency at Vikso, but was absent from Svalbard. While the genotype 228231 was the most frequent genotype for the populations from Vikso, Dicksonfjorden and Vestpynten, and genotype 228228 was found at low frequencies in all populations. Genotype 228231 was the most common genotype in all populations except Bukken.

Table 3.1: Genotype frequencies for the four analysed loci calculated for each population. Question mark indicates missing values.

	31074 Genotypes	7734 Genotypes			8540 Genotypes			4846 Genotypes			
Population	194	114114	114117	117117	214214	214233	233233	216219	219219	228228	228231
Bukken	?	0,16	0,16	0,68	?	?	?	0,05	0,45	0,15	0,35
Vikso	1,00	0,00	0,00	1,00	1,00	0,00	0,00	0,00	0,13	0,22	0,65
Dicksonfjorden	?	0,27	0,14	0,59	?	?	?	0,00	0,00	0,20	0,80
Vestpynten	1,00	0,48	0,19	0,33	0,07	0,21	0,71	0,00	0,00	0,11	0,89

The geographical distribution of the genotype frequencies for the analysed loci was shown in Figure 3.6. There were little clear differences between regions, but there were differences between the populations. For the 4846 locus Vestpynten and Dicksonfjorden were quite similar, showing highest frequencies of genotype 228231. While Vikso and Bukken showed some differences in genotype frequencies, the 219219 genotype was completely absent from Svalbard, but it is found in Vikso and is the most common genotype at Bukken. For the 7734 locus the difference between Svalbard and Bergen was not as clear, both Bukken and Dicksonfjorden had highest frequency of the 117117 genotype, while in Vestpynten and Vikso the highest frequency was of genotype 114114, for Vikso it was the only genotype. For the 8540 locus there was only data from two of the localities. But there was a clear difference in genotype frequency between Vestpynten and Vikso, were the 214214 genotype was not found in the Vikso population. Overall there are differences in genotype frequencies between Svalbard and Bergen, but also between the two populations at each Bergen and Svalbard.

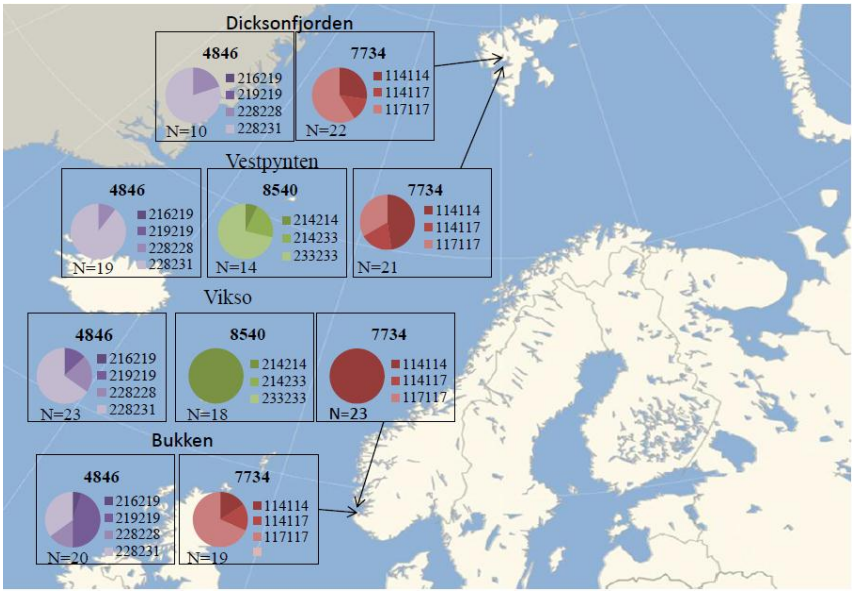

Figure 3.6: Genotype frequencies from Table 3.1 represented as pie charts. The genotype frequencies were plotted for each locus, where 7734 is red, 8540 is green and 4846 is purple, for the four populations Dicksonfjorden, Vestpynten, Vikso and Bukken. N represents the number of individuals analysed for the loci at that location.

Discussion:

Methodology:

The reason for the problems of getting usable sequence data as well as microsatellite amplifications for *P. rubens* is not entirely understood. Most of the extracted DNA was apparently in good condition, although the problems were most likely due to PCR inhibitors being co-extracted with the DNA. Both the dilute DNA template used and the improved results after DNA cleanup support this suggestion. Denatured DNA and extraction byproducts are known for causing problems when amplifying it in a PCR (Hoarau *et al* 2007b) Since microsatellites loci represent short DNA fragments they are usually possible to amplify even from somewhat denatured DNA (Selkoe and Toonen 2006). Problems with the extracted DNA are why the sample size for both DNA sequence data and microsatellite data set are smaller than the initial plan.

Direct PCR of both the cox and ITS1 regions would sometimes give double bands. The extra bands were shorter than the expected length of ITS1, about 350-400 bp. It is hard to say what the extra bands are; most likely it is because the primers are not designed for *P. rubens* and were therefore not always amplifying the whole gene. It's likely that this problem is also because of sample conservation, PCR inhibitors and the extraction methods of the DNA. Saunders and Moore (2013) also reported problems with the R1S primers for amplifying ITS1, and there solution was to modify the primer to make it more universal. So this might also be a problem with regards to the *P. rubens* when using this ITS primer. The rubisco marker did not give usable results for *P.rubens,* it would have needed more PCR optimization or primer modification.

When methods interpreting sequence and microsatellite data there may also potentially be problems to be aware of, for instance since the scoring and alignments were checked by hand, which may introduce biases and error. All methods used will have problems and pitfalls that are hard to avoid, and it is important to remember this when interpretation the results. Homoplasy could be a problem, and it is when two sequences have the same nucleotide in a position due to chance, and not by common ancestry can occur (San Mauro *et al* 2009). With the methods used it is impossible to know if this is the case. However for populations with relative short history homoplasy is in general often a minimal source of bias, this is because; homoplasy is proportional to the genetic distance between individuals or populations (Selkoe

and Toonen 2006). When using microsatellites it is hard to say if low levels of detectable polymorphisms reflect limited genetic diversity, or if this comes from methodological limitations (Reush *et al* 2000). The methods assessing allele peaks are not perfectly exact, meaning that the number of genotypes could be underestimated. Also in microsatellite analyses occurrence of homoplasy can be a problem, observed similarity can be from convergence, parallelism and reversal, and not common ancestry (Selkoe and Toonen 2006).

Postglacial colonization of Norway and Svalbard:

Two European lineages of *P. rubens* were identified by van Oppen *et al* (1995). The East Atlantic lineage was found in France, Ireland and the Barents Sea, and the Baltic lineage was found in Denmark (Kolding) and Germany (Kiel and Helgoland). All the ITS1 sequences obtained in this study belonged to the East Atlantic lineage of van Oppen *et al* (1995), and their distribution fits well with the East Atlantic lineage. In the study by van Oppen *et al* (1995) it was concluded that the East Atlantic region was colonized from an Atlantic gene pool. Since the sequences obtained in this study belong to the same lineage, Norway and Svalbard were probably also recolonized after the Pleistocene glaciations from the same Atlantic gene pool. In general, the cox2-3 spacer and ITS1 data showed similar genetic patterns, with mostly closely related haplotypes/sequence type occurring in Svalbard and Norway, suggesting that the sites were recolonized from the same gene pool. Although Svalbard possibly could have been colonized from elsewhere than the Atlantic there is no evidence for this seen in the sequence data, and there is no evidence of ITS1 sequence types in Svalbard or Norway resulting from the second trans-Atlantic dispersal event suggested by van Oppen *et al* (1995). However, Svalbard did have a private ITS1 sequence type (F) and for the cox2-3 spacer there was two haplotypes (I and H) only found in Svalbard and Northern Norway. This could indicate that there is currently little genetic exchange between Svalbard and Northern Norwegian locations of *P. rubens* and more southern Norwegian locations, or genetic group sorting during the northward migration leading to fixation of different haplotypes/sequence type in different populations. The most likely reasons for this are isolation of populations after the postglacial re-colonization. The sharing of ITS1 sequence types between populations in Svalbard and Oslofjorden is probably a result of re-colonization from the same Atlantic gene pool; since the Pleistocene glaciation is not so long ago evolutionary speaking. The ITS sequence obtained from *P. rubens* in Sweden by Goff *et al* (1997) this sequence was very different (around 10%) from the other studied individuals (Figure 3.5). A large genetic difference between populations is often because of geographic

isolation, and the North Atlantic glacial refugia are believed to have isolated populations for a long period (Maggs *et al* 2008). Separation from the rest of the East Atlantic lineage during the last glaciation could explain why the individual from Sweden analysed by Goff *et al* (1997) was different from all the other ITS1 sequence types.

Phylogeographical studies on other macroalgae have also shown patterns of range-wide genetic diversity mostly explained by long-term processes such as postglacial survival and re-colonization (Provan *et al* 2013). Genetic analyses of *Palmaria palmata* by Provan *et al* (2005) indicated that the species survived in a refugium in the English Channel with possible secondary refugia in Ireland, North America and Iceland. Refugial areas are often characterized by higher levels of nucleotide diversity, which was observed in the genetic analyses of *P.palmata*. The *P. palmata* alga is an arctic-cold temperate species, and is today very common on Svalbard (Provan *et al* 2005). Since *P. palmata* and *P. rubens* grow under similar conditions it is reasonable to assume that if *P. palmata* could survive in the English Channel during the LGM this is a possible refugium also for *P. rubens.* In line with the results of the present study a recent population expansion was suggested also for *P. palmata* (Provan *et al* 2005). For *Fucus* spp. the English Channel is also a possible refugium, and Coyer *et al* (2011) found based on microsatellite markers for *F. spiralis* patterns that suggest a refugium in the Hurd Deep area, and patterns of low haplotype diversity throughout the North Atlantic, pointing to a recent colonization event. Coyer *et al* (2003) hypothesized the occurrence of refugia in the English Channel and the Iberian Peninsula based on microsatellites variation in *F. serratus.* This study also showed no loss of genetic variation for more northern locations (Novea Scotia), which would be expected if there was a founder effect or a bottleneck during the re-colonization (Coyer *et al* 2003). A similar pattern was found for *P. rubens* in the present study, there was no evidence for a founder effect or bottleneck happening while *P. rubens* migrated northwards. In addition Hoarau *et al* (2007a) studied the phylogeography of *F. serratus* further and found, however, a different pattern than previously (Coyer *et al* 2003). Based on mtDNA markers Hoarau *et al* (2007a) identified the South-West Ireland refugium as being important for postglacial re-colonization, based on that the Ireland genetic signature was present in all Northern European locations. The English Channel refugium was less important for postglacial expansion, and the Iberian refugium not important at all (Hoarau *et al* 2007a). A phylogeographic study of *Ceramium tenuicorne* hypothesized based on mtDNA haplotypes that *C.tenuicorne* in the Baltic region probably had colonized twice from a diverse Atlantic gene pool (Gabrielsen *et al* 2002). Based on my data I also suggest that *P. rubens*

colonized Northern Europe from a diverse eastern Atlantic gene pool. However in this study there was no evidence that there was a second colonization for *P. rubens*, Norway and Svalbard were most likely colonized once. Marine phylogeographic patterns in the North Atlantic are complex. There is however, often consistency in regards of refugia (Hoarau *et al* (2007a). The literature of phylogeographic studies on macroalgae shows that post-glacial patterns of genetic variation based on molecular tools can be difficult to interpret. However the overall studies all point to macroalgae surviving in the southern range in ice free refugia, and post-glacial re-colonizing from one or more refugia.

Several glacial refugia have been identified in the North Atlantic and Mediterranean Sea (Maggs *et al* 2008). Based on the ITS sequence MSN (Figure 3.4) there is a possibility that *P. rubens* survived in one of the refugia during the glaciated periods. Since the ITS sequence types and cox2-3 spacer haplotypes showed one lineage in the data and no apparent loss of genetic variation, it is likely that all the sequences obtained came from one large refugium. Northern Norway (Coyer *et al* 2011) has been suggested as a refugium, as well as Iceland and Faroe Island area (Wares 2001, Roman and Palumbi 2005). There is no evidence in my data to suggest that *P. rubens* survived in refugia in Iceland or Northern Norway. If so, then it would be expected that populations from Svalbard, Northern Norway and Iceland would have more genetic differentiated from the rest of the locations. Maggs *et al* (2008) reviewed some other refugia that could have been survival areas for *P. rubens.* The Iberian Peninsula was shown too been refugia for brown algae (Hoarau *et al* 2007a). Also the English Channel and South-West Ireland is believed to have been refugia for red and brown algae based on genetic evidence (Provan *et al* 2005, Hoarau *et al* 2007a). The most likely refugia for *P.rubens* in the Atlantic based on other macroalgae studies are the English Channel, Ireland or the Iberian Peninsula It is possible that any of these three areas could have been the southern overwintering area for *P. rubens.* The locations of the refugia suggested make it likely that *P. rubens* could migrate with the currents from the refugial area up the Norwegian coast and to Svalbard. More sequence data from the possible refugia would be necessary to study what refugium *P. rubens* survived in.

Interpopulation differentiation of *Phycodrys rubens* in the North Atlantic:

Based on the seven ITS1 sequence types found in the 36 individuals analysed, and with a genetic variation among the sequences of around 0.1%, there is little ITS1 sequence variation in the Norwegian and Svalbard region. Van Oppen *et al* (1995) found a 7% genetic divergence between different lineages of *P. rubens*, this was however over a large

geographical area, within the East Atlantic lineage the genetic variation within the ITS1 and ITS2 was 0.6%. A study on *Corallinales* spp. by Hind and Saunders (2013) showed for ITS a 0% to 0.6% genetic divergence within species. This point to that the variation found within *P. rubens* is as expected, both for expected levels for a red alga as well as expected for the species.

The MSN of the nrDNA ITS1 sequences (Figure 3.4) showed one very common (14 individuals) ITS1 sequence type(A) that had several connected ITS1 sequence types (B-G), this and that ITS1 sequence type A was the most common indicates that this is the basal ITS1 sequence type. The little variation between ITS1 sequence types indicates that they all have the same and fairly recent origin. A less likely explanation involves current genetic exchange between populations; *P. rubens* individuals or thallus could theoretically be transported between populations. ITS1 sequence type A was found in every location, except Bergen, Horta, Iceland and Svalbard. For the Horta location the lack of A could be because of lacking data, there is only one ITS1 sequence from that location in the data set. The Vikso population had two individuals with ITS1 sequence type B, this sequence type was only found in two locations in Western Norway, this is probably also due to the limited data available. It is interesting that the ITS1 sequence type A does not occur on Svalbard, although this could also be because of the few sequence obtained. ITS1 sequence type F is private for Svalbard, and private ITS1 sequence types often indicate genetic isolation. So this supports that there might be low genetic exchange between Svalbard and Norway, this limitation of genetic exchange could be isolation after the re-colonization. Limited sample size might be why ITS1 sequence type F was not found in the Norwegian locations. ITS1 sequence type D and E were also found on the east coast of Norway and Svalbard, but not between, this indicates that the ITS1 sequence types have the same origin and that the genetic variation comes from a diverse Atlantic gene pool. It is far less likely that Svalbard and Oslofjorden populations have had genetic exchange in the current population locations. The otherwise lack of geographical isolation of ITS1 sequence types indicates recent genetic exchange between populations. Genetic exchange is however difficult to time, indication of genetic exchange could date back to when the populations could have been in joint location further south. Comparing to other ITS1 sequences gives a broader perspective of how the Svalbard and Norwegian genetic variation fits with the genetic patterns of *Phycodrys rubens* from other studies.

The ITS sequence from Ireland identified by Goff *et al* (1997) were quite similar to ITS1 sequence type A, meaning it belongs to the same lineage and probably have the same origin.

Their ITS sequence from Sweden, however, neither belonged clearly to the East Atlantic nor the Baltic lineage of van Oppen *et al* (1995). This could be because of geographical isolation of the Swedish coast. Since much of the Swedish coastline is somewhat geographically isolated, and it is not known exactly where the sequenced material was collected. ITS1 sequence type L from Ireland and Norris Point (Canada) identified by van Oppen *et al* (1995), also belonged to the East Atlantic lineage, van Oppen *et al* (1995) explains the Norris Point sequence with shipping traffic, this seem reasonable since marine species can spread with ships, for example by growing on hulls. ITS1 sequence type J of Goff *et al* (1996) from Sweden had some common variation with van Oppen *et al* (1995) East Atlantic and the Baltic genetic type (ITS1 sequence type K, M-O); this might indicate a common ancestry, even though the sequence from Sweden was very different from anything else in the alignment. The West Atlantic lineage (ITS1 sequence type O) and *P.riggi* (ITS1 sequence type P) from van Oppen *et al* (1995) was very different (around 5%) from any of the other sequences obtained in this study, this indicates that there are no sequences from Svalbard or Bergen that belong to or have variation in common with the Baltic or West Atlantic lineages of van Oppen *et al* (1995) Atlantic lineage (Baltic). The alignment and MSN of van Oppen *et al* (1995) sequences in this study are different from the ones in the paper itself, this is because in this study only ITS1 was considered and not also ITS2 van as Oppen *et al* (1995) did.

The cox2-3 sequences showed a more complex network then the ITS1 data. Based on the sequences it was identified one lineage, and 10 distinct haplotypes for 19 individuals. The genetic variation shows that cox2-3 spacer is highly variable for *P. rubens*. There was many shared nucleotide changes in the haplotype groups. The nucleotide changes that are shared by many of the haplotypes could indicate recent exchange and a common origin, most likely from the Pleistocene ice age. However, shared nucleotides could also be because of homoplasy, for instance cox2-3 spacer haplotype D also shared one nucleotide change with the E-G cox2-3 haplotype group, but since this is the only one and there is no other evidence that these haplotypes have other variation in common, this is most likely homoplasy. Many localities have private haplotypes and this could indicate population isolations, however since the dataset is limited it is impossible to say if the haplotypes really are private or if is because of low sampling size. Haplotype A was the basal haplotype and the most common; it was found on Svalbard and Iceland in many of the sequenced individuals, this indicates a current low genetic exchange between the Svalbard and Iceland area and the mainland of Norway. This fits with the ITS1 data, were ITS1 sequence type A was not found on Svalbard and

Iceland. This suggestion of little genetic exchange is supported by none of the haplotypes found in Western or Eastern parts of Norway were found on Svalbard. Haplotypes A, I and H were only found on Iceland, Svalbard and Northern Norway. Based on cox2-3 and ITS1 data there is some genetic difference between Svalbard and Norway, with could indicate populations isolation after the re-colonization.

Population structure of *P. rubens* in Svalbard and Bergen.

Based on the microsatellites there is different population structure found in the four populations, for instance in differences in genotype frequencies (Figure 3.6). The genotype frequency varied between genotypes for each locality. Since the dataset is small there is limited conclusions that can be made, but the data available did show some populations structure (Figure 3.6). For Vikso there were few genotypes found in comparison to the other sites, two of the loci scored for this location were homozygote in the population, meaning Vikso was less variable than the other populations in the study. Bukken had one genotype (116119) in the 4846 locus only found in this location, also indicating differences between sites. The genotype 116119 was however only found in one individual, so based on this dataset it is impossible to say how common this genotype is, is could have also be a scoring error. For the 8540 locus Vestpynten also had two genotypes (214214 and 214233) not found on Vikso, there is however no information found on this locus from Bukken or Hotelneset. So it is impossible to say what pattern would be seen if there was a larger data set on this locus, including the other two populations as well as more individuals. There were also differences in genotype frequencies between populations at Svalbard and Bergen (Table 3.1), for instance there was two genotypes only found in Bergen. For the 7734 one of the genotypes 114114 was more common on Vestpynten then any of the other sites, this illustrates that there were also differences between the populations on Svalbard. These results demonstrate that there are differences between populations of *P. rubens* in population structure.

Microsatellite studies on other algae have found complex patterns between populations. For instance a study of *C. crispus* showed that geographically close populations were more genetically similar then those spatially separated (Provan *et al* 2013). This could be because of the limited dispersal potential of red algae associated with various life-stages (Provan *et al* 2013). In this study it was also hypothesized that there was differences between populations from Svalbard and Bergen; this is most likely because of geographical distance, because of the limitation in dispersal could be who the two populations in Bergen and Svalbard were also different from each other. A further study on *C.crispus* (Krueger-Hadfield *et al* 2011) also

showed genetic differences between populations in Northern Europe and suggesting significant inbreeding. However the article highlights the need for more testing of spatial separation on different scale, to be able to conclude about this using microsatellite (Krueger-Hadfield *et al* 2011). Microsatellites have also been used in *Lessonia nigrescens* in a study by Martinez *et al* (2005) where they found large genetic differences in populations in Northern Chile when comparing these with populations in the southern locations, as well as a lack of heterozygosity on the Northern locations. They hypothesize that this could be because of mass mortality due to historical weather events (Martinez *et al* 2005). It is possible that extreme events could shape the population structure also of *P. rubens,* but this is very hard to investigate. Overall it does seem like differences in population structure like I found in my data is not unusual for macroalgae. The theory based on microsatellite genotype frequency that there are differences between populations, both between Svalbard and Bergen but also within these main sites fits with the hypothesize based on sequence ITS1 and cox2-3 data, that states that Svalbard seem to be currently genetic isolated from the more southern parts of mainland Norway.

Usefulness of the different genetic markers used *for P. rubens*:

Since different markers can show different levels of variation and different genetic patterns combining two or more markers could better reveal genetic structure of the species studied (Provan *et al* 2005). Studies on the usefulness of the cox2-3 marker for red algae have shown that the marker can be very variable even within populations, for instance for *Caloglossa leprieurii* four haplotypes were found within one population (10m radius sampling site) (Zuccarello *et al* 1999). This means that the cox2-3 spacer can pick up genetic variation well. ITS1 is also believed to be a useful marker in species discrimination and in some cases useful at the population level (Saunder and Moore 2013). Different markers sometimes find different patterns, so it is possible that there are genetic differences that is found in cox2-3 spacer, does not show up in ITS1 sequences. Based on this it is probable that the cox2-3 spacer in this study found more variable haplotypes in the same data set that ITS1 found less variable haplotypes, and looking at them together might give a clearer picture of the genetic variation of *P. rubens*.

Marker variability has to be considered when assessing genetic patterns based on molecular work. Considering the data set there are more sequence data for ITS1 then there is for Cox2-3, therefor ITS haplotypes also have more certainties, since there was several ITS1 sequences for each haplotype. Some individuals were sequenced for both markers (Table 3.1). Based on

this the markers do show differences between individuals. For instance in the Dicksonfjorden location, where four individuals gave the same haplotype for cox2-3 spacer, but had two different ITS1 sequence types. Another example is Nordøyan here cox2-3 spacer finds two haplotypes in two individuals; based on ITS1 the same two individuals have the same haplotype. This might indicate that the markers within the species have different variability between individuals, some individuals have nucleotide variation in the cox2-3 spacer and not in ITS1, and for other individuals it is the other way around. The two markers therefore have given different patterns for the same sequence data; this illustrated the importance of using several markers to investigate genetic variation.

For the microsatellite markers the results varied, and therefor also the usefulness of the markers. Based on the results the 4846, 7734 and 8540 loci are good microsatellite markers to use in populations based studies of *P. rubens.* The 31074 locus did not give usable results in this study, because for the material tested it was homozygote, however it might be heterozygote for other populations of *P. rubens* are closely related species. Meaning that for other studies of *P. rubens* studying other populations it might be usable. Since optimization did give better results for the microsatellite loci used, it is possible that with more optimization the other loci will work as well. Microsatellites can have high mutations rates this means that loci must be selected based on the questions in interest, low mutations rate means that signatures of important events will last longer, while the loci with higher mutations rate will be more informative (Selkoe and Toonen 2006). So also when it comes to microsatellite loci it is important to keep on mind marker variability when selecting markers and when analyzing data.

Conclusions:

Red algae DNA can be very difficult to work with; the methods needed much optimization to give usable results. The cox2-3 spacer and ITS1 are useful markers for interspecific studies on *P. rubens*. However since the cox2-3 spacer and ITS1 showed partly different patterns, combining several markers can be good. While the cox2-3 spacer showed several private haplotypes, ITS1 had one dominant basal ITS1 sequence type and Svalbard had a privateITS1 sequence type. There is little geographic structure in the data, this indicates recent genetic exchange between the populations, and this could be during the last glacial maximum. However there was some geographical differences that could indicate that Svalbard is

currently isolated from the Norwegian mainland. The ITS1 sequence data showed that populations of *P. rubens* from Svalbard and Norway have the same Atlantic origin as the East Atlantic lineage identified by van Oppen *et al* (1995). *Phycodrys rubens* most likely survived the LGM in refugia in the East Atlantic, and migrated northwards once an re-colonized Norway, Iceland and Svalbard. Five of the microsatellite loci gave usable results for *P. rubens*, and could therefore potentially be used in other studies of *Phycodrys* species. The microsatellite data set is small, and it is therefore limited what conclusion can be drawn from it. There seems like there is population structure among the analysed populations of *P. rubens*, and this is something that can be studied further.

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References:

Altschul S.F, Schuler G.D and Lipman D.J (1991) A workbench for multiple alignment construction and analysis. *Proteins: Structure, Function, and Bioinformatics, 9(3), 180-190.*

Bennet K.D and Provan J (2008). What do we mean by 'refugia'? *Quaternary Science Reviews 27, 2449–2455.*

Coyer J.A , Hoarau B, Costa J.F, Hogerdijk B, Serrão E.A, Billard E, Valero M, Pearson G.A, Olsen J.L (2011) Evolution and diversification within the intertidal brown macroalgae *Fucus spiralis/F. vesiculosus* species complex in the North Atlantic. *Molecular Phylogenetics and Evolution, 58, 283-296.*

Coyer J.A , Hoarau G, Van Schaik J, Luijckx P and Olsen J.L (2010) Trans-Pacific and trans-Arctic pathways of the intertidal macroalga Fucus distichus L. reveal multiple glacial refugia and colonizations from the North Pacific to the North Atlantic. *Journal of Biogeography (J. Biogeogr.) 38, 756–771.*

Coyer, J.A, Hoarau G, Stam W.T, Olsen J.L (2003) Post-ice age recolonization and differentiation of Fucus serratus L. (Phaeophyceae; Fucaceae) populations in Northern Europe. *Molecular Ecology, 12, 1817–1829.*

Hewitt G.M (1996). Some genetic consequences of ice ages, and their role, in divergence and speciation. *Biological Journal of the Linnean Society, 58: 247-276.*

Hind K.R and Saunders G.W (2013) Molecular markers from three organellar genomes unravel complex taxonomic relationships within the coralline algal genus *Chiharaea* (*Corallinales*, Rhodophyta). *Molecular Phylogenetics and Evolution, 67, 520-540.*

Hoarau G, Coyer J. A, Veldsink J. H, Stam W. T, and Olsen J. L (2007a) Glacial refugia and re-colonization pathways in the brown seaweed *Fucus serratus. Molecular Ecology, 16, 3606–3616.*

Hoarau G, Coyer J. A, Wytze T, Stam W. T, and Olsen J. L (2007b) A fast and inexpensive DNA extraction/purification protocol for brown macroalgae. *Molecular Ecology Notes, 7, 191.*

Gabrielsen T.M, Brockmann C and Rueness J (2002) The Baltic Sea as a model system for studying postglacial colonization and ecological differentiation, exemplified by the red alga *Ceramium tenuicorne. Molecular ecology, 11(10), 2083-2095.*

Goff L.J, Ashen J and Moon D (1997). The evolution of parasites from their host: A case study in the parasitic red algae. *Evolution, 51(4), 1068-1078.*

Kennett JP (1982) Marine Geology. *Prentice-Hall, Englewood, Cliffs, New Jersey.*

Knowlten N (2000) Molecular genetic analyses of species boundaries in the Sea. *Hydrobiologia 420, 73-90.*

Krueger-Hadfield S.A, Collèn J, Daguin-Thièbaut C and Valero M (2011) Genetic population structure and mating system in *Chondrus crispus* (Rhodophyta). *Journal of Phycolology, 47, 440–450.*

Lin S.M and Nelson W.A (2009) Systematic revision of the genus Phycodrys (Delesseriaceae, Rhodophyta) from New Zealand, with the descriptions of three new species, *P.novae-zelandiae* sp. nov., *P. franiae* sp. nov. and *P.adamsiae* sp. Nov. *European Journal of Phycology 45(2), 200–214.*

Lawver, L.A, Muèller, R.D, Srivastava, S.P and Roest W (1990) The opening of the Arctic Ocean. Geological history of the polar oceans: Arctic vs. Antarctic. *Kluwer Academic Publishers, The Netherlands.*

Lindstrom S.C (2002) The Bering Strait connection: dispersal and speciation in boreal macroalgae. *Journal of Biogeography, 28, 243-251.*

Maggs C.A, Castilho R, Folts D, Henzler C, Jolly M.T, Kelly J, Olsen J, Perez K.P, Stam W, Rista V, Viard F and Wares J (2008) Evaluating signatures of glacial refugia for North Atlantic benthic marine taxa. *Ecology 89(11), 108-122.*

Martinez E.A, Càrdenas L, Figueroa C, Vidal R.O and Billot C (2005) Microsatellites of *Laminaria digitata* tested in *Lessonia nigrescens*: Evaluation and improvement of cross amplification between kelps of two different families. *Journal of Applied Phycology, 17, 245–253.*

Pielou, E. C (1991) After the Ice Age: the return of life to glaciated North America. *University of Chicago Press, Chicago, Illinois, USA.*

Provan J, Glendinning K, Kelly R and Maggs C.A (2013) Levels and patterns of population genetic diversity in the red seaweed Chondrus crispus (Florideophyceae): a direct comparison of single nucleotide polymorphisms and microsatellites. *Biological Journal of the Linnean Society, 2013, 108, 251–262.*

Provan J, Wattier R.A, Maggs C.A (2005) Phylogeographic analysis of the red seaweed Palmaria palmate reveals a Pleistocene marine glacial refugium in the English Channel. *Molecular Ecology 14, 793–803.*

Rasmussen T.L, Forwick M and Mackensen A (2012) Reconstruction of inflow of Atlantic Water to Isfjorden, Svalbard during the Holocene: Correlation to climate and seasonality. *Marine Micropaleontology, 94-95, 80-90.*

Roman J and Palumbi S.R (2005) A global invader at home: population structure of the green crab, *Carcinus maenas*, in Europe. *Molecular Ecology, 13, 2891–2898.*

Rueness J (1998) Alger i Farger. *Oslo, Almater Forlag.*

San Mauro D and Agorreta A (2009), Molecular systematics: a synthesis of the common methods and the state of knowledge. *Cellular & Molecular Biology Letters 15, 311-341.*

Saunders G.W and Moore T.E (2013) Refinements for the amplification and sequencing of red algal DNA barcode and RedToL phylogenetic markers: a summary of current primers, profiles and strategies. *Algae, 28(1), 31-43.*

Selkoe K.A and Toonen R.J (2006) Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters, 9, 615–629.*

Selivanova O.N and Zhigadlova G.G (2002) *Phycodrys valentinae* sp. nov. (Delesseriaceae, Rhodophyta) with Discussion of Other Species of the Phycodrys Genus from the North Pacific. *Russian Journal of Marine Biology, 29(4), 206–215.*

Schoschina E.V. (1996) Seasonal and age dynamics of growth and reproduction of *Phycodrys rubens* (Rhodophyta) in the Barents and White Sea. *Aquatic Botany, 55, 13-30.*

Taberlet P (1998) Biodiversity at the intraspecific level: the comparative phylogeographic approach. *Journal of Biotechnology, 64, 91–100.*

van Oppen M.J.H, Draisma S.G.A Olsen J.L and Stam W.T (1995) Multiple trans-Arctic passage in the red alga *Phycodrys rubens*: evidence from nuclear rDNA and ITS sequences. *Marine Biology, 123, 179-188.*

Vik U, Halvorsen R, Ohlson M, Rydgren K, Carlsen T, Korpelainen H and Kauserud H (2012) Microsatellite markers for *Hylocomium Splendens* (*Hylocomiaceae*). *American Journal of Botany, 344–346*

Wares J. P (2001) Intraspecific variation and geographic isolation in *Idotea balthica* (Isopoda:Valvifera). *Journal of Crustacean Biology 21, 1007–1013*

Wattier R, Dallas J.F, Destombe C, Saumitou-Laprade P and Valero M (1997) Single locus microsatellites in *Gracilariales* (Rhodophyta); High level of genetic variability within *Gracilaria Gracilis* and conservation in related species. *Journal of Phycology, 33, 868-880.*

Whittick A (1983) Spatial and Temporal Distribution of Dominant Epiphytes on the stipes of *Laminaria hyperborea* (Gunn.) Fosl. (Phaeophyta: Laminariales) in S.E Scotland. *Journal of Experimental Marine Biology and Ecology, 73, 1-10.*

Zuccarello G. C, Burger G, West J. A and King R. J (1999). A mitochondrial marker for red algal intraspecific relationships. *Molecular Ecology, 8, 1443–1447.*

Appendix

Appendix 1: Overview of samples used in sequencing and Microsatellite analyses, and assigned genotypes.

					PCR product	Phylotypes			Msats ind nr
Sample code	Locality	Region	Sample date	DNA extraction	Cox	ITS1	Cox	ITS	
DF21	Dicksenfjorden (Isfjorden)	Longyearbyen, Svalbard	06.08.2012	11.06.2013	271, 1	291, 1	A	F	D-14
DF22	Dicksenfjorden (Isfjorden)	Longyearbyen, Svalbard	06.08.2012	11.06.2013	271, 2	297, 5	A	F	D-15
DF23	Dicksenfjorden (Isfjorden)	Longyearbyen, Svalbard	06.08.2012	11.06.2013	271, 3	35.1	A	D	D-16
DF24	Dicksenfjorden (Isfjorden)	Longyearbyen, Svalbard	06.08.2012	11.06.2013	271, 4	35.2	A	D	D-17
HN36	Hotelneset (Isfjorden)	Longyearbyen, Svalbard	05.08.2012	05.06.2013		297, 6		E	H-9
HN37	Hotelneset (Isfjorden)	Longyearbyen, Svalbard	05.08.2012	05.06.2013		318,5		D	H-10
HN31	Hotelneset (Isfjorden)	Longyearbyen, Svalbard	05.08.2012	11.06.2013	271, 5	35.3	H	D	H-12
HN32	Hotelneset (Isfjorden)	Longyearbyen, Svalbard	05.08.2012	11.06.2013	271, 6	35.4	A	F	H-13
HN33	Hotelneset (Isfjorden)	Longyearbyen, Svalbard	05.08.2012	11.06.2013	286, 2		I		H-14
BV23	Berlevåg	Berlevåg, Finnmark	21.08.2012	05.06.2013	286, 3	291,5	I	A	
BV17	Berlevåg	Berlevåg, Finnmark	22.08.2012	17.06.2013		339, 5		C	
HO3	Horta	Leka, Nord-Trøndelag	16.06.2011	20.08.2013		358, 1		G	
N2	Nordøyan	Vikna, Nord-Trøndelag	14.06.2011	20.08.2013	286, 5	320, 2	E	A	
N3	Nordøyan	Vikna, Nord-Trøndelag	14.06.2011	20.08.2013	325, 8	312, 2	D	A	
HI1	Hitra	Hitra, Sør-Trøndelag	16.06.2011	20.08.2013		292, 1		A	
F20	Møre og Romsdal	Ålesund, Møre og Romsdal	17.06.2012	17.06.2013		292, 3		C	
F21	Møre og Romsdal	Ålesund, Møre og Romsdal	16.06.2012	16.06.2013	299, 3	312, 7		A	
F22	Møre og Romsdal	Ålesund, Møre og Romsdal	17.06.2012	17.06.2013	299, 4	312, 8	J	C	
ER1	Erkna	Ørsta, Møre og Romsdal	10.06.2011	19.06.2013		292, 6		A	
ER2	Erkna	Ørsta, Møre og Romsdal	10.06.2011	19.06.2013		316, 3		C	
ER4	Erkna	Ørsta, Møre og Romsdal	10.06.2011	19.06.2013	326, 2	316, 4	G	A	
FR3	Frilsøyene	Fedje, Hordaland	06.06.2011	19.06.2013		323, 1		A	
FR4	Frilsøyene	Fedje, Hordaland	06.06.2011	19.06.2013	326, 3	316, 5	F	A	
E28	Espeland (Raunefjorden)	Bergen, Hordaland	06.06.2012	12.06.2013		318,6		B	-
E29	Espeland (Raunefjorden)	Bergen, Hordaland	06.06.2012	12.06.2013		323, 5		B	-
B22	Bukken (Raunefjorden)	Bergen, Hordaland	04.09.2012	12.06.2013	276, 3	318, 7	K	H	BUK-8
B23	Bukken (Raunefjorden)	Bergen, Hordaland	04.09.2012	12.06.2013	280, 1		B	A	BUK-9
B24	Bukken (Raunefjorden)	Bergen, Hordaland	04.09.2012	12.06.2013		35.19		A	-
J1	Jæren	Kragerø, Telemark	04.06.2011	19.06.2013		316, 7		A	
J2	Jæren	Kragerø, Telemark	04.06.2011	19.06.2013	306, 6		C		
J3	Jæren	Kragerø, Telemark	04.06.2011	19.06.2013		35.22		B	
JF4	Jomfruland	Rogaland	10.07.2011	20.08.2013		317,1		A	
JF5	Jomfruland	Rogaland	10.07.2011	20.08.2013		320, 4		D	
OF1	Ytre oslofjorden	Telemark	22.05.2012	15.08.2013		294, 2		A	
OF2	Ytre oslofjorden	Telemark	22.05.2012	15.08.2013	288, 4	317, 2	E	A	
OF3	Ytre oslofjorden	Telemark	22.05.2012	15.08.2013		35.25		E	
OF4	Ytre oslofjorden	Telemark	22.05.2012	15.08.2013		35.26		A	
R1	Reykjavik	Seltjarnarnes, Island	12.01.2013	24.08.2013	281, 7	320, 7	A	G	
R2	Reykjavik	Seltjarnarnes, Island	12.01.2013	24.08.2013	281, 8	324, 4	A	G	
R3	Reykjavik	Seltjarnarnes, Island	12.01.2013	24.08.2013		35.29			
R4	Reykjavik	Seltjarnarnes, Island	12.01.2013	24.08.2013		35.30			

Appendix 2: nrDNA ITS1 ITS1 sequence types observed in *P. rubens* based on the ITS1 regions. Nucleotide position refers to the sequences of ITS1 sequence type A.

Appendix 3: mtDNA haplotypes observed in *P. rubens* based on the Cox2-3 spacer regions. Nucleotide position refers to the sequences of haplotype A.

	Nucleotide position																		
Haplotype	461	467	485	509	554	557	582	601	615	663	684	693	709	722	723	724	739	814	817
A	A	T	A	T	A	C	A	G	T	A	T	T	A	A	A	A	T	G	A
B	G	G	.	.	.	T	G	A	C	.	.	A	.	.	.	.	C	.	.
C	G	G	.	.	.	T	G	A	C	.	.	A	.	T	T	T	C	C	T
D	.	.	C	C	.	T	G	.	C	.	.	A	.	.	.	.	.	.	.
E	.	.	.	C	.	T	.	.	.	.	.	A	.	.	.	.	.	.	.
F	.	.	.	C	.	T	.	.	.	T	.	A	.	.	.	.	.	.	.
G	.	.	.	C	.	T	.	.	.	.	.	A	G	.	.	.	.	.	.
H	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.
I	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.
J	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.

Appendix 4: Allele peaks observed for each individual for each locus. Where BUK is Bukken populations. ESP is Vikso, D is Dicksonfjorden and H is Vestpynten. The number code is the loci and allele, and 1 means the individual has this allele and 0 means is does not, and question mark indicates missing value.

Ind nr	antall	4846-216	4846-219	4846-228	4846-231	08540-214	08540-233	7734-114	7734-117	31074-194
BUK-1	1	0	1	0	0	?	?	0	1	?
BUK-2	2	0	1	0	0	?	?	0	1	?
BUK-3	3	0	0	1	0	?	?	1	1	?
BUK-4	4	0	1	0	0	?	?	0	1	?
BUK-5	5	0	0	1	1	?	?	0	1	?
BUK-6	6	0	0	1	0	?	?	1	1	?
BUK-7	7	0	1	0	0	?	?	0	1	?
BUK-8	8	0	0	1	1	?	?	1	1	?
BUK-9	9	0	1	0	0	?	?	?	?	?
BUK-10	10	1	1	0	0	?	?	?	?	?
BUK-11	11	0	1	0	0	?	?	0	1	?
BUK-12	12	?	?	?	?	?	?	0	1	?
BUK-13	13	0	0	1	1	?	?	1	0	?
BUK-14	14	0	0	1	0	?	?	1	0	?
BUK-15	15	0	1	0	0	?	?	0	1	?
BUK-16	16	0	0	1	1	?	?	0	1	?
BUK-17	17	0	0	1	1	?	?	1	0	?
BUK-18	18	0	0	1	1	?	?	0	1	?
BUK-19	19	0	1	0	0	?	?	0	1	?
BUK-20	20	0	0	1	1	?	?	0	1	?
BUK-21	21	0	1	0	0	?	?	0	1	?
ESP-1	1	0	0	1	1	1	0	0	1	1
ESP-2	2	0	0	1	0	1	0	0	1	?
ESP-3	3	0	0	1	1	1	0	0	1	?
ESP-4	4	0	0	1	1	1	0	0	1	?
ESP-5	5	0	0	1	1	1	0	0	1	?
ESP-6	6	0	0	1	1	1	0	0	1	?
ESP-7	7	?	?	?	?	?	?	0	1	?
ESP-8	8	0	0	1	1	?	?	0	1	?
ESP-9	9	0	0	1	1	1	0	0	1	?
ESP-11	10	?	?	?	?	1	0	0	1	?
ESP-12	11	0	0	1	0	0	?	0	1	?
ESP-13	12	0	0	1	1	1	0	0	1	?
ESP-14	13	0	0	1	1	1	0	0	1	?
ESP-15	14	0	0	1	1	1	0	0	1	?
ESP-16	15	0	1	0	0	?	?	0	1	?
ESP-17	16	0	0	1	1	1	0	0	1	?
ESP-18	17	0	0	1	1	1	0	0	1	?
ESP-19	18	0	0	1	1	1	0	0	1	?
ESP-20	19	0	0	1	1	1	0	0	1	?
ESP-21	20	0	0	1	0	1	0	0	1	?
ESP-22	21	?	?	?	?	1	0	0	1	?
ESP-23	22	0	0	1	0	1	0	0	1	?
ESP-24	23	0	0	1	0	?	?	?	?	?
ESP-25	24	0	0	1	1	?	?	?	?	1
ESP-26	25	0	1	0	0	?	?	?	?	?
ESP-27	26	0	1	0	0	?	?	?	?	?

Continue of Appendix 4:

Ind nr	antall	4846-216	4846-219	4846-228	4846-231	08540-214	08540-233	7734-114	7734-117	31074-194
D-2	2	0	0	1	1	?	?	1	0	?
D-3	3	?	?	?	?	?	?	1	0	?
D-4	4	0	0	1	1	?	?	1	1	?
D-5	5	0	0	1	0	?	?	1	1	?
D-6	6	?	?	?	?	?	?	0	1	?
D-7	7	0	0	1	1	?	?	0	1	?
D-9	8	?	?	?	?	?	?	1	1	?
D-11	9	0	0	1	1	?	?	0	1	?
D-12	10	?	?	?	?	?	?	0	1	?
D-13	11	?	?	?	?	?	?	0	1	?
D-14	12	0	0	1	1	?	?	0	1	?
D-15	13	?	?	?	?	?	?	0	1	?
D-16	14	?	?	?	?	?	?	1	0	?
D-17	15	?	?	?	?	0	1	1	0	?
D-18	16	0	0	1	0	?	?	0	1	?
D-19	17	?	?	?	?	?	?	0	1	?
D-21	18	?	?	?	?	?	?	0	1	?
D-22	19	?	?	?	?	?	?	1	0	?
D-23	20	0	0	1	1	?	?	0	1	?
D-24	21	?	?	?	?	?	?	1	0	?
D-25	22	?	?	?	?	?	?	0	1	?
D-26	23	0	0	1	1	?	?	?	?	?
D-27	24	0	0	1	1	?	?	?	?	?
H-6	1	?	?	?	?	1	1	?	?	1
H-7	2	?	?	?	?	?	?	0	1	?
H-8	3	?	?	?	?	?	?	0	1	?
H-9	4	?	?	?	?	?	?	1	1	1
H-10	5	?	?	?	?	1	0	?	?	1
H-11	6	?	?	?	?	0	1	1	0	?
H-12	7	?	?	?	?	?	?	1	0	?
H-13	8	?	?	?	?	?	?	1	0	?
H-14	9	?	?	?	?	?	?	1	0	?
H-15	10	0	0	1	1	1	1	1	0	?
H-16	11	0	0	1	1	0	1	0	1	?
H-17	12	0	0	1	1	0	1	1	0	?
H-18	13	0	0	1	1	0	1	1	0	?
H-19	14	0	0	1	1	?	?	0	1	?
H-20	15	?	?	?	?	?	?	1	1	?
H-21	16	0	0	1	0	1	1	?	?	?
H-22	17	0	0	1	1	0	1	1	1	1
H-23	18	0	0	1	1	0	1	1	0	1
H-24	19	0	0	1	1	0	1	?	?	1
H-25	20	0	0	1	1	0	1	?	?	1
H-27	21	0	0	1	1	0	1	?	?	1
H-28	22	0	0	1	1	0	1	?	?	1
H-32	23	0	0	1	1	?	?	?	?	?
H-36	24	0	0	1	1	?	?	1	1	?
H-37	25	0	0	1	1	?	?	0	1	?
H-38	26	0	0	1	1	?	?	1	0	?
H-39	27	0	0	1	0	?	?	1	0	?
H-40	28	0	0	1	1	?	?	0	1	?
H-41	29	0	0	1	1	?	?	0	1	?

Appendix 5: Genotypes observed for the four different microsatellite loci analysed, for each of the four populations.

	31074 Genotypes	7734 Genotypes			8540 Genotypes			4846 Genotypes				8003 Genptypes		
Population	194	114114	114117	117117	214214	214233	233233	216219	219219	228228	228231	188188	188196	196196
Bukken	0	3	3	13	0	0	0	1	9	3	7	1	1	8
Vikso	1	0	0	23	18	?	?	?	3	5	15	?	?	?
Dicksonfjorden	0	6	3	13	0	0	1	0	0	2	8	?	?	?
Vestpynten	9	10	4	7	1	3	10	0	0	2	17	2	0	0