Polyploidy and ecotype variation in *Cochlearia officinalis* L. and related species

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"*Her gror skjørbuksurt i sanden, lenger opp enn floa når*"

Moddi – Kjerkegård ved havet, words by Helge Stangnes

Preface

This master has been a wonderful journey, I'm only sad that it is over so soon. There are many I would like to thank for being a part of this. First of all, I would like to thank my main supervisor, Anne - for your kindness, enthusiasm and knowledge. You have been important in every stage of this master, from the very beginning when you suggested the project. In the 1980's, before I was even born, Inger (and Odd) worked with *Cochlearia*. Work that set the stage for this master thesis. I would like to thank Inger, Odd and Charlotte for being my additional supervisors, Inger – for being the ultimate field expert in our *Cochlearia* field work, Odd – for helping me with numbers as well as creating maps and Charlotte – for *Cochlearia* collections.

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Lastly, I would like to thank my friends outside UiO and my family, for being so understanding, caring and supportive.

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In between the stones and the seaweed in the arctic shoreline, exposed to tide and weather, is where you'll find *Cochlearia*. This is also where you'll find my feet, I hope again, someday soon.

Marie.

Contents

Abstract **5**

Introduction **6**

Cochlearia 6

Cochlearia officinalis s. lat. 8

Research questions. 9

Material and methods **13**

Plant Material 13

Flow cytometry 15

DNA isolation 16

Microsatellite analyses 16

RAD-seq analyses 18

Creating maps 20

Microsatellite data analyses 20

RAD-seq data analyses 24

Results **25**

Flow cytometry 25

Microsatellite analyses 27

RAD-seq analyses 35

Discussion **42**

The closely related species in section *Cochlearia* 42

The genetic aspect of the subspecies of *C. officinalis* 43

The polyploid origin of *C. officinalis* in Northern Scandinavia 46

Further research 50

Conclusion 51

References **52**

Appendix I

Abstract

Polyploidy and ecotypic differentiation are important aspects of plant evolution. The present study has applied molecular methods to investigate the polyploid origin and ecotypic differentiation of the tetraploid *Cochlearia officinalis* in Northern Scandinavia (comprising three subspecies/ecotypes), in the context of related species in section *Cochlearia*. The genetic results from six microsatellites markers (cross-amplification of markers developed for other species in Brassicaceae) and thousands of single nucleotide polymorphisms (SNPs) retrieved from restriction-site associated DNA sequencing (RAD-seq) are largely congruent, although displaying different levels of resolution.

The species investigated, representing different ploidal levels from diploid to octoploid (*C. pyrenaica*, *C. aestuaria*, *C. groenlandica*, *C. officinalis* and *C. anglica*, as well as the hybrid *C. x hollandica*), seems to be genetically closely related, reflecting a section consisting of recently derived species where gene flow may occur both between ecotypes and across ploidal level. The genetic structure detected in *Cochlearia officinalis* in Northern Scandinavia can only to some extent be explained by the different subspecies/ecotypes per se. Both geographical distance and population affiliation are also to a large extent responsible for the patterns found. *Cochlearia officinalis* ssp. *integrifolia* (the spring ecotype) is the most distinct of the three subspecies, whereas the two other subspecies, *C. officinalis* ssp. *norvegica* (the estuary ecotype) and *C. officinalis* ssp. *officinalis* (the beach ecotype) are more genetically overlapping.

A single (auto)polyploidization event resulting in *C. officinalis* in Northern Scandinavia is proposed, based on the genetic data. The subspecies/ecotypes of *C. officinalis* constitute a single group, distinguished clearly from other species and ploidal levels, based on the high-resolution RAD-seq data. The events leading to ecotypic differentiation in *C. officinalis* within Northern Scandinavia are discussed, but cannot be fully elucidated by the genetic results in the present study. Most evidence (including previous studies on morphology and physiology) seems to support a single ecotypic differentiation event with subsequent dispersal for the spring ecotype, whereas the ecotypic differentiation of the coastal subspecies (the estuary and the beach ecotypes) is less evident from the available data. Both repeated ecotypic differentiation, as well as a single ecotypic differentiation can be argued for.

Keywords: plant evolution, polyploidy, ecotypic differentiation, flow cytometry, microsatellites, restriction-site associated DNA sequencing, Brassicaceae, *Cochlearia*

Introduction

Polyploidy, duplication of the genome, is of major impact in evolution, especially of plants, and is an important mechanism of adaptation and speciation (Ramsey & Schemske 1998). Doubling of the genome might in many cases lead to rapid sympatric speciation, because the putative hybrid between a parental species and the polyploid would have an uneven number of chromosomes and often be less viable or sterile (Otto & Whitton 2000, Mallet 2007). Polyploidization does, however, not necessarily result in immediate reproductive isolation from its parental species (Slotte et al. 2008), and interploidal gene flow is observed in many plant genera e.g through triploid bridges (Husband 2004). Studies about the formation of polyploids encompass mode of polyploidization; (1) autopolyploidization, i.e. genome duplication within a single species, or (2) allopolyploidization, i.e. genome duplication in combination with hybridization (Ramsey & Schemske 1998), as well as number of polyploidization events; (1) single, or (2) recurrent (Soltis & Soltis 1999, Soltis et al. 2004b).

In addition to drastic events like polyploidization, species adapt and evolve in relation to their environment, and species variability related to habitat differentiation has been studied by many (e.g. Turesson 1922, Ghalambor et al. 2007, Lowry 2012). According to Turesson (1922), phenotypic variability can be a result of (1) phenotypic plasticity, the ability of an organism to alter its phenotype after the habitat, and (2) genetic adaptation to the habitat, i.e. ecological differentiation which may result in recognition of e.g. ecotypes. Recent studies suggest that also epigenetics, regulation of gene expression which can be heritable, might affect phenotype variability (Jaenisch & Bird 2003, Bossdorf et al. 2008, Biémont 2010). The genus *Cochlearia* L. comprises a group of recently, and in some cases not yet fully, differentiated taxa that exhibit complex variation with regard to ploidy, ecology and morphology (Gill 1971, Gill 1973, Nordal & Stabbetorp 1990, Nordal & Laane 1996, Koch et al. 1996, Koch et al. 1998, Gill 2007). The genus is, thus, a suitable system for studying ecological adaptation, speciation and polyploid evolution, by the use of molecular markers.

Cochlearia

The genus *Cochlearia* belongs to the Brassicaceae and contains annual to perennial herbs with flowers characteristic for the family; four petals forming a cross, two short and four long stamens and the fruit develops into a silicule (Torkelsen & Østern 1982, Lid & Lid 2005, Judd 2008). Representatives of the genus are often somewhat fleshy (succulent). Because of the high C-vitamin (ascorbic acid) content, *Cochlearia* species have traditionally been an important remedy used to cure scurvy, hence the common name Scurvy grass (Høeg 1976, Jonsson & Jonsson 1980, Torkelsen & Østern 1982, Buckland et al. 1991). The species inhabit coastal and inland habitats, distributed from lowland to alpine-arctic (Koch et al. 2003, Lid & Lid 2005). While the genus *Cochlearia* has a worldwide distribution, section *Cochlearia* O.E. Schulz (= *Eucochlearia* Prantl) is widely distributed in Europe as well as being circumpolar (Nordal & Laane 1990) and contains about 12-16 recognized species depending on taxonomic treatment (Appendix Table A1). The focus of this study is on section *Cochlearia* and, if not specified, further mention of *Cochlearia* refers to this section.

It is believed that after the last Pleistocene glaciation, diploid *Cochlearia* species spread northwards from refugia South of the glaciated areas (both South of the Alps/Pyrenees and in

the South of England), colonized the vacant habitats adjacent to the retreating glaciers and diversified (Koch et al. 1996). Similar colonization stories are found in other plants (e.g. Hurka & Neuffer 1997, Skrede et al. 2006, Alsos et al. 2009). During the evolutionary history of *Cochlearia* several events of polyploidization have occurred (Gill 1973, Koch et al. 1996, Koch 2002). Earlier studies indicate that the polyploid speciations in *Cochlearia* are of post-glacial origin (Koch et al. 1996). Subsequent events of hybridization and polyploidization can build up species complexes with complicated evolutionary histories such as found in *Cochlearia* and in several other plant taxa within previously glaciated regions of Arctic and North Atlantic region (e.g. Brochmann et al. 2004, Brysting et al. 2007).

The taxonomy of *Cochlearia* has gone through many changes due to the difficulty of delimiting taxa (Gill 2007). Different naming, delimitations and ranking of the taxa have been proposed (Saunte 1955) due to the complex variation present with regard to cytology, ecology and morphology in combination with considerable phenotypic plasticity (Nordal & Laane 1996). In addition, few sterility barriers seem to occur; the crossing of cytotypes and ecotypes results in offspring with little reduced fertility (Nordal & Laane 1996). These are features indicating that *Cochlearia* is a group that has quite recently diversified (Koch et al. 1996, Gill 2007).

Two basic chromosome numbers are found in *Cochlearia*; x=6 and x=7 (Saunte 1955). Diploids occur in both series, however, they are geographically separated (Gill 1971). In Central Europe and Britain we find diploids with 2n=12, while in the Arctic all diploids have 2n=14 (Appendix Table A1, Gill 1971, Elven 2011.). The only geographical overlap of these two cytotypes is in Iceland (Nordal & Laane 1990, Koch et al. 1996). Tetraploid *C. officinalis* with 2n=24 is widely distributed in Europe and many infraspecific taxa are recognised (Appendix Table A1, Gill 2007). Hexaploid species exist, with both 2n=36 and 2n=42 (Appendix Table A1, Gill 1976, Abs 1999, Cieslak et al. 2007, Paschke et al. 2002a, Paschke et al. 2002b, Paschke et al. 2003). On the octoploid level (2n=*48*), we find *C. anglica* L. and *C. borzaeana* (Coman et Nyár.) Pobed. (Appendix Table A1, Elkington 1984, Nordal & Laane 1996, Kochjarová et al. 2006). *Cochlearia anglica* has rarely been reported as decaploid (2n=60) as well.

Species in *Cochlearia* inhabit a wide range of habitats, but are often divided into two broad ecogeographical elements; whether they are coastal or inland species (Koch et al. 2003). Coastal species have adapted to habitats like bird cliffs, estuaries and beaches including coastal grassland, sand dunes, stony sea shores and beach cliffs (Nordal & Stabbetorp 1990, Gill 2007). Inland species have adapted to grow in alpine habitats, on easily weathered, mineralic soils, by springs/fountains and by streams. In addition to these primary habitats some *Cochlearia* species (especially the annual *C. danica*) have also been observed colonizing and spreading along roadsides (e.g. in Scotland, Welch & Welch 1998, Welch 2001). Ecology is a web of many influencing factors, but nutrient levels (nitrogen) and day length have been pointed out as factors that Scandinavian ecotypes of *Cochlearia* respond differently to (Eriksen & Nordal 1989).

Various authors have examined the morphology of *Cochlearia*, both of hybrids and non-hybrids (e.g. Crane & Gairdner 1923, Saunte 1955, Fearn 1977, Nordal & Stabbetorp 1990, Gill 2007). Many morphological features in *Cochlearia* are phenotypically plastic to environmental conditions (Elkington 1984). Cultivation studies using similar growing conditions in a

greenhouse have documented this plasticity (Eriksen & Nordal 1989). Morphological characters used to identify species are e.g. shape of leaves, as well as flower/fruit characters (Lid & Lid 2005). However, it has been noted by several that morphological differentiation is low and often poorly defined (Koch et al. 1996). Especially, infraspecific taxa can be difficult to separate in the field, due to overlapping morphology (Wyse Jackson 1991, Gill 2007).

From crossing experiments, it is known that cytotypes and ecotypes of *Cochlearia* produce offspring with little loss of fertility, thus they hybridize freely when they meet (Gill 1971, Gill 1973). Hybrid populations have been observed in nature, and the frequently occurring hexaploid *C. officinalis* x *C. anglica* has been named *C.* x *hollandica* Henrard (Appendix Table A1, Koch et al. 1996, Nordal & Laane 1996). Hybridization and introgression between hybrid and parental populations has also been noted between *C. officinalis* and *C. danica* (Fearn 1977). Hybridization and introgression are due to weak reproduction barriers between species and may result in gene flow between ploidal levels (Koch et al. 1996). Species originating from hybridization are also reported; e.g. *C. bavarica* Vogt, which is an allopolyploid; the result of hybridization and genome doubling (Abs 1999, Koch 2002).

***Cochlearia officinalis* s. lat.**

Cochlearia officinalis is a cold-tolerant, coastal halophyte and is with few exceptions the only resident *Cochlearia* in Norway (Nordal et al. 1986, Welch & Welch 1998, Gill 2007). The exceptions are *C. groenlandica* in Spitzbergen and Bear Island (Zmudczyńska-Skarbek et al. 2013), and a few short-lived *C. anglica* individuals observed in the southernmost of Norway, probably dispersed from overseas (Pedersen 2009). Previous morphological, cytological, crossing and growth studies at the University of Oslo found that the morphological variation in *C. officinalis* in Northern Scandinavia is related to the ecology and that three subspecies comprising four ecotypes can be recognized (Nordal et al. 1986, Eriksen & Nordal 1989, Nordal & Stabbetorp 1990). *Cochlearia officinalis* ssp. *norvegica* Nordal & Stabbetorp corresponds to the estuary ecotype, *C. officinalis* ssp. *integrifolia* (Hartm.) Nordal & Stabbetorp corresponds to the spring ecotype and *C. officinalis* L. ssp. *officinalis* comprise the beach and bird cliff ecotypes.

Cochlearia officinalis ssp. *officinalis* (a-b in Fig. 1 and Fig. 2) comprises the beach ecotype occuring on gravel beaches and salt marshes, as well as the bird cliff ecotype (Nordal & Stabbetorp 1990, Lid & Lid 2005). The bird cliff ecotype is especially adapted to exploit the high nutrient levels at manured cliffs and shows vigorous growth at high nutrient levels (Eriksen & Nordal 1989). The taxon is normally biennial and morphologically very variable. Rosette leaves are more or less reniform with cordate/truncate basis. The diameter of leaves can range from 5 mm (in exposed beaches) to 5 cm (on bird cliffs). The silicules are sub-globose (Nordal & Stabbetorp 1990).

Cochlearia officinalis ssp. *norvegica* (c-d in Fig. 1 and Fig. 2) is the estuary ecotype and grows in sheltered habitats near outlets of large rivers in innermost fjords (Nordal & Stabbetorp 1990, Lid & Lid 2005). These are habitats that are inundated by brackish water at flood-tide. This taxon is adapted to handle nutrient poor habitats and shows very little increase in growth when presented with higher nutrient levels, i.e. nitrogen (Eriksen & Nordal 1989). The taxon is biennial, the rosette is overwintering. It is the morphologically most distinct of the ecotypes.

Rosette leaves are oblong/rhomboid/ovate with cuneate/truncate basis, short teeth can occur. This taxon has the fleshiest leaves and the largest flowers (longest petals and sepals). The silicules are oblong (up to 1 cm) and compressed laterally (Nordal & Stabbetorp 1990).

Cochlearia officinalis ssp. *integrifolia* (e-f in Fig. 1 and Fig. 2) is the spring ecotype and is growing inland in more or less base-rich cold springs, along streams and brooks or in snow beds (Nordal & Stabbetorp 1990, Lid & Lid 2005). It is perennial, with the rhizome often branching and giving rise to more rosettes and developing buds before the snow has melted. This means that flowering is induced earlier in this ecotype compared to the others, if exposed to the same conditions. Rosette leaves are reniform with cordate basis and are mostly broader than they are long. This taxon has the thinnest and darkest leaves. The silicules are sub-globose to oblong (Nordal & Stabbetorp 1990).

The same habitat preferences are also found on other ploidal levels in *Cochlearia* (Appendix Table A1, Nordal & Laane 1996). *Cochlearia officinalis* is believed to be of an autopolyploid origin from an ancestral diploid from Central Europe (Gill 1973). Three diploid species that are closely related to each other are found in Central Europe; *C. pyrenaica* DC. (a in Fig. 3), *C. macrorrhiza* Pobed. and *C. excelsa* Zahlbr. ex Fritsch (Koch et al. 2003). They all share the same ecology, which is similar to the spring ecotype found in Northern Scandinavia (Nordal & Laane 1996). The two latter are especially rare. The diploid *C. aestuaria* (Lloyd) Heywood (b in Fig. 3), also from Central Europe, has estuary ecology. *Cochlearia officinalis* is believed to be the progenitor of the octoploid *C. anglica* (d in Fig. 3) through autopolyploidy (Koch et al. 1998). *Cochlearia anglica* is also associated with estuaries (Nordal & Laane 1996). The ecological preferences are reflected in the morphology. The taxa sharing the same habitat also share morphological characters to some degree. This is most evident for the taxa with estuary ecology. These taxa seem to have leaves with more or less cuneate basis and relatively large flowers (Nordal & Laane 1996).

Research questions

Genetic studies in *Cochlearia* so far have not included all the three subspecies of *C. officinalis* in Northern Scandinavia (Koch et al. 1996, Koch 2002, Cieslak et al. 2007, Gill 2007, Cires et al. 2011, Rucińska & Puchalski 2011). Given the ecotypical and morphological differentiation found in previous studies, we will investigate to what degree the subspecies/ecotypes in Northern Scandinavia are genetically differentiated. We would also like to investigate the origin of the ecotypes.

Specifically we ask:

- Are the three subspecies/ecotypes of *C. officinalis* genetically differentiated?
- Did *C. officinalis* originate from a single polyploidization event and then differentiated into three ecotypes?
- Did the three ecotypes originate independently from different genotypes with different ecology?

Figure 1. Habit and habitat of the three subspecies/ecotypes of *Cochlearia officinalis* (2*n*=24). a-b: ssp. *officinalis* (the beach ecotype), Sjøvassbotn and Skittenelv, Troms, Norway; c-d: ssp. *norvegica* (the estuary ecotype), Skibotn, Troms, Norway; e-f: ssp. *integrifolia* (the spring ecotype), Kvaløysletta, Troms, Norway. (Photo: M.K.Brandrud).

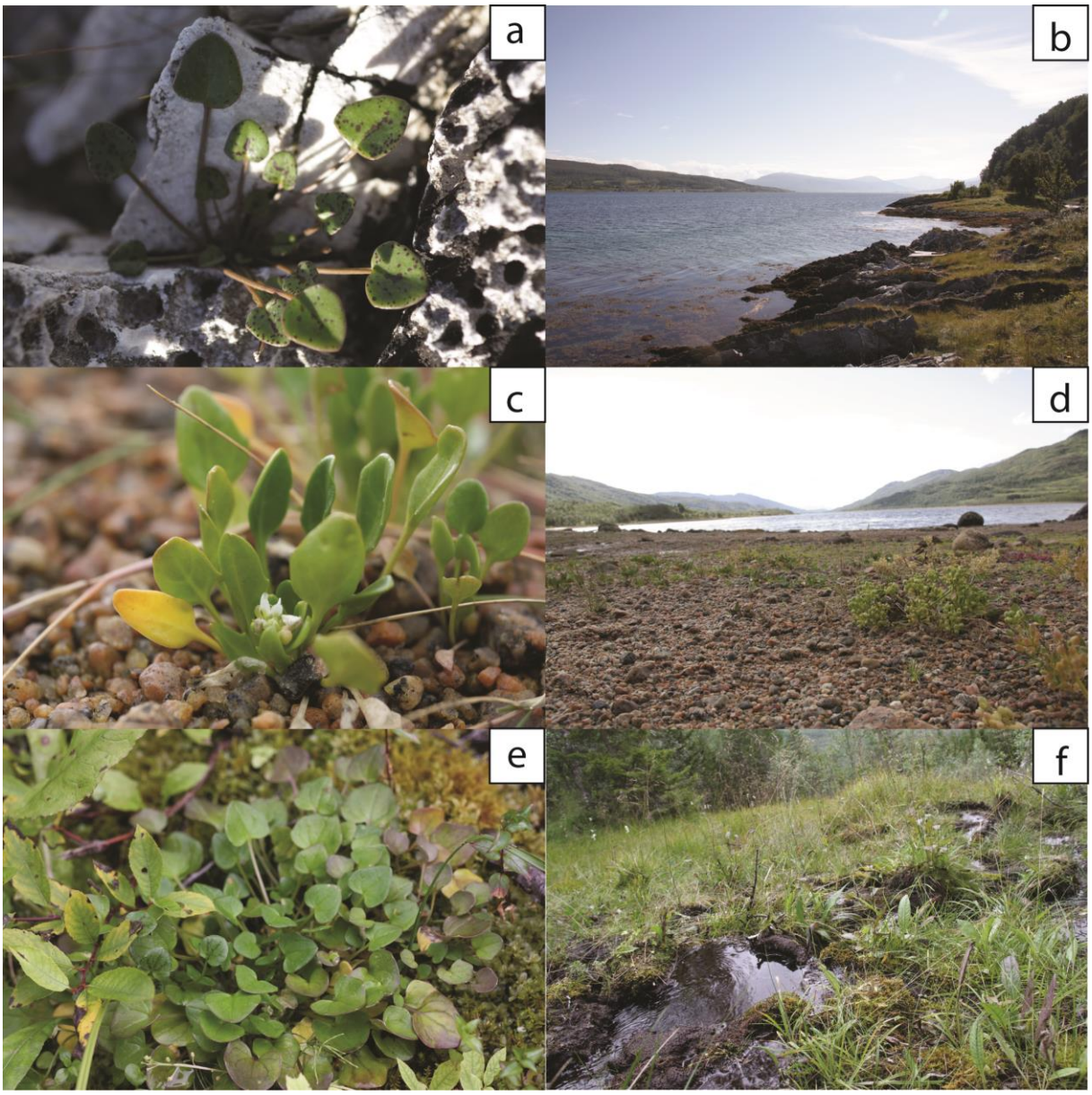

Figure 2. Habit and habitat of the three subspecies/ecotypes of *Cochlearia officinalis* (2*n*=24). a-b: ssp. *officinalis* (the beach ecotype), Tjeldsundet, Troms, Norway; c-d: ssp. *norvegica* (the estuary ecotype), Kanstadbotnen, Nordland, Norway; e-f: ssp. *integrifolia* (the spring ecotype), Sørfjorddalen, Nordland, Norway. (Photo: M.K.Brandrud).

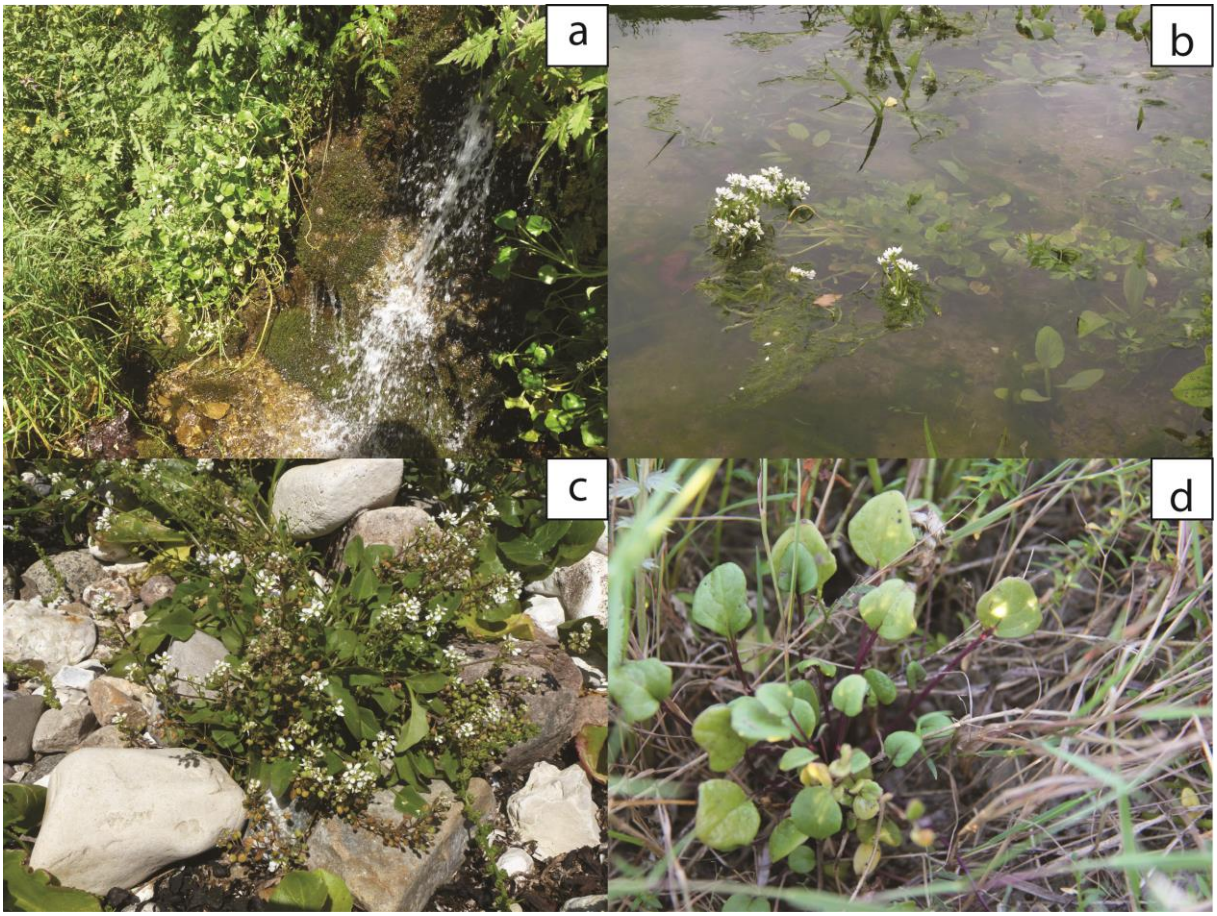

Figure 3. a: *Cochlearia pyrenaica* (2n=12), Asturias, Spain (Photo A.K.Brysting); b: *C. aestuaria* (2*n*=12), Asturias, Spain (Photo A.K.Brysting); c: *C.* x *hollandica* (2*n*=36), Fyn, Denmark (Photo: A.K.Brysting); d: *C. anglica* (2*n*=48), Scania, Sweden (Photo: K.H.Brandrud).

Materials and methods

Plant Material

The main sampling of this study included the three subspecies of *C. officinalis* sensu Lid & Lid (2005). Three of the four ecotypes that the subspecies comprise were sampled; beach, estuary and spring. Since all three subspecies co-occur in Northern Norway, we selected two areas within this region for our main sampling; Tromsø-Skibotn and Lofoten (Fig. 4). A minimum of two populations of each subspecies were collected from each of these areas, i.e. six populations from each area. These populations were selected based on previous locality records reported in Nordal & Stabbetorp (1990) and voucher labels from the Herbarium of Oslo. For more information on sampling sites, see Appendix Table A2.

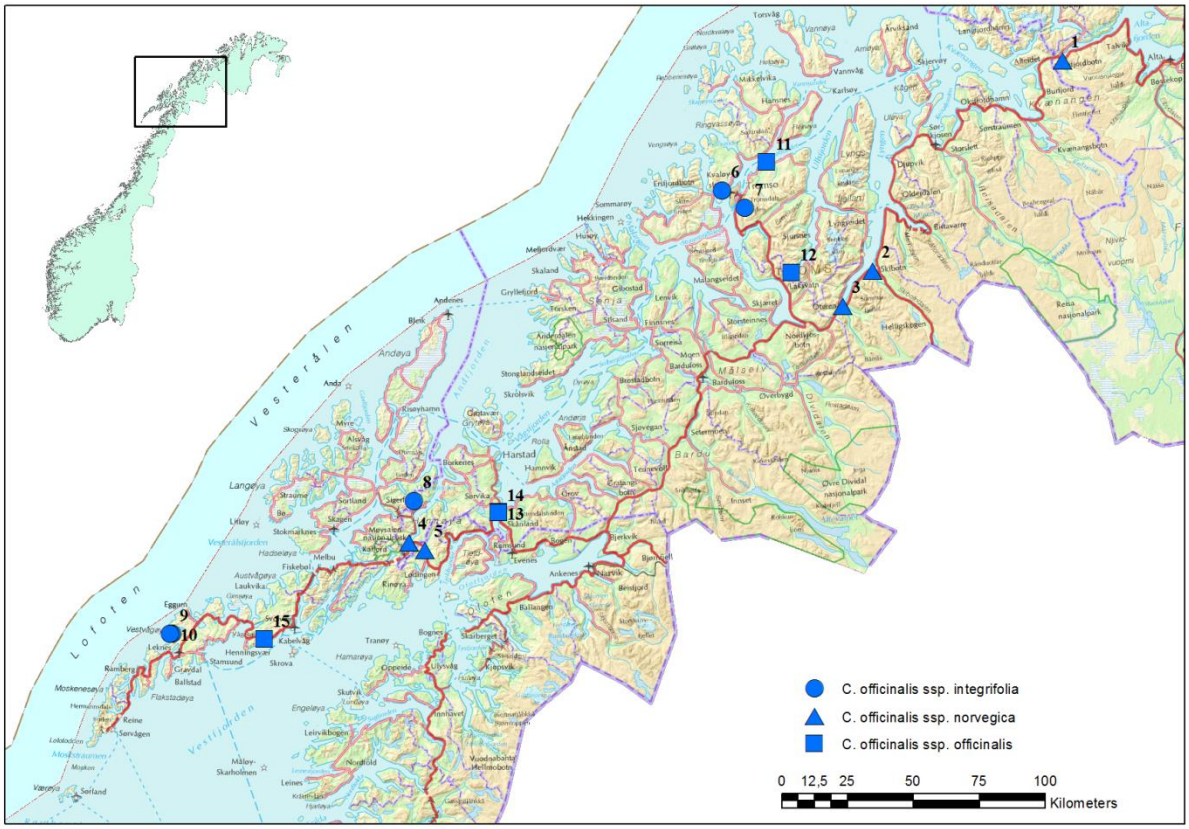

Figure 4. *Cochlearia officinalis* populations collected in Northern Norway for this study. Two populations of each subspecies of *C. officinalis* were collected from two geographical regions, Troms in the North and Lofoten further South. Different subspecies are indicated by different symbols. See Appendix Table A2 for further locality information.

This study also included samples of *C. pyrenaica* and *C. aestuaria* from South Western Europe as well as samples of *C. anglica* from Southern Scandinavia (Fig. 5). Three populations of *C. aestuaria* and one population of *C. pyrenaica* were sampled in the county of Asturias, Spain (Appendix Table A2). One population of *C. anglica* was sampled in the county of Scania, Sweden (Appendix Table A2). The Spanish sampling was based on known populations from the Spanish plants information system database Anthos (http://www.anthos.es/) and a similar database was used to find the Swedish population (http://artportalen.se/). The present study

also included two Spanish and two French populations of *C. pyrenaica* collected and used in the study by Cires et al. (2011). Additional *Cochlearia* material, used as reference in the molecular analyses, came from various locations in Europe (Appendix Table A2, Fig. 5) and included e.g. *C.* x *hollandica* (c in Fig. 3) and *C. groenlandica*.

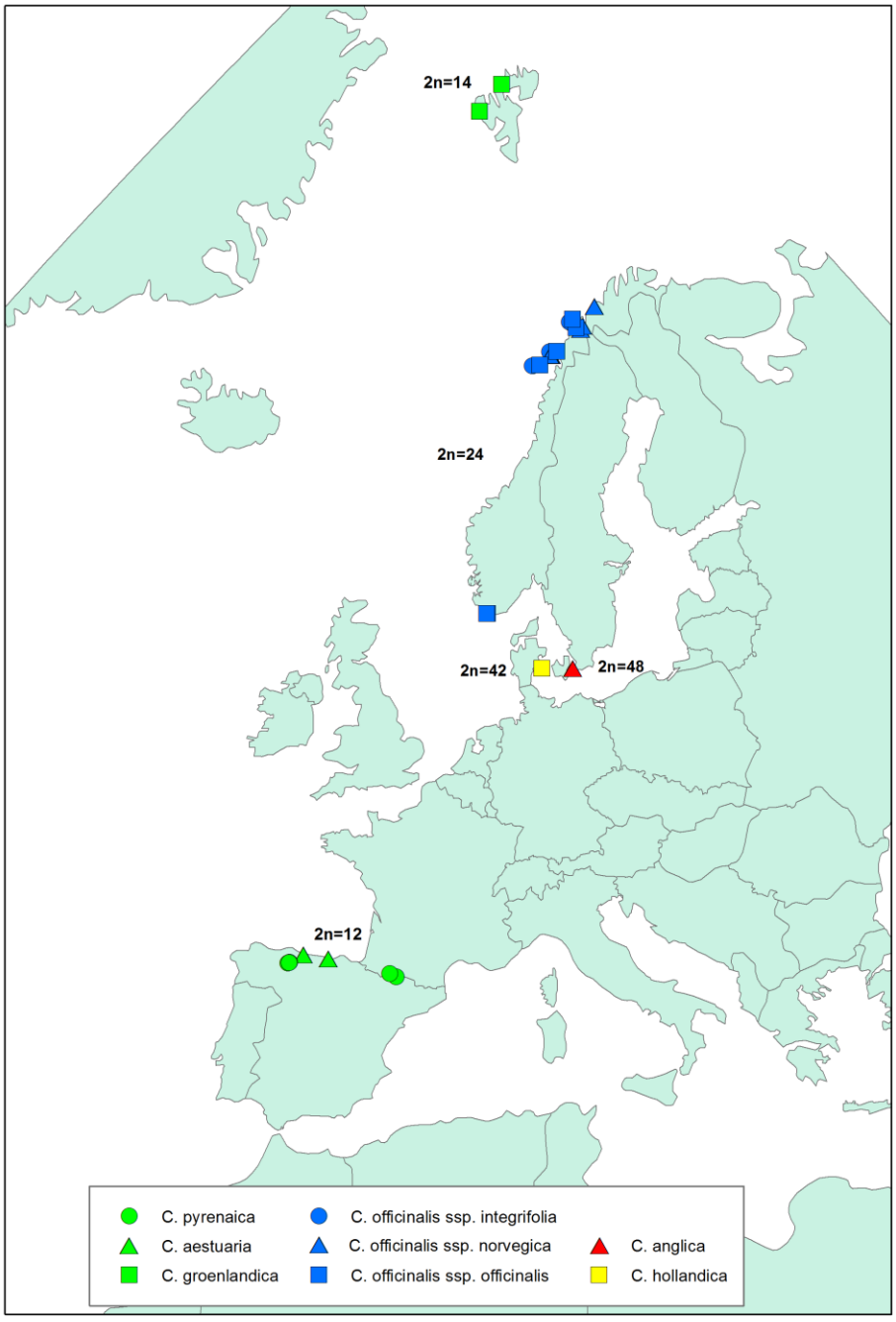

Figure 5. The European *Cochlearia* populations analysed in this study. Different taxa are shown by different symbols and colours, and chromosome numbers are indicated on the map.

In field, leaves of 10 different individuals from each population were dried instantly on silica gel for later DNA isolation. When available, seeds were collected from the same or other individuals, and five representative and complete individuals from each population were collected as herbarium vouchers and will be deposited in the Herbarium of Oslo. Due to few available seeds, a few living plants from one of the *C. officinalis* ssp. *integrifolia* populations from Troms (offinttro1) were brought from the field and replanted in greenhouses at the University phytotron. Seeds of *C. pyrenaica* were not available in field.

Seeds were stored dry and cold (at about 3°C) before germinated in a phytontron at the University of Oslo. Due to a low germination rate (2 %) after two weeks, a treatment of wet and cold conditions was tested. The seed were placed in petri dishes with; 10 pieces of cell paper, 5 ml distilled water, filter paper, about 10 seeds, filter paper, and 2 ml distilled water. The seeds were then kept cold (3°C) for 3 weeks or longer before transferred to standard soil. Seedlings were allowed to grow tall in summer conditions (18 hours of 18°C in light and 6 hours of 10°C in darkness) for about 5 months before moved to winter conditions (10 hours of below 9°C in light and 14 hours of below 9°C in darkness) for about 5 months.

Flow cytometry

Flow cytometry can be used to estimate ploidal level and genome size (DeLaat et al. 1987, Arumuganathan & Earle 1991, Doležel & Bartoš 2005). Fresh leaves are cut and mixed with a buffer containing dye. Then they are exposed to light, and the fluorescence is measured. Individuals are sorted by ploidal level by using a reference.

Two to three fresh leaves from individuals growing in the phytotron were packed in plastic bags with a wet filter paper and sent to G. Geenen, Plant Cytometry services in the Netherlands (http://www.plantcytometry.nl/), who performed the ploidy estimation by flow cytometry. Altogether 86 individuals representing 16 different populations were sent in the first batch, which were analysed without an internal standard.

In the protocol used by G. Geenen, 1-2cm^2/50-100 mg leaf material was cut in ice-cold buffer to isolate and dye the nuclei from the plant cells. The buffer, which contained 5 mM Hepes, 10 mM magnesium sulphate heptahydrate, 50 mM potassium chloride, 0.2% Triton X-100, 0.1% DTT (dithiothreitol), 1.0% PVP-40, and 2 mg/l DAPI (4',6-diamidino-2-phenylidole), is a modified version of the buffer used in Arumuganathan & Earle (1991), where DAPI is the dye. The buffer was then sent through a nylon filter with mesh size 50 µm and then through the flowcytometer CyFlow Space (Partec GmbH, Müster, Germany) with a UV high power led (365) lamp, objective of 40 x N.A. 0.8 air (Partec), dichroic mirrors TH 420A and emission-filter GG 435E. To obtain the DNA histograms the software Flomax version 2.8 (Partec) was used.

A second batch of 21 individuals from 16 populations were sent once more for analysing of the relative fluorescence intensities compared to an internal standard; *Vinca minor*, which was cut together with the *Cochlearia* sample. Due to no seeds, and hence, no living material of *C. pyrenaica*, it was not possible to include this species in the flow cytometry analysis.

DNA isolation

Before DNA isolation the leaves were normally dried on silica for at least one week to be sure that they were completely dry. DNA was extracted from the dried leaves using the E.Z.N.A.® SP Plant DNA Kit (Omega bio-tek, Norcross, USA), following the protocol for dry samples with a few modifications. In preparing for the isolation, instead of grinding the samples with a pellet pestle, two tungsten carbide beads, 3 mm (Qiagen, Venlo, Netherlands), were added to the eppendorf tube with the sample before it was crushed for 1-2 min at 20 Hz in a tissuelyser, Retsch MM01 (Qiagen). About 30 mg leaf material were used from each individual. In most cases, elution with 50 µl (run through once or twice) was used**.** At first isolated DNA was stored in normal eppendorf tubes at -20 °C, however, to prevent DNA to be lost due to binding to the plastic surface of these tubes, DNA LoBind tubes (Eppendorf, Hamburg, Germany) were used for later isolations. A ND-1000 Spectrophotometer (Thermo scientific (NanoDrop products), Wilmington, USA) was used to check quantity and quality of the isolated DNA.

Microsatellite analysis

Microsatellites (also referred to as STRs, i.e. short tandem repeats or SSRs, i.e. simple sequence repeats) are usually considered to be neutral evolving non-coding DNA (Ellegren 2004). They are highly variable and polymorphisms mainly arise from variability in length (insertions/deletions). This is thought to mainly happen during the replication, by disassociation followed by incorrect realigning (aligning to a previous or later repeat) of the DNA strand that is being synthesized and the template; replication slippage. The number of nucleotides that are repeated varies; however, the main types are mono-, di-, tri- and tetra-nucleotide repeats, where di-nucleotide repeats are most common.

To amplify the microsatellites, PCR (polymerase chain reaction) with buffer and polymerase from the HotStarTaq *Plus* DNA Polymerase kit (Qiagen) was performed using the M13-tailing approach from Schuelke (2000). This was performed with a total volume of 10 µl, containing 1 µl CoralLoad PCR buffer, 1 µl 2mM dNTP (Thermo Fisher Scientific, Waltham, US), 0.2 µl 5 µM forward primer, 0.8 µl 5 µM reverse primer, 0.8 µl 5 µM fluorescently-labelled M13 primer, 0.05 µl HotStarTaq, 4.15 µl mqH_2O and 2 µl 5X diluted DNA. The following cycling conditions were used: initial denaturation for 5 min at 95°C, followed by 30 cycles [30 sec 95°C, 45 sec T_a, 45 sec 72°C], 8 further cycles [30 sec 95°C, 45 sec 53°C, 45 sec 72°C], 30 min final elongation at 72°C, and incubation at 10°C, stored at 4°C until further processing. T_a is the specific annealing temperature used for each of the microsatellites after optimalization (Table 1).

To make it possible to co-load the microsatellites in the fragment analysis, M13 tails (Schuelke 2000) with four different fluorescent dyes were used; 6-FAM (IDT, Coralville, USA), NED, PET and VIC (all three from Life Technologies/Applied Biosystems, Carlsbad, USA).

The PCR products were tested for successful amplification by gel electrophoresis using a 1% TAE agarose solution of Seakem LE Agarose (Lonza, Basel, Switzerland) and GelRed (VWR, Radnor, US). Successfully amplified products were diluted by 10X and pooled into two co-loading mixes, each containing PCR products with different fluorescent dyes and/or different expected fragment length.

Fragment analysis was prepared with a mastermix containing 8.85 µl Hi-Di formamide and 0.15 µl Genescan LIZ 500 size standard (both Life Technologies/Applied Biosystems) and was carried out with an ABI 3730 analyzer (Life Technologies/Applied Biosystems).

Twenty primers (IDT) developed for other Brassicaceae taxa (*Arabidopsis*, *Brassica* and *Draba*), used in the study by Skrede et al. (2009) were tested for amplification on 15 *Cochlearia* individuals (see Table 1 for further information of primers). After an initial test, the successfully amplifying microsatellites were run for 177 *Cochlearia* individuals, comprising all ploidal levels and ecotypes (Appendix Table A2). At least five replicates and at least one negative control were included per plate (96 wells).

Table 1. Microsatellite primers developed for other Brassicaceae species, selected from Skrede et al. (2009), and tested for cross-amplification in the genus *Cochlearia.* Primers developed by: [1]Skrede et al. (2009), [2]Bell & Ecker (1994), [3]Clauss et al. (2002), [4]Suwabe et al. (2002), [5]Ponce et al. (1999), [6]Uzunova & Ecke (1999). Primers marked * were used in the final microsatellite analysis of 177 *Cochlearia* individuals. Annealing temperature (Ta) resulting in successful amplification is given for the microsatellites. Fragment length is given for the microsatellites that were scorable in GENEMAPPER.

Name	T_a	Forward primer	Reverse primer	Fragment length
*DnA222[1]	48	GTGGCAATTTGCTTCCAACC	GCGCAGTGAGATGGATTTCTGG	142-144
*DnB101[1]	48	TGGCTTACCATTGCTGTCC	CCGCATTGTGTTGTTCTTG	123-288
DnB207[1]	-	GGACGGCTGCATTTTCAC	TCAGCTTCACACCAAACAATTC	
DnB220[1]	-	GCAAAGCAGAGCGTAGAATGG	ACTCGGACGTCTCAATCAGC	
*AthCTRI[5]	51	TATCAACAGAAACGCACCGAG	CCACTTGTTTCTCTCTCTAG	135-143
*AthSO392[2]	51	GTTGATCGCAGCTTGATAAGC	TTGGAGTTAGACACGGATCTG	148-203
BRMS008[4]	-	AGGACACCAGGCACCATATA	CATTGTTGTCTTGGGAGAGC	
AthGAPAb[3]	51	CACCATGGCTTCGGTTACTT	TCCTGAGAATTCAGTGAAACCC	
BRMS033[4]	51	GCGGAAACGAACACTCCTCCCATGT	CCTCCTTGTGCTTTCCCTGGAGACG	
BRMS037[4]	-	CTGCTCGCATTTTTTATCATAC	TACGCTTGGGAGAGAAAACTAT	
*MR187[6]	51	GAGTTTTGGTTCCACCATTA	CCCTTCAGCCTTTGATAAAT	143-243
SSL2[3]	51	CATGTACTGGGATTCAGTGTCC	CGTCCTTTGTGTGGTTACACG	
nga129[2]	-	TCAGGAGGAACTAAAGTGAGGG	CACACTGAAGATGGTCTTGAGG	
AthSO191[2]	-	TGATGTTGATGGAGATGGTCA	CTCCACCAATCATGCAAATG	
DnB123[1]	-	CAGTGCAAAATGCGTGAAT	GCGTGGAGATAGAGAAAGAGC	
DnB106[1]	-	TGCGCGCAGAGACAAAGGAG	GAATCCGCCATAGCCGAGGTTG	
DnA8[1]	-	CTTTGGTGGTCTTCCTTG	ATACGATTCCGAGTATTACCTC	
DnB3[1]	-	GCCGTTGTATTGTAGAGTGAG	ACTGGGTCCTCGCTAAAC	
*DnA117[1]	48	TTGTATTCATCGGTTGTGTATC	ACCTGGAAGCACTGGTTC	232-242
DnA138[1]	-	CTTCCTGCGACATCACTCAAAC	TACGGATTGGAGAGAATTCTGAGC	

RAD-seq analysis

RAD-seq, i.e. restriction-site associated DNA sequencing, is a way of reducing the genome to a desired amount of fragments with a restriction enzyme and sequencing these fragments of which polymorphisms can be searched for (Davey & Blaxter 2010). A reference genome can be applied, but is not necessary. The procedure is performed with a restriction enzyme of choice, and a specific molecular identifier (MID) for each individual, attached to the fragments, so individuals can be recognized later.

In the present study RAD-seq was prepared following the RAD-seq protocol from Baird et al. (2008) with modifications by Ovidiu Paun and Clemens Pachschwöll (University of Vienna). This modified protocol comprised (1) single digest (2) double barcoding (3), and size selection with beads. Barcodes with at least three nucleotides difference were used. Two libraries of 60 individuals were prepared and sequenced; one with a rare cutter (Sbfı) and one with a frequent cutter (PstI). The amount of DNA used in the libraries was 62.5 ng per diploid, 125 ng per tetraploid and 250 ng per octoploid. Normally the same amount of DNA should be used when the sublibraries are pooled together. However, when dealing with individuals of different ploidal levels the DNA amount should be proportional to the ploidal level of the individual. This had to be taken into consideration when pooling the sublibraries. In total 12 different P1 adapters (Sigma-Aldrich, St. Louis, US) and five different P2 adapters (Sigma-Aldrich) were used (Table 2).

Prior to RAD-seq DNA samples were assessed with two quantity measures; ND-1000 Spectrophotometer (NanoDrop) and Qubit fluorometer (Life Technologies/Invitrogen) with dsDNA BR Assay Kit, that specifically binds to double stranded DNA. Because the nanodrop values were more or less the double of the Qubit values, all samples used for the RAD-seq libraries (Appendix Table A2) were first cleaned with general NucleoSpin® gDNA Clean-up (Macherey-Nagel, Düren, Germany) and quantified once more.

The first RAD-seq library: From the quantification values each sample was diluted to obtain correct amount of DNA in the digestion step; 50 µl DNA solution (x µl DNA and 50-x µl water), 1 µl 15 U Sbfı (CCTGCA/GG) (NEB, Ipswich, USA) and 5 µl 10X SmartCutBuffer were incubated at 37°C for 45 min. To heat inactivate the enzyme the samples were exposed to 80°C for 20 min. For ligation of the P1 adapter 1.25 µl 100 mM P1 adapter, 1 µl 100 mM rATP (Promega, Fitchburg, USA), 1 µl NEB buffer 2, 0.5 µl 200 000 U T4 ligase (NEB) and 6.25 µl water were added to each sample and incubated at 16°C over night. The reaction was exposed at 10 min of 65°C to heat inactivate the enzyme before samples with different P1 adapter were pooled together in 5 mixes and sonicated (stochastic shearing) by the Bioruptor® Pico (Diagenode, Seraing, Belgium). A short test was performed and checked by the 2100 Bioanalyzer (Agilent), which showed that 3 cycles of 45 sec on and 60 sec off were appropriate for *Cochlearia*. Samples were purified with Mini elute reaction cleanup kit (Qiagen) and size selection with SPRI (Agencourt, Beverly, USA), i.e. solid phase reversible immobilization, was performed on both the left and the right side. To polish the ends a Quick blunting kit (NEB) was used; 2.5 µl Buffer, 2.5 µl 100mM dNTP and 1 µl enzyme was added to 19 µl DNA and left for room temperature for 30 min. Another purification with the Qiagen kit was performed like before, dATPs were added in a reaction containing 2 µl 15 U klenow exo- (NEB), 1 µl 100mM dATP, 2 µl NEB Buffer 2 and 15 µl DNA. The samples were incubated at 37°C for 30 min and after another

purification with the Qiagen kit, quantified by using a ND-3300 fluorospectrometer (Thermo scientific, NanoDrop products) with the 2.8.0 software using the dsDNA PicoGreen option.

Considering the amount of diploid, tetraploid and octoploid individuals in each sublibrary the correct amount of DNA was calculated and pooled together. Ligating the P2 adapter was done by adding 5 µl P2 adapter, 1 µl 100mM rATP (Promega), 3 µl NEB Buffer 2, 0.5 µl 200 000 U T4 ligase to 20.5 µl DNA solution (x µl DNA + 20.5-x µl water). One P2 adapter was used for each sublibrary, thus giving each individual a unique combination of P1 and P2 adapters (double barcoding). After the ligation the samples were size selected on the left side with SPRI (Agencourt). PCR was, used to amplify the fragments in a reaction containing 25 µl Phusion Master Mix (NEB), 0.2 µl 100 mM P1-PCR primer (Table 2), 0.2 µl 100 mM P2-PCR primer (Table 2), 23 µl water and 2 µl DNA, with the following cycling conditions; 30 sec at 98°C, followed by 18 cycles [10 sec 98°C, 30 sec 65°C, 30 sec 72°C], 5 min at 72°C, and incubation at 4°C. PCR products, as well as a positive control (sample not included in the PCR), were run on a 1 % TBE gel (1.2 % agarose and 0.5 % TBE) to see if amplification was successful. Samples were then size selected on the left side with SPRI (Agencourt). A high sensitivity DNA chip (Agilent, Santa Clara, USA) was prepared with sample from before and after SPRI, and run on the 2100 Bioanalyzer (Agilent) to see if adapters were gone after SPRI.

The library (containing 47 tetraploid *C. officinalis*, 5 diploid *C. pyrenaica*, 5 diploid *C. aestuaria* and 3 octoploid *C. anglica* individuals, see Appendix Table A2) was sent to paired-end (100 bp) sequencing in one Illumina HiSeq2000 lane at Campus Science Support Facilities (CSF) Gmbh, Vienna, Austria (http://www.csf.ac.at/).

The second RAD-seq library: The second library was prepared with a similar protocol as the first one, but another, more frequent cutting, enzyme was used; PstI (CTGCA/G) (NEB), and a few modifications of the protocol was necessary. The digestion of the samples was set to two hours instead of 45 min. After digestion three additional steps were added; cleaning with the SPRI with no selection (1.8X) because PstI cannot be heat inactivated, quantification with PicoGreen and another normalization by concentration to be sure that the same DNA amounts used in further steps were equal. Because we cleaned the samples after the digestion we had to add the SmartCutBuffer again for the ligation of the P1 adapter, and 3 µl SmartCutBuffer, 1.25 µl P1 adapter, 1 µl rATP, 1 µl NEB buffer 2, 0.5 µl T4 ligase and 3.25 µl water were added to 30 µl DNA solution (x µl DNA + 30-x µl water). After pooling we cleaned with SPRI with no selection (1.8x) instead of cleaning with the Qiagen kit.

The library (containing 46 tetraploid *C. officinalis*, 5 diploid *C. pyrenaica*, 5 diploid *C. aestuaria* and 4 octoploid *C. anglica* individuals, see Appendix Table A2) was sent to paired-end sequencing (100 bp) in one Illumina HiSeq2000 lane at the Norwegian Sequencing Centre (NSC), Oslo, Norway (http://www.sequencing.uio.no/). Due to low quality at barcode sites in the retrieved reads from the sequencing centre, the library was sequenced once more.

Table 2. P1 adapters, P2 adapters and PCR primers used for the RAD-seq protocol. xxxxx indicate where the barcode is

Name	Sequence
P1_T_SbfI/PstI	5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTxxxxxTGC*A-3'
P1_B_SbfI/PstI	5'-Phos-xxxxxAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT*T-3'
P2_T_PE:	5'-Phos-xxxxAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCAGAACAA-3'
P2_B_PE:	5'-CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTxxxx*T-3'
P1-PCR-primer F:	5'-AATGATACGGCGACCACCGA-3'
P2-PCR-primer R:	5'-CAAGCAGAAGACGGCATACGA-3'

Creating maps

Maps of studied localities (Fig. 4 and Fig. 5) as well as visualization of some on the results were made in ARCMAP 10.3 (ESRI) using GPS (Global positioning system) values with coordinates in decimal degrees (see Appendix Table A2).

Microsatellite data analysis

Processing the raw data: Microsatellite genotypes, with alleles varying in size, were assessed in GENEMAPPER version 3.7 (Life technologies/Applied Biosystems). The automated scoring performed by the software was manually edited to make sure that the scoring was plausible, i.e. diploids had no more than two alleles for each microsatellite, and tetraploids no more than four alleles etc. Replicates were checked to ensure that they had identical profiles and were scored in the same way. To evaluate the uncertainty added to the data by using this scoring approach, two separate scorings were performed. A multilocus binary data matrix (presence/absence) was created in addition to the matrix containing allele sizes.

An issue when assigning polyploid genotypes to codominant data is that of partial heterozygotes (e.g. AB or ABC in a tetraploid organism) (Dufresne et al. 2014). Full allelic configuration would be preferable to extract as much information as possible from the data and to be able to perform various population genetic analyses. Esselink et al. (2004) describes a method to attain this by using peak intensities (calculating the ratio between the peak heights). This method requires high quality and easily scorable data, and even when these criteria are fulfilled, one should be aware that other factors could be present and influence the ratios. This method was tested for two of the microsatellites (data not shown), but proved to be too time consuming and introducing too much uncertainty to be used for the purpose of this study. However, even without full allelic configuration population genetic analyses can be performed using several recent programs that have been designed to treat polyploid data containing partial heterozygote genotypes (Hardy & Vekemans 2002, Clark & Jasieniuk 2011).

The allele size data matrix was used as input file for POLYSAT version 1.3 (Clark & Jasieniuk 2011) in Rstudio (http://www.rstudio.org/). POLYSAT is an R package designed to analyze polyploid microsatellite data. The program assumes ambiguous allele copy number in partial heterozygotes and ploidal levels are estimated based on maximum alleles counted. The estimated ploidal levels were compared with the flow cytometry results and adjusted according to these. Additional information about population affiliation was added as well as information about repeat types from Skrede et al. (2009), Bell & Ecker (1994), Uzunova & Ecke (1999) and

Clauss et al. (2002). Input files for STRUCTURE 2.3.4 (Pritchard et al. 2000) and SPAGEDI 1.4 (Hardy & Vekemans 2002) were created in POLYSAT.

Multivariate analyses: An ordination is a scaling technique, commonly used in multivariate analyses, that can be used to visualize distances or dissimilarities by representing the samples in a low dimensional space (Greenacre & Primicerio 2014). Several ordinations with different distance measures were performed. From the binary data matrix a PCoA (Principal coordinates analysis) was created in PAST 3.01 (Hammer et al. 2001) with the Dice coefficient, which is a binary similarity measure, which puts more weight on shared presence of markers than on mismatches (Dice 1945). For each of the two independent scorings from GENEMAPPER, a PCA (Principal component analysis) was created in POLYSAT from a distance matrix using Bruvo distance (Bruvo et al. 2004). Bruvo distance is a distance measure designed for microsatellites that can be used for mixed ploidal levels. It assumes that the microsatellites evolve in: (1) a stepwise fashion, i.e. that alleles are created by addition or reduction of repeats, and (2) that mutations mainly arise through slipped-strand mispairing which usually are single-step mutations, e.g. assuming that the fewer repeats in difference the more similar are the alleles. The two ordinations were compared by using the R package VEGAN 2.0-10 (Oksanen et al. 2013) to perform a Procrustes Rotation, which rotates the ordination to obtain maximum fit to another ordination, and a Protest, which tests the significance (non-randomness) between the two ordinations (by repeatedly performing Procrustes analyses). The axes were also tested for correlation with Kendall's Tau (τ), which is a measure of association that is rank-based.

Population structure: In addition to the ordinations described in the previous paragraph, population structure was also investigated with the program STRUCTURE (Pritchard et al. 2000). Based on the Hardy-Weinberg (HW) assumption, STRUCTURE uses Bayesian clustering in order to find the optimal number of groups (=K) that the dataset can be divided into, and then assigns individuals to these groups. The program allows for ambiguity in polyploid heterozygotes by using the recessive allele option (Falush et al. 2007). The recessive allele option (recessivealleles=1) was chosen in addition to a tetraploid ploidal level (ploidy=4) because no individuals possessed more than four different alleles. Because we are dealing with closely related species, the admixture model and correlated frequencies were chosen, assuming that individuals can have originated from more than one group and allowing that the allele frequencies in the different populations can be quite similar (Falush et al. 2003). STRUCTURE was run with 10 runs for each K from K=1 to K=10 and from K=11 to K=20, using the Lifeportal at the University of Oslo (https://lifeportal.uio.no/). A total of 1 000 000 iterations was used with a burn-in of 100 000. For comparison STRUCTURE was also run for the binary matrix with ploidy=1 and without the recessive allele setting, but otherwise similar settings. To summarize the results from STRUCTURE a compressed (.zip) output file was uploaded to STRUCTURE HARVESTER (Earl & vonHoldt 2012). STURCTURE HARVESTER delivers a graph of mean likelihood and variance per K as well as having implemented the Evanno method to produce others graphs; e.g. delta K (Evanno et al. 2005). From the output of STRUCTURE HARVESTER, the optimal number of clusters was chosen. Most emphasis was put on delta K when choosing K, as it seems to be a reliable method for inferring the true K value (Evanno et al. 2005). Further, a run with the chosen K value was processed in DISTRUCT (Rosenberg 2004) to visualize the results. MICROSOFT EXCEL (2010) was also used to create a triangle diagram to visualize the STRUCTURE results.

Population differentiation: At the very heart of population genetics is the F-statistics described by Wright (1943). These statistics use the allele frequencies to calculate e.g. the genetic differentiation between populations (F_{ST}) (Wright 1949). Weir & Cockerham (1984) proposed an estimate of F_{ST} using an Analysis of Variance (ANOVA) approach (e.g. variance within and among populations), which is used in this study. While the main principles of F-statistics can be extended to autotetraploids with polysomic inheritance, there might still be a problem to estimate the allele frequencies if there is dosage uncertainty in the partial heterozygotes (Dufresne et al. 2014). In addition, it might be an issue with possible violations of polysomic inheritance (and assumption of HW).

Chromosomes in polyploids normally follow disomic or polysomic inheritance (Ronfort et al. 1998). Disomic inheritance is normally the presumed inheritance mode for allopolyploids and means that the two genomes, that the allopolyploid consists of, are inherited separately, i.e. with non-random segregation in meiosis. Polysomic inheritance is normally the presumed mode of inheritance for autopolyploids and implies that all e.g. four chromosomes in a tetraploid can pair in meiosis, i.e. with random segregation. Non-random versus random segregation in the meiosis require different estimations of e.g. allele frequencies and a model assuming wrong mode of inheritance would not return correct estimations. Rho (ρ) has proved to be the only population differentiation measure that is independent of ploidal level and type of inheritance (Meirmans & Van Tienderen 2013). An ANOVA approach has been proposed for the estimation of Rho_{ST} (Ronfort et al. 1998), which was used in this study.

SPAGEDI (Spatial Pattern Analysis of Genetic Diversity) offers a way to estimate the allele frequencies in polyploids by assuming that each of the alleles in a partial heterozygote has an equal likelihood of being present more than once (Dufresne et al. 2014) and can be used to compute genetic distances between populations or relatedness coefficients between individuals from codominant genotypes (Hardy & Vekemans 2002). By adding spatial coordinates in UTM format spatial distances can also be calculated. Spatial coordinates were converted from decimal degrees to UTM format by using a geographic/UTM Coordinate Converter (http://home.hiwaay.net/~taylorc/toolbox/geography/geoutm.html) and added to the input file.

On the population level pairwise F_{ST} and Rho_{ST} values were calculated in addition to the pairwise genetic distances Ds (Nei 1978) and dm2, i.e $(\delta\mu)^2$ (Feldman et al. 1997), and pairwise spatial distance. The latter is by default calculated in Euclidean distance (Hardy & Vekemans 2002). Both Ds and dm2 are measures of genetic differentiation between two populations, but while Ds is calculated on the assumption of the infinite allele model like F_{ST} and Rho-st, dm2 is based on the stepwise mutation model like Bruvo (Hardy et al. 2003). Ds and dm2 are developed for diploids, but have been applied for polyploid codominant data in Lo et al. (2009).

Isolation by distance: Isolation by distance as described by Wright (1943) assumes that when comparing pairs of populations, as geographic distance increases, so will genetic distance increase as a consequence of decreased dispersal. This was tested by comparing the genetic distances with the geographic distances using a mantel test (1000 randomizations) and performing reduced major axis (RMA) regression, using the ISOLATION BY DISTANCE web service

Version 3.23 (Jensen et al. 2005). The mantel test is testing the significance of the relationship and the RMA regression is measuring the strength of the relationship.

Genetic variation and diversity: Genetic diversity was assessed by counting the number of unique alleles as well as calculating expected heterozygosity (H_E) and the inbreeding coefficient (F_{IS}) for each population, using SPAGEDI. The expected heterozygosity is in SPAGEDI corrected for by sample size according to Nei (1978). Total number of alleles for each population was calculated in POLYSAT.

In an Analysis of Molecular Variance, AMOVA, the variance is hierarchically divided to the levels specifically defined. Two AMOVAs were performed in ARLEQUIN ver. 3.5.1.2 (Excoffier & Lischer 2010) for the binary matrix, one for regions, and one for subspecies. MICROSOFT EXCEL (2010) was used to create pie charts of the AMOVA results.

Trees and networks: SPLITSTREE4 is a software designed to infer phylogenetic networks and trees from e.g. distances (Huson & Bryant 2006). From the distances splits are calculated, meaning that the largest splits represent the largest distances. In the present study distances from SPAGEDI were applied to perform neighbour nets in SPLITSTREE4, with each end node representing a population.

RAD-seq data analysis

Processing the raw data: Raw Illumina reads returned from the sequencing lab were processed with STACKS version 1.19, which is a pipeline program consisting of components to build loci and identify SNPs i.e. single nucleotide polymorphisms (Catchen et al. 2011, Catchen et al. 2013). To find the individuals/barcodes (demultiplex) and remove the low quality data (clean) the program PROCESS_RADTAGS.PL was used. Next the program DENOVO_MAP.PL was used. DENOVO_MAP.PL executes the task of three programs with one command; unique stacks (USTACKS), catalog stacks (CSTACKS) and search stacks (SSTACKS). USTACKS aligns the reads in so-called stacks. The stacks are compared to find loci. In the loci SNPs are detected by a method using maximum likelihood. CSTACKS creates a catalog with consensus loci from the output of USTACKS. SSTACKS searches the stacks created in USTACKS against the catalog created in CSTACKS. In the next step the individuals were linked to their respective populations and output files in STRUCTURE and PHYLIP format were created with the program POPULATIONS. POPULATIONS also calculates population statistics, like F_{ST} values. These could, however, not be used for the *Cochlearia* dataset because STACKS assumes that the individuals are diploids. For more information about what options used in the STACKS pipeline see Appendix Table A3.

For the network construction, the entire PHYLIP file was used. For the STRUCTURE analyses and ordination analyses, the STRUCTURE output file was used after reducing the number of SNPs in the following way: (1) for the tetraploids, only SNPs with data for at least four individuals in each population were used further, and (2) for all individuals, only SNPs with data for at least four individuals in each of the following groups: *C. aestuaria*, *C. pyrenaica*, each of the tetraploid populations and *C. anglica* were used further. In the STRUCTURE output file from STACKS there is two lines for each individual with putatively different character states, for the ordination one of these alleles were chosen at random for each SNP.

Multivariate analyses: The reduced and randomized SNP dataset was used to create a PCoA in PAST. As distance measure Jukes Cantor was used. This is a distance that is measured for sequence data by assuming similar probability for each nucleotide change (no difference between transversions and transitions) and taking into account the probability of reversals.

Population structure: For STRUCTURE the reduced datasets were run for each K from K=1 to K=10 by using the Lifeportal at the University of Oslo. Ambiguity in polyploid heterozygotes was allowed for by using the recessive allele option. All individuals were considered as tetraploids (ploidy=4). The admixture model and correlated frequencies were chosen. In total 1 000 000 iterations and burn-in of 100 000 was used. Results were summarized in STRUCTURE HARVESTER and after the evaluation of the likelihood graphs and graphs produced by the Evanno method, visualized in DISTRUCT.

Trees and networks: SPLITSTREE4 can also infer networks and trees from SNP or sequence data. From these characters a distance matrix is created from which the splits further are created. For the SNP data distances were calculated by Uncorrected_P distance. For all individuals a network was performed with each end nodes representing a population. For the tetraploids alone, a network was performed with each end node representing an individual.

Results

In the result part of the present study these abbreviations will be used: (1) *officinalis* for *C. officinalis* ssp. *officinalis,* (2) *norvegica* for *C. officinalis* ssp. *norvegica,* and (3) *integrifolia* for *C. officinalis* ssp. *integrifolia.*

Flow cytometry

Altogether 86 individuals from 16 populations had ploidal level successfully estimated by flow cytometry. Ploidal level is reported for populations only (Table 3), because all individuals in each population, with one exception, had the same ploidy level. The exception is one *C. officinalis* individual in the population from Finnmark (offnorfin), which was noted as aneuploid. The ploidy estimations are in agreement with what was expected based on morphology and ecology. A high amount of endopolyploidy, i.e. within-individual occurrence of cells with higher ploidy, often as a result of endoreduplication where chromosomes replicate without subsequent nucleus and cell division (Barow 2006), was also noted. A tetraploid individual could e.g. have cells with ploidy of 4x, 8x, 16x etc., with the lowest peak in the histogram i.e. the lowest ploidal level, corresponding to the ploidy of the individual. In some of the samples examined less than 10 % of the cells had the ploidy that corresponded to the individual.

For the reduced number of samples (21 individuals from 16 populations), which were analysed for relative fluorescence intensities using an internal standard, ploidal levels are indicated by the graph in Fig. 6. Compared to the internal standard, diploids had a ratio between 0.43 and 0.48, tetraploids (without the presumed aneuploid) a ratio between 0.82 and 0.95, hexaploids a ratio between 1.24 and 1.43, and the only octoploid individual included had a ratio of 1.72. The aneuploid individual had a ratio of 1.15. Most variation in relative fluorescence intensity was found among the hexaploids (1.24-1.43). Coefficient of variation (CV) of the histogram peaks was less than 5 %.

Table 3. Ploidy estimation based on relative flourescence intensities of 16 *Cochlearia* populations (86 individuals) from flow cytometry analysis without an internal standard. For detailed locality information, see Appendix Table A2. *Population offnorfin contained one aneuploid.

Population	Ploidy	Taxon	No. of individuals
aesES1	2x	*C. aestuaria*	4
aesES2	2x	*C. aestuaria*	3
aesES3	2x	*C. aestuaria*	5
angSE	8x	*C. anglica*	4
holDK	6x	*C.* x *hollandica (officinalis x anglica)*	5
offinttro1	4x	*C. officinalis* ssp. *integrifolia*	3
offinttro2	4x	*C. officinalis* ssp. *integrifolia*	5
offnorfin*	4x	*C. officinalis* ssp. *norvegica*	8
offnortro1	4x	*C. officinalis* ssp. *norvegica*	9
offnortro2	4x	*C. officinalis* ssp. *norvegica*	10
offnortro3	4x	*C. officinalis* ssp. *norvegica*	8
offofflis1	4x	*C. officinalis* ssp. *officinalis*	1
offofflis2	4x	*C. officinalis* ssp. *officinalis*	1
offofftro1	4x	*C. officinalis* ssp. *officinalis*	7
offofftro2	4x	*C. officinalis ssp. officinalis*	8
offofftro3	4x	*C. officinalis* ssp. *officinalis*	5

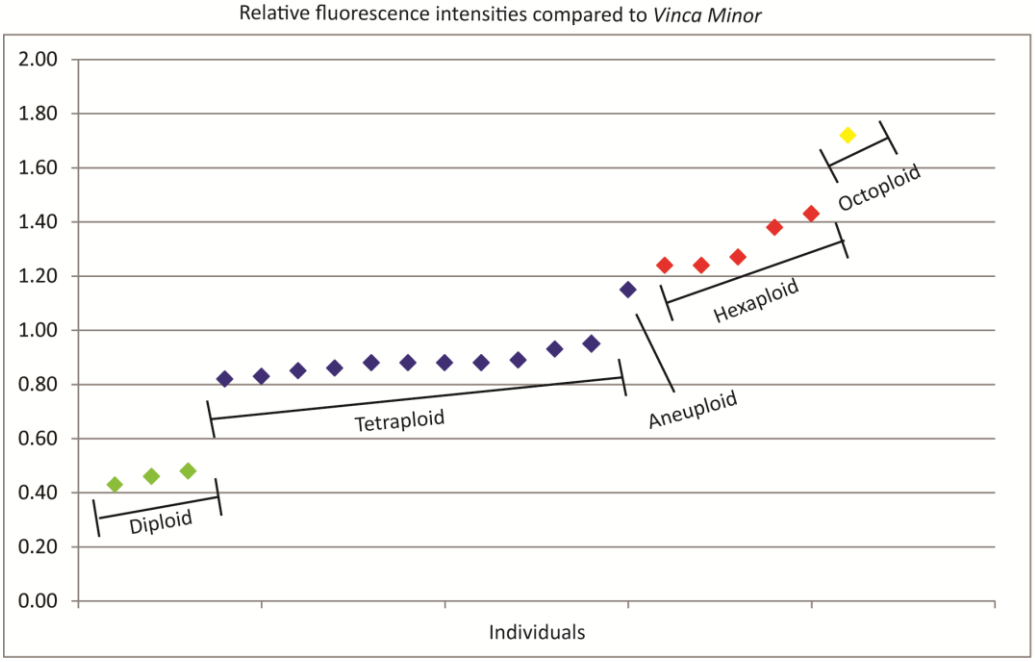

Figure 6. Ploidy estimation based on relative flourescence intensities of 16 *Cochlearia* populations (21 individuals) from flow cytometry analysis with an internal standard. For detailed locality information, see Appendix Table A2. Ploidal levels are indicated.

Microsatellite analyses

Nine of the 20 microsatellites tested were successfully PCR amplified and visible after the gel electrophoresis. Of these only six were both polymorphic and possible to score reliably with GENEMAPPER. These six microsatellites possessed from two to 41 alleles. In total 105 alleles were scored for 177 individuals.

Multivariate analyses: The PCA analyses based on the two datasets from the two different scorings of the microsatellite allele sizes (Appendix Fig. A1) are clearly correlated, but not entirely identical (which would mean a Procrustes Sum of Square of 0). The highest correlation values are between the two first axes and the second highest is between the two second axes, where the former is a quite strong correlation (0.67τ) and the latter is a weak correlation (0.30τ) (Appendix Fig. A1).

The different ordinations performed (PCoA and PCA) showed similar patterns, hence only the PCoA will be presented further.

The PCoA performed for all individuals on the binary data matrix, shows that all the samples collected are genetically close, with no distinct groupings, and little variation explained by the two first axes (11.0% and 6.1%, respectively). Nevertheless, some trends can be inferred. Although overlapping, samples with the same ploidal level are more or less grouped together (Fig. 7). The tetraploids show the greatest variation (but this is also the ploidal level with most samples in this study). When ecology of the different samples is imposed onto the plot, samples sharing the same ecology are not grouped together (Appendix Fig. A2). Individuals from the same population are situated close to each other, but are extensively overlapping with individuals from other populations (Appendix Fig. A3).

In the PCoA analyses performed for the tetraploids alone, there is a tendency that the three subspecies of *C. officinalis* can be distinguished (Fig. 8). Though there is also much overlap in this ordination and low variation explained along the two first axes (8.1% and 6.9% respectively). Subspecies *integrifolia* is most distinct with many samples located toward the right side of the plot, whereas the most of the *officinalis* and *norvegica* samples are found at the left side of the plot and are quite overlapping.

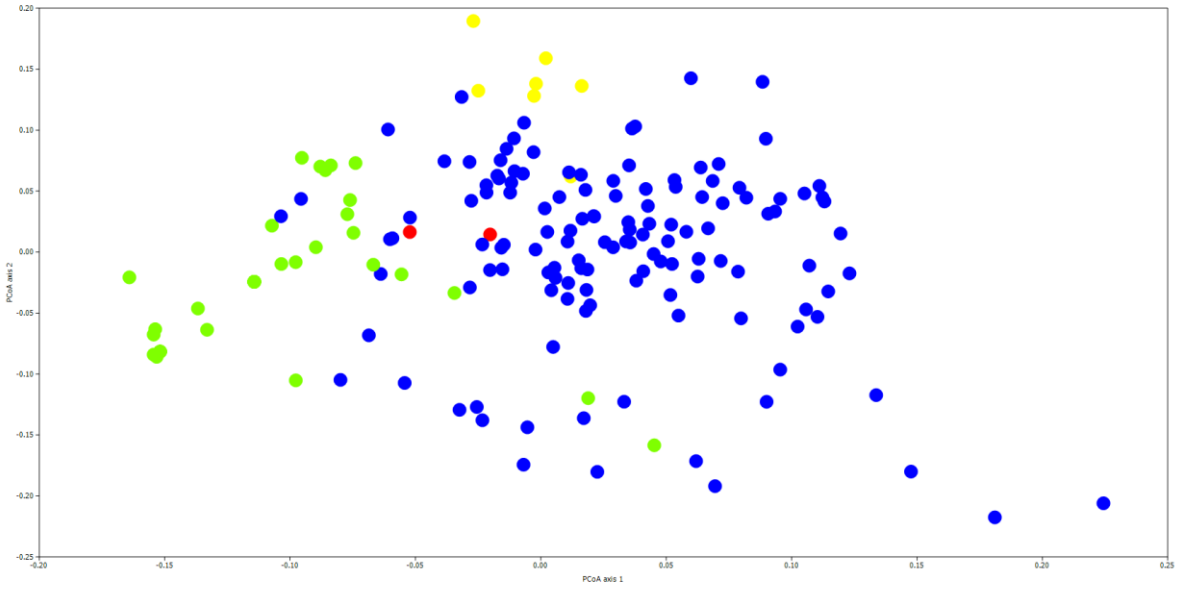

Figure 7. PCoA performed in PAST for all 177 *Cochlearia* indiviuals, using the binary microsatellite dataset and Dice similarity. The first PCoA axis explains 11.0 % of the total variation in the dataset, the second axis 6.1 %. Colour representation is according to ploidal level: diploids – green, tetraploids – blue, hexaploids – red and octoploids – yellow.

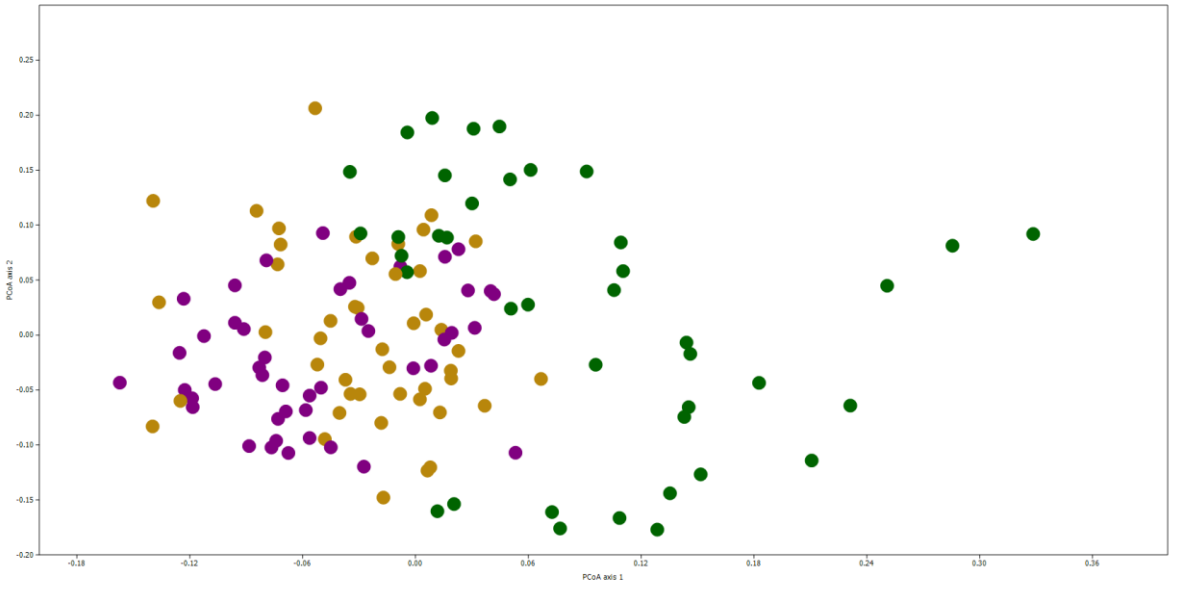

Figure 8. PCoA performed in PAST for 125 tetraploid *Cochlearia officinalis* individuals, using the binary microsatellite dataset and Dice similarity. The first PCoA axis explains 8.1 % of the total variation in the dataset, the second axis 6.9 %. Colour representation is according to subspecies: *norvegica* – purple, *officinalis* – dark yellow and *integrifolia* – dark green.

Population structure: In the Bayesian clustering analysis performed by STRUCTURE for all individuals on the dataset with allele sizes, K=1 to K=10, both K=2 and K=6 were chosen to be visualized by DISTRUCT based on the delta K graph and the mean likelihood of K graph (a-b in Appendix Fig. A4). K=2 has the highest delta K value, while K=6 (which has a minor peak in the delta K graph) has a higher value in the likelihood of K graph. When two groups are selected (K=2), the tetraploids are suggested as one group, and the diploids and the octoploids are suggested as another group (Fig. 9, top). The only exceptions are most of the individuals in one of the tetraploid *C. officinalis* populations from Lista, Southern Norway (offofflis1), as well as a few other single tetraploid individuals, which is grouped together with the octoploid, hexaploid and diploid populations. When six groups are selected (K=6), the tetraploid group is split into four groups (which will be further dealt with below), and the diploid/hexaploid/octoploid group is split into two groups. Of these, one group (orange) includes diploid *C. pyrenaica* and *C. aestuaria* from Spain, whereas the other group (brown) includes individuals of diploid *C. pyrenaica* from France, diploid *C. groenlandica*, hexaploid *C.* x *hollandica* and octoploid *C. anglica* (Fig 9, middle).

When even higher Ks (K=11 to K=20) were tested on the same dataset, K=14 was selected based on delta K (c in Appendix Fig. A4). The highest mean likelihood of K is reached with K=17 (d in Appendix Fig. A4). As a minor peak is found in the delta K graph at K=17 (c in Appendix Fig. A4), both values of K were visualized with DISTRUCT, but K=17 did not add much meaningful structure in comparison to the groups already presented with K=14 and is not displayed or discussed. When 14 groups are selected (K=14) one of the *C. aestuaria* populations from Spain (aesES2) is separated from the other diploid individuals from Spain (Fig. 9, bottom), and the group (from K=6) consisting of *C.* x *hollandica*, *C. groenlandica*, *C. anglica* and *C. pyrenaica* from France is split into three groups; *C.* x *hollandica* and *C. pyrenaica* from France group together, the two *C. groenlandica* populations group together and *C. anglica* constitute a group on its own (Fig. 9, bottom).

When the tetraploids were analysed alone (dataset with allele sizes, K=1 to K=10, K=3 was chosen based delta K (e in Appendix Fig. A4). The result is visualized in three ways; with DISTRUCT (Fig. 10, top), pie charts on a map over Northern of Norway (Fig. 10, middle) and as a cluster triangle (Fig 10, bottom). It is not full correspondence between the three STRUCTURE groups and the three subspecies. Several individuals in some of the populations show high admixture to two or three of the groups. Admixture is especially high in *officinalis* from Lofoten and one of the *officinalis* populations from Troms (offofftro2). Most of the individuals of the second *officinalis* population from Troms (offofftro1) mainly allocate to the yellow group, which is also highly represented in the admixed *officinalis* population from Troms. Furthermore the individuals from the two *norvegica* populations from Troms also mainly allocate to the yellow group. The individuals of the *norvegica* populations from Lofoten mainly allocate to the green group together with one of the *integrifolia* populations from Lofoten (offintlof2). This latter population has the least degree of admixture of all population. The other *integrifolia* population from Lofoten (offintlof1) is highly admixed, among others the red group is represented, which is the group that the individuals of the *integrifolia* populations from Troms mainly are allocated to.

When the six groups are selected (K=6) in the analysis including all individuals (Fig. 9, middle), the tetraploids constitute four groups, where two groups are more or less unique for Troms and the other two are more or less unique for Lofoten. Overall similar patterns are observed here. The main difference is that the *norvegica* populations in Lofoten and offintlof2 is not grouped together like before, offintlof2 is distinguished (green group) and individuals of the *norvegica* populations in Lofoten are mainly allocated to another group (light blue).

When the three STRUCTURE groups are imposed onto the map, the geographical structure of the genetic structure is visualized more clearly (Fig. 10, middle). Starting furthest North, the red group is dominating in the two *integrifolia* populations in Troms, but is also represented in the geographically close *officinalis* population (offofftr02). The yellow group is highly represented in the two *norvegica* populations in Troms, but also in the geographically close *officinalis* population (offofftr01). Further South the yellow group is also relatively highly represented in the admixed *officinalis* populations from Lofoten as well as in the admixed *integrifolia* population (offintlof1). The green group is highly represented in the two *norvegica* populations and one of the *integrifolia* populations in Lofoten (offintlof2), all of which are geographically closely related, but otherwise found with some degree of admixture throughout all populations.

In the cluster triangle (Fig. 10, bottom) the *norvegica* and *integrifolia* individuals are found towards the corners of the triangle, while the *officinalis* individuals occupy positions towards the middle of the triangle.

The STRUCTURE analyses run for all individuals or tetraploids alone, on the binary dataset (K=1 to K=10), show almost identical patterns to the corresponding analyses based on allele sizes and the results are not displayed.

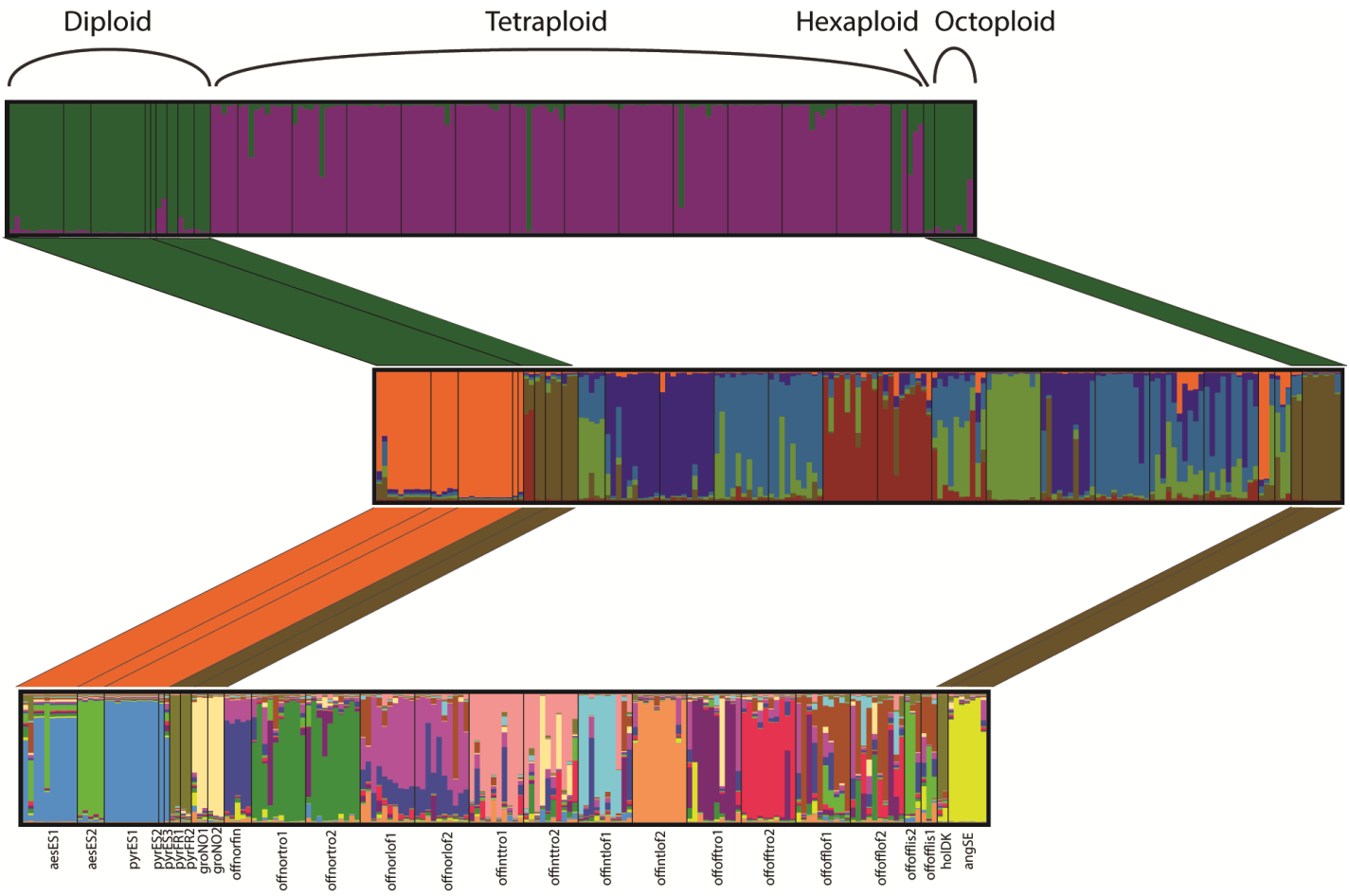

Figure 9. STRCUTURE runs visualized by DISTRUCT for K=2 (top), K=6 (middle) and K=14 (bottom) for all 177 *Cochlearia* individuals using the microsatellite dataset with allele sizes. Each individual is represented by a bar and colours represent the proportional assignment to the STRUCTURE groups (K). Populations are separated by a black line. See Appendix Table A2 for detailed population information. Ploidal levels are indicated on the figure. Coloured lines between top, middle and bottom indicate how the non-tetraploids group in K=2 is split in further groups in K=6 and K=14; from the top figure to the middle figure, the green group is split into two groups; orange and brown. From the middle figure to the bottom figure the orange group is split into two groups; blue and green, while the brown group is split into three groups; light brown, offwhite and yellow.

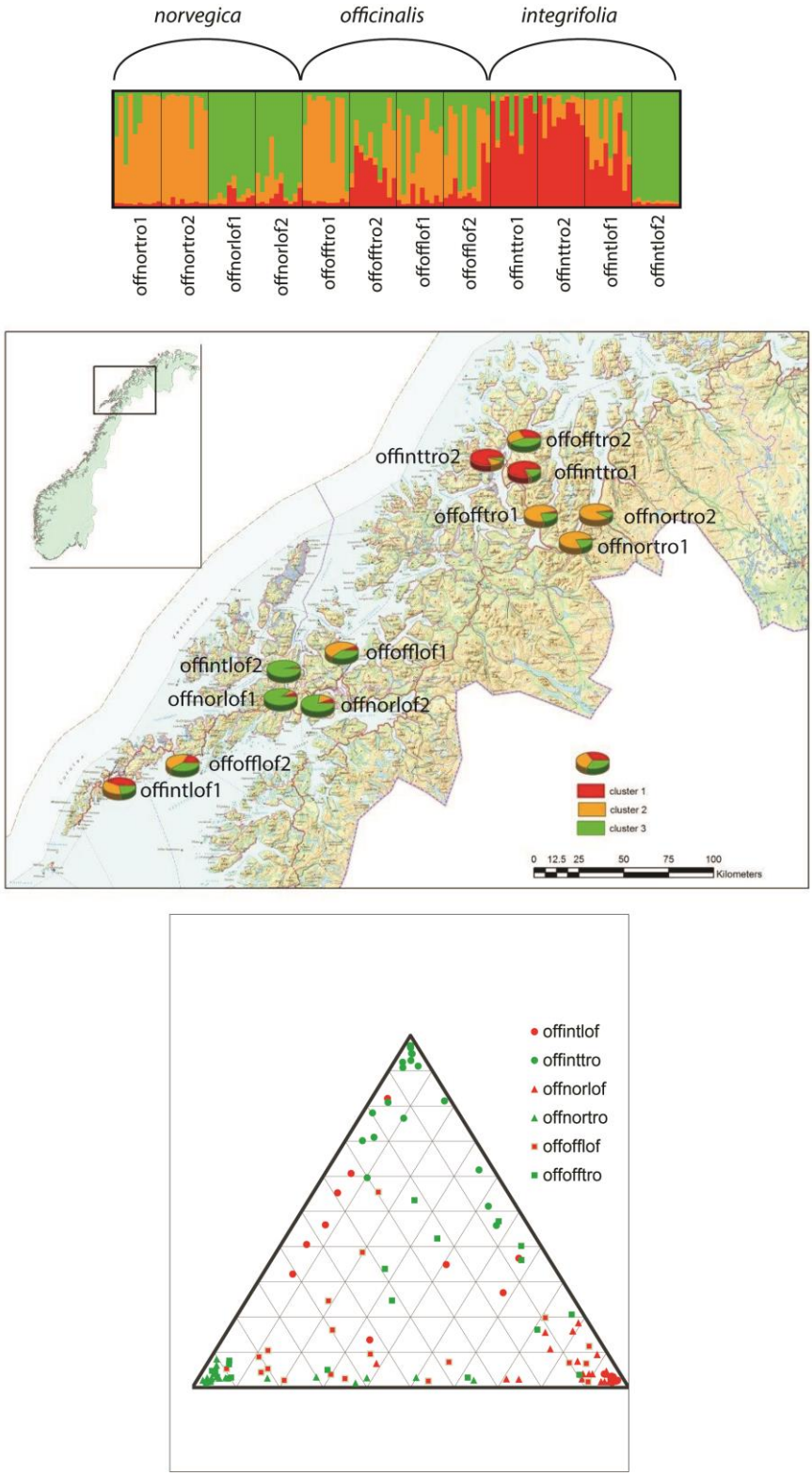

Figure 10. STRUCTURE run for K=3 for 120 tetraploid *Cochlearia officinalis* individuals, using the microsatellite dataset with allele sizes. Top: visualized by DISTRUCT. Each individual is represented by a bar and colours represent the proportional assignment to the STRUCTURE groups. Populations are separated by a black line. See Appendix Table A2 for detailed population information. Middle: visualized on the map of Northern Norway with ARCMAP. Each population is represented by a pie chart, which shows the allocation to the three STRUCTURE groups. Bottom: visualized with a cluster triangle created in MICROSOFT EXCEL. Subspecies (*integrifolia*, *norvegica* and *officinalis*) are indicated by different symbols, whereas regions (Lofoten and Troms) are indicated by different colours.

Population differentiation: For measuring population differentiation the mean Rho_{ST} and the mean F_{ST} values for each population are presented (Table 4). Population differentiation seem to be highest for the *integrifolia* populations from Troms (Rho_{ST}: 0.75 and 0.64, F_{ST}: 0.29 and 0.26) and one of the *integrifolia* populations from Lofoten (Rho_{ST}: 0.85, F_{ST}: 0.35), when compared to the other populations.

Table 4. Genetic diversity and differentiation indices calculated for 120 tetraploid *Cochlearia* officinalis individuals, using the microsatellite dataset with allele sizes. N=number of samples; TA=total number of alleles; U=number of unique alleles; Mean F_{ST}= mean population differentiation; F_{IS}=inbreeding coefficient; Mean Rho_{ST}=mean population differention; H_E=gene diversity, corrected by sample size (Nei 1978).

Pop	N	TA	U	Mean F_{ST}	F_{IS}	Mean Rho_{ST}	H_E
offofftro1	10	25	0	0.171	0.052	0.415	0.5480
offnortro1	10	28	0	0.140	0.138	0.329	0.6078
offnortro2	10	30	0	0.133	-0.184	0.385	0.6379
offinttro1	10	35	3	0.292	-0.214	0.755	0.6688
offinttro2	10	39	1	0.257	0.071	0.641	0.6350
offofftro2	10	26	1	0.187	-0.051	0.444	0.5633
offofflof1	10	36	2	0.139	0.031	0.343	0.5744
offintlof1	10	39	6	0.139	-0.066	0.338	0.6538
offnorlof1	10	36	3	0.145	-0.119	0.388	0.5783
offintlof2	10	18	1	0.346	0.005	0.846	0.4196
offnorlof2	10	33	1	0.172	-0.252	0.485	0.5905
offofflof2	10	40	5	0.108	0.116	0.254	0.6721

Isolation by distance: The comparison of the genetic and the geographic distances (Appendix Table A4) by a mantel test did not show a significant correlation (r=0.1192, p=0.2190). The RMA regression present a positive relation between genetic and geographic distance ($y=3.294e^{-6}x$ - 0.09930; Appendix Fig. A5). However, r^2 ,which is a goodness of fit measure for the regression, is close to zero (0.0142), indicating that the regression is not a very good fit and not explaining much of the variation observed. The dm2 distance did not give a significant correlation with geographic distance either and is not shown.

Genetic variation and diversity: The estimated heterozygosity (H_E) for the tetraploids shows quite similar values for the different populations (Table 4). Most of the values are between 0.55 and 0.65. The lowest value (0.42) is found for one of the *integrifolia* populations from Lofoten (offintlof2), and the highest value (0.67) is found for one of the *officinalis* populations from Lofoten (offofflof2). The inbreeding coefficient (F_{IS}) shows positive or negative values close to zero in the tetraploid populations. Total number of alleles is varying between 18 and 40. Some populations have unique alleles, with six unique alleles found at most in one of the *integrifolia* populations from Lofoten (offintlof1).

The AMOVA performed to test the effect of regions on the genetic variation shows that the largest proportion of the molecular variance in the tetraploids is found within populations (70%), whereas the 25% is found among populations and 5% among regions (Appendix Fig. A6). The AMOVA performed to test the effect of subspecies on the genetic variation shows that the largest proportion of molecular variance in the tetraploids is within populations (71%),

whereas 24% is found among populations and 5 % is found among subspecies (Appendix Fig. A7).

Trees and networks: The networks created with dm2 distance didn't give any geographical or taxonomical structure and is not shown.

Looking at the neighbour net performed by SPLITSTREE for tetraploids (using Ds distance), *integrifolia* is found at one side of the network, and *officinalis* and *norvegica* are found at the other (Fig. 11). The largest split in the network separate the two *integrifolia* populations from Troms and one of the *integrifolia* populations from Lofoten (offintlof2), respectively, from the remaining populations. The second *integrifolia* population from Lofoten (offintlof1) is closer to the *officinalis – norvegica* group.

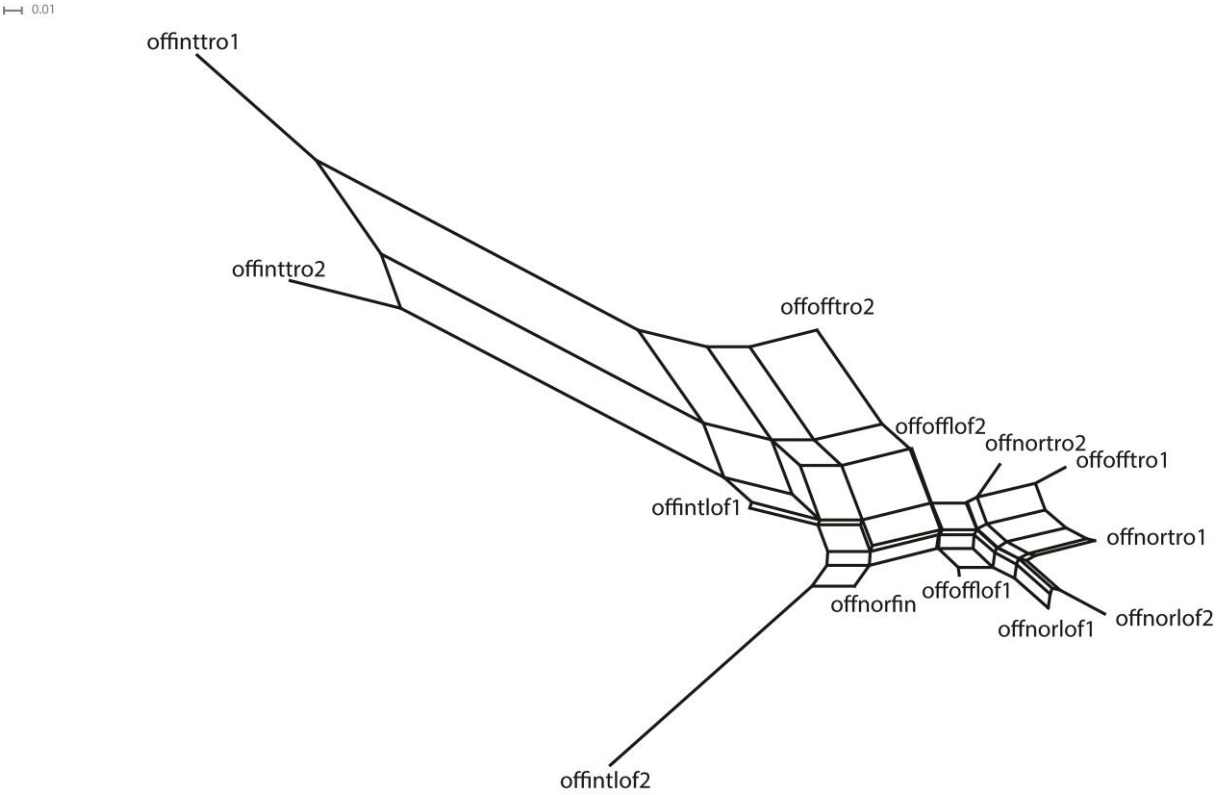

Figure 11. Neighbour net performed in SPLITSTREE for 125 tetraploid *Cochlearia officinalis* individuals, using Ds genetic distances produced in SPAGEDI from the microsatellite dataset using allele sizes. Each end node represents a population.

RAD-seq analyses

The first RAD-seq library (with rare cutter SbfI) contained only 601 SNPs after processing in STACKS and as initial network and ordination analyses showed no relevant structure, this dataset was not used further.

From the second RAD-seq library (with frequent cutter PstI) the STRUCTURE output file from STACKS for all individuals contained 85 193 SNPs, and the PHYLIP output file contained 5782 SNPs (only including nucleotides that are fixed within or variable among populations). The STRUCTURE output file from STACKS for the tetraploids contained 28 963 SNPs and the PHYLIP output file contained 51 929 SNPs (including variable sites).

Multivariate analyses: The ordinations performed from the RAD-seq dataset group the data in distinct groups. In the PCoA performed for all individuals (reduced dataset with 11 223 SNPs), the ploidal levels are visible as clear groups; diploid, tetraploid and octoploid (Fig. 12) and variation explained by the first axes are 13.3% and 12.1% respectively.

In the PCoA performed for the tetraploids alone (reduced dataset with 8 661 SNPs), the populations come out as non-overlapping groups in the ordination, with one of the *integrifolia* populations from Lofoten (offintlof2) distinguished markedly from the remaining populations along the first axis. The *officinalis* populations are placed adjacent to each other, whereas the two *norvegica* populations are separated along the second axis (Fig. 13). Variation explained by the two first axes are 16.2% and 13.8% respectively.

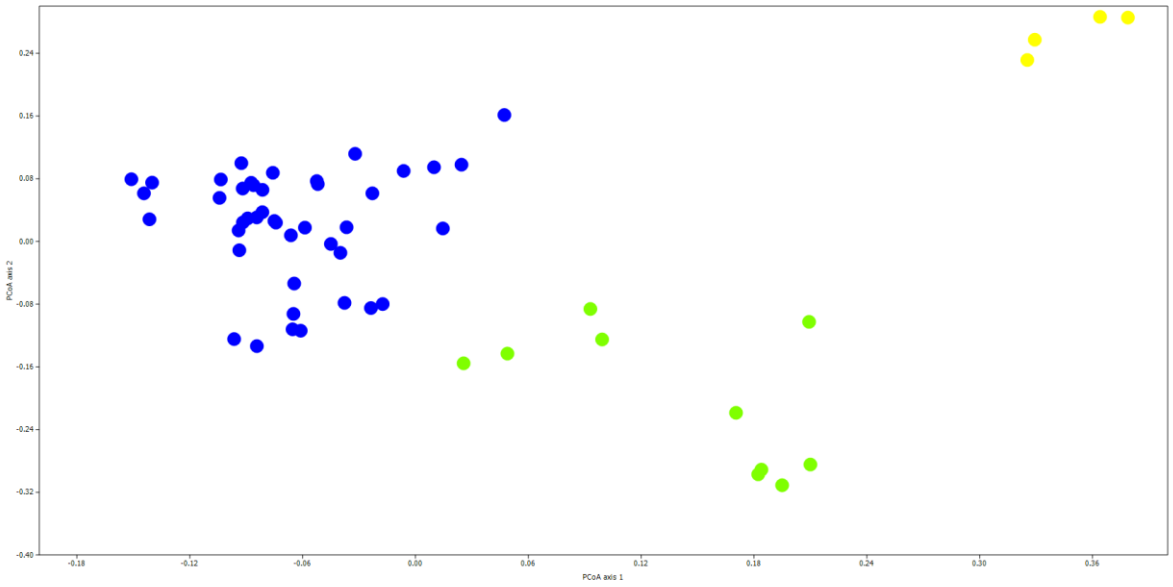

Figure 12. PCoA performed in PAST for 57 *Cochlearia* individuals using the reduced dataset (11 223 SNPs) from RAD-seq and Jukes-Cantor distance measure. The first PCoA axis explains 13.3 % of the total variation in the dataset, the second axis 12.1 %. Colour representation is according to ploidal level: diploids – green, tetraploids – blue and octoploids – yellow.

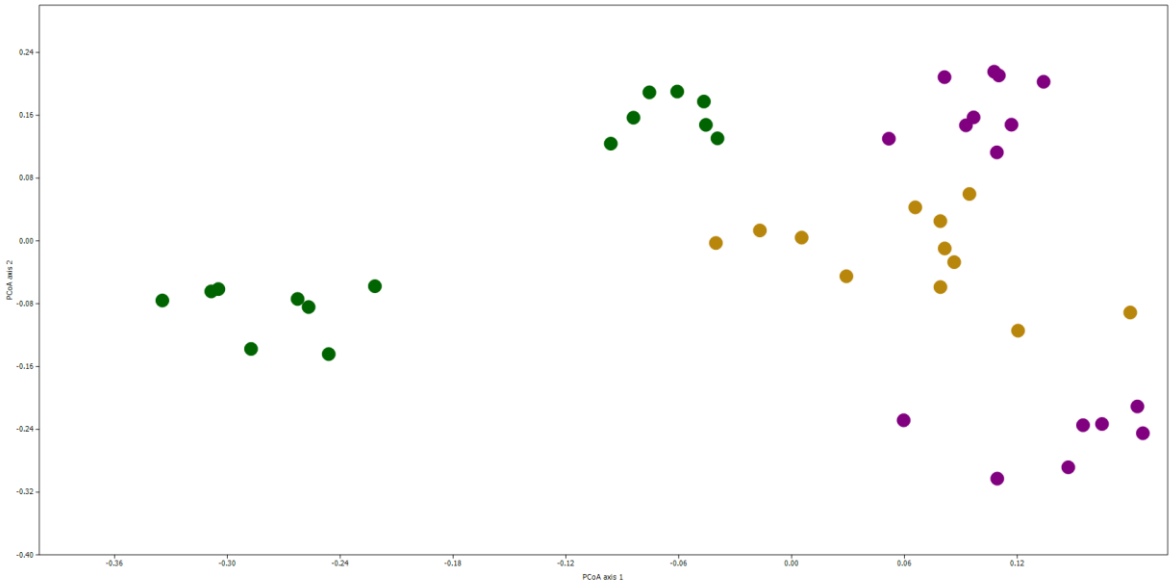

Figure 13. PCoA performed in PAST for 43 tetraploids *Cochlearia officinalis* individuals, using the reduced the dataset (8 661 SNPs) from RAD-seq and Jukes-Cantor distance measure. The first PCoA axis explained 16.2 % of the total variation in the dataset, the second axis 13.8 %. Colour representation is according to subspecies: *norvegica* – purple, *officinalis* – dark yellow and *integrifolia* – dark green.

Population structure: In the Bayesian clustering analysis performed in STRUCTURE on all individuals (using the reduced dataset with 11 223 SNPs, K=1 to K=10), K=2 was selected based on delta K (a in Appendix Fig. A8). As a minor peak is seen in the delta K graph for K=9 (with higher mean likelihood value, b in Appendix Fig. A8), both values of K were visualized by DISTRUCT. When two groups are selected (K=2) (Fig. 14, left), one group comprises all diploids as well as the octoploids, although some individuals (especially *C. pyrenaica* individuals) display admixture of both groups. The other group contains mainly all the tetraploid populations. When nine groups are selected (K=9), the diploid/octoploid group is split into one (gray) group for *C. aestuaria*, one (yellow) group for *C. anglica*, one (light green) group for *C. pyrenaica* from Spain and one (red) group for *C. pyrenaica* from France, although again with considerable admixture with other groups (Fig. 14, right). The tetraploid group is further divided into five groups, with the four populations of *integrifolia* and *norvegica* largely allocated each to a specific group, whereas the two *officinalis* populations are highly admixed to all other tetraploid groups in addition to a specific fifth group.

In the separate analysis for the tetraploids (using the reduced dataset with 8 661 SNPs, K=1 to K=10), K=4 was selected based on delta K and the mean likelihood value (c-d in Appendix Fig. A8). As the delta K graph also had a high peak for K=2, both values of K were visualized by DISTRUCT. When two groups (K=2) are selected, the *integrifolia* population from Lofoten is distinguished as one group, whereas the remaining populations mainly allocate to the other group, although with some admixture in most populations (Fig. 16, right). When four groups are selected (K=4), a similar pattern is seen as in the analyses for all individuals (K=9): the four populations of *integrifolia* and *norvegica* are each allocated to a specific group, while the two *officinalis* populations are highly admixed, allocated to all four of the groups (Fig. 16, left).

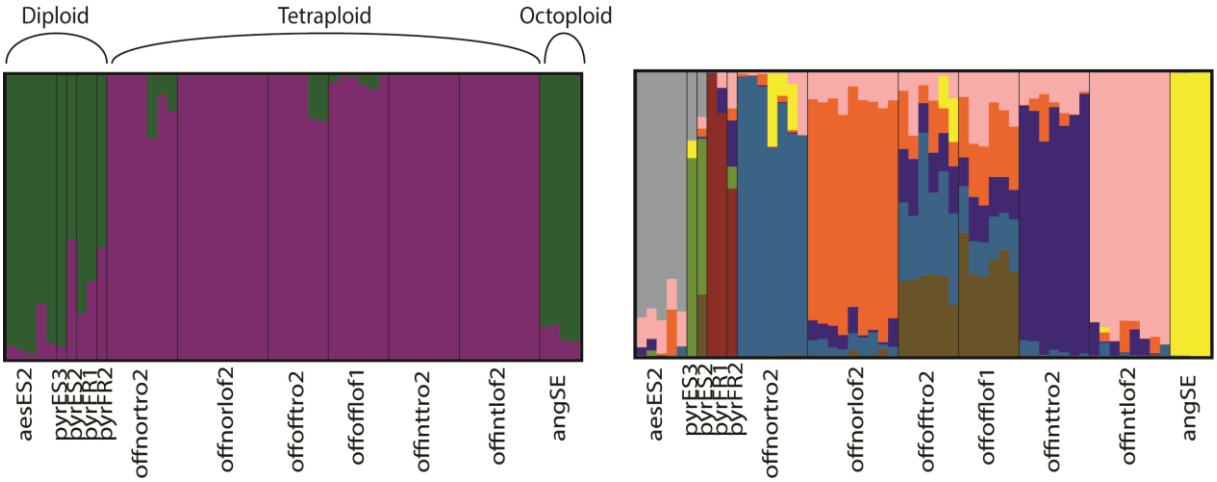

Figure 15. STRUCTURE run visualized by DISTRUCT for K=2 (left) and K=9 (right), for 57 *Cochlearia* individuals, using the reduced dataset (11 223 SNPs) from RAD-seq. Each individual is represented by a bar and colours represent the proportional assignment to the STRUCTURE groups. Populations are separated by a black line. See Appendix Table A2 for detailed population information.

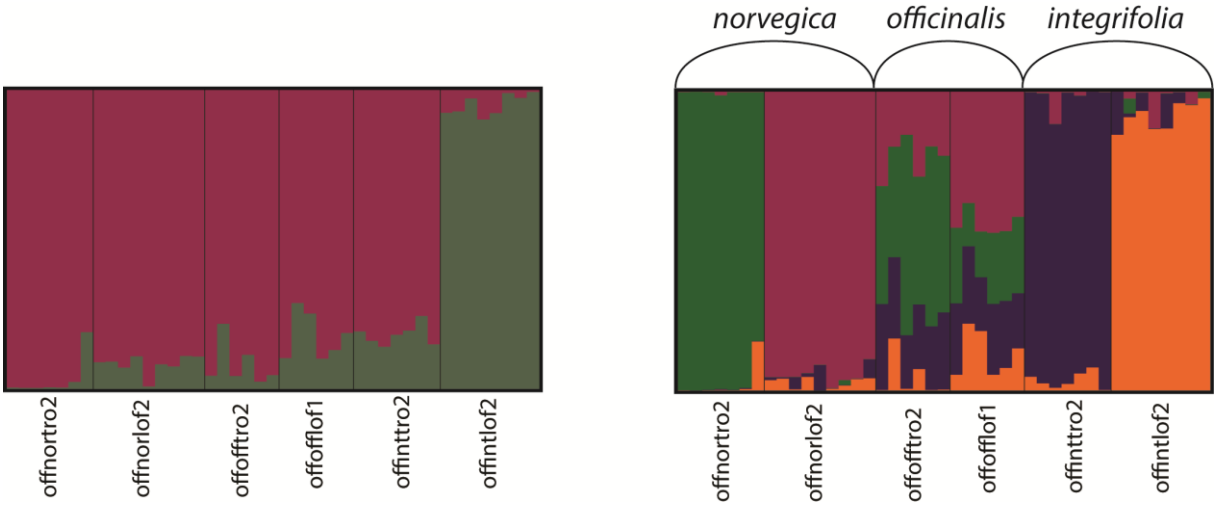

Figure 16. STRUCTURE run visualized by DISTRUCT for K=2 (left) and K=4 (right), for 43 tetraploids *Cochlearia officinalis* individuals using the reduced (8 661 SNPs) dataset from RAD-seq. STRUCTURE run visualized by DISTRUCT for K=2 (left) and K=9 (right). Each individual is represented by a bar and colours represent the proportional assignment to the STRUCTURE groups. Populations are separated by a black line. See Appendix Table A2 for detailed population information.

Trees and networks: In the neighbour net performed by SPLITSTREE for all individuals (5782 SNPs), a clear split separates the tetraploid populations from the diploid and octoploid populations (Fig. 17). Within the diploid/octoploid group there are long splits supporting four groups; *C. aestuaria*, *C. pyrenaica* from Spain, *C. anglica* and *C. pyrenaica* from France. There is also a smaller split supporting the diploids from Spain as a group (*C. pyrenaica* and *C. aestuaria*).

A separate neighbour net was produced for the tetraploids alone (51 929 SNPs), in which the six largest splits support groups corresponding to each of the six tetraploid populations (Fig. 18). No other large splits are apparent, e.g. a separation of the two regions (Troms and Lofoten) is only supported by a minor split.

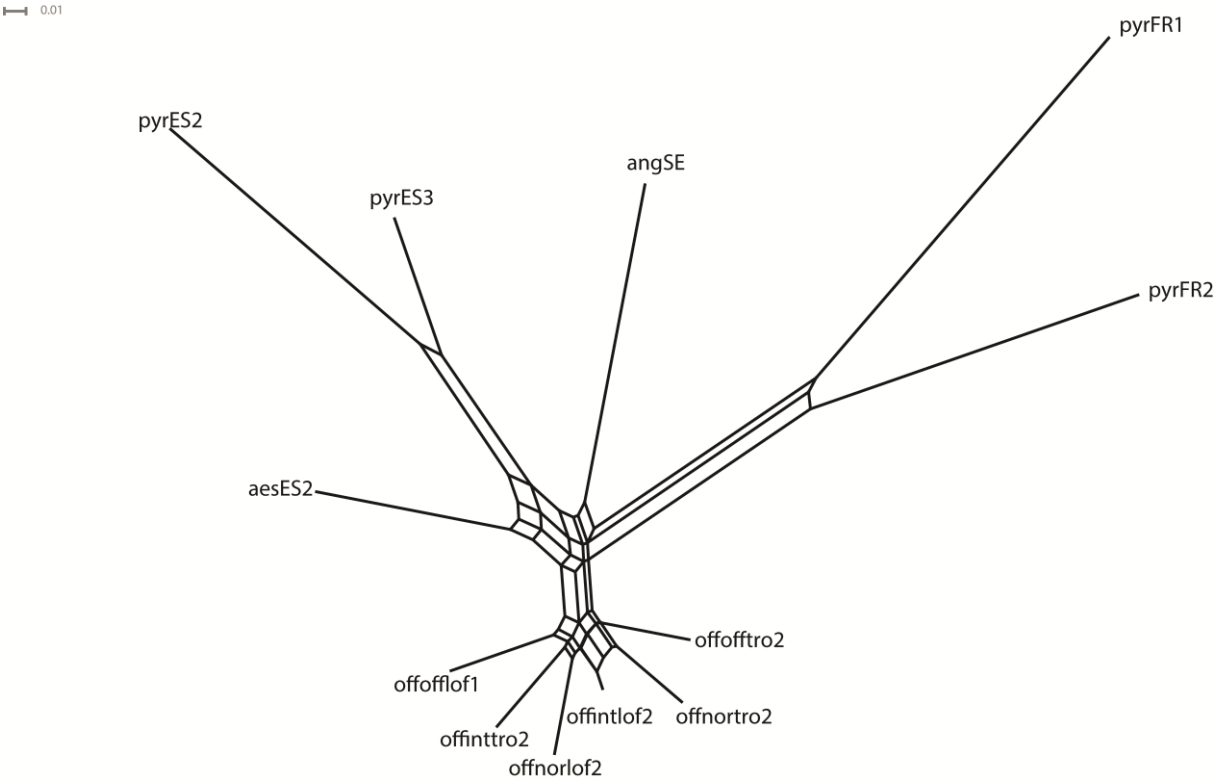

Figure 17. Neighbour net performed in SPLITSTREE for 57 *Cochlearia* individuals using the full dataset (5782 SNPs) from RAD-seq and the Uncorrected_P distance measure. Each end node represents a population.

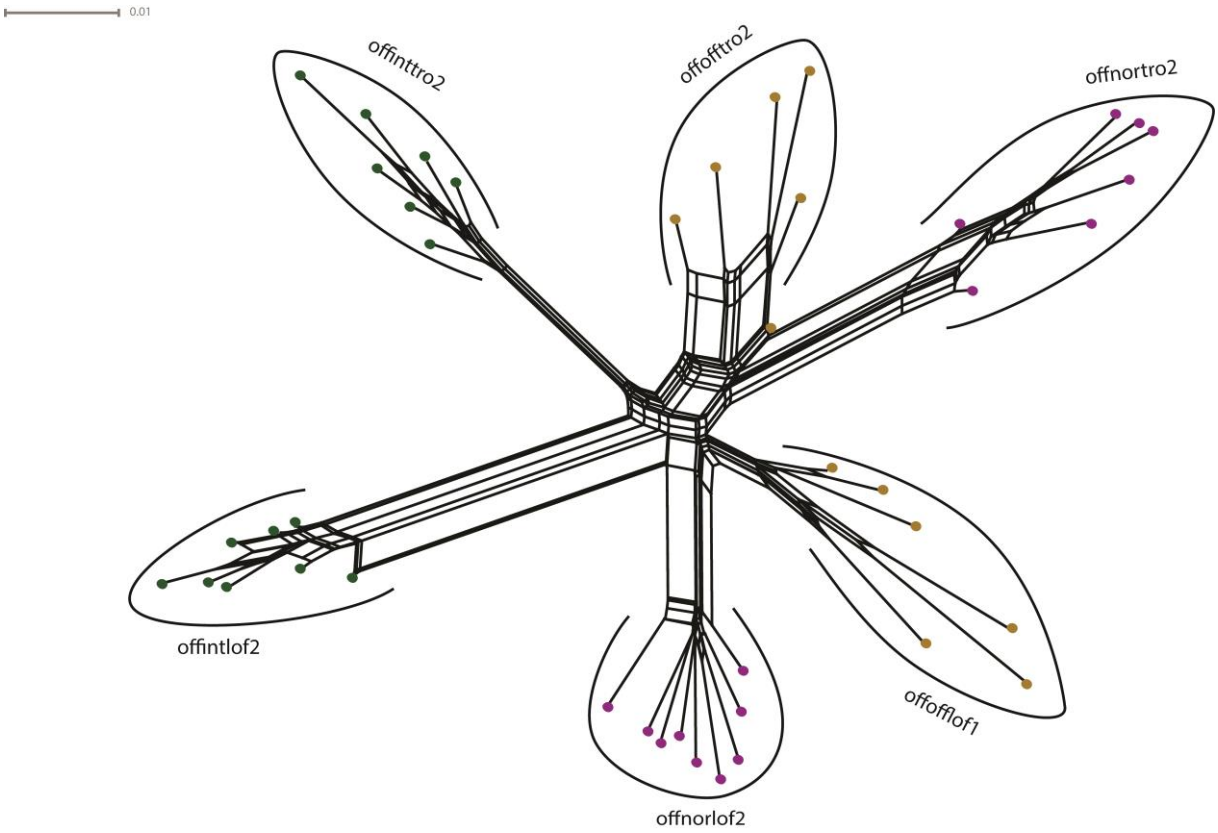

Figure 18. Neighbour net performed in SPLITSTREE for 43 tetraploid *Cochlearia officinalis* individuals, using the full dataset (51 929 SNPs) from RAD-seq and the Uncorrected_P distance measure. Each end node represents an individual, and groups corresponding to populations are indicated.

Summarizing RAD-seq and microsatellite results

Officinalis: Three of the four *officinalis* populations in the microsatellite analyses (two from Lofoten and one from Troms) are genetically very similar and appear internally heterogeneous. Two of these populations (one from Lofoten and one from Troms) were analysed by RAD-seq and appear also in these analyses very similar (and heterogeneous). The second *officinalis* population (from Troms) is somewhat deviating and resemble the two neighbouring populations of *norvegica* (from Troms).

Norvegica: In the microsatellite analyses the two *norvegica* populations from Troms (geographically close) are genetically very similar to each other. The two *norvegica* populations from Lofoten (geographically close) are also very similar to each other, but *norvegica* populations from the two regions appear genetically more distant. Results from the RAD-seq analyses, where only one population from Troms and one from Lofoten are included, support that *norvegica* from the two regions are not genetically very similar.

Integrifolia: In the microsatellite analyses the two *integrifolia* populations from Troms (geographically close) are very similar to each other, and do also show some resemblance with the geographically close *officinalis* population. The two *integrifolia* populations from Lofoten differ both from each other as well as from the two Troms populations. One of the *integrifolia* populations from Lofoten (offintlof2) is in one of the STRUCTURE analysis, based on microsatellites, grouped together with the two geographically adjacent *norvegica* populations. However, all other analyses (both microsatellites and RAD-seq) suggest this population as the most distinct of all the *C. officinalis* populations included in this study. The other *integrifolia* population (offintlof1) from Lofoten is not included in the RAD-seq analyses, but is in the microsatellite analysis shown to be genetically closer to populations of the other subspecies.

Discussion

While microsatellites already is a well-established and well-used molecular marker for population genetics (Ellegren 2004, Selkoe & Toonen 2006), RAD-seq is a recently developed method that has been introduced to population genetics (Miller et al. 2007). Microsatellites amplify specific fragments that differ in allele size and would normally need primer development (Ellegren 2004). RAD-seq cuts the genome in fragments to be sequenced, aligned and searched for SNPs, and can be performed both on species with and without a reference genome (Davey & Blaxter 2010). In the present study six microsatellites, from cross-amplified primers of other Brassicaceae species (Skrede et al. 2009), as well as RAD-seq performed with a frequent cutter (PstI) was performed. The microsatellite analysis was performed with few loci and many individuals, while the RAD-seq was performed with many loci, but with a more limited selection of populations. The results from the two methodologies are largely congruent. The RAD-seq results seem to show similar patterns as those found in the microsatellite analyses, but with higher differentiation between populations and subspecies, most likely a result of the higher resolution obtained with so many markers (SNPs).

The closely related species in section *Cochlearia*

Overall, the *Cochlearia* species and subspecies included in the present study appear to be genetically closely related across ploidal levels, supporting the view that they are a result of recent speciation events, like other studies have concluded (Gupta 1981b, Koch et al. 1996). Individuals with different ploidal levels (2x, 4x, 6x and 8x) are separated in the analyses based on RAD-seq (ordination and STRUCTURE analysis based on 11 223 SNPs and network based on 5782 SNPs), whereas some more overlap between ploidal levels are found in the analyses based on microsatellite data (six microsatellites developed for other Brassicaceae species). The molecular analyses and the flow cytometry results correspond to the presumed ploidal levels based on morphological identification of the species, and confirm previously reported ploidal levels for these species (Appendix Table A1): *C. aestuaria* and *C. pyrenaica* are diploid (2*n*=12), *C. officinalis* (including all subspecies) is tetraploid (2*n*=24) and *C. anglica* is octoploid (2*n*=48). The hybrid between tetraploid *C. officinalis* and octoploid *C. anglica*; *C.* x *hollandica*, which is frequently occurring (Koch et al. 1996, Nordal & Laane 1996), was confirmed to be hexaploid (2*n*=36).

The diploid material in the present study included *C. pyrenaica* (populations from Spain and France), *C. aestuaria* (populations from Spain) as well as *C. groenlandica* (populations from Svalbard, Norway). Some of the *C. pyrenaica* material were previously used in the study by Cires et al. (2011). A geographical differentiation of *C. pyrenaica* and *C. aestuaria* was observed in the present study: The populations from Spain (both *C. pyrenaica* and *C. aestuaria*) group together and the populations from France (only *C. pyrenaica*) group together. *Cochlearia aestuaria* was not included in the study by Cires et al. (2011), but the study support the regional divergence of *C. pyrenaica*. The two *C. groenlandica* populations included (comprising six individuals) show similarity to each other as well as to the other diploid populations (even though the other diploid populations are 4 000 km away geographically). A thesis is under preparation by L.N.Olsen, which will further investigate the taxonomical relationships of *C. groenlandica*, especially *C. groenlandica* in Iceland which is cytologically heterogenous.

The genetic aspect of the subspecies of *C. officinalis*

The focus of this *Cochlearia* study has been on the subspecies of *C. officinalis* in Northern Scandinavia. Based on morphological, physiological and ecological differentiation, three subspecies (comprising four ecotypes) are recognised (Nordal et al. 1986, Eriksen & Nordal 1989, Nordal & Stabbetorp 1990); (1) *Cochlearia officinalis* ssp. *officinalis* (hereafter *officinalis*) comprises the beach and bird cliff ecotype. (2) *Cochlearia officinalis* ssp. *norvegica* (hereafter *norvegica*) corresponds to the estuary ecotype. (3) *Cochlearia officinalis* ssp. *integrifolia* (hereafter *integrifolia*) corresponds to the spring ecotype. The present study has applied molecular methods to investigate the genetic aspects of the three subspecies of *C. officinalis*. One of the main questions in this study has been whether the subspecies/ecotypes are genetically differentiated.

Genetic differentiation of the subspecies: The subspecies/ecotypes of *C. officinalis* show genetic differentiation to some degree; *integrifolia* is distinguished in most analyses, and *officinalis* and *norvegica* are (more or less) separated from each other in some of the analyses (e.g. in the PCoA ordination performed on the binary microsatellite dataset, Fig. 8).

Ecotypic differentiation of plants in coastal versus inland habitats, like we find in *Cochlearia*, is found in many other plant species (e.g. Turesson 1922, Lowry et al. 2008, Moore et al. 2014). The genus *Grindelia* in Asteraceae represents a similar system to *Cochlearia* with ecotypes of coastal and inland affiliation, and in a microsatellites study (Moore et al. 2014) these ecotypes (coast, inland and intermediate) show very similar genetic differentiation to that found in *C. officinalis*.

While the present study has addressed the genetic aspect of the taxa belonging to *C. officinalis* in Northern Scandinavia, Gill (2007) performed a genetic study on the taxa belonging to *C. officinalis* in Britain. In contrast to the present study, genetic markers (AFLP and chloroplast DNA) could not distinguish the taxa of *C. officinalis* s. lat. found in Britain, including taxa both of coastal and inland ecology (see Appendix Table A1) (Gill 2007). In the study by Gill (2007), the coastal and the inland taxa were also investigated separately, showing that the coastal taxa were entirely overlapping genetically.

In the present study the two coastal taxa (*norvegica* and *officinalis*) also show genetically overlap to some degree, while the inland taxon (*integrifolia*) is most distinct. A possible explanation for this pattern is the fact that *officinalis* and *norvegica* are more likely to exchange genes because of their ecology; beach and estuary, which both are in connection to the sea. At least some of the *integrifolia* populations appear to be more isolated and grow in springs or streams that have a long way to the sea. For example one of the *integrifolia* populations from Lofoten (offintlof2) was growing in a spring that was located in an open forest far from the sea. The other *integrifolia* population from Lofoten (offintlof1), which was genetically less distinct, was on the other hand growing in a stream near the outlet to the sea (pers. field observation).

Strong population affiliation and limited dispersal: The *Cochlearia* individuals show strong population affiliation, especially in analyses based on the RAD-seq data. Furthermore, on a local geographical scale, closely located populations seem to be genetically similar, also populations of different subspecies. The fact that most populations in the vicinity of each other

(20-30 km apart) are similar, whereas populations separated by longer distances in many cases are less similar, could indicate limited dispersal.

According to the seed morphology (no apparent adaptation for distant dispersal), Quinn et al. (1994) presumed that coastal *Cochlearia* (*C. scotica*) has low dispersal ability (short distance). On the other hand, Nordal et al. (1986) suggested that *Cochlearia* seeds can be dispersed by sea currents and birds. Coastal taxa, like *officinalis* and *norvegica* have the potential of using the ocean to disperse their seeds. Some coastal species like *Mertensia maritima* have diaspores that float well and could be dispersed a long way with sea currents (Skarpaas & Stabbetorp 2001). Others have seeds that do not float that well and dispersal are much dependent on the speed of the sea current (Curle et al. 2004, Solås et al. 2004). Dispersal of *Cochlearia* seeds has not been studied extensively. However, in floating experiments performed by Praeger (1913), coastal *Cochlearia* (*C. officinalis*, *C. danica* and *C. anglica*) seeds float about one minute, which could indicate that dispersal with sea currents is probably limited to shorter distances. With putative limited ocean dispersal, it is not surprising that gene exchange between geographically close populations, e.g. within the same fjord-system, potentially is larger, resulting in similarity between neighbouring populations. Dispersal by birds is another possibility. Previous studies have shown that birds can be important in dispersal of seeds of coastal and wetland taxa (Morton & Hogg 1989, Nogales et al. 2001). *Cochlearia officinalis* has been suggested as one of the first seabird dispersed plants to arrive the recently established volcanic Island Surtsey (Magnússon et al. 2009), which could indicate that bird dispersal may be important also for seeds of *Cochlearia*.

Strong population affiliation, like in the present study of *C. officinalis*, is also found in the previously mentioned study of *Grindelia* (Moore et al. 2014). Moore et al. (2014) calls this pattern local differentiation, explained either by few opportunities of gene exchange or selection against migrants. Fragmented or patchy populations will potentially suffer from reduced gene flow between populations and increased genetic differentiation (Young et al. 1996). Habitats like estuaries and springs are typically not occurring continuously, e.g. spring vegetation types is described as "small islands in the landscape" (Fremstad & Moen 2001). If dispersal of *Cochlearia* seeds is limited to short distances, habitats like springs and estuaries could be considered patchy or isolated.

While the habitat of *norvegica* and *integrifolia* can be considered to have a patchy distribution and geographically distant populations are genetically distinct, the populations of *officinalis* show a slightly different pattern. The *officinalis* populations are genetically similar, also when geographically distant. This is shown both in microsatellite and RAD-seq data. Compared to the populations of *norvegica* and *integrifolia*, the *officinalis* populations can be considered genetically intermediate and show admixture in STRUCTURE analyses. This is visualized particularly well in the cluster triangle of the STRUCTURE results for the tetraploids (K=3). One explanation of this pattern could be that the *officinalis* habitats are not as patchily distributed as the habitats of *integrifolia* and *norvegica*, or at least with shorter distance between patches. *Officinalis* is the most common of the three subspecies and also has the least specific habitat (Lid & Lid 2005), meaning that gene flow between *officinalis* populations could potentially be higher.

In addition to the *officinalis* populations from Northern Norway, two populations of *officinalis* from the Southern Norway (geographically very distant from the Northern Norway populations) are also included in the present study. At least one of these populations show tendencies to group with the Southern non-tetraploids rather than with the Northern *officinalis* in STRUCTURE (microsatellite) analyses based on the whole dataset. However, in an ordination including only *officinalis* from Northern Norway and Southern Norway, neither of the Southern Norway populations is actually especially deviating (data not shown). More *officinalis* (from mid and South of Norway), should be sampled to be able to explain the genetic structure of *officinalis* fully, including effects of geographical distance.

Regardless of subspecies, the tetraploid populations of *C. officinalis* seem to have similar values of genetic diversity (based on microsatellite data), with the exception of one of the *integrifolia* populations from Lofoten (offintlof2) that has a lower number of alleles and lower expected heterozygosity than other populations. This population was a small and particularly isolated population as mentioned earlier. A potential effect of small and isolated populations is loss of genetic diversity by genetic drift (Young et al. 1996, Pardo et al. 2005). *Cochlearia officinalis* is self-incompatible and genetic diversity loss by inbreeding should normally not be a problem (Nordal & Laane 1990). Furthermore, it should be kept in mind that the duplicated genome of polyploids implies an increased effective population (Parisod et al. 2010), and hence slows down the process of genetic drift.

Morphology due to phenetics, genetics and epigenetics? Morphology of *C. officinalis* in Northern Scandinavia was extensively studied by Nordal & Stabbetorp (1990). They proposed the following distinguishing morphological characters; (1) *officinalis* has rosette leaves that are more or less reniform with cordate to truncate basis. Silicules are sub-globose; (2) *norvegica* has rosette leaves that are oblong to rhomboid to ovate and have cuneate to truncate basis. This subspecies has larger flowers than the other subspecies and oblong silicules that are compressed laterally; (3) *integrifolia* has reniform rosette leaves that are usually broader than they are long, with cordate basis. Silicules are sub-globose to oblong. The root is often branching to more than one rosette, showing its perenniality (the others are biannuals).

The study by Gill (2007) comprised both morphological and genetical analyses of *C. officinalis* in Britain. While some of the morphological characters varied significantly between taxa, all the morphological characters combined, as well as the genetical data, could not distinguish the taxa. This led Gill to propose that "genetic adaptation and phenotypic plasticity have led to the occurrence of eco-morphotypes in *Cochlearia*", and concluded that the British taxa are the result of local ecotypic differentiation. The present genetic study found the taxa of *C. officinalis* in Northern Scandinavia to be genetically differentiated to some degree. Morphology was not specifically studied, though it was noted that the morphological characters proposed by Nordal & Stabbetorp (1990) may be variable (in field, as well as in the material grown in the phytotron; data not shown). According to Nordal & Stabbetorp (1990), *norvegica* is the morphologically most distinct, whereas the present study indicate that *integrifolia* is the genetically most distinct of the three subspecies. As proposed by Gill (2007) for the British *Cochlearia*, a combination of genetics and phenotypic plasticity may be responsible for the morphological differences between the subspecies/ecotypes found in Northern Scandinavia, as well. However,

a study combining morphology and genetics should be performed for *C. officinalis* in Northern Scandinavia to investigate this further.

In the present study no attempt has been made to distinguish neutral and non-neutral markers in the genetic data. Microsatellites are believed to be neutral (Ellegren 2004), while SNPs from all over the genome potentially could contain both neutral and non-neutral markers (Nielsen 2005). A continuation of the present study could attempt to separate the neutral and the putative selective variation of the SNPs to detect if there is a selective element to the genetic differentiation of *C. officinalis* in Northern Scandinavia (that could reflect ecotypic differentiation).

Another possible factor in ecotype differentiation could be epigenetics, influencing the gene expression, which can result in ecological divergence (Paun et al. 2010). Epigenetic signals, in form of methylation of DNA, modifications of histones and small RNAs, can be inherited across generations (Zhang 2008, Jablonka & Raz 2009). Epigenetics influence is frequently found after an instance of genomic stress, e.g. polyploidy (Chen 2007). Both Nordal & Stabbetorp (1990) and Gill (2007) found that some morphological differences between the taxa of *C. officinalis* were maintained when cultivated in common garden experiments, in addition to evidence of phenotypic plasticity. The morphological differentiation between the taxa of *C. officinalis* could potentially be explained partly by (1) phenotypic plasticity, (2) genetics and (3) epigenetics. Untangling to what degree genetics and epigenetics are explaining the phenotype variation have been studied in *Viola cazorlensis* in the Violaceae, which is a non-model organism like *C. officinalis* (Herrera & Bazaga 2010, Herrera & Bazaga 2011). Only genetics has been investigated in the present study. However, in a future study this could be expanded to include epigenetics.

The polyploid origin of *C. officinalis* in Northern Scandinavia

Section *Cochlearia* contains polyploid variation, with series of polyploid taxa, similar to other Brassicaceae taxa like *Capsella* (Hurka & Neuffer 1997), *Draba* (Grundt et al. 2005) and *Cardamine* (Jørgensen et al. 2008). Origins of polyploids have been investigated in many other plant groups (e.g. Soltis & Soltis 1991, Gutiérrez et al. 1994, Yang et al. 2006, Brysting et al. 2007). A duplication of the genome may within a few generations result in reproductive isolation from the parental species and sympatric speciation (Otto & Whitton 2000). This is because a cross between a newly synthesized polyploid (e.g. 4x) and its parent (2x) will result in a putative hybrid with uneven chromosome set number (e.g. 3x), which is often less viable or sterile (with aneuploid gametes) (Mallet 2007). Such sterile interploidal hybrids are found e.g. in *Viola* in Violaceae (Brandrud & Borgen 1986). Sympatric speciation does, however, not necessarily follow immediately after a polyploidization event (Slotte et al. 2008). Reproductive isolation is not always complete, enabling gene flow between ploidal levels (Petit et al. 1999, Husband 2004). Low reproductive barriers are also found between ploidal levels in *Cochlearia* and gene flow is possible, e.g. between *C. officinalis* and *C. anglica*, where hybridization and introgression have been observed (Koch et al. 1996, Nordal & Laane 1996).

Broadly speaking, polyploidization can be divided into (1) autopolyploidization; genome doubling within the same species, which normally results in a polyploid cytotype assumed to have polysomic inheritance, and (2) allopolyploidization; genome doubling in combination

with hybridization between two species, which normally results in a polyploid offspring assumed to have disomic inheritance (Ramsey & Schemske 1998, Rieseberg & Willis 2007). Depending on the genomic distance between the parental species, and on what level hybridization is defined (interspecific or interpopulational, Rieseberg & Carney 1998), allopolyploids and autopolyploids can be considered relative terms (Ramsey & Schemske 2002). Assuming interspecific hybridization and a taxonomical species concept, both autopolyploids and allopolyploids are found in *Cochlearia* (Gupta 1981a, Pegtel 1999, Koch 2002). Apart from the mode of polyploidization, many polyploids might be the result of, not only a single polyploidization event, but recurrent formations (Soltis & Soltis 1999). Recurrent formation is fairly common and found in many polyploid species (Soltis & Soltis 1995, Soltis & Soltis 1999, Soltis et al. 2010), e.g. polyploid species in the genera *Chrysanthemum* and *Tragopogon* in Asteraceae, and in the genera *Arabis* and *Draba* in Brassicaceae (Soltis & Soltis 1991, Brochmann et al. 1992, Sharbel & Mitchell-Olds 2001, Soltis et al. 2004a, Yang et al. 2006).

With respect to the origin of tetraploid *C. officinalis*, an autopolyploidization from an ancestral diploid from Central Europe has been proposed (Gill 1973, Koch et al. 1998). A subsequent autopolyploidization of *C. officinalis* has been proposed for the origin of octoploid *C. anglica* (Koch et al. 1998). Koch et al. (1996) found infraspecific variation in chloroplast DNA of *C. officinalis* material from Britain, Denmark and Norway, linking the *C. officinalis* populations to different taxa of other ploidal levels. Koch et al. (1996) suggested that this could be explained by either (1) recurrent formation, (2) differentiation within the species, or (3) interspecific hybridization, meaning interploidal gene flow, e.g. with *C. anglica* or *C. danica*.

The diploid taxa in Central Europe have either spring ecology (*C. pyrenaica*, *C. excelsa* and *C. macrorrhiza*) or estuary ecology (*C. aestuaria*) (Koch et al. 2003). Assuming autopolyploidization, there are at least two hypotheses for the origin of *C. officinalis* in Northern Scandinavia (comprising three subspecies and four ecotypes):

(1) One polyploidization event from a Central European diploid parental species (resulting in tetraploid *C. officinalis*), followed by ecotypic differentiation into beach, bird cliff, spring and estuary ecotypes.

(2) Several independent polyploidization events involving diploid parental species with different ecology resulting in the ecologically differentiated taxa at higher ploidal levels. This scenario, which would mean that taxa with similar ecology, across different ploidal levels, constitute monophyletic groups, was originally proposed by Nordal & Laane (1996).

Assuming that the latter hypothesis of several independent polyploidizations is correct, we would expect that taxa with the same ecological habitat, across ploidal levels, are closely related to each other and would group together in the molecular analyses.
No such groups are observed in any of the results from the present study (neither in ordinations, STRUCTURE clustering nor networks). Based on these results, the hypothesis, originally proposed by Nordal & Laane (1996), that several independent polyploidization events are responsible for the ecotypical differentiation seen today, seems less likely.

The present study supports the hypothesis of one polyploidization event of *C. officinalis* in Northern Scandinavia, supported by the fact that the *C. officinalis* subspecies/ecotypes in

Northern Scandinavia appear as one group genetically. This is especially evident in the network for all taxa based on 5782 SNPs from RAD-seq (Fig. 17). The major split in the network separates the tetraploids from the diploids (and the octoploid). Polyploid taxa evolved from a single polyploidization event, and not recurrent as otherwise seems to be the rule rather than the exception (e.g. Soltis & Soltis 1999), have been found for example in the genus *Galeopsis* in Lamiaceae, as well as the genera *Draba* and *Arabidopsis* in Brassicaceae (Widmer & Baltisberger 1999, Säll et al. 2003, Bendiksby et al. 2011). A wider genetical study of *C. officinalis* and its potential parental diploid species is needed to be able to conclude whether *C. officinalis* has originated from a single or recurrent polyploidizations, throughout its entire geographic distribution.

Assuming that *C. officinalis* in Northern Scandinavia originated from a single polyploidization event, we cannot be sure about the habitat preferences of this newly synthesized polyploid. We have no certain evidence for its parental species (e.g. *C. aestuaria*, *C. pyrenaica* or a diploid ancestor of *C. aestuaria* and *C. pyrenaica*), and it is not uncommon that a newly synthesized polyploid is able to colonize a different habitat than its parental species (Ramsey 2011, e.g. te Beest et al. 2012). However, we can assume that *C. officinalis*, when colonizing Norway, had a broad coastal ecology/ beach ecology, which is the most common ecological habitat found for *C. officinalis* in Norway as well as in the rest of Europe (Lid & Lid 2005, Gill 2007). Based on these assumptions a following question would be whether the subspecies and ecotypes of *C. officinalis* are the result of: (1) repeated ecotypic differentiation in various geographical locations for each of the ecotypes, i.e. parallel evolution of ecotypes, or (2) a single ecotypic differentiation event for each of the ecotypes, with subsequent dispersal to their present habitat and current distribution.

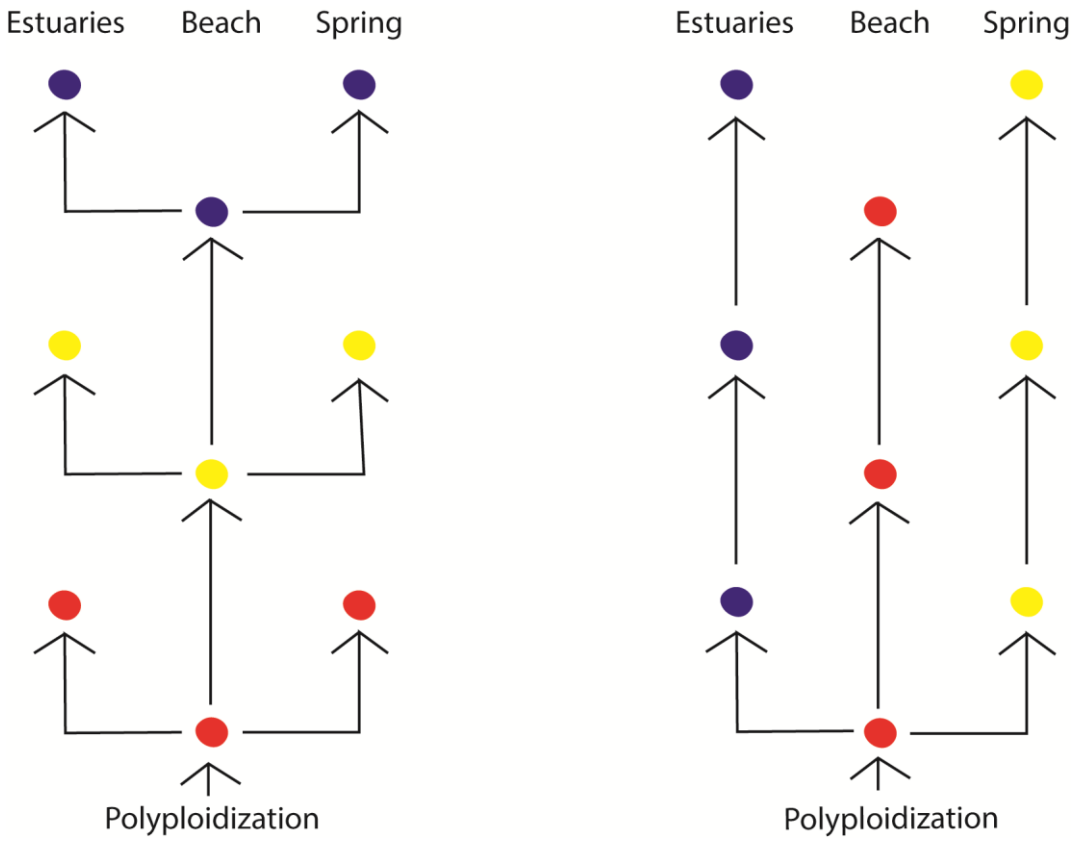

Figure 19. Illustrating different scenarios for subsequent ecotypic differentiation after polyploidization. Repeated ecotypic differentiation events (left) and single ecotypic differentiation events with subsequent dispersal (right). Populations with similar colour are expected to have relatively high genetic similarity compared to populations of different colour. Sidewise arrows are indicating ecotypic differentiation. Straight arrows are indicating dispersal to a new geographical area.

In a scenario with repeated ecotypic differentiation events, i.e. parallel evolution of ecotypes (Fig. 19, left), geographically distant populations of an ecotype (that have originated from independent ecotypic differentiation events) would be expected to have low genetic similarity (e.g. Foster et al. 2007, Roda et al. 2013). Gill (2007) detected low genetic (AFLP) similarity between populations of the same taxa (ecotype) in British *Cochlearia*. She proposed repeated ecotypic differentiation, when the genetic similarity between populations of the same taxa was no higher than the genetic similarity between populations of different taxa. While *officinalis* populations in Northern Norway show genetic similarity also when geographically distant, populations of *norvegica* and *integrifolia* show lower similarity to geographically distant populations of their respective subspecies (ecotype). This low similarity between geographically distant populations of the same subspecies (ecotype) could, thus, be explained as a result of repeated ecotypic differentiation.

In a scenario with a single event of ecotypic differentiation and subsequent dispersal of the ecotypes to their current distribution (Fig. 19, right), populations (both geographically close and distant) of an ecotype, would be expected to be relatively similar to each other genetically,

and less similar to populations of other ecotypes (e.g. Lowry et al. 2008, Moore et al. 2014). In the present study, genetic analyses distinguish the *Cochlearia* subspecies (ecotypes) to some degree, i.e. populations of the same subspecies (ecotypes) show similarity to each other, even though the pattern is not clear-cut as mentioned above. The low genetic similarity observed between geographically distant populations of *integrifolia* and *norvegica* can, in a one origin scenario be explained by low levels of gene flow because of patchy, isolated habitats, as discussed earlier. And the genetic overlap between *norvegica* and *officinalis* could in the same line be explained by more frequent gene flow between *norvegica* and *officinalis* because they both are coastal subspecies.

Nordal et al. (1986) investigated the glucosinolates of the ecotypes of *C. officinalis* in Northern Scandinavia and found that the southern estuary and beach ecotypes contained the 2-butyl glucosinolate, while the northernmost populations lacked this glucosinolate. All the populations of the spring ecotype had the glucosinolate, also the populations collected in the Northern area where the estuary and the beach ecotype lacked it. Based on these results, Nordal et al. (1986) suggested that the spring populations, in the area where the estuary and the beach ecotype lack the glucosinolate, have arrived there independently of the estuary and beach populations. A local ecotypic differentiation scenario would have involved regaining the lost glucoinolate, which seems more unlikely. Extending this to a broader scale, this could suggest that the distribution of the spring ecotype most likely is due to one ecotypic differentiation event and subsequent dispersal (independent of the coastal ecotypes), rather than repeated ecotypic differentation in various geographical areas. Furthermore the two coastal ecotypes, estuary and beach, could have dispersed independently along the coast of Norway, and both lost the glucosinolate on their way North. Nordal et al. (1986) mentioned that glucosinolate is putatively of selective value by preventing herbivory. If there is less herbivory at more Northern latitudes, the glucosinolate would be less needed, and parallel loss more likely. Alternatively the beach and the estuary ecotypes dispersed along the coast of Norway as one coastal group and along the way repeated ecotypic differentiation occurred. From the present genetic results, it is not possible to take these alternative scenarios apart.

Further research

To better understand the phylogeographical patterns of *C. officinalis*, a study including more Southern material (a wider geographical range) of *C. officinalis* should be performed. To fully understand what underlies the morphological and ecological differentiation of *C. officinalis* in Northern Scandinavia, a study including morphology, genetics and epigenetics should be performed. Inclusion of the fourth ecotype of *C. officinalis*; bird cliff (belonging to *officinalis* along with the beach ecotype) would be another possible extension of the present study. It would also be interesting to compare the coastal and inland taxa of *C. officinalis* considered in the present study (in Northern Scandinavia) with the coastal and inland taxa of *C. officinalis* in Britain. The hybrid between *C. officinalis* and *C. anglica*; *C* x *hollandica*, represents a bridge between these two ploidal levels (tetraploid and octoploid respectively). In a study including the a few hybrid populations and a few populations of the parental species gene flow (by introgression) could be addressed. To infer more about the origin of *C. officinalis*, more diploid genotypes from Central Europe as well as more *C. officinalis* from various places in Europe should be included. At the moment the genome of *C. pyrenaica* is being sequenced, which will

provide a basis for further studies of the evolution and adaptation of the subspecies of *C. officinalis*, e.g. genetic, epigenetic and transcriptome studies, as well as whole-genome duplications within the genus.

Conclusion

The two molecular markers used in the present study; microsatellites and RAD sequencing, arrive at largely congruent results, even though with different levels of resolution. Genetic differentiation is found at different levels within *C. officinalis* in Northern Scandinavia; (1) between the subspecies/ecotypes (2) between geographical groups (=populations located close to each other geographically), and (3) between populations. At the subspecies level *integrifolia* (the spring ecotype) is the genetically most distinct of the three subspecies, whereas *officinalis* has the least distinct populations. At the level of geographical groups, populations of *integrifolia* and *norvegica* show similarity to populations of the same subspecies if geographically close, but are less similar to populations of the same subspecies, when geographically distant. Overall closely located populations show some similarity, also across subspecies. At the population level all individuals show strong population affiliation.

One polyploidization event of *C. officinalis* in Northern Scandinavia is proposed. This is based on the genetic data supporting the tetraploids as one group, distinguished from other species and ploidal levels, which is especially evident in the RAD-seq data. Further, the genetic results of the present study in combination with results from previous studies present more than one possible scenario for the subsequent ecotypic differentiation resulting in *integrifolia*, *norvegica* and *officinalis*. The combined evidence might favour a scenario with a single ecotypic origin of the genetically distinct spring ecotype, with subsequent dispersal to account for its current distribution, while repeated ecotypic differentiation along the coast might be the best explanation for the genetic patterns found for the estuary and the beach ecotypes. However, the alternative scenarios can be argued for in both cases, and the genetic data cannot be used to conclusively take the different alternatives apart.

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Appendix

Appendix Table A1. Overview of section *Cochlearia* in Europe. Taxa in red are the species considered by Gill (2007) in her study of British *Cochlearia*. Taxa in green are the Northern Scandinavian *Cochlearia* considered in the present study. Ecology elaboration; spring: mountain springs or along brooks, estuary: estuaries and sheltered beach meadows, beach: exposed beaches and beach rocks.

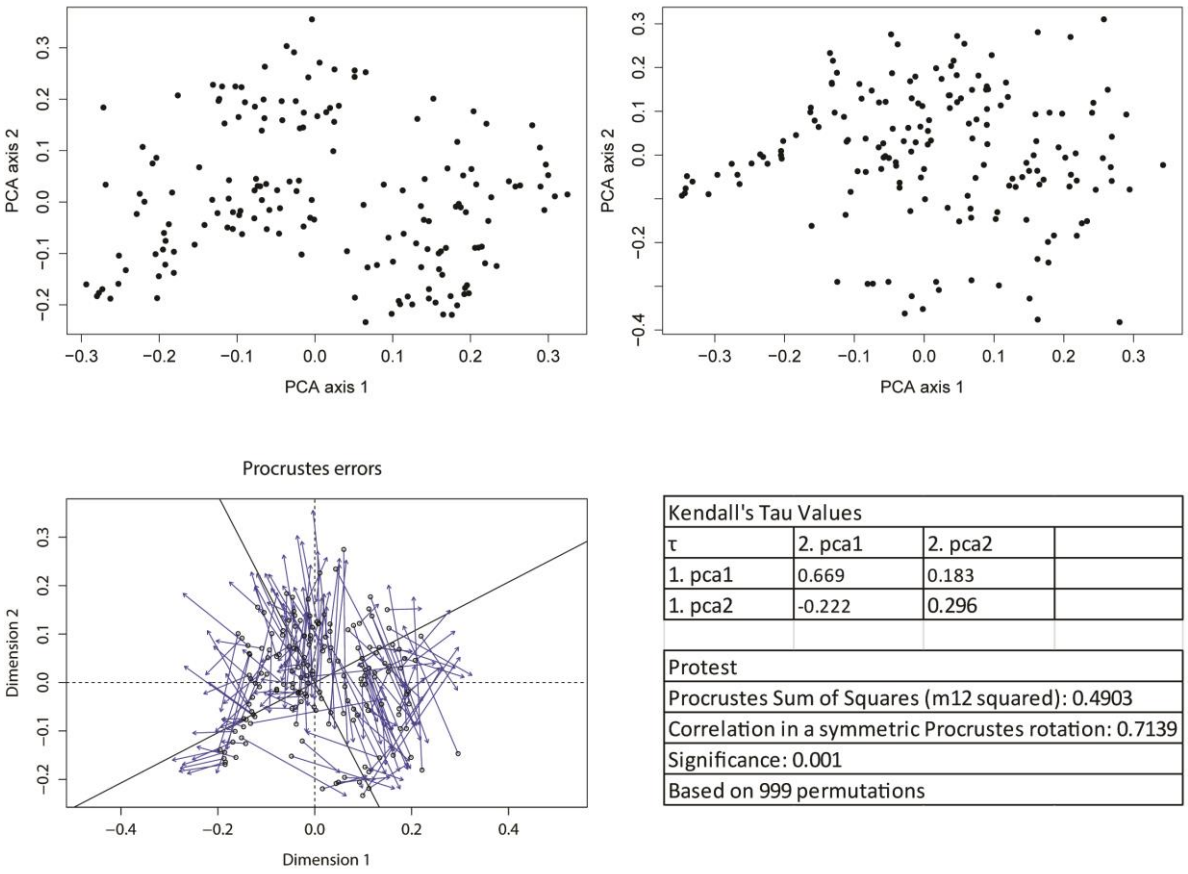
Names	Cytology	Ploidy	Ecology	References
Cochlearia groenlandica L.	2*n*=12	Diploid	Several arctic habitats	Nordal & Laane 1990
	2*n*=14	Diploid		Saunte 1955, Gill 1971, Elkington 1984
Cochlearia arctica Schltdl.	2*n*=14	Diploid	Several arctic habitats	Gill 1971
Cochlearia lensis Adams ex Fisch.	2*n*=14	Diploid	Several arctic habitats	pan arctic flora
Cochlearia aestuaria (Lloyd) Heywood	2*n*=12	Diploid	Estuary	Gill 1971, Nordal & Laane 1996
Cochlearia macrorrhiza (Schur.) Pobed	2*n*=12	Diploid	Spring	Koch et al. 2003, Nordal & Laane 1996
Cochlearia excelsa Zahlbr.	2*n*=12	Diploid	Spring	Koch et al. 2003, Nordal & Laane 1996
Cochlearia pyrenaica DC.	2*n*=12	Diploid	Spring	Nordal 1988, Nordal & Laane 1996
Cochlearia pyrenaica DC. ssp. *pyrenaica* Druce	2*n*=12	Diploid	Spring	Gill 2008
Cochlearia pyrenaica DC. ssp. *alpina* (Bab.) Dalby	2*n*=24	Tetraploid	Spring	Gill 2008
Cochlearia atlantica Pobed.	2*n*=24	Tetraploid	Beach	Gill 2008
Cochlearia micacea Marshall	2*n*=26	Tetraploid	Spring	Gill 1973, Elkington 1984, Gill 2008
Cochlearia officinalis L. s.str.	2*n*=24	Tetraploid	beach, bird cliff, estuary	Gill 1973, Saunte 1955, Gill 2008
C. officinalis L. ssp. *scotica* (Druce) Wyse-Jackson	2*n*=24	Tetraploid	Beach	Gill 2008
C. officinalis L. ssp. *officinalis*	2*n*=24	Tetraploid	Beach and bird cliff	Nordal et al. 1986, Nordal & Laane 1996
C. officinalis L. ssp. *integrifolia* (Hartm.) Nordal & Stabbetorp	2*n*=24	Tetraploid	Spring	Nordal et al. 1986, Nordal & Laane 1996
C. officinalis L. ssp. *norvegica* Nordal & Stabbetorp	2*n*=24	Tetraploid	Estuary	Nordal et al. 1986, Nordal & Laane 1996
C. officinalis L. ssp. *islandica (Pobed.)* Nordal & Bjorå ined.	2*n*=14	Diploid	Several arctic habitats	pan arctic flora
Cochlearia x *hollandica* Henrard (*C. officinalis* x *C. anglica*)	2*n*=36	Hexaploid	Estuary/beach	Nordal & Laane 1996, pers. obs.
Cochlearia bavarica Vogt	2*n*=36	Hexaploid	Spring	Paschke et al. 2003, Nordal & Laane 1996
Cochlearia polonica Fröhl.	2*n*=36	Hexaploid	Spring	Cieslak et al. 2007, Nordal & Laane 1996
Cochlearia danica L.	2*n*=42	Hexaploid	Beach, (motorways)	Gill 1976, Saunte 1955, Welch 1998
Cochlearia tatrae Borb.	2*n*=42	Hexaploid	Spring	Koch et al. 2003, Cieslak et al. 2007
Cochlearia borzaeana (Coman et Nyár.) Pobed.	2*n*=48	Octoploid	Spring	Kochjarová et al. 2006
Cochlearia anglica L.	2*n*=48	Octoploid	Estuary	Saunte 1955, Elkington 1984, Nordal & Laane 1996
	2*n*=60	Decaploid		Elkington 1984, Nordal & Laane 1996

Appendix Table A2. Collection data for the *Cochlearia* populations used in this study. GPS coordinates are given in degrees. Collectors; AKB: A.K.Brysting, MKB: M.K.Brandrud, KHB: K.H.Brandrud, PBE: P.B.Eidesen, EM: E.Müller, IN: I.Nordal, CSB: C.S.Bjorå, RE: R.Elven, TMP: T.M.Pedersen, KH: K.Høiland, EC: E.Cires, JH: J.Homet and JAFP: J.A.F.Prieto. Number of individuals included from each population is indicated for flow cytometry, microsatellite analyses (Microsat), the first RAD-seq library (RAD-seq 1) and the second RAD-seq library (RAD-seq 2).

Pop.	Taxon	Country	Locality	GPS North-South	GPS East-West	M.a.s.l.	Collection date	Collector	Flow cytometry	Microsat	RAD-seq 1	RAD-seq 2	Number on map	Collection number
aesES1	*C. aestuaria* (Lloyd) Heywood	Spain	Asturias, Ria de Tina Mayor	43.37944	-3.48750		04.07.2013	AKB	4	10	-	-	-	AB13-1
aesES2	*C. aestuaria* (Lloyd) Heywood	Spain	Asturias, Ribadesella	43.46222	-4.93583		04.07.2013	AKB	3	5	5	5	-	AB13-2
aesES3	*C. aestuaria* (Lloyd) Heywood	Spain	Asturias, Navia	43.55111	-5.278055556		05.07.2013	AKB	5	-	-	-	-	AB13-3
angSE	*C. anglica* L.	Sweden	Scania, Vellinge, Skanör with Falsterbro	55.42500	12.83991	0	25.08.2013	MKB & KHB	4	7	3	4	-	MKB13-10
groNO1	*C. groenlandica* L.	Norway	Svalbard, Gustav V land, Murchisonfjorden, Storsteinhalvøya, Kinnvika	80.05006	18.21990	-5	16.07.2013	MKB & PBE	-	3	-	-	-	MKB13-1
groNO2	*C. groenlandica* L.	Norway	Svalbard, Haakon VII Land, Krossfjorden, Fjortende Julibukta	79.13038	11.84877		18.07.2013	MKB, PBE & EM	-	3	-	-	-	MKB13-2
holDK	*C.* x *hollandica* Henrard	Denmark	Fyn, Kerteminde, Langø	55.58875	10.59916	0.5	26.07.2012	AKB	5	2	-	-	-	AB12-1
offintlof1	*C. officinalis* L. ssp. *integrifolia* (Hartm.) Nordal & Stabbetorp	Norway	Nordland, Vestvågøy, Himmeltind	68.21663	13.52911-13.54613	70-345	12.08.2013	MKB, AKB & IN	-	10	-	-	9-10	MKB13-4
offintlof2	*C. officinalis* L. ssp. *integrifolia* (Hartm.) Nordal & Stabbetorp	Norway	Nordland, Sortland, Sørfjorddalen	68.67430	15.77752	80	13.08.2013	MKB, AKB & IN	-	10	8	8	8	MKB13-7
offinttro1	*C. officinalis* L. ssp. *integrifolia* (Hartm.) Nordal & Stabbetorp	Norway	Troms, Tromsø, Tromsdalen	69.62466	19.04558	40	18.08.2012	MKB, AKB & IN	3	10	-	-	7	MKB12-6
offinttro2	*C. officinalis* L. ssp. *integrifolia* (Hartm.) Nordal & Stabbetorp	Norway	Troms, Tromsø, Kvaløysletta	69.68858	18.83241	65-70	18.08.2012	MKB, AKB & IN	5	10	8	7	6	MKB12-7
offnorfin	*C. officinalis* L. ssp. *norvegica* Nordal & Stabbetorp	Norway	Finnmark, Alta, Langfjordbotn: Bognelvosen	70.02291	22.29173		11.08.2012	CSB, RE & TMP	8	5	-	-	1	CSB1200
offnorlof1	*C. officinalis* L. ssp. *norvegica* Nordal & Stabbetorp	Norway	Troms, Kvæfjord, Gullesfjordbotnen	68.53358	15.72755	0	13.08.2013	MKB, AKB & IN	-	10	-	-	4	MKB13-6
offnorlof2	*C. officinalis* L. ssp. *norvegica* Nordal & Stabbetorp	Norway	Nordland, Lødingen, Kanstadbotnen	68.50711	15.87602	0	14.08.2013	MKB, AKB & IN	-	10	8	10	5	MKB13-8
offnortro1	*C. officinalis* L. ssp. *norvegica* Nordal & Stabbetorp	Norway	Troms, Storfjord, Melneset, Oteren	69.26708	19.92058	0	16.08.2012	MKB, AKB & IN	9	10	-	-	3	MKB12-2
offnortro2	*C. officinalis* L. ssp. *norvegica* Nordal & Stabbetorp	Norway	Troms, Storfjord, Skibotn	69.37866	20.23541	0	16.08.2012	MKB, AKB & IN	10	10	7	7	2	MKB12-4
offnortro3	*C. officinalis* L. ssp. *norvegica* Nordal & Stabbetorp	Norway	Troms, Storfjord, Skibotn	69.3795	20.2192	0	16.08.2012	MKB, AKB & IN	8	-	-	-	-	MKB12-3
offoffli1	*C. officinalis* L.	Norway	Vest-Agder, Farsund, Havika	58.06808	6.72960		14.07.2012	KH	1	3	-	-	-	MKB12-10
offoffli2	*C. officinalis* L.	Norway	Vest-Agder, Farsund, Einarsneset, Grønnodden	58.06276	6.78419		18.07.2012	KH	1	3	-	-	-	MKB12-9
offoffof1	*C. officinalis* L. ssp. *officinalis*	Norway	Troms, Harstad, The Hinnøy side of Tjeldsundet, by Tjeldsundbrua	68.62775-68.62966	16.56533-16.56900	0	11.08.2013	MKB, AKB & IN	-	10	8	6	13-14	MKB13-3
offoffof2	*C. officinalis* L. ssp. *officinalis*	Norway	Nordland, Vågan, Ørsnes	68.20463	14.39763	0	14.08.2013	MKB, AKB & IN	-	10	-	-	15	MKB13-9
offofftro1	*C. officinalis* L. ssp. *officinalis*	Norway	Troms, Tromsø, Sjøvassbotn, innermost of Sørfjord	69.39397	19.45308	0	16.08.2012	MKB, AKB & IN	7	10	-	-	12	MKB12-1
offofftro2	*C. officinalis* L. ssp. *officinalis*	Norway	Troms, Tromsø, Skittenelv	69.77505	19.29294	0	18.08.2012	MKB, AKB & IN	8	10	8	8	11	MKB12-8
offofftro3	*C. officinalis* L. ssp. *officinalis*	Norway	Troms, Tromsø, Selnes, Kvaløya	69.6865	18.8534	0	17.08.2012	MKB, AKB & IN	5	-	-	-	-	MKB12-5
pyrES1	*C. pyrenaica* DC.	Spain	Astuarias, Somiedo, Villar de Vildas	43.07861	-5.66472	1040	06.07.2013	AKB	-	10	-	-	-	AB13-4
pyrES2	*C. pyrenaica* DC.	Spain	Asturias, Between Villar de Vildas and La Pornacal	43.06666	-5.68333			JH & JAFP	-	1	1	1	-	VIL
pyrES3	*C. pyrenaica* DC.	Spain	Asturias, Ascent to Puerto de Somiedo	43.03333	-5.76666			EC, JH & JAFP	-	1	1	1	-	SOM
pyrFR1	*C. pyrenaica* DC.	France	Haute-Garonne, Between Oô and Lac d'Oô	42.76666	0.50000			EC, JH & JAFP	-	2	2	2	-	LOÔ
pyrFR2	*C. pyrenaica* DC.	France	Hautes-Pyrénées, Col du Tourmalet, west face	42.90000	0.11666			EC, JH & JAFP	-	2	1	1	-	TOU

Appendix Table A3. Commands and options applied to process the RAD-seq raw reads in STACKS. Options: -r: rescue barcodes and RAD-tags, -c: clean data and remove reads with an uncalled base, -q: discard reads with low quality, -m: minimum number of identical raw reads required to create a stack, -M: number of mismatches allowed between loci of a single individual and –n: number of mismatches allowed between loci when creating the catalog

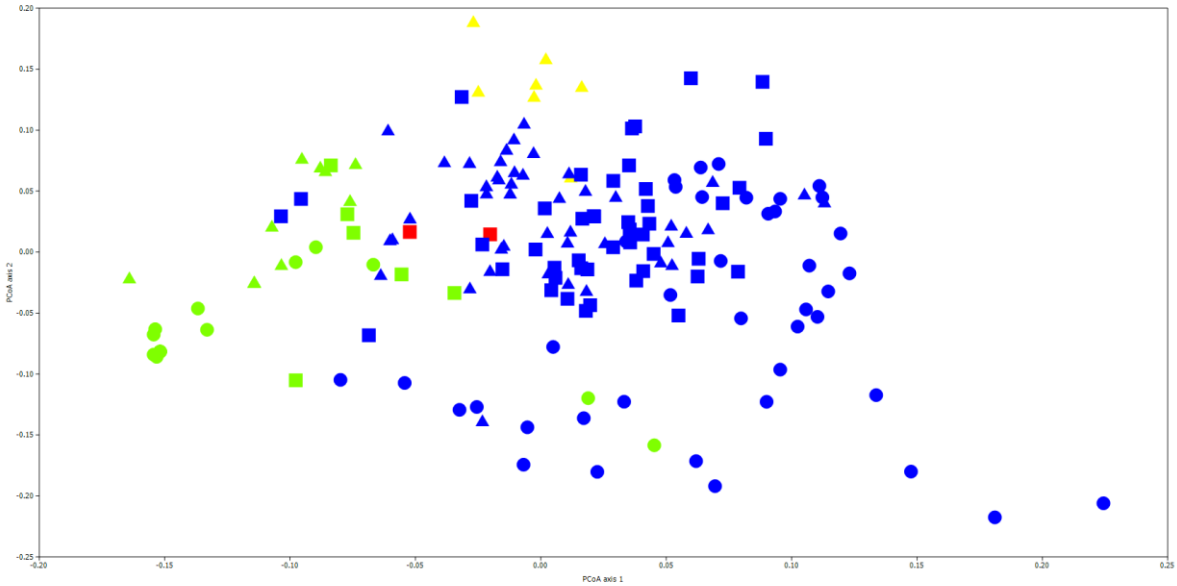
Commands used in STACKS	Options used in final run
process_radtags	-r -c -q
denovo_map.pl	-m 10 -M 1 -n 1
populations	--structure --phylip (for all individuals) --phylip_var (for the tetraploids only)



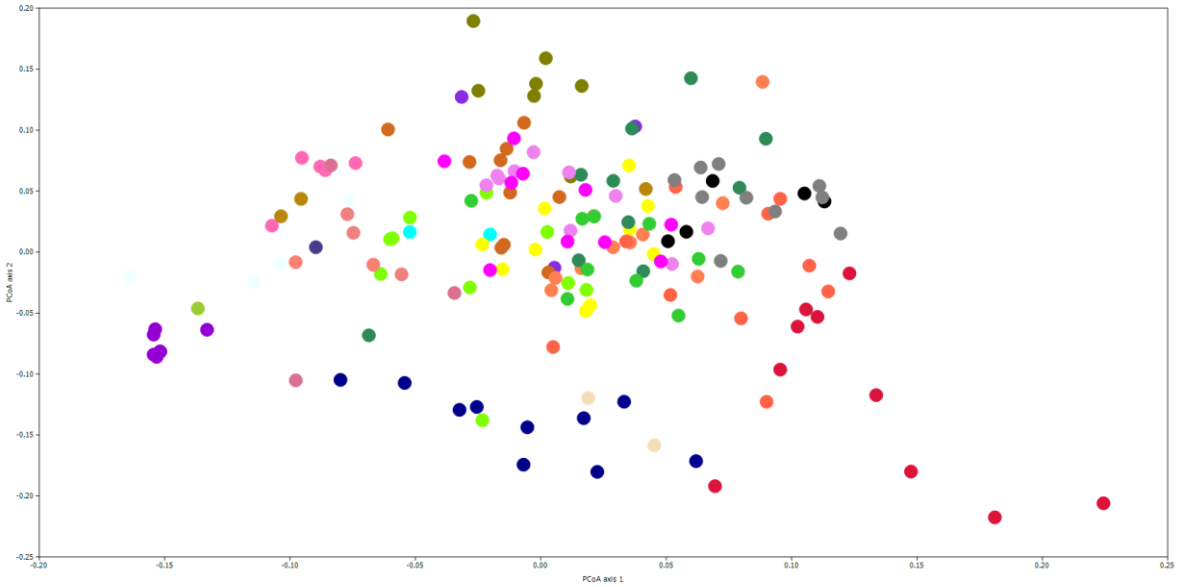
Kendall's Tau Values			
τ	2. pca1	2. pca2	
1. pca1	0.669	0.183	
1. pca2	-0.222	0.296	

Protest
Procrustes Sum of Squares (m12 squared): 0.4903
Correlation in a symmetric Procrustes rotation: 0.7139
Significance: 0.001
Based on 999 permutations

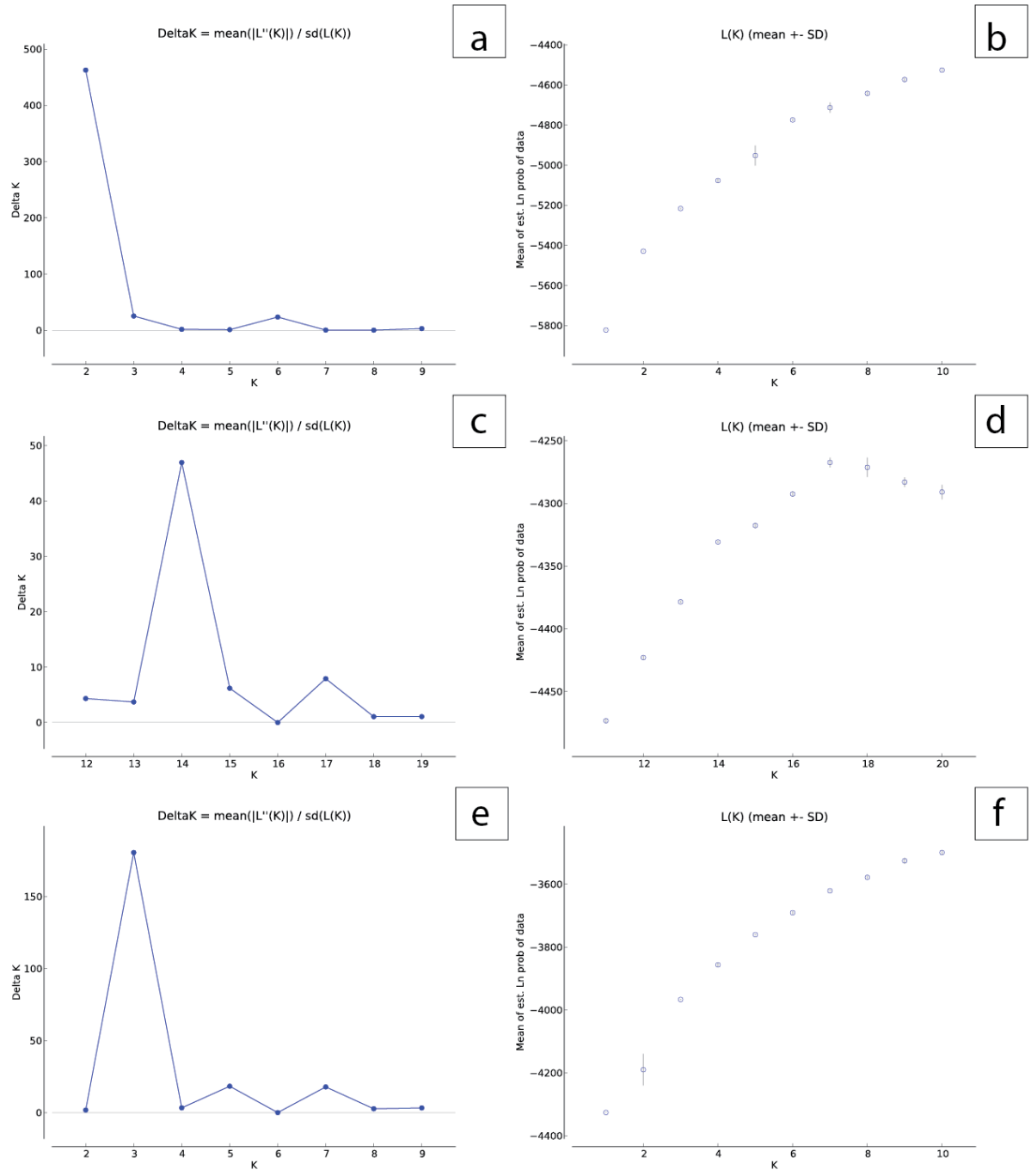
Appendix Figure A1. Top: two PCA performed in POLYSAT for all 177 *Cochlearia* individuals, using the microsatellite dataset with allele sizes and Bruvo distance measure. Analysis based on the second scoring (allele calling) in GENEMAPPER (left), and analysis based on the first scoring (allele calling) in GENEMAPPER (right). Bottom: visualization of Procrustes rotation (left) performed between the first and the second scoring in VEGAN. Protest performed between the first and the second scoring in VEGAN as well as Kendall's Tau correlation between the axes (right).



Appendix Figure A2. PCoA performed in PAST for all 177 *Cochlearia* individuals using the binary microsatellite dataset and Dice similarity. The first PCoA axis explained 11.0 % of the total variation in the dataset, the second axis 6.1 %. Colour representation according to ploidal level: diploids – green, tetraploids – blue, hexaploids – red and octoploids – yellow. Symbol representation is according to ecotype: beach (or bird cliff) ecology – square, estuary ecology – triangle and spring ecology – circle. These are the same symbol and colour representation as used in the map (Fig. 5).



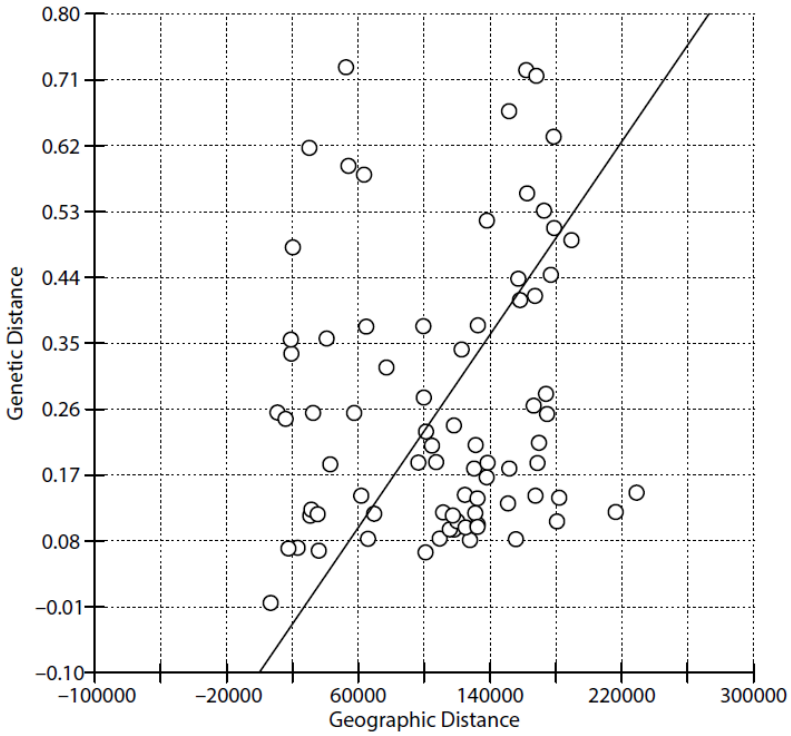
Appendix Figure A3. PCoA performed in PAST for all 177 *Cochlearia* indiviuals, using the binary microsatellite dataset and Dice similarity. The first PCoA axis explains 11.0 % of the total variation in the dataset, the second axis 6.1 %. Colour representation is according to population: each colour represents one population.



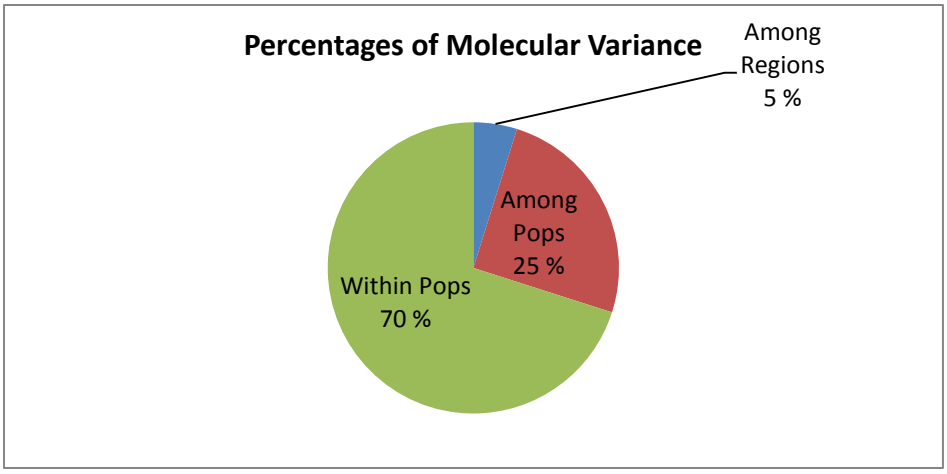
Appendix Figure A4. Delta K (left) and mean likelihood of K (right) values, as calculated in STRUCTURE HARVESTER, for the STRUCTURE runs using the microsatellite dataset with allele sizes. a-b: K=1 to K=10, for all 177 *Cochlearia* individuals, c-d: K=11 to K=20, for all 177 *Cochlearia* individuals and e-f: K-1 to K=10 for 120 tetraploid *Cochlearia* individuals.

Appendix Table A4. Pairwise genetic distances (lower, Ds) and spatial distances (upper, Euclidean, in km) between 13 tetraploid *Cochlearia officinalis* populations from Northern Norway, analysed in SPAGEDI using the microsatellite dataset with allele sizes. See Appendix Table A2 for detailed population information.

	offnorfin	offofftro1	offnortro1	offnortro2	offinttro1	offinttro2	offofftro2	offoffslof1	offintslof1	offnorslof1	offintslof2	offnorslof2	offoffslof2
offnorfin	-	130.3	124.8	107.2	132.6	138.1	118.3	155.7	229.1	167.6	151.7	169.8	216.3
offofftro1	0.1792	-	23.2	30.8	30.3	40.8	43.0	150.9	131.3	132.3	122.7	138.6	138.0
offnortro1	0.1432	0.0706	-	17.6	52.6	63.4	61.7	127.7	118.1	109.4	99.5	115.6	120.0
offnortro2	0.1876	0.1145	0.0697	-	54.0	64.7	57.5	125.2	132.5	111.6	99.8	117.3	131.0
offinttro1	0.3748	0.6171	0.7271	0.5926	-	10.9	19.3	178.7	158.3	161.9	151.7	168.0	167.4
offinttro2	0.5178	0.3564	0.5803	0.3727	0.2556	-	20.2	189.6	166.6	172.8	162.6	179.0	177.0
offofftro2	0.0957	0.1848	0.1418	0.2549	0.3359	0.4811	-	182.1	174.1	168.8	157.3	174.7	180.6
offoffslof1	0.0823	0.1313	0.0813	0.0986	0.6323	0.4911	0.1391	-	132.3	35.9	32.5	31.4	100.9
offintslof1	0.1462	0.2111	0.2377	0.1025	0.4091	0.2649	0.2810	0.0994	-	96.4	104.7	101.2	35.3
offnorslof1	0.1418	0.1382	0.0833	0.1191	0.7232	0.5312	0.1864	0.0669	0.1871	-	15.8	6.7	65.9
offintslof2	0.1791	0.3416	0.3734	0.2759	0.6670	0.5548	0.4382	0.2550	0.2101	0.2467	-	19.1	77.1
offnorslof2	0.2140	0.1869	0.0959	0.1146	0.7155	0.5076	0.2534	0.1228	0.2295	-0.0000	0.3551	-	69.6
offoffslof2	0.1197	0.1671	0.1071	0.1181	0.4148	0.4436	0.1068	0.0644	0.1168	0.0831	0.3168	0.1171	-

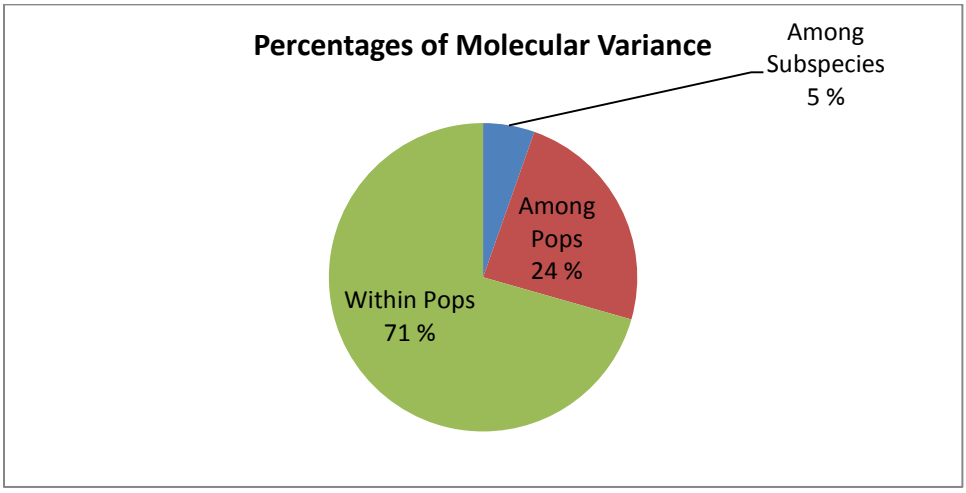


Appendix Figure A5. Isolation by distance analysis performed in ISOLATION BY DISTANCE from the pairwise genetic (Ds) and spatial (Euclidean) distances for 13 tetraploid *Cochlearia officinalis* populations from Northern Norway (Appendix Table A4).



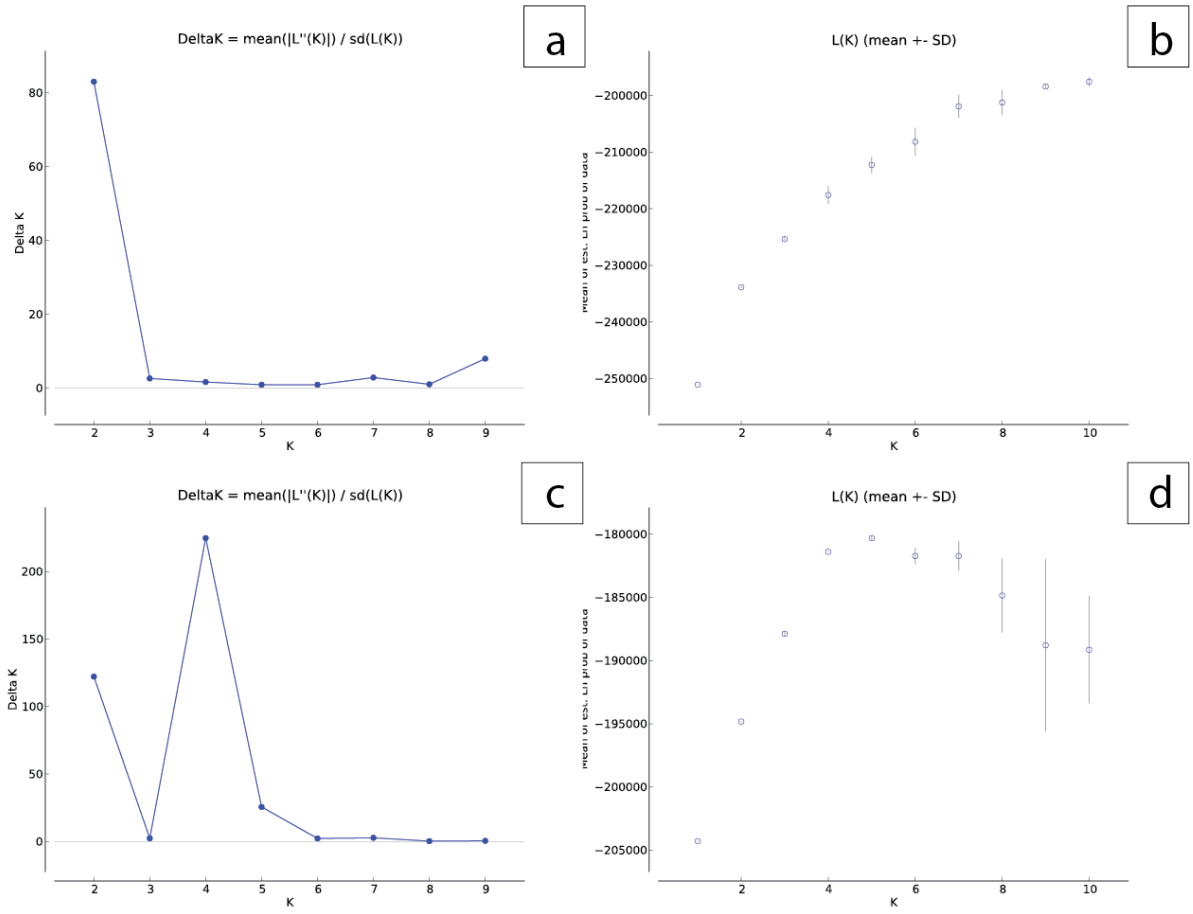
Source	Degrees of freedom	Sum of Squares	Variance component	Variance %	p-values
Among Regions	1	31.15	0.24667	5 %	0.01369 + - 0.00367
Among Pops	10	163.5	1.27815	25 %	0.00000 + - 0.00000
Within Pops	108	385.4	3.56852	70 %	0.00000 + - 0.00000
Total	119	580.05	5.09333	100 %	

Appendix Figure A6. AMOVA performed in ARLEQUIN to test for the effect of regions (Troms vs. Lofoten) on the genetic diversity of 120 tetraploid *Cochlearia officinalis* individuals, using the binary microsatellite dataset.



Source	Degrees of freedom	Sum of Squares	Variance component	Variance %	p-values
Among Subspecies	2	53.25	0.27285	5 %	0.00782 + - 0.00280
Among Pops	9	141.4	1.21426	24 %	0.00000 + - 0.00000
Within Pops	108	385.4	3.56852	71 %	0.00000 + - 0.00000
Total	119	580.05	5.05563	100 %	

Appendix Figure A7. AMOVA performed to test for the effect of subspecies on the genetic diversity of 120 tetraploid *Cochlearia officinalis* individuals using the binary microsatellite dataset.



Appendix Figure A8. Delta K (left) and mean likelihood of K (right) values, as calculated in STRUCTURE HARVESTER, for the STRUCTURE runs a-b: K=1 to K=10, for all 57 *Cochlearia* individuals using the reduced dataset (11 223 SNPs) from RAD-seq, c-d: K=1 to K=10, for 43 tetraploid *Cochlearia* individuals using the reduced dataset (8 661 SNPs) from RAD-seq.