Postglacial Colonization and Parallel Evolution of Metal Tolerance in the Polyploid *Cerastium alpinum*

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Cover image of *Cerastium alpinum* growing in serpentine soil is modified from a photo taken by A.-B. Nyberg Berglund.

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Abstract

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The Fennoscandian flora is characterized by a high frequency of polyploids, probably because they were more successful than diploid plants in colonizing after the last Ice Age. The first postglacial colonizers were likely poor competitors and became displaced from the lowlands as forests advanced. Consequently, many of these pioneers are currently found only above tree line. However, some have persisted within the forests on open habitats such as naturally toxic serpentine soils where succession is arrested at the pioneer stage. These populations represent relicts of former widely distributed plants. The polyploid *Cerastium alpinum* L. (Caryophyllaceae) grows on serpentine soils throughout Fennoscandia. *C. alpinum* populations on different soil types provide a model system for the study of the early postglacial colonization history of Fennoscandia.

Genetic markers showed that *C. alpinum* populations in western Fennoscandia differ genetically from eastern populations, suggesting a two-way colonization. The two lineages meet in a hybrid zone in Northern Scandinavia where a high degree of genetic variation was found. Plants from Fennoscandia and the Western Arctic (Canada, Greenland and Iceland) shared many AFLP fragments, which suggests they originate from common refugia. The Fennoscandian populations were more distantly related to the populations in potential refugia in southern Europe. In fact, the northern populations contained AFLP fragments not found in populations in the Pyrenees and the Alps. Lack of chloroplast DNA variation indicates fast postglacial range expansions and/or a recent origin of *C. alpinum*. Crosses were made to establish the inheritance of enzyme markers. The results strengthen the evidence for an allopolyploid origin of *C. alpinum*.

Adjacent serpentine and non-serpentine populations of *C. alpinum* provide a model system of natural replicates to test whether adaptation to serpentine is constitutive (common for all populations) or locally evolved. A growth experiment with high concentrations of nickel and magnesium, two metals that limit the fertility of serpentine soils, showed that the degree of metal tolerance reflects site-specific soil conditions. Since local adaptation was found in both the eastern and the western immigration lineages, the postglacial colonization of Fennoscandia has involved parallel evolution of metal tolerance in *C. alpinum*.

Key words: adaptation, AFLP, *Cerastium*, enzyme electrophoresis, heavy metal, metal tolerance, parallel evolution, polyploidy, postglacial colonization, serpentine

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To

my children Linnea and David

and

Dr. Olof Rune
in recognition of his pioneering studies
on the Scandinavian serpentine vegetation

Contents

Appendix

Papers I-V

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Nyberg Berglund, A.-B., Saura, A. & Westerbergh, A. 2001. Genetic differentiation of a polyploid plant on ultramafic soils in Fennoscandia. S*outh African Journal of Science 97*, 533-535.

II. Nyberg Berglund, A.-B. & Westerbergh, A. 2001. Two postglacial immigration lineages of the polyploid *Cerastium alpinum* (Caryophyllaceae). *Hereditas 134*, 171-183.

III. Nyberg Berglund, A.-B., Saura, A. & Westerbergh, A. Electrophoretic evidence for disomic inheritance and allopolyploid origin of the octoploid *Cerastium alpinum* (Caryophyllaceae). Submitted.

IV. Nyberg Berglund, A.-B., Dahlgren, S. & Westerbergh, A. 2004. Evidence for parallel evolution and site-specific selection of serpentine tolerance in *Cerastium alpinum* during the colonization of Scandinavia. *New Phytologist 161*, 199-209.

V. Nyberg Berglund, A.-B. & Westerbergh, A. Postglacial colonization of *Cerastium alpinum*: inference from AFLP and cpDNA-SSR markers and comparison with plants from potential refugia. Manuscript.

Introduction

In 1600, the Italian philosopher Giordano Bruno was burned on stake as a heretic. Among his heresies was a claim that there is life outside of Earth. At this very moment, high-tech machines are digging the ground of Mars and Titan, searching for evidence for conditions that may support life. The environment of our own planet has brought forth life in seemingly endless forms. Abiotic forces such as climate and geology put the power of life to test without an end. In general, life wins. Biotic forces such as competition of resources between different life forms add another dimension of challenge. Genetic variation is the key: the lucky recombinants or mutants survive. Infectious bacteria in humans that survive treatments with antibiotics are one example of survivors exposed to what seems to be insurmountable environmental challenges. The study of evolution aims at throwing light on processes that explain the variation among living things at different taxonomic levels. The words of Dobzhansky (1973) "*nothing in biology makes sense except in the light of evolution*" are a way of putting it: evolution explains the question "why" in the study of life.

In this thesis, I aim to contribute to our knowledge on plant evolution in general and plant colonization of serpentine soils in Fennoscandia (roughly Scandinavia and Finland) in particular. The natural serpentine soils are characterized by low concentrations of most plant nutrients and high concentrations of iron, magnesium and heavy metals like nickel. These conditions are toxic to most plant species and consequently the serpentine flora is scarce. I have focused on one species that grows on serpentine throughout Fennoscandia, the common alpine herb *Cerastium alpinum* (Caryophyllaceae). This pioneer plant grows also on other soil types but as it is a weak competitor, it is mainly restricted to open habitats: above the tree line on alpine heaths and in the boreal forests on serpentine, steep slopes and riverbanks. Large parts of the world, including Fennoscandia, were covered with ice only 10 000 years ago. The present flora is therefore the result of plant colonization from regions that were not glaciated. The Fennoscandian flora is characterized by a high frequency of polyploid plants, that is, plants with multiple sets of chromosomes. This can be taken as evidence that polyploid plants probably were more successful than diploids in colonizing the newly deglaciated areas.

I have used genetic methods to elucidate the postglacial colonization history of the polyploid *C. alpinum* in Fennoscandia. In addition, I have used growth experiments to study the tolerance of *C. alpinum* to toxic factors connected to serpentine soils. The outline of the thesis is written for a target group of graduate students in biology and other people with a general interest in plant biology and evolution.

Evolution of plant populations

Evolution is a change of the genetic composition (proportion of individuals with different genotypes) of populations over time. Various evolutionary processes such as mutation, recombination, genetic drift, gene flow and natural selection influence the genetic composition. These processes act on different levels of

organization: nucleotide, gene, genome, individual and population (Figure 1). In the following, numbers in parenthesis correspond to numbers used in Figure 1.

General evolutionary processes

Mutations change the chemical substance of genes, deoxyribonucleic acid (DNA), and result in new genetic variants, alleles (1). When the gametes are formed, the process of recombination during meiosis can result in new combinations of genes. These new gene combinations can be transferred to new zygotes either through the combination of gametes from different individuals, that is, outcrossing (2) or through selfing (3). In addition, asexual reproduction (apomixis) can produce offspring through unreduced gametes. Some genetic variants or gene combinations may be lost from the population by the random process of genetic drift (4). Such stochastic events can have major effects in small populations. Stochastic events such as fires and storms take place without regard to population size, but their consequences vary depending of it, so that small populations are more vulnerable (Frankham, Ballou & Briscoe, 2002). Even if change in the environment may be stochastic over time, each new environmental condition imposes a force in a certain direction. Environmental factors drive the process of natural selection: individuals with inferior attributes of fitness such as low viability and poor reproduction in a habitat lose, while individuals with higher fitness become parents to the next generation (Darwin, 1859). The forces of natural selection often vary in space. The fitness of each plant genotype is therefore dependent on in which environment it happens to live and reproduce. This genotype × environment interaction corresponds to the process of local adaptation of populations, which promotes genetic differentiation of populations. Divergent selection and local adaptation are widely accepted to be the important driving forces for population differentiation. Further, directional selection (selection in one direction) is probably a primary cause of species differentiation and speciation (Rieseberg *et al.*, 2002). Species differentiation may not, however, be prevented when the direction of selection changes over time (*e.g.* Westerbergh & Doebley, 2002, 2004). Gene flow (5) through dispersal of seeds or pollen between plant populations has a homogenizing effect that counteracts that of differentiation. Gene flow may also have a creative role as a mechanism for the spread of advantageous mutations (Rieseberg & Burke, 2001).

Hybridization and polyploidization

Linnaeus (1759) argued that most plant species originate through hybridization, that is, crosses between species. Hybridization is indeed a major creative force in evolution and gives rise to new combinations of genetic material from divergent evolutionary lineages (Arnold, 1997). However, hybridization often results in sterile offspring, mainly because of a requirement of precise pairing of chromosomes during meiosis. If the hybridization has involved parents with different numbers of chromosomes or chromosomes that differ in length or order of genes there may be a lack of homology with defective gametes as a result.

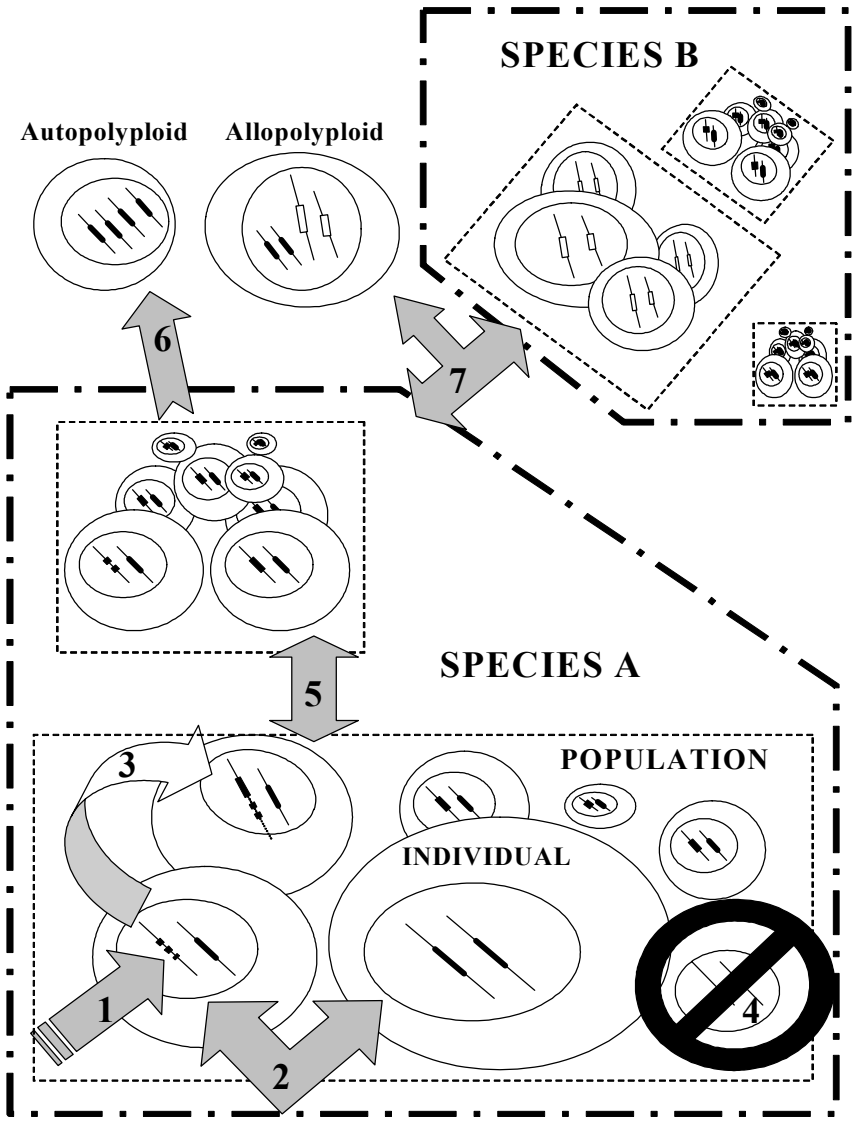

Fig. 1. Evolutionary processes acting on different levels of organization: the individual, the population and the species. Mutations (1) result in new alleles and recombination (during gamete formation) gives new genetic combinations that are transferred to next generation through out-crossing (2) or selfing (3). Some alleles are lost (4) due to random genetic drift or selection. The degree of gene flow between populations (5) that occur through dispersal of seeds and/or pollen affects population differentiation. A doubling of an identical chromosome set, autopolyploidization (6) can occur through the union of two unreduced gametes. Hybridization between species accompanied with genome doubling is allopolyploidization (7).

There may also be individual genes that do not match such incompatibility results in sterility. Since many hybrid species are not only viable but also fertile, there must be processes that act to overcome the above difficulties. Recombination may

result in increased homology between chromosomes of different parental origin. The emerging hybrid species will have the same chromosome numbers as its parents (Rieseberg, 1998). The most important process stabilizing hybrids is genome doubling or polyploid speciation. The doubling of the chromosomes of each parent, either before or after the hybridization event, provides each chromosome with a precise pairing partner, thus allowing fertility and persistence of the hybrid. A hybrid polyploid is called an allopolyploid (see (7) in Figure 1). Polyploidization may also occur without hybridization, that is, autopolyploidy (see (6) in Figure 1). Even though allopolyploidy prevails in nature, autopolyploids are known from natural populations of several plant species (*e.g.* in Soltis & Soltis, 1993; Borgen & Hultgård, 2003).

Polyploidizations have a major effect on the evolution of plants (Arnold, 1997). More than 70% of angiosperm plants may have a polyploid origin (Masterson, 1994) and polyploidization is estimated to represent 2 to 4 % of speciation events in them (Otto & Whitton, 2000). New reproductively isolated plant taxa can emerge almost instantaneously through hybridization accompanied with genome duplication. The fertility of both newly formed auto- and allopolyploids may be low (Ramsey & Schemske, 2002). However, even a very low proportion of functional gametes will be selected for to give rise to functional sexual reproduction (Ramsey & Schemske, 2002). It has been shown that polyploidization may occur repeatedly so that even within relatively small regions and on short time scales both auto- and allopolyploid taxa may have multiple origins (*e.g.* Brochmann, Soltis & Soltis, 1992ab; Brysting, Elven & Nordal, 1997; Brysting, Holst-Jensen & Leitch 2000; Soltis & Soltis, 1993; Borgen & Hultgård, 2003; Brysting *et al.*, 2004).

Inheritance of genes in polyploids

The inheritance of genes in polyploids can be much more complex than in diploids. When a diploid plant produces gametes, the reduction in chromosome number during meiosis is based on pairing of two similar chromosomes (bivalent pairing of homologous chromosomes). If a diploid heterozygous plant with genotype *ab* is selfed the plant will produce *a* and *b* gametes in a 1:1 proportion and the offspring will contain *aa*, *ab* and *bb* individuals in a 1:2:1 proportion. This corresponds to disomic ("two bodies") inheritance in diploids. The presence of highly similar repetitive sequences on all chromosomes of a diploid complicates the recognition and pairing of homologous chromosomes. In polyploid species, this situation is even worse (Moore, 2002).

In autopolyploids, identical chromosome sets have been doubled and in the course of meiosis, both bivalents and multivalents may be formed. If symmetry of the chromosome pairing is achieved, meiosis can be completed without problems. If an autotetraploid heterozygous plant with genotype *aabb* is selfed, this plant will produce three types of gametes: *aa*, *ab* and *bb* in a 1:2:1 proportion and the offspring will contain *aaaa*, *aaab*, *aabb*, *abbb*, *bbbb* individuals in a 1:4:6:4:1 proportion. This is an example of polysomic inheritance. Haldane (1930) developed the theoretical framework for inheritance in autopolyploids.

An allopolyploid plant contains multiples of genes that are located on chromosomes that originate from different parental genomes, that is, *homoeologous* chromosomes. If the parental chromosome sets are so different that pairing does not occur between *homoeologous* chromosomes but only between homologous chromosomes during meiosis, the gametes will be formed as in a diploid (*i.e.* only bivalents are formed resulting in disomic inheritance). The diploid-like meiotic behavior in allopolyploids is, in fact, an immensely important question for all bread and pasta eaters. The allopolyploidization that gave rise to valuable properties of bread wheat (allohexaploid *Triticum aestivum* ssp. *aestivum*) involved major genetic changes of the involved parental genomes that physically stabilized the chromosome paring during meiosis (Levy & Feldman, 2004). Genetic changes that may stabilize chromosome pairing in new polyploids are structural changes such as translocations, insertions and deletions of DNA sequences (*e.g.* Song *et al.*, 1995). In fact, a survey of plant C-values (amount of DNA in the unreplicated gametic nucleus) suggests that loss of DNA following polyploid formation or genome downsizing may be a widespread phenomenon (Leitch & Bennett, 2004). An additional mechanism that ensures correct chromosome pairing in polyploids (not in diploids) is the premeiotic pairing of centromere regions, which may improve the efficiency of the sorting process (Moore, 2002). Stebbins (1950) mentioned cases where plants behaved as intermediates between auto- and allopolyploids, that is, segmental allopolyploids. Wu *et al.* (2001) formulated a general polyploid model for gene segregation that combines meiotic behavior of both bivalent and multivalent pairings.

Evolution and expression of duplicated genes in polyploids

The effects of polyploidy on individual genes and their expression is a complex matter, just in the beginning to be elucidated by scientists. What may happen when multiple numbers of genes with the same function are present? Wendel (2000) identifies three different outcomes: (i) all gene copies may retain their gene function, (ii) some copies may lose function or (iii) the duplicated genes diverge in function. The central idea of the two latter cases is that multiples of genes allow a relaxed pressure of selection so that additional copies mask the accumulation of mutations. Nevertheless, duplicated genes are often preserved in evolution (Otto & Whitton, 2000). Recent studies of gene expression in allopolyploid cotton (*Gossypium*; Adams *et al.*, 2003; Adams, Percifield & Wendel, 2004) show that some duplicated genes (*i.e. homoeologous* genes) are silenced (*i.e.* not expressed) in one organ while expressed in others. This may explain why duplicated genes can be retained for long time following polyploidization. Selection may act upon all duplicated genes but in different stages in the life of the plant. Otto (2003) writes; "*in polyploids, one plus one does not equal two*". This highlights the creative and innovative role of polyploidy, which produces novelty in flowering plants (Levin, 1983).

Duplicated genes are also present in diploids. However, in a polyploid, all genes are subjected to new genetic backgrounds where interactions of alleles at other loci (epistasis) are formed. The genetic effects of polyploidization as well as repeated polyploidization events within a species will increase the number of potential pathways for the evolution of different adaptations.

Genetic methods for evolutionary studies in plants

Several interesting cases of polyploidy were discussed by Stebbins (1950) in his landmark book "Variation and evolution in plants". He summed up the progress in the study of evolution during the previous 20 years: "*The direction and speed of the evolution of any group of organisms at any given time is the resultant of the interaction of a series of reasonably well-known factors and processes, both hereditary and environmental. The task of the evolutionists, therefore is to seek out and evaluate all these factors and processes in respect to as many different groups of organisms as possible, and from the specific information thus acquired to construct such generalizations and hypothesis as he can. This requires the broadest possible knowledge of biology…*". At this time, DNA was not identified yet as the universal hereditary material. Morphological and cytological characters were used to study the patterns of variation, and evolutionary processes were deduced using this information. Since then, biologists have experienced an explosion of new methods for the study of variation. Genetic variation is the raw material for evolutionary change, let it be random, selective neutral or adaptive. Using genetic markers, we can study the patterns of genetic variation directly, that is, without influence of the environment, which is the case in morphological traits (*e.g.* the color of a plant can be modified by variation in soil chemistry and does not reflect solely inherited factors). If the markers are neutral and not subject to selection, the pattern of genetic variation can be used to study the genetic relationship among populations.

Examples of different genetic markers

Enzyme markers (allozymes) studied by protein electrophoresis have been in use since the late 1960s and still contributes significantly to plant studies (>300 scientific papers concerning plant evolution during the past 5 years involved allozymes; Science Citation Index Expanded (SCIE), search the past 5 years for "allozyme* AND plant* AND evolution*"). The method is not species-specific but readily available for a wide range of organisms as the enzymes used are conserved through evolution. Enzymes are extracted from fresh tissue, separated based on size and charge differences with gel electrophoresis (starch or polyacrylamide gels) and visualized with enzyme specific staining reagents. The result is a banding pattern, that is, the enzyme phenotype. Most of the informative enzyme systems are coded by nuclear genes. Some enzyme markers used for plants, however, are also coded by genes in the haploid (only one copy) chloroplast genome. Just how neutral different enzyme variants are may be an open question. Nuclear enzyme markers have a major advantage in that they are co-dominant, that is, if an individual carries two different genetically determined variants of the enzyme, it can be distinguished as a heterozygote from the banding pattern. The two major drawbacks of enzymes are that rather much fresh plant tissue is needed for analysis and the limited degree of polymorphisms that can be found in protein coding genes.

The two problems with enzyme analysis are easily overcome by using PCR (polymerase chain reaction) based DNA methods such as amplified fragment length polymorphism (AFLP), random amplified polymorphism (RAPD) and

amplification of simple sequence repeats (SSR or microsatellites). Extracted DNA is the template to which the PCR reactions work. These techniques are, in general, based on three steps: (1) selection of primers, that is, small (10-20 bp) molecules of single stranded DNA that binds to specific sequences on the template DNA, which initiate the building of DNA fragments (usually 50-500 bp) by a DNA polymerase, (2) separation of fragments that differ in length (*e.g.* with electrophoresis) and (3) detection of fragments with DNA staining (*e.g.* ethidium bromide or silver staining) or by using labeled primers (*e.g.* radioactive or fluorescent). These methods are now commonly used in plant evolutionary studies (>1200 scientific papers concerning plant evolution during the past 5 years used DNA-based methods; SCIE, search the past 5 years for "(AFLP OR RAPD OR SSR) AND plant* AND evolution*"). Both AFLP and RAPD can be used without any previous knowledge about the genome. As most of the DNA is found in the nucleus the majority of the randomly selected AFLP and RAPD fragments are examples of nuclear markers. Both AFLP and RAPD result in the presence (1) or absence (0) of fragments. It is almost impossible to distinguishing heterozygotes (diploid genotype *01* for a certain fragment) from the homozygotes (genotype *11*) and therefore AFLP and RAPD are considered as dominant markers. Dominant markers have a major disadvantage that not all genotypes can be directly seen. Nuclear microsatellites, that is, simple sequence repeats (SSR) markers have the advantages of PCR-methods (*i.e.* small amounts of DNA needed, high degree of polymorphism) and the advantage of co-dominance. Here the polymorphism is not based on presence/absence of fragments but rather fragments with minor size differences. It is possible to distinguish a heterozygote that carries alleles that differ in length by as little as one nucleotide. The microsatellites have, however, to be developed individually for each species, which often requires a substantial investment in time and money.

Another way to study genetic variation is to look at the DNA sequence level. The number of studies of DNA sequences of both haploid genomes (chloroplast and mitochondrial DNA) and diploid nuclear genomes has rapidly grown (>1500 scientific papers concerning plant evolution during the past 5 years involved sequence data; SCIE, search the past 5 years for "DNA sequence* AND plant AND evolution*"). Mutations at any site in the DNA result in traceable unique genetic variants, which offer a highly polymorphic raw material. However, most important, and in contrast to all the above-mentioned methods, the polymorphism is ordered in a pattern that reflects the relationship among the variants.

In addition to the above-mentioned methods for the analysis of nuclear genetic variation, variation in chloroplast DNA (cpDNA) has been studied for many plants. CpDNA represents a haploid genome (within an individual that might be diploid or polyploid at the nuclear level) that is uniparentally inherited. This allows a separate analysis of gene flow through seed dispersal (when maternally inherited as in angiosperms) versus pollen (when paternally inherited as in many gymnosperms). CpDNA is in general characterized by a relative low degree of variation. Variation in intron (sequences of unknown function) and simple sequence repeats (cpDNA-SSR) has, however, been found within populations of several species (*e.g.* King & Ferris, 1998; Mengoni *et al.*, 2001; Mengoni *et al.*, 2003; Kitamoto *et al.*, 2005).

Population genetic analysis of co-dominant and dominant markers

The goal of the data analysis of genetic markers is to get parameters that contribute to the description of how populations are structured and differentiated. The analysis of co-dominant markers in diploids results in individual genotypes for each locus. This data is used to estimate population genetic parameters such as allele frequencies, proportion of polymorphic loci, average number of alleles per locus and average proportion of individuals being heterozygous per locus. Observed genotype frequencies can be compared with expected genotype frequencies calculated from the allele frequencies (p and q) under the assumption of Hardy-Weinberg equilibrium (the sum of the proportion of homozygous and heterozygous genotypes are $p^2 + q^2 + 2pq = 1$, and $p + q = 1$). Eventual deviations can be quantified and statistically tested and used to infer the breeding structure in the populations (*e.g.* selfing that is a deviation from random mating causes a deficiency of heterozygotes). The amount of variance in allele frequencies that is attributed to variation within (*e.g.* F_{IS}) and among populations (*e.g.* F_{ST}; Wright, 1965) can also be estimated. Further, allele and genotype frequencies can be used to calculate different genetic distance measures (*e.g.* Nei, 1972; Hedrick, 1971) between all pairs of populations that may help in the interpretation of the relationship among populations.

The raw data from analyses of dominant markers such as AFLP and RAPD describes each individual with absence/presence (0/1) of each genetic locus (fragment) scored. Because heterozygotes cannot be observed for dominant markers only the expected heterozygosity in a population can be calculated by using the Hardy-Weinberg equilibrium as described above. This is a major drawback because it is not possible to evaluate eventual deficiencies of heterozygotes, which make it more difficult to study the within-population structure. As only the *00* genotype can be scored unambiguously (as a presence of a fragment may indicate either a *01* or an *11* genotype) the frequency of *0* is determined as p. An important point is that only one sequence (that matches the selected primers) leads to the presence of a fragment, but the absence of a fragment can be due to several distinct mutations that are all pooled in the ´null allele´ state. Several measures of genetic distances or similarities can be calculated from presence/absence data. These are in general based on the number of shared fragments between two individuals (a), the number of fragments found in only one individual (b) or in the other (c) and sometimes also includes the number of fragments not found in either of the two (d). As an example, the Jaccard similarity index (S) is formulated as $S = a / (a + b + c)$. If two individuals have no fragments in common ($a = 0$) they are not similar at all and $S = 0$. On the other hand, if all fragments are shared between the two $S = 1$. Both AFLP and RAPD analyses often gives many (*e.g.* 25-100) informative characters. This makes it appropriate to use multivariate analyses such as cluster analysis and ordination. Cluster analysis is based on the calculation of pairwise genetic distances between all individuals (or populations). Different techniques for clustering is then used to group those individuals that are most similar together which results in a hierarchical structure that usually is illustrated with a dendrogram (a “tree”). The ordination techniques that usually are used for the analysis of presence/absence data include correspondence analysis (CA), principal component analysis (PCA) and principal

coordinate analysis (PCoA or PCO). Depending on the structure in the data, one or the other may be more suitable to use. Even though the mathematical algorithms behind these techniques differ, they all reduce the number of interpretable variables into a few ordination axes that can be used to interpret the data in two or three dimension graphs (instead of numerous dimensions in the original data that correspond to the number of variables studied). The two techniques of cluster analysis and ordination are commonly used together to explore genetic data.

The possibility to use sequence informative markers required major technical developments. However, at least as important as the technical advantages has been the development of the analysis of sequences under the conceptual framework of coalescence theory (Schaal & Olsen, 2000). In this theory, all alleles for a gene that differ by mutations that have randomly occurred through time can be derived from a single common ancestral allele that existed at some point in the past (Nordborg, 2001). The possibility to reconstruct gene trees (genealogical relationships among alleles) has opened up new possibilities to study evolutionary processes. Unlike classical population genetic models that rely on assumptions of equilibrium conditions (large population sizes, no selection, no migration) coalescent theory can be used to study processes like selection and gene flow without such assumptions. This is especially important for species in which recent historical processes (such as range expansions and bottlenecks) has influenced the population structure (Schaal & Olsen, 2000).

Assessment of genetic diversity with various markers

Comparison of genetic variation from allozyme data across a number of species (Hamrick & Godt, 1989) showed that about 50% of allozyme loci in diploid plant species are polymorphic (more than one allele is found). Within an average plant population, approximately 34% of the loci are polymorphic. Hamrick & Godt (1989) stressed that the proportion of polymorphic loci is more important than the numbers of alleles at individual loci to determine the levels of allozyme diversity. If the goal is to describe differentiation of populations, it is therefore of major concern to make efforts to get information from as many allozyme loci as possible.

About 20% of the individuals within an average plant population are expected to be heterozygous for both allozymes (Hamrick & Godt, 1989) and RAPD markers (Nybom & Bartish, 2000). These markers reveal that long-lived, outcrossing, late successional taxa retain most of their genetic variability *within* populations. By contrast, annual, selfing and/or early successional taxa are characterized by genetic variability *among* populations (Nybom & Bartish, 2000; Nybom, 2004). Nybom (2004) reports that dominant markers such as RAPD and AFLP yield similar results of genetic variability estimates within and among populations, which probably indicates that each one of these markers covers variability from the whole genome. However, there is some evidence that AFLP markers show clustering on genetic maps around centromeric regions where repetitive regions are present (Fay, Cowan & Leitch, 2005). The values for within-population diversity are higher with co-dominant markers in comparison with the dominant markers, whereas among-population diversity estimates are more similar (Nybom, 2004).

Genetic markers and consequences of polyploidy

In a polyploid more than two alleles may be present at a locus but not all may be expressed. This affects the usage of different genetic markers in polyploids. Enzyme markers are dependent on gene expression. If all alleles are expressed and have different mobility the number of alleles can be counted directly and all polyploid genotypes (both homo- and heterozygotes) can be distinguished. If two or more gene copies code for the same enzyme variant, the staining intensity could theoretically be used to infer the numbers of each allele. This can in practice, however, be difficult (*e.g.* Lokki & Saura, 1980). Enzymes may be coded by *homoeologous* genes that do not segregate at meiosis. This gives rise to fixed heterozygous banding patterns, which is common in polyploid plants (*e.g.* Brochmann *et al.*, 2004). Genetic analysis of segregation patterns among offspring from crosses may help in the interpretation of polyploid enzyme electrophoretic data. The observed enzyme patterns can always be scored as presence/absence characters. In this case, enzyme markers are not co-dominant, since not all genotypes can be determined.

For AFLP and RAPD markers, polyploidy may increase the number of fragments because of larger amount of DNA compared to a diploid relative. There seems however, at least for AFLP, that the genome size *per se* (*i.e.* all the genes carried by a single gamete) is more important for the quality and utility of the AFLP fragments than the degree of ploidy (Fay, Cowan & Leitch, 2005).

For the study of variation in DNA sequences in allopolyploid species, one must be able to identify and characterize *homoeologous* DNA regions. This is no trivial task. If the parental species can be found, the different PCR products that occur can be cloned and analyzed separately. This method has been applied to separate variation attributable to genes at different *homoeologous* chromosomes in *Silene* (Popp & Oxelman, 2001; Popp, *et al.*, 2005).

One model system – two evolutionary questions

The analysis of genetic diversity is fundamental in plant evolutionary studies. However, as pointed out by Stebbins (1950), the study of evolution requires a broad knowledge in biology because the genetic patterns become interesting only within a context that makes a living plant more than just an accumulation of its genes. Depending on the evolutionary question, different aspects such as historical, physiological and ecological factors make up the context that provides a suitable model system.

I have studied the polyploid *C. alpinum*, which is a perennial herb common and abundant in the alpine region in Fennoscandia. *C. alpinum* is also found in the boreal region but only scattered on open habitats. Following the retreat of the ice sheet, starting about 10 000 years ago, the newly exposed land areas were colonized by pioneer plants such as alpine herbs, grasses as well as mosses and lichens (Liljegren & Lagerås, 1993; Vasari, 2004). Many of the pioneers were likely poor competitors and became extinct in the lowland areas. The plants that we call *alpine* retreated above the tree line where they now grow within relatively restricted areas in the mountains (Sjörs, 1967). Some alpine pioneer plants such as

C. alpinum, however, have persisted on open habitats within the forest where they have a scattered distribution on naturally toxic soil types like serpentine and also on steep slopes where succession is arrested at a pioneer stage. Such populations show the footsteps of history, as they are the relicts of a former widely distributed pioneer. The interesting distribution of *C. alpinum* on different soil types throughout Fennoscandia provides a model system that can be used to address two different evolutionary questions.

The model system that involves adjacent serpentine and non-serpentine *C. alpinum* populations in both the alpine and the boreal region can be used (1) to study the postglacial colonization history of a polyploid pioneer plant in Fennoscandia and (2) to test for local adaptation in natural independent replicates.

The historical and ecological factors that are central in this model system are (i) the glacial history of Fennoscandia that has had important consequences for plant colonization in general and (ii) the ecology of serpentine soils that require specific adaptations of the plants.

Glaciation history of Fennoscandia

The latest Pleistocene glaciation, called Weichsel in northern Europe and Wisconsin in North America, extended from about 120 000 to 10 000 years ago (Table 1). The Weichsel was not a uniform cold period but the maximum extensions of the ice sheet covered all parts of Fennoscandia (Figure 2).

Table 1. *A time scale for events during the Quaternary, ya is years ago and Mya millions of years ago*

PERIOD	EPOCH	TIME	EVENTS
Quaternary ”The Age of Man”	**Holocene**	7 000 ya	Development of human cultures in northern Europe
		9 000 ya	Spruce colonizes Fennoscandia from the east
		10 500 ya	Younger Dryas
	Pleistocene ”The Last Ice Age”	0.12 Mya	Weichsel (Northern Europe) Wisconsin (North America) Würm (the Alps)
		0.44 Mya	Humans evolve Saber tanded cats and mammoths walk around
		1.48 Mya	Proposed origin of arctic-alpine *Cerastium*
		1.8 Mya	

Fig. 2. The maximum ice cover (hatched area) during the last Pleistocene glaciation (Hewitt, 1999): the last phase of the last Pleistocene glaciation some 18.000 years BP. The central European mountains had ice caps and between these and the continental ice sheet in the north, there was permafrost, tundra and steppe.

Ice started retreating from southernmost Sweden at about 14 000 radiocarbon years ago (BP, before present) and the melting was relatively rapid until at least 12 000 BP (Björck & Möller, 1987) when large parts of southern Fennoscandia were ice-free. During this period, the Norwegian coast was also ice-free. The melting ice produced huge volumes of melt-water and the reduced mass of the ice resulted in land uplift. During about 400 years, a land bridge connected southern Sweden with the European continent (Björck, 1995). Both plants and animals could colonize the ice-free areas but they probably went extinct during the cold Younger Dryas period (about 11 000 – 10 500 BP). The land bridge between Denmark and southern Sweden was destroyed when the ice re-advanced, which made the Baltic Ice Lake rise and be drained over the former land bridge. When the climate became again warmer a new prolonged land contact between Fennoscandia and the European land mass (Björck, 1995) enabled a massive immigration of animals and plants (Jaarola, Tegelström & Fredga, 1999). The process of land uplift slowed down in the south and increased in the north, with the result that this land bridge was submerged and from about 8 200 BP southern Sweden was permanently cut off from the continent by the Öresund strait. The expansion of plants and animals continued to the north along with the retreat of the ice.

The deglaciation of Finland began at about 10 000 BP (Ignatius, Korpela & Kujansuu, 1980; Koivisto, 2004) and at about 9 000 BP an ice-free north-south corridor enabled contact between southern and northeastern immigration routes (Björck, 1995; Jaarola, Tegelström & Fredga, 1999). The earliest findings of human culture, the Komsa culture in northernmost Norway, have been dated to this time (Meinander, 1984). The last remnants of the glacial ice were in the central parts of southern Norway and in Swedish Lapland (Björck, 1995). These ice sheets probably acted as temporarily barriers for different colonization routes to come into contact.

Postglacial re-colonization of Scandinavian biota

Botanists and geologists agree that ice has covered northwestern Europe in a not very distant past. Plants that now grow on areas once covered with ice must have colonized from ice-free refugia. Here the consensus ends: the location of these refugia has been debated for a long time. Particular emphasis has been placed on endemics and species with a disjunctive distribution (Nordhagen, 1933; *e.g.* Sjörs, 1967; Dahl, 1987). Some alpine plants have a unicentric distribution and are only found on the high mountains of Dovre and Jotunheimen in southern Norway or in the high mountains from 65°N up to about 69°N in northern Fennoscandia. Bicentric plants grow in both of these areas but not in the area in between (Sjörs, 1967). Two alternatives have been put forward to explain these patterns of distribution: the hypothesis for "in situ *glacial survival*" (the "*nunatak*" hypothesis, *i.e.* refugia located within the ice sheet) and the "*tabula rasa*" hypothesis (refugia located outside the ice-sheet). A recent review (Brochmann *et al.*, 2003) of accumulated evidence since the 1960s suggests that endemism and disjunctions in the northwestern European flora can be explained with the "*tabula rasa*" hypothesis.

The potential European refugia of both plants and animals are thought to be found south of the southern limit of the permafrost, in Portugal-Spain, in Italy and in the Balkans (Huntley & Birks, 1983; Hewitt, 1999). Parts of northern Russia were also ice-free during the latest and coldest phase of the Weichsel glaciation (25.000-18.000 BP; Aleksev, 1997; Svendsen *et al.*, 1999). As it has been shown that some species found on both sides of the Atlantic are genetically very similar (*e.g. Lychnis alpina*, Haraldsen & Wesenberg, 1993; *Phippsia algida*, Aares, Nurminiemi & Brochmann, 2000; *Cerastium arcticum*, Hagen, Giese & Brochmann, 2001) the North Atlantic must not have been a strong barrier for dispersal during the Quaternary. Therefore, there may also be potential refugia in the west that may have been connected to the large Beringian refugium, which was ice-free during the Wisconsin glaciation (Abbot & Brochmann, 2003).

Hypotheses of different postglacial immigration routes of plants into Fennoscandia have been based on studies of present distributions as well as from analyses of fossil records like pollen and macrosubfossils such as seeds and wood fragments. Wind pollinated species like birch and spruce produce huge amounts of pollen that can be found in sediments with a relatively high probability. Pollen analysis, however, does not give an adequate view on the presence of insect pollinated plants that produce and disperse much less pollen. The application of genetic methods for the study of variation among extant populations of species has proven to be a powerful tool for the evaluation of alternative immigration hypotheses for all kinds of species (*i.e.* intra-specific phylogeography; Avise *et al.*, 1987).

The ground exposed by the retreating ice had both more calcium and other soluble minerals available for plants than nowadays (Vasari, 2004). Nitrates that result from bacterial action, were, however in short supply. The first periglacial vegetation was sparse, made up of grasses and herbs such as *Artemisia* and *Chenopodium* and sedges (*Carex*). This community, an intermediate between tundra and steppe with no modern counterpart was transient and short-lived,

lasting for some decades (Vasari, 2004). It was replaced with a much more rich and closed heath community characterized by *Dryas octopetala* and early leguminous plants that, like *Dryas*, harbor nitrogen-fixing bacteria. The result was an environment that could support *Salix polaris* and *S. herbacea*, several Caryophyllaceae and Brassicaceae, *Potentilla crantzii* and grasses, sedges and *Juncus* species. There was light, minerals and vacant sites to be occupied. This pristine period lasted for about two centuries (Vasari, 2004). The soils became more acid with accumulating organic material. The light-loving pioneer plants had to give way to treeless heaths that had the dwarf birch, *Betula nana*, as a main ingredient with other common shrubs. The pioneers retreated to the mountains or steppes, but held on to steep slopes, dolomite and serpentine. Here they may still live as living reminders, relicts, of an age past long ago.

The climate became warmer and trees or large bushes like *Hippophaë rhamnoides*, *Juniperus communis*, *Sorbus aucuparia*, *Populus tremula*, *Salix* spp. and finally birch (*Betula pendula)* pine (*Pinus sylvestris)* and hazel (*Corylus avellana)* extended their ranges fast into the former glaciated areas (Huntley & Birks, 1983). Genetic studies of cpDNA variation in hazel and birch (Palmé & Vendramin, 2002; Palmé *et al.*, 2003) indicate colonization of Fennoscandia from one refugium in the south for hazel and a two-way colonization from an eastern and a western refugium for birch. Black alder (*Alnus glutinosa*) also showed a two-way immigration into Fennoscandia (King & Ferris, 1998). A two-way colonization has been demonstrated in several mammals, for example with molecular markers in the field vole (*Microtus agrestis*; Jaarola & Tegelström, 1995) and with chromosome studies in the common shrew (*Sorex araneus*; Fredga, 1996). Other terrestrial mammals have shown a unidirectional colonization pattern - either from the south or from the northeast (*e.g.* wood lemming, *Myopus schisticolor*, Jaarola, Tegelström & Fredga, 1999; Siberian jay, *Perisoreus infaustus*, Uimaniemi *et al.*, 2000; Siberian tit, *Parus cinctus* and the flying squirrel, *Pteromys volans*, Uimaniemi *et al.*, 2003). Norway spruce (*Picea abies*) arrived early (some 8000 years ago) to some localities in northern Finland and Sweden (Kullman, 1996; Giesecke & Bennett, 2004; Vasari, 2004). The main colonization of spruce started, however, later from the White Sea some 7000 years ago and it is still spreading in western Norway (pollen data; Tallantire, 1972; 1977; allozyme data Lagercrantz & Ryman, 1990; cpDNA-SSR, Vendramin *et al.*, 2000). Three immigration routes have been proposed for *Viola rupestris* (Nordal & Jonsell, 1998).

The Bothnian Bay has a subset of a pioneer flora of its own (Jonsell, 1988). Some of these species such as *Arabis petraea* have highly disjunct distributions with the closest occurrences in western Norway and Lake Onega in Russia. It has been proposed that these species spread through the "White-Sea – Baltic Corridor" during the early postglacial times. Allozyme studies of *A. petraea* (*Arabidopsis lyrata* ssp *petraea*) showed that isolated populations of the species on coastal cliffs in Ångermanland, Sweden was more closely related to populations located in western Norway than with Russian allies (Jonsell, Kustås & Nordal, 1995). Using molecular techniques, however, van Treuern *et al.* (1997) showed that the White Sea population of *A. petraea* and the Ångermanland population were as distant or similar to one another as to American populations of *A. lyrata*.

Serpentine soils – a sanctuary for weak competitors

The serpentine soils (also named ultrabasic or ultramafic soils) usually are easily recognized in nature as "islands" of barren soil or cliffs surrounded by more close vegetation (Figure 3a). Serpentine is a ferromagnesian silicate mineral ($(Fe,Mg)_3Si_2O_5(OH)_4$), which is a transition product of the mineral olivine (Brooks, 1987). Olivine is one of the main ingredients in peridotite that is found in igneous rocks and in the mantle of the earth. The process of serpentinization takes place during mountain folding. Depending on the specific composition of the olivine mineral (*i.e.* the relative concentrations of Fe and Mg) and the conditions during the transition (*e.g.* temperature and water availability) serpentine is composed of either of the minerals lizardite, antigorite and chrysotile (Roberts & Proctor, 1992). The latter is also called asbestos. Serpentine soils are characterized by low concentrations of plant nutrients such as nitrogen (N), phosphorous (P), potassium (K) and calcium (Ca) and high, potentially toxic, concentrations of magnesium (Mg) and the heavy metals nickel (Ni), chromium (Cr) and cobalt (Co) (Brooks, 1987; Proctor, 1999). The granular texture of the soil and the lack of organic material make the soil also dry and exposed (Figure 3b). Accordingly, the "serpentine syndrome" is a multifactorial phenomenon (Kruckeberg, 1984). However, high concentrations of Mg in relation to Ca and elevated concentrations of Ni are considered to have major influence on plant growth and survival (Proctor, 1971; Marrs & Proctor, 1976; Johnston & Proctor, 1981; Gabrielli & Pandolfini, 1984; Proctor & Nagy, 1992; Nagy & Proctor, 1997).

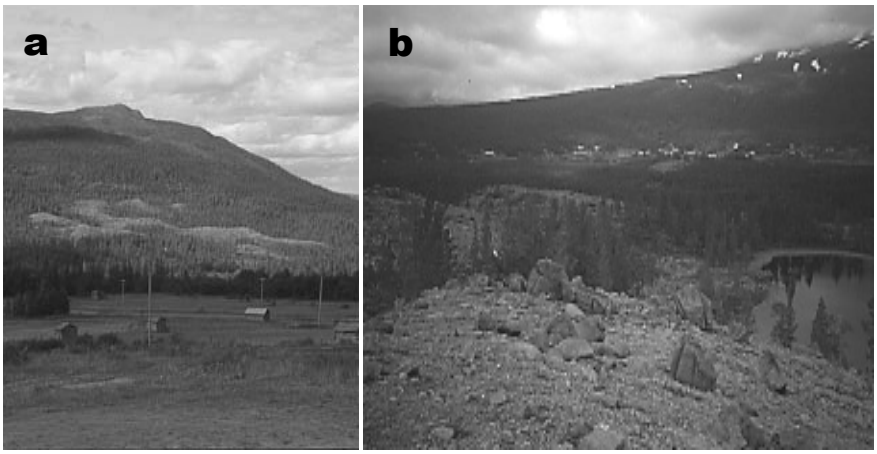

Fig. 3.a) The serpentine soils are easily recognized as islands of barren soil in the surrounding vegetation. b) The granular texture and the lack of organic material make the serpentine soils dry and exposed. Kittelfjäll, Sweden. Photo A.-B. Nyberg Berglund.

Plant adaptation to serpentine – growth strategies and metal tolerance

Plants on serpentine generally are dwarfish and have enlarged root systems compared to conspecific taxa outside serpentine (Rune, 1953; Brooks, 1987; Roberts & Proctor, 1992). Several of these traits have been shown to be genetically determined (Westerbergh, 1994a). Growth studies of the obligate

outcrosser *Silene dioica* (Caryophyllaceae; Westerbergh, 1994b) showed that serpentine plants had a higher root/shoot dry weight ratio than non-serpentine plants. Perennial plants on nutrient-poor and dry soils in general invest a proportionally high amount of resources into the root system (Fitter & Hay, 2002). This limits the resources available for the above ground plant parts. A reduced amount of photosynthetically active tissue lowers the net assimilation, which results in a slow growth rate. In other words, adaptation to stress entails a cost in the form of reduced growth rate. The optimal growth strategy of a plant, however, essentially maximizes fitness (Fitter & Hay, 2002). Plants adapted to serpentine soils are typical examples of plants with a stress-tolerant growth strategy according to Grime (1977; Figure 4). The quality of the environment and the degree of disturbance are two dimensions that can be used to distinguish three different growth strategies. At the most favorable sites with a high degree of disturbance, successful plants are the ones with fast growth and high investment in reproduction - a ruderal growth strategy exemplified by many annual herbs. If the degree of disturbance is low, successful plants invest more resources in vegetative growth to compete for resources - a competitive growth strategy exemplified by trees and shrubs. At the least favorable environments, a stress-tolerant growth strategy is needed (Grime, 1977).

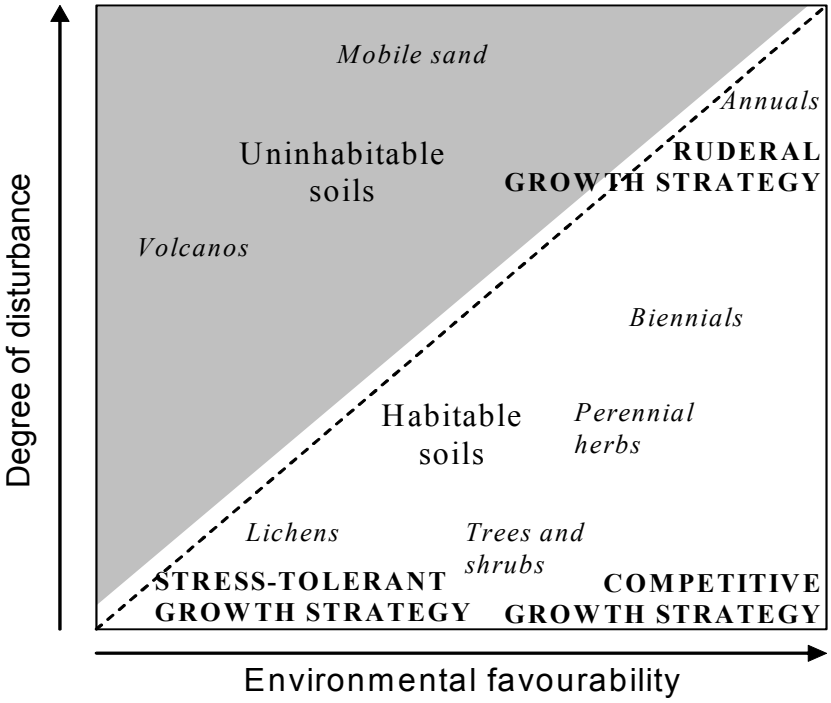

Fig. 4. A two-dimensional graph that illustrates successful growth strategies (ruderal, competitive and stress-tolerant) in relation to the quality of the environment (x-axis) and the degree of disturbance (y-axis) (after Grime, 1977).

Plants adapted to serpentine soils must have strategies to tolerate metal toxicity. In some plants, the metal content in the tissues reflects metal concentrations in the soils. These plants may be called *indicators* (Baker, 1981). Other plants, *accumulators*, have mechanisms for accumulation of metals in non-toxic forms in certain plant compartments. Extreme accumulators like the Ni-hyperaccumulator *Thlaspi montanum* have concentrations > 4000 ppm dry mass in above ground plant tissue (Boyd & Martens, 1998). Finally, *excluders* are plants that can restrict the uptake of metals so that the metal content in plant shoot tissue does not reflect the elemental composition of the soil (Baker, 1981).

Plant diversity on serpentine in Fennoscandia

Rune (1953) made the pioneering studies on the species composition on serpentine in the Scandinavian Mountain Range. A compilation of the data presented in that study shows that >70% (93 species) of the about 130 species that were recorded on 35 typical serpentine sites, are rare with occurrences on less than five sites (Figure 5). These are the plants that Rune (1953) classified as "*serpentine-accidental*" (*e.g. Woodsia alpina*, *Phippsia algida* and *Thalictrum alpinum*).

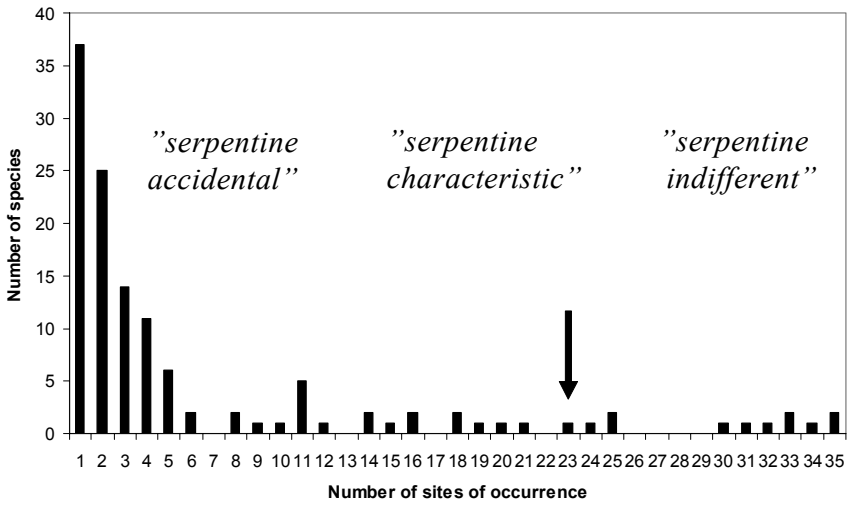

Fig. 5. A frequency diagram showing the number of species found in different number of sites (data from Rune, 1953). The arrow indicates the number of sites where *C. alpinum* was found. The terms "serpentine accidental", "characteristic" and "indifferent" are arbitrarily placed in the graph.

On the contrary, less than 15% (17 species) of the recorded species were found on more than 50% of the sites (Figure 5). I have calculated that the species occurrences are not randomly distributed among the studied serpentine sites (significant ($P < 0.001$) deviations from randomness; test for nestedness on 0/1 matrix of species by site occurrences; data not shown). Instead, the species assemblages show a nested subset pattern (Patterson & Atmar, 1986), that is, species found on sites with few species are the most common species in all sites.

Moreover, the rarest species are only found in species-rich sites. Most insular biota exhibits a high degree of nestedness (Wright *et al.*, 1998). The ecological factors that usually are invoked to explain this pattern are habitat area (larger area, more species), habitat richness (more available niches, more species) and habitat quality (*e.g.* more nutrients, more species). All these factors were discussed by Rune (1953). Rune (1953) called some of the species that almost always are present on serpentine (*e.g. Empetrum hermaphroditum*, *Festuca ovina*, *Vaccinum uliginosum*, *Deschampsia flexuosa*, *Betula nana*, *Salix lapponum*, *S. glauca*, *Juncus trifidus*, *Juniperus communis*, *Calluna vulgaris*) "*serpentine-indifferent*" mainly because they grow abundantly outside serpentine and/or lack obvious morphological differences between the serpentine and non-serpentine plants. On the contrary, most of the "*serpentine-characteristic*" plants (*e.g. C. alpinum* sp*., Lychnis alpina* sp., *Rumex acetosa* and *S. dioica* sp.) express morphological characters that differ from non-serpentine plants of the same species and form subspecies or varieties on serpentine (Rune, 1953, 1988; Rune & Westerbergh, 1992).

Cerastium alpinum

C. alpinum is one of the characteristic serpentine plants in Fennoscandia (Rune, 1953, 1957, 1988; Jonsell, 2001). It is also found outside Fennoscandia, in alpine and arctic regions on both sides of the Atlantic as well as in the southern and central European Mountain Ranges (Figure 6).

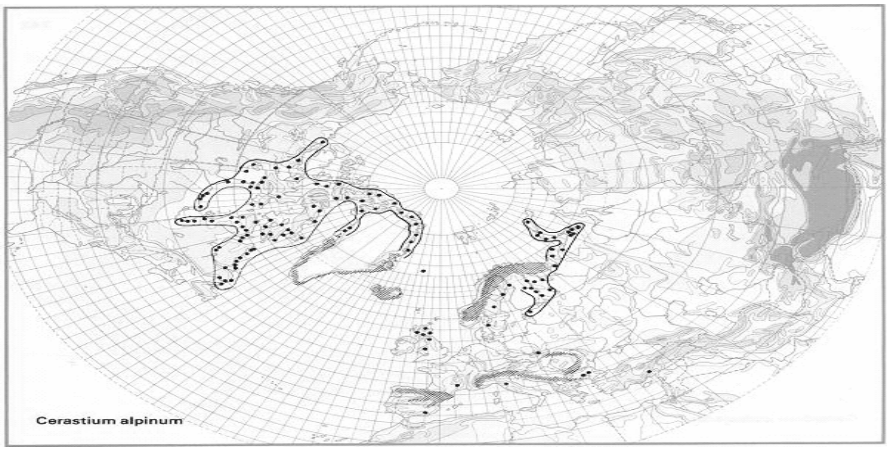

Fig. 6. The native growth sites of *C. alpinum* in the Northern Hemisphere (from Hultén, 1956).

The plant is hermaphroditic with a potential of a mixed mating system of selfing and outbreeding (Grundt, Borgen & Elven, 1999; Totland & Schulte-Herbrüggen, 2003). It has attractive white flowers (Figure 7a) that develop to capsules that usually contain about 10-50 seeds (Figure 7b). Grundt, Borgen & Elven (1999) who quantified seed set in *C. alpinum* growing on different soil types showed that plants on serpentine soils allocated less to reproduction than plants on calcic soils. The seeds have no adaptation for wind dispersal but dispersal over long distances may occur despite of that (*cf. C. arcticum*, Hagen, Giese & Brochmann, 2001).

Long distance dispersal of seeds may occur through animals that move long distances and that have eaten the seeds or a prey that has eaten them. Interestingly, *C. alpinum* seeds had higher germination rate after the passage of the gut of Arctic fox (*Alopex lagopus,* Graae, Pagh & Bruun, 2004). In addition to sexual reproduction, *C. alpinum* plants can produce vegetative shoots or runners from the main stem. The runners appear close to or under the soil surface and the plants form small mats at favorable sites (personal observation).

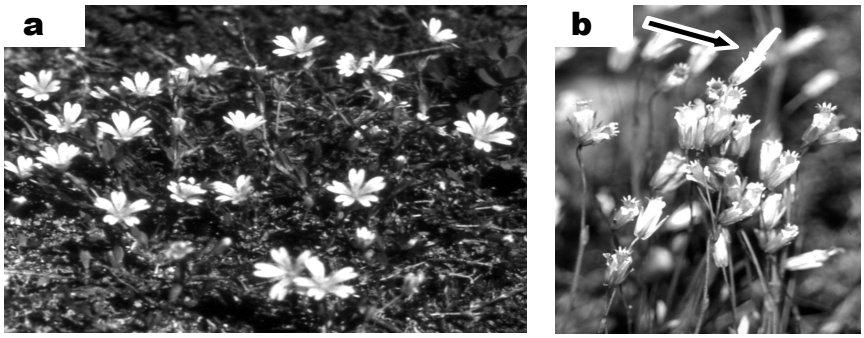

Fig. 7. a) *C. alpinum* produces attractive white flowers and b) capsules that open up with mature seeds. The arrow indicates a capsule where a moth or butterfly larvae has made a chrysalis (pupa). The larvae may consume most of the *C. alpinum* seeds (personal observation). Photo A.-B. Nyberg Berglund.

The frequency of polyploid plants increases with latitude (*e.g.* Stebbins, 1984). This is reflected by the high number of polyploid plants in the Fennoscandian flora (Löve & Löve, 1975). A recent study (Brochmann *et al.*, 2004) of many circumarctic plants supports the view that polyploids have been more successful than diploids in colonizing areas exposed at the end of the latest Ice Age. The genus *Cerastium* is circumpolar and includes several polyploid groups, among them the *C. alpinum* – *C. arcticum* complex (Hultén, 1956). The chromosome numbers of *C. alpinum* and *C. arcticum* are 72 and 108, respectively (Löve & Löve 1975; Brysting, 2000; Jonsell 2001). The taxonomy of the genus *Cerastium* in general and the *C. alpinum* - *C. arcticum* complex in particular has challenged botanists for a long time (*e.g.* Grenier 1841; Murbeck 1898; Hultén 1956; Böcher 1977). Natural hybridization between the taxa in the genus *Cerastium* followed by repeated backcrossing to the parental populations might have created the complex with many intermediate forms. The taxonomic difficulties in this species complex are most pronounced in the arctic-alpine region (Hagen *et al.*, 2002). *C. alpinum*, however, is quite well differentiated from *C. arcticum* in non-arctic areas. Nevertheless, the morphological variation within *C. alpinum* is extensive. Many taxa have been distinguished based on differences in hairiness, leaf morphology and the presence of subterranean shoots (Rune 1957; Jalas & Suominen 1987; Jonsell 2001). These taxa are at present treated as subspecies of *C. alpinum*, *i.e.* ssp. *alpinum*, ssp. *glabratum* and ssp. *lanatum* (Jonsell, 2001).

A classical polyploid complex comprises basic diploid (2n = 2x) taxa, which are morphologically and reproductively isolated. The diploids have hybridized and

produced a swarm of polyploids (2n = 4x, 6x, 8x…; Stebbins, 1950). The *C. alpinum*-*C. arcticum* complex, however, has only high levels of polyploidy (2n = 8x, 12x) and lack close living diploid relatives. One diploid *Cerastium* species is known: *C. lithospermifolium* (2n = 2x = 18) from the Altai Mountains (Krogulevich, 1971). Based on morphological and enzymatic similarities, the tetraploid (2n = 4x = 36) *C. eriophorum* now growing in the central European Mountains has been suggested to be the closest extant relative of *C. alpinum* (Boşcaiu, Vicente & Ehrendorfer, 1999; Brysting & Borgen, 2000).

Scheen *et al.* (2004) have given a molecular phylogeny of the genus *Cerastium* based on sequences of non-coding chloroplast DNA regions. They analyzed one individual each from 57 *Cerastium* taxa. The results suggested an Old World (Europe-Asia-Africa) origin of the genus. The origin of the arctic and alpine high-polyploid group including *C. alpinum* and *C. arcticum* was estimated to about 1.48 – 0.44 Mya (million years ago; Table 1). These two species formed a polytomic group (the species in the group could not be resolved from each other) together with *C. arvense* and some other species from the *C. tomentosum* group. The time frame for the origin of *C. alpinum*, accordingly, would be within the Pleistocene climate oscillations.

Objectives

In this thesis, I strive to elucidate the immigration history and the evolution of serpentine tolerance in the polyploid *C. alpinum* in Fennoscandia. More specifically, my objectives in the papers I-V were the following:

Paper I Investigate the genetic relationship among serpentine populations of *C. alpinum* in Fennoscandia.

Paper II Study the genetic differentiation and verify the ploidy levels among serpentine and non-serpentine populations of *C. alpinum* in Fennoscandia.

Paper III Determine the inheritance of enzyme markers in the polyploid *C. alpinum*.

Paper IV Test if metal tolerance is constitutive (common to all populations) or locally evolved in *C. alpinum*.

Paper V Compare genetic patterns obtained from enzyme markers with DNA markers. Study the genetic relationship between *C. alpinum* populations in Fennoscandia and potential refugia. Compare variation in *C. alpinum* with other *Cerastium* species.

Material and methods

Detailed descriptions of all studies are found in the papers I-V. The following is to summarize the material and methods that I have been working with. I have done most of the fieldwork and laboratory experiments myself or in collaboration with others. Analyses performed solely by other laboratories are marked with *.

Studied populations of *C. alpinum* - plant and soil samples

Plant material from a total of 43 *C. alpinum* populations have been used in paper I – V (Table 2). Maps over the localities and more detailed information such as surrounding vegetation and population sizes are given in the papers.

Table 2. *Serpentine (s) and non-serpentine (n-s) populations of C. alpinum from different regions within and outside of Fennoscandia (W= western, C=central, N=northern, S=southern, E=eastern) used in papers I-V*

Region	Locality (coordinates)	Altitude (m)	Paper
W Norway (s)	Raudberget (60º55'N/6º19'E)	1100	I, II, III, V
W Norway	Gryteberg (60º55'N/6º20'E)	900	II
W Norway	Vikdal (60º58'N/6º25'E)	1000	II
C Norway	Grimsdalen (62º02'N/9º30'E)	1100	II
C Norway	Grimsdalshyttan (62º05'N/9º40'E)	1000	II
C Norway	Grimsån (62º05'N/9º48'E)	870	II
C Norway (s)	Tollevshaug (62º05'N/9º49'E)	980	I, II, III
C Norway (s)	Skårhammerdalen (62º34'N/11º19'E)	700	II
C Norway (s)	Gråberget (62º32'N/11º28'E)	860	II, III
N Norway (s)	Rånafältet, Arnesfjellet (68º10'N/17º10'E)	420	I – V
N Norway	Arnesfjellet (68º10'N/17º11'E)	420	II
W Sweden (s)	Gäddede, Muruhatten (64º30'N/14º10'E)	450	I, II, III
W Sweden (s)	Graipesvaare (65º10'N/15º10'E)	1100	I
W Sweden	Kittelfjället (65º15'N/15º29'E)	520	II, IV, V
W Sweden (s)	Kittelfjäll village (65º14'N/15º31'E)	520	I, II, IV, V
W Sweden	Henriksfjäll (65º15'N/15º37'E)	700	II
N Sweden	Bävvilåptå (66º40'N/16º20'E)	700	II
N Sweden	Björkliden (68º20'N/18º30'E)	1000	II, IV, V
N Sweden (s)	Lingonberget (66º50'N/21º20'E)	400	I, II
E Sweden	Öberget (62º30'N/16º15'E)	300	II
E Sweden	Bodviksberget (64º15'N/17º20'E)	400	II
E Sweden	Storåliden (64º55'N/20º10'E)	250	II
E Sweden	Lögdeåkullen (64º25'N/18º25'E)	400	II
E Sweden	Halvvägsberget (64º35'N/18º30'E)	350	II
NW Russia	Pasvik (69º10'N/29º10'E)	200	II, V
N Finland	Sodankylä (67º20'N/26º35'E)	200	II, IV, V
N Finland (s)	Kuttusvaarat (67º50'N/28º50'E)	330	I
N Finland (s)	Takkaselkätunturi (67º28'N/29º42'E)	560	I
N Finland (s)	Tarpomapää (67º50'N/25º55'E)	340	II, IV, V
N Finland (s)	Tammakkosuvannonmaa (67º30'N/28º50'E)	220	I, II, III
N Finland	Kiutaköngäs (66º10'N/29º10'E)	160	II
C Finland (s)	Kellojärvi (64º16'N/29º10'E)	170	I, II
S Finland (s)	Kokanlampi (63º20'N/28º49'E)	130	I, II, V
S Finland (s)	Louhilampi (63º10'N/28º51'E)	140	I, II, V
W Alps	Tirol (47º04'N/10º53'E)	2700	V
W Alps	Tirol (47º12'N/10º29'E)	2500	V
W Alps	Öst-tirol (46º59'N/12º41'E)	2710	V
Pyrenees	Aragon (42º39'N/0º16'E)	2500	V
Pyrenees	Aragon (42º35'N/0º39'E)	2800	V
N Iceland	Eyjafjarðarsýsla (65º39'N/18º14'W)	250	V
E Greenland	Jameson Land (71º35'N/23º58'W)		V
N Canada	Nunavut, Rankin inlet (62º48'N/92º06'W)		V
N Canada	Manitoba, Churchill (58º45'N/93º51'W)		V

Plants or cuttings of *C. alpinum* collected from 13 serpentine populations during the summer of 1997 were used in paper I (Table 2). The plants were cultivated at room temperature illuminated with fluorescent tubes for 16 h light at Mid Sweden University, Härnösand, Sweden. All plants died during the winter-spring 1998. The new plant material collected from 30 populations of different soil types in Norway, Sweden and Finland during the summer of 1998 and received cuttings from one population in northwestern Russia were used in paper II (Table 2).

Each population was sampled arbitrarily at even intervals along one or two transects. Cuttings were placed in plastic bags with moist paper for three weeks to produce roots. All plants were cultivated in standard garden soil mixed with sand in proportion 1:1. The plant material was maintained at Nordvik Garden, Sweden (62°51'50"N, 18°00'40"E), with approximately 16 h light (400 W sodium vapor lamps) at 15 ºC. The plants were watered when necessary and given a standard nutrient solution once a week. From July 1999, all plants were planted outdoors at Nordvik. These plants were used as parental plants in the crosses made during 2000 and 2001 (Table 1, paper III). In September 2001, all original plants were lost due to a catastrophic event. Seeds from the crosses (III) were germinated on constantly moist filter paper and seedlings were cultivated in standard low nutrient soil: sand (1:1) and cultivated in a laboratory at +20 °C with 16 h light (400 W sodium vapor lamps) at Mid Sweden University, Härnösand, Sweden. An infection killed > 70% of the seedlings. Survived plants were later moved to Mid Sweden University, Sundsvall, Sweden where they were grown under similar conditions.

Seeds from open pollinated *C. alpinum* plants collected from three serpentine and three non-serpentine populations during 1999 were used in the tolerance study in paper IV (Table 2). Soil samples were taken from all six populations (IV) and whole plants from the three serpentine sites were collected for the analysis of metal content in shoots, roots and runners.

During the summers 2002 and 2003 all plants were planted outdoors 1 km W of Mid Sweden University at Sundsvall. Plant samples of survived plants from the tolerance study (IV) were put in -80 °C in zip-lock bags or eppendorf tubes and were used in the molecular study in paper V. For this study (V) I collected new plant material (during 2002) from two populations in southern Finland, grow seeds from the population in NW Russia, received silica dried plant material of *C. alpinum* and other *Cerastium* species from colleagues in Austria, Norway, Switzerland and Slovakia (during 2003). Received seeds from Iceland, Greenland and Canada were germinated and planted as described above. Herbarium specimens from the original populations, silica dried and frozen plant material and extracted DNA (V) are at present deposited at Mid Sweden University, Sundsvall.

Genetic markers and cytological methods

C. alpinum is a wild polyploid plant for which the polyploidization history and what parental genomes that have been involved remain to be worked out. This has influenced the choice of marker systems. I started by using starch gel enzyme electrophoresis as it works for almost all species without any requirements of knowledge about the genome. The initial screening of enzyme systems was done at Umeå University. I then set up a lab at Mid Sweden University (I, II, III). Later on, I chose universal primers for chloroplast microsatellites (cpDNA-SSR) which also work across a wide range of species and since the chloroplasts are haploid, it did not matter that the parental species were unknown. The study of cpDNA-SSRs variation (V) was done at the Department of Plant Biology and Forest Genetics, SLU, Uppsala where also the first trials to use amplified fragment length polymorphism (AFLP) markers were done. I did the AFLP analyses presented in paper V at Mid Sweden University, Sundsvall. The fluorescent PCR products were analyzed with an automated sequencer* (V). To verify if all studied populations of *C. alpinum* had the same ploidy level, the DNA content was analyzed with flow cytometry* and the numbers of chromosomes were determined with cytological standard methods (II).

Study of metal tolerance

Soil and plant sample analyses

The collected soil samples were analyzed for plant available concentrations of different elements* (IV) and used to characterize the soils of the different populations and to choose proper nutrient solutions for a growth study of *C. alpinum* seedlings. The whole plant samples were washed and dried before an analysis of their metal content in shoots, roots and runners, respectively*.

Nutrient culture experiment

C. alpinum seedlings were subjected to a growth study in nutrient solutions. The experiment (performed at Mid Sweden University, Sundsvall) was designed as a full factorial experiment arranged in blocks with Ni and Mg as fixed factors. The response was estimated as the growth of the roots which was measured manually (longest root) and with image analysis (total root growth from scanned pictures).

Data analysis

I have used different multivariate analyses: PCA, PCoA and cluster analysis with UPGMA to analyze discrete genetic marker data (0/1 matrices; I, II, V) as well as quantitative soil chemistry data (IV). The correlation between geographic and genetic distances was analyzed with Mantel statistics (V).

For the analysis of the soil and plant samples I also used one-way ANOVA, non-parametric Kruskal-Wallis test and the growth experiment was analyzed with a general linear model including blocks as random and Ni and Mg as fixed factors and their interactions (IV).

Results and discussion

A two-way colonization of *C. alpinum* in Fennoscandia

Caryophyllaceous plants must have been common in the pioneer plant community that invaded Fennoscandia after the last Ice Age. There is, in fact, transient peak of Caryophyllaceae pollen in the southern Swedish pollen diagrams immediately after the ice sheet had retreated (Sjörs, 1967). Since the pollen cannot be determined species-specifically, there is no way to prove directly the presence of *C. alpinum*. I have studied the postglacial immigration history indirectly by using genetic markers in extant populations of *C. alpinum*.

The genetic variation among *C. alpinum* populations studied with enzyme electrophoresis (I, II) showed that about 50% (8 out of 15) of the studied enzyme loci were polymorphic. This is higher than the average proportion found for diploid plant populations (Hamrick & Godt, 1989). Both study I and II showed that populations from eastern Fennoscandia formed a group genetically different from populations in the west. A cluster analysis of 13 serpentine populations (Figure 2, paper I) showed that the populations of *C. alpinum* in eastern Sweden and Finland formed one group and the western Swedish and the Norwegian populations another. A principal component analysis (PCA) of plants from both serpentine (12) and non-serpentine (19) populations (Figure 1, paper II) showed that plants from populations in eastern Fennoscandia were separated from populations in the west (Figure 6, paper II). This suggested that *C. alpinum* has colonized Fennoscandia through two independent immigration routes. This is now seen in an eastern and a western lineage of descent.

To test the hypothesis of a two-way immigration of *C. alpinum* in Fennoscandia as found with enzyme markers we used AFLP markers, which, in general, show a high degree of polymorphism (V). Once again, populations in eastern Fennoscandia formed a group that differed genetically from plants in the western parts (Figure 8). Populations in southern Finland formed a distinct group, as did the populations in the W Swedish Mountains. Consequently, the AFLP markers strengthen the evidence for a two-way colonization of Fennoscandia by *C. alpinum*.

Secondary contacts

Plants from populations located in eastern Sweden and in the northernmost part of Scandinavia (populations 10-11, 17-18, 21-23; Figure 1, paper II) showed either enzyme multilocus phenotypes with a combination of enzyme phenotypes common in eastern with typical western or some quite rare enzyme phenotypes which shared some enzyme bands from phenotypes frequent in plants from both the eastern and the western population groups. This suggested a zone of secondary contact from the alpine region in the north downward to the lowlands in eastern Sweden (Figure 9).

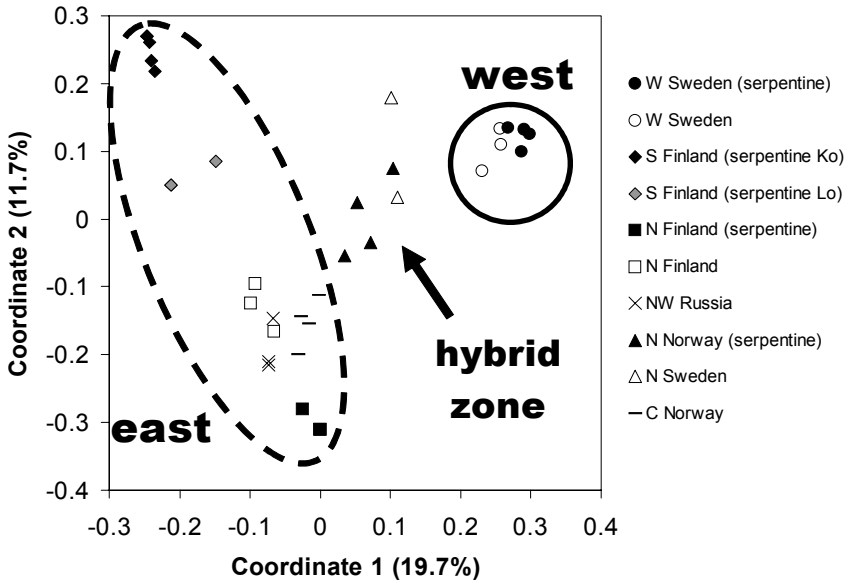

Fig. 8. A PCoA score plot based on AFLP data that shows the first two principal coordinates (explaining 19.7% and 11.7%, respectively of the total variation) for *C. alpinum* plants from Fennoscandia (W = western, S = southern, N = northern, NW = northwestern and C = central). Interpreted clusters of plants from west and east are indicated with bold and dashed circles, respectively. The arrow indicates the plants in the proposed hybrid zone located in northern Norway and Sweden.

When two populations with different allele frequencies meet, the resulting hybrid zone can be expected to have elevated levels of heterozygosity. As has been shown for other hybrid zones (*e.g.* Arnold, 1997) we found a high genetic variability in some of these populations. The genetic variability was measured as the expected number of multilocus phenotypes (enzyme phenotype constitution over all loci) in samples of different sizes using the distribution-free rarefaction analysis (Simberloff, 1979) to avoid the difficulty of dissimilar sample size. The genetic variability was especially high in the northern populations. The northern populations are surrounded by open alpine heaths, where the pollinators forage over large areas (Lundberg & Ranta, 1980). This, along with a recent or ongoing hybridization process is a plausible explanation for the high genetic diversity in the northern part of Fennoscandia. The populations located in the southern part of the hybrid zone showed lower degree of variability than the northern ones. These populations grow on patches of suitable open habitats surrounded by a dense spruce forest that effectively isolates the *C. alpinum* populations from each other. The contact that the two lineages had with each other probably ended when forests invaded the area during the Boreal period for about 8000 years ago. This was followed by population differentiation and reduced effective population size, which probably has resulted in lower genetic variability.

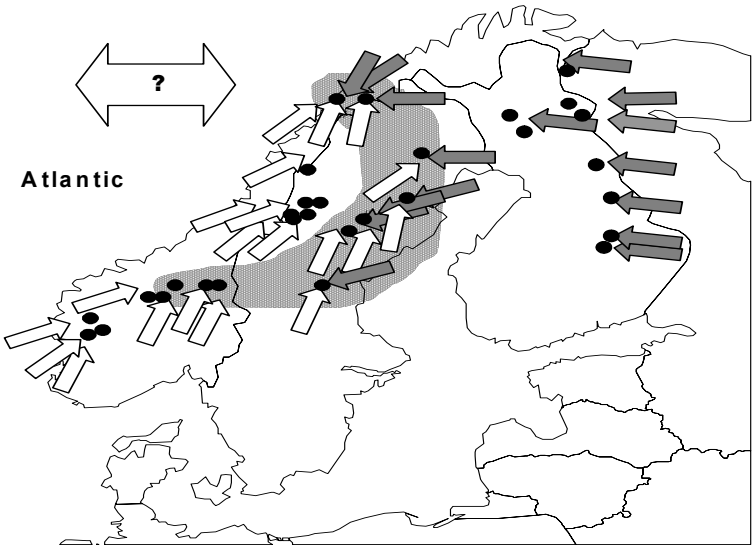

Fig. 9. The postglacial colonization of *C. alpinum* reconstructed through enzyme and AFLP analyses of populations in Fennoscandia. The western and eastern immigration lineages are shown as open and gray arrows. The shaded area indicates the proposed hybrid zone. The double-arrow with a question mark corresponds to the discussion about the genetic similarities that were found between Fennoscandian *C. alpinum* and plants form Iceland, E Greenland and Canada.

Enzymatic similarities between the eastern and the western genetic group were also found in a few populations (population 4-7; Figure 1, paper II) in an alpine valley in central Norway, which indicated another contact zone between the two lineages. The AFLP study (V) that included plants from northern Norway and northern Sweden showed high genetic variability and genetic similarities with both the eastern and western lineage (V), which supports a zone of secondary contact in northern Scandinavia. Interestingly, *C. alpinum* plants from Dovre, located in another alpine valley in central Norway, clustered together with the northern Finnish populations (Figure 1, paper V). This strengthens the evidence for a contact zone between the eastern and western immigration lineages in this region as found for enzyme markers (II).

Large genetic distances among populations

An UPGMA dendrogram (Figure 5, paper II) showed that the genetic distances (based on enzyme phenotype frequencies) within both the western and eastern population groups were large. In the eastern group, the largest distance was found between two serpentine populations located only a few kilometers apart in southern Finland. The finding that these closely located populations were fixed for different enzyme patterns, suggested that the gene flow is highly restricted and that the populations are isolated from each other. Restricted gene flow could be explained by a high degree of selfing and inbreeding as well as limited pollinator flight between populations. In order to discriminate between these alternatives, we wanted to estimate the degree of inbreeding by a study of the genetic structure

within the populations. This can be done by using a co-dominant marker for which observed and expected (under random mating) heterozygosity can be compared. As *C. alpinum* is polyploid, complex banding patterns were obtained (I, II). We therefore first needed to understand the inheritance of the enzyme markers. Analysis of crosses demonstrated disomic inheritance at the *Mr-1* locus (III). This allowed us to use a diploid model to analyze the population genetic structure of the polyploid *C. alpinum* (III).

Only two alpine populations showed fixed homozygous patterns for the MR enzyme while more than half of the populations that are surrounded by forests were fixed for one allele (III). The fixation of closely located populations for different alleles shows that forests effectively isolate *C. alpinum* populations. The importance of the surrounding vegetation in influencing gene flow patterns among populations has also been shown for the related and dioecious *S. dioica* in the Scandinavian mountains (Westerbergh & Saura 1992, 1994). Accordingly, the isolating effect of vegetation may be invoked to explain the large genetic differences found in the eastern immigration lineage. The populations representing the western lineage, however, are mostly located in the open alpine region. Large genetic distances were found even among adjacent alpine populations: populations 1, 2 and 3 in western Norway, populations 5, 6 and 7 in central Norway, populations 13 and 14 in the Swedish mountains and populations 21 and 22 in northern Norway. The observed number of homozygotes at *Mr-1* greatly exceeded Hardy-Weinberg expectations in at least one population in each of these regions (population 1, 2, 7, 13 and 21), Table 2, paper III). The deviation from random mating can be explained by subdivision of populations into restricted neighborhood groups caused by a high degree of selfing and/or restricted pollinator flight and limited seed dispersal. Inbreeding may therefore help explain the large genetic distances found in the alpine region. Polyploid plants are expected to tolerate high degrees of selfing (Soltis & Soltis, 2000).

In the study of inheritance of enzyme markers (III) we found fixed heterozygous banding patterns for all enzyme systems except for MR. One reason for the large genetic distances that were found (Figure 5, paper II) is that several enzyme systems showed fixed heterozygous banding patterns. For the enzymes ACN, MDH and MR, we distinguished phenotypes from different staining intensity on the different enzyme bands (I, II). Variation in band intensity may depend on a variety of reasons like number of gene copies, gene silencing or difference in kinetic activity. The study of inheritance of enzyme markers (III) showed that the intensity differences in MR were not completely reliable so that all heterozygous banding patterns rather should be interpreted as one genotypic class. A re-analysis of the data in paper II did not change the results much so that the major pattern of genetic relationships among populations was the same.

Large genetic distances would be expected if there were differences in ploidy levels among populations. As different ploidy levels are known in the genus *Cerastium* and since hybridization takes place between taxa within the genus (Hultén, 1956; Jonsell, 2001), we wanted to ascertain that the studied plant populations had the same level of ploidy. We therefore analyzed the relative DNA content and the chromosome number of *C. alpinum* plants with different

morphology and from different soil types (II). No difference was found in relative DNA content among 80 plants (II). It was difficult to determine the exact number of chromosomes since *C. alpinum* has many and small chromosomes and we identified between 60 to 78 chromosomes. One plant from a Swedish serpentine population had about 42 chromosomes and two plants in a Finnish serpentine population had about 36 chromosomes each. Other *C. alpinum* plants from these populations that were analyzed with flow cytometry did not, however, deviate in their relative DNA content. We conclude that the majority of plants from the studied populations of *C. alpinum* have the same ploidy level irrespective of morphology and soil type. This agrees with previous chromosome investigations of *C. alpinum* (Boşcaiu, Vicente & Ehrendorfer, 1999; Brysting, 2000).

Serpentine colonization

Plant species have different abilities to evolve serpentine tolerant populations depending on their genetic resources. If *C. alpinum* in general has the genetic prerequisites to colonize serpentine, serpentine and non-serpentine populations would respond to serpentine stress in a similar way. Alternatively, the immigrating populations may have had genetic variability in tolerance so that tolerant individuals would have been selected upon the colonization of serpentine on each site.

No evidence for a serpentine adapted enzyme genotype

The serpentine plants did not form a common cluster separated from the non-serpentine populations. Both of the two major population groups (eastern and western; Figure 5, paper II) contained both serpentine and non-serpentine populations and no soil type-related pattern was found. There is, accordingly, no evidence for a separate genetic group among serpentine populations. In other words, *C. alpinum* has been able to colonize various soil types repeatedly irrespective of the genetic background. Dynesius & Jansson (2000) argue that generalists have an advantage in areas with varying glacial and warm periods. The climatic oscillations at higher latitudes should favor powers of dispersal and reduce specialization. In fact, few plant species are restricted to serpentine soils at high latitudes while in the tropics the number of serpentine endemics is very high (Brooks, 1987; Jaffré, Reeves & Becquer, 1997). The time factor, of course, may be of great importance for the evolution of serpentine endemics. The Scandinavian flora is young and the serpentine plant populations have evolved relatively recently. The strength of selection exerted by serpentine also affects the evolution of serpentine populations. In fact, serpentine soils in the tropics often have a higher content of heavy metals than serpentines in northern Europe (Brooks, 1987).

Parallel evolution of metal tolerance

To test if metal tolerance in *C. alpinum* is constitutive or locally evolved, we made a growth experiment (IV) using plants originating from one serpentine and one non-serpentine population in each of the two immigrating lineages, as well as in the presumed hybrid zone (Figure 1, paper IV). We focused on Ni and Mg and

analyzed the individual and combined effects of these metals in a nutrient solution culture experiment with one low and one high concentration of each metal. The responses of plants measured in terms of root growth showed that plants from serpentine populations in general had a higher tolerance against both Ni and Mg compared to plants from adjacent non-serpentine populations (Figure 3, paper IV). This suggests that Ni and Mg tolerances are adaptive rather constitutive traits in *C. alpinum*. Two other plants of the same family, Caryophyllaceae, *S. dioica* in Sweden (Westerbergh, 1994b) and *Cerastium fontanum* in Scotland (Nagy & Proctor, 1997) did not show any significant difference in Ni tolerance between populations on and off serpentine. In addition, the Ni hyperaccumulator *T. montanum* var. *montanum* (Brassicaceae) was shown to be constitutively adapted (Boyd & Martens, 1998). However, local adaptations of metal tolerance such as that found for *C. alpinum* have been reported perhaps more frequently (*e.g. Agrostis tenuis*, Nichols & McNeilly, 1982; *A. capillaris*, Al-Hiyaly, McNeilly & Bradshaw, 1988; *Mimulus guttatus*, Macnair, 1983; *Silene vulgaris*, Schat, Voijs & Kuiper, 1996). In general, for those plant species that are found in metalliferious soils, low frequencies of tolerant individuals are found in non-metalliferious populations of the species (Gartside & McNeilly, 1974; Ingram, 1987).

Different degrees of tolerance were found among the serpentine populations. The populations from the western and eastern immigration lineages (1 S and 5 S; Figure 1, paper IV) showed a similar response to Ni and Mg stress, while the serpentine population in the hybrid zone (3 S) differed in response. The latter population showed a high tolerance to Ni, whereas root growth was clearly reduced in the two other populations. Plants from 3 S responded negatively to high Mg concentrations while the growth of plants from the two other populations was not affected by this treatment. Why do the serpentine populations differ in their tolerance to Ni and Mg stress? The answer can be found in the different compositions of the serpentine soils. The degree of tolerance to Ni and Mg reflects differences in intensities of selection, that is, different plant available concentrations of these metals in the serpentine sites (Table 1, paper IV). The serpentine populations in the two independent immigrating lineages grow in soils with similar metal concentrations and they also showed similar response to Ni and Mg stress. Mg ameliorated the effect of Ni in these two populations where the Ni tolerance was rather low. However, in the serpentine population in the hybrid zone (3 S) where the Ni tolerance was much higher, Mg did not have that positive effect. Analysis of the Ni content in shoots from plants in these three serpentine populations reflected the soil Ni (Figure 10 and Table 1, paper IV). The highest Ni concentration (mean 0.02% = 200 ppm) was found in population 3 S. The plants from this population had also the highest Ca concentrations and the highest Ca/Mg ratios. Interestingly, the content of Ca was much higher in plants from 5 S than in 1 S, even though the soils contain about the same amounts Ca (Table 1, paper V). These results indicate that *C. alpinum* populations may achieve their tolerance to serpentine somewhat differently.

This growth experiment was designed to evaluate the phenotypic response of plants from serpentine and non-serpentine in a single environment. The plants could of course have showed a different response in another environment (*e.g.*

enriched by nitrogen, other Ca/Mg ratios). What we study is the variation at the phenotypic level (V_P) which is made up of genetic variation (V_G), environmental variation (V_E) and the interaction between the two (V_{GE}): $V_P = V_G + V_E + V_{GE}$. If both the growth strategy of the plant (slow or fast growing) and the metal tolerance mechanisms *per se* have a genotype-environment interaction it is impossible to disentangle the two components within a single experiment. The metal tolerance of a plant population is usually measured as a proportional increase or decrease of root growth in comparison with growth in parallel controls. The proportional growth tends to indicate a higher tolerance of the plants with lower growth rate compared to faster growing plants (Macnair, 1990, 1993; Bannister & Woodman, 1992). As *C. alpinum* plants from non-serpentine populations showed a higher growth rate in the control solution compared to serpentine plants, we based all conclusions on absolute growth measurements, which do not over-estimate the degree of tolerance.

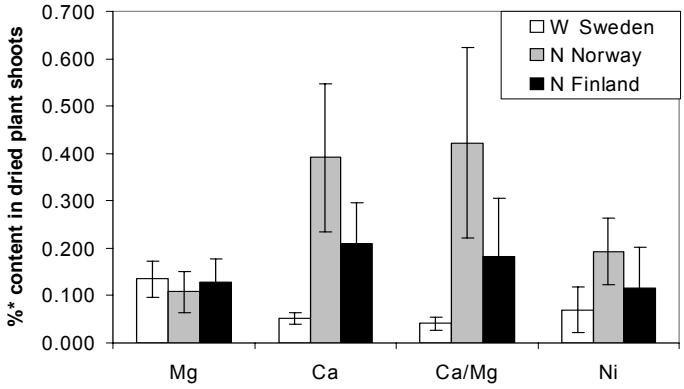

Fig. 10. The content of magnesium (Mg), calcium (Ca), the Ca/Mg ratio and the nickel (Ni) content in dried *C. alpinum* shoots from 10, 10 and 8 plants, respectively, from serpentine populations in western (W) Sweden, northern (N) Norway and northern (N) Finland. The asterisk * in the label of the y-axis, indicates that the percentage values have been divided by a factor 10 for Mg and multiplied with a factor 10 for Ni to obtain a useful scale for all elements. Error bars corresponds to standard deviations (unpublished).

In conclusion, our results suggest that Ni and Mg tolerance have evolved in parallel in the two genetic lineages of *C. alpinum* during the postglacial colonization of Scandinavia. The degree of tolerance may, however, differ which reflects adaptation to site-specific soil characteristics. Parallel evolution of metal tolerance has been suggested for *S. vulgaris* (Caryophyllaceae; Schat, Voijs & Kuiper, 1996) and parallel evolution of other physiological traits has recently been shown in *Lasthenia californica* (Asteraceae; Rajakaruna *et al.*, 2003). In fact, recurrent formation of ecologically distinctive races and species due to parallel selective pressures may not be unusual (Levin, 2001). The question whether the parallel evolution of the adaptive traits is caused by changes in the same genes remains to be proved. The strongest evidence so far for parallel genotypic adaptations comes from artificial selection experiments involving bacterial populations (Wood, Burke & Rieseberg, 2005). It would be very interesting to

investigate if the same genetic changes have occurred in both the eastern and the western lineage of *C. alpinum* in Fennoscandia. The genetic basis of metal tolerance can have a simple genetic background as has been shown for Cu tolerance in *M. guttatus* (Macnair, 1983) and *S. vulgaris* (Schat & ten Bookum, 1992) and Zn tolerance in *Arabidopsis halleri* (Macnair *et al.,* 1999). A simple genetic background increases the likelihood that parallel phenotypic changes have a common genetic basis.

Evolution of hairy and glabrous morphotypes

Hultén (1956) stressed that the lanate ("woolly") hairs of the leaves were an important characteristic to recognize *C. alpinum* while the lack of hairs made *C. glabratum* a clearly separated species. Grundt, Borgen & Elven (2000) who studied the infraspecific variation in *C. alpinum* in central Norway confirmed that hair characters, and especially the lack of hairs, are important in the delimitation of taxa within *C. alpinum*. However, as they pointed out, it may be questionable to put too large weight on a character that have been claimed to have a quite simple genetic basis. Through controlled crosses of glabrous and hairy plants, Westerbergh (1992) and Westerbergh & Nyberg (1995) showed that glabrousness in *S. dioica* was due to alleles at a single locus. Glabrous morphotypes are often found in serpentine populations of many plant species (Brooks, 1987). Hairs function among other things as a defense against herbivores and drought. Westerbergh & Nyberg (1995) showed that hairs make *S. dioica* less palatable for snails. Snails are less frequent on the dry and exposed serpentine habitats and the absence of hairs on serpentine could be a consequence of low herbivory. In the course of this study, I have observed both hairy and glabrous forms growing next to each other within a single serpentine population, which may be explained by a genetic polymorphism.

The enzyme (II) and AFLP (V) studies that involved both hairy and glabrous *C. alpinum* plants from different regions in Fennoscandia show that the genetic structure reflects geography and not morphology. In other words, the glabrous and hairy morphotypes are members of the same population at each locality. Brysting & Borgen (2000) found also a closer enzymatic relationship between ssp. *alpinum* and ssp. *lanatum* within regions than between populations of one subspecies from different regions. A subspecies in a particular geographical region is expected to be genetically distinguishable from other subspecies of the same species.

The different subspecies within the *C. alpinum* complex have been described to prefer different habitats (Hultén, 1956; Grundt, Borgen & Elven, 2000; Jonsell, 2001). It could therefore be appropriate to use the ecotype concept here. According to Turesson (1922) an ecotype is a local genetic adaptation to a particular environment. Turesson (1922) used transplantation experiments to determine if a genetic component was responsible for observed morphological differences between geographical races of species. I have not made transplantation experiments. However, I have cultivated collected plants and plants grown from seeds collected from different sites in a common garden environment and found that morphological differences such as hairiness, leaf forms and plant size are preserved over several seasons. Offspring from populations that are characterized

by different morphological characters showed distinct morphotypes. Further, serpentine and non-serpentine plants differed significantly in growth rate under controlled growth conditions (IV). There is without doubt a genetic component involved in the determination of different plant characteristics observed within *C. alpinum*. However, both extremely hairy and completely glabrous plants grow in serpentine habitats. I therefore conclude that none of the described subspecies in *C. alpinum* (*e.g.* ssp. *glabratum*) represents a true serpentine ecotype, at least not one that can be defined by its morphology. Jonsell (2001) summarizes the extensive morphological variation found on different soil types and points out that "*...It thus seems as if evolution of ultrabase variants has taken place along many lines*....". Indeed the results of the present thesis strengthen this view.

The three taxa are highly cross-compatible (Grundt, Borgen & Elven, 2000). The lack of reproductive barriers probably reflects recent differentiation. The tolerance experiments (IV) suggested that local adaptation of metal tolerance in serpentine populations have evolved within the last 10 000 years. Given that selective forces outside serpentine also may impose local adaptation in *C. alpinum*, the infraspecific variation in morphology within this species is likely to be the result of recurrent evolution of adaptive traits during the postglacial colonization of Fennoscandia.

Glacial refugia?

The results of two different immigration routes of *C. alpinum* in to Fennoscandia may indicate an expansion of plants from two different refugia. We attempted to use cpDNA variation to analyze plants from different potential refugial areas to test the hypothesis of immigration from east and west. However, almost no variation was found within *C. alpinum* among the seven cpDNA-SSR markers that were used (V). This may be interpreted as an effect of a fast and recent spread of *C. alpinum* from a common refugium. The same cpDNA haplotypes, however, were also found for *C. arvense* and *C. arcticum*. This suggests a common and recent origin of these species, in agreement with the *Cerastium* phylogeny of Sheen *et al.* (2004).

Where do we find the most probable glacial refugia of *C. alpinum*? According to Flora Europea (Jalas & Suominen, 1987) and Hultén (1956), *C. alpinum* grows in Fennoscandia, Northern Russia, Iceland, the British Isles, the Pyrenees, the Alps, the Balkans, the Transylvanian Alps, and the Carpathians and in the high mountains of Poland and Slovakia. It is also found in North America, Greenland and Iceland (Figure 6). To explain the presence of the same plant species in the central and south European Mountain Ranges (csEMRs) and in northern Europe and the Arctic, three scenarios have been proposed (Schönswetter, *et al.*, 2003): (i) postglacial colonization of formerly glaciated northern Europe from csEMRs; (ii) glacial survival in northern European refugia and postglacial colonization of csEMRs; and (iii) glacial survival both in northern refugia an in csEMRs. The Central European Mountains were extensively glaciated during the ice age (called Würm here) and between those and the Weichsel ice sheet there was a plain of permafrost, tundra and cold steppe (Sjörs, 1967; Vasari, 2004). It has been proposed that alpine taxa like *C. alpinum* had a continuous distribution between

the csEMRs and the Weichsel ice sheet during the last glacial period but the evidence is questionable (see references in Schönswetter *et al.*, 2003).

Hewitt (1999) reviews several cases where the southern peninsulas of Iberia, Italy and the Balkans are confirmed as major refugia for both plants and animals. The variation in topography, climate and habitat in the southern Europe provides great opportunities for a species to find appropriate habitats through climatic cycles. During warm periods alpine plants spread to the north but as weak competitors, they also avoided extinction in the south retreating higher up in the mountains. On the opposite, during colder periods these alpine plants find suitable habitats on lower altitudes. Such refugia with an altitudinal variation in distribution have been found in the Alps for example for the alpine plant *Saponaria pumila* (Tribsch, Schönswetter & Stuessy, 2002).

The genetic diversity of Fennoscandian populations was compared with plants from Arctic regions in the west (Iceland, Greenland and Canada), the Alps and the Pyrenees. The Fennoscandian populations were most distantly related to the populations in the Alps and the Pyrenees, while they showed genetic similarity with the populations in Canada, eastern Greenland and Iceland (Figures 3, 4, paper V). We may note that the highest number of shared fragments was found between the hybrid zone in northern Scandinavia and plants form Greenland and Canada. Arctic regions in the west may have been colonized by the same source populations as the eastern and western lineages in Fennoscandia. Alternatively, these regions have had recent contact. Nevertheless, the genetic similarity between the populations in Arctic regions in west and Fennoscandia may suggest that these populations originate from common refugia in the circumarctic region. Based on geographical distributions of plant taxa Hultén (1937) and Nimis *et al.* (1998) independently identified Beringia as a major glacial refugium for arctic plants. *C. alpinum* is not found in this area (Figure 1, paper V) so the glacial refugia for Fennoscandian *C. alpinum* are probably found in more closely located regions. At least some parts of Arctic Canada were ice-free during late Wisconsin (Steig, Wolfe & Miller, 1998) and probably served as glacial refugia for arctic plants (*e.g. Dryas integrifolia*, Tremblay & Schoen, 1999). Caryophyllaceae plants are insect pollinated plants, so that finding their pollen in pollen records are unexpected and can be taken as evidence for high plant densities. Rundgren & Ingólfsson (1999) found Caryophyllaceae pollen in Icelandic pollen records and argued that plant species with high tolerance to climate fluctuations may have survived the whole Weichsel in northern Iceland. Significant amounts of Caryophyllaceae pollen has also been found in East Siberian Arctic (Sher *et al.*, 2005) and on the northern Taymyr Peninsula, Arctic Russia (Andreev *et al.*, 2003). Accordingly, potential Arctic refugia are found in both the east and the west. But can we exclude that the Fennoscandian populations have originated from source populations in central or southern Europe? Schönswetter *et al.* (2003) studied the postglacial immigration of *Ranunculus glacialis* in northern Europe. They found a high number of specific AFLP fragments in the Alps while the northern European populations (*i.e.* Scandinavia, Iceland, E Greenland) harbored no specific fragments and only a subset of the ones found in the eastern Alps. They therefore concluded that *R. glacialis* colonized northern Europe from source populations in the eastern Alps. Interestingly, an AFLP study of the arctic-alpine annual *Comastoma tenellum*

(Gentianaceae) suggested a recent immigration in to the Alps from Scandinavian populations (Schönswetter, Tribsch & Niklfeld, 2004).

The general expectation is to find higher genetic diversity in refugia that have been unglaciated for long times (*e.g.* Hewitt, 1996; Comes & Kadereit, 1998). Evidently, glaciated areas that have been colonized from many source populations will also be characterized by high genetic diversity (Widmer & Lexer, 2001; Petit *et al.*, 2003). We found that northern populations of *C. alpinum* harbored more specific fragments than the Alps and the Pyrenees. However, detailed studies of numerous populations of other alpine plants in the Alps have shown large genetic differentiation among populations (*e.g. Eryngium alpinum*, Gaudeul, Taberlet & Till-Bottraud, 2000; *Eritrichium nanum*, Stehlik, Schneller & Bachmann, 2001; *Saponaria pumila*, Tribsch, Schönswetter & Stuessy, 2002). Since we have only studied a few *C. alpinum* populations in the Alps and the Pyrenees, we may have only detected a fraction of the genetic variation in these areas. This would then explain the low number of specific fragments found in the Alps and the Pyrenees and the present results cannot exclude the hypothesis involving source populations in potential refugia in southern Europe. Brysting & Borgen (2000) found that the most common enzyme multilocus phenotype among *C. alpinum* plants from southern Norway, Iceland and East Greenland was also found in a few plants from the Alps. A denser sampling of *C. alpinum* populations both in southern and central Europe and in the west and east Arctic may help tracing the source populations of the eastern and western immigration lineages in Fennoscandia.

High number of polyploids in the arctic - alpine flora

In the course of repeated glaciations when species distributions contract and expand, founder effects, inbreeding and genetic drift operate and depress the genetic diversity (Hewitt, 1996; Pamilo & Savolainen, 1999). The species range-dynamics driven by the climatic oscillations also decrease the gradual speciation rates but increase the proportion of species formed by abrupt speciation, mainly polyploidy (Dynesius & Jansson, 2000). Stebbins (1950) pointed out that the availability of new ecological niches can favor the establishment of polyploids. Based on observations of North American material, Stebbins (1984) argued that the frequency of polyploidy is correlated with the degree of glaciation rather than with latitude *per se*. Brochmann *et al.* (2004) surveyed the whole flora in the arctic region and did not find a general correlation between degree of glaciation and frequency of polyploid plants. When only arctic specialist taxa are considered, however, the frequency of polyploids clearly exceeds that of diploids. When plant and animal populations invaded the new areas exposed by the melting ice, the population densities were initially low. The probability for male and female gametes to meet and accomplish was therefore reduced and successful sexual reproduction decreased. Consequently, there was possibly a selection for asexual reproduction and as polyploidy is tied with apomixis (asexual reproduction) in plants (Asker & Jerling, 1992) the increase in polyploidy may be an indirect effect of selection for apomixis. However, for insects characterized by *geographical polyploidy* (*e.g.* Stenberg *et al.*, 2000) the success of asexual and clonal

populations is argued to be a by-product of the advantages of hybridity and polyploidy rather than the opposite (Lundmark & Saura, unpublished).

Recurrent polyploidizations?

In areas where polyploidizations are facilitated it is likely that several polyploidization events occur within the same genus. In fact, more than 30 polyploid angiosperms species have been reported to have a multiple origin (Soltis & Soltis, 1993). Recurrent polyploidization has also been shown to occur in rather small geographical areas and over a relatively short time, for example within arctic-alpine *Draba* (Brochmann *et al.*, 1992ab) and *Dupontia* (Brysting *et al.*, 2004).

The study of inheritance of enzyme markers in *C. alpinum* (III) showed fixed heterozygous systems, which strenghthen the evidence for an allopolyploid origin of *C. alpinum*. We found two different fixed patterns for the PGI enzyme: one found in almost all plants in the eastern and the other found in the western genetic lineage (II). This may indicate a bi-directional colonization from two source populations that have involved different polyploidization events. The circumstance that representatives of the two lineages are easily crossed, argues (weakly) against this view.

Genetic relationship between *C. alpinum* and other *Cerastium*

The study of cpDNA-SSR markers of *C. alpinum* (V) involved also plants from 10 related *Cerastium* species collected in Europe. The UPGMA dendrogram showed three major groups of taxa (Figure 5, paper V). The first group consisted of the *C. arcticum* and *C. arvense* plants that clustered together with *C. alpinum*. The second group was a cluster of *C. carinthiacum*, *C. eriophorum*, *C. julicum*, C. *latifolium* and *C. uniflorum* plants. The third group, most distant from the *C. alpinum* group, consisted of *C. cerastoides* and *C. pedunculatum* plants together with the one plant analyzed of *C. lithospermifolium*. This relationship among *Cerastium* taxa corresponds well to the recently published phylogenies by Sheen *et al.* (2004) that are based on sequence data from non-coding chloroplast regions. The circumstance that the three species: *C. arvense*, *C. arcticum* and *C. alpinum* have similar chloroplast genotypes suggests a common maternal lineage and a recent polyploid origin during Pleistocene (Scheen *et al.*, 2004).

In the search for species that potentially have been ancestors in the polyploidization history of *C. alpinum*, it seems logical to look at species with lower ploidy levels. Both cpDNA-SSRs and AFLPs show that the diploid *C. lithospermifolium* (2n = 18) and the tetraploid *C. cerastoides* (2n = 36) only are distantly related to *C. alpinum*. The AFLP phylogeny (Figure 6, paperV) showed a closer relationship between the tetraploid *C. eriophorum* (2n = 36) and *C. alpinum* than did the cpDNA phylogeny (Figure 5, paper V). Brysting & Borgen (2000) found a close enzymatic relationship between *C. eriophorum* and *C. alpinum*. *C. eriophorum* that grows in the central European Mountains shows also morphological similarities with *C. alpinum* and Boşcaiu, Vicente & Ehrendorfer (1999) suggested it to be the closest extant relative of *C. alpinum*. The AFLP

phylogeny strengthens the evidence that *C. eriophorum* is one of the closest extant relatives of *C. alpinum*. I included one *C. arvense* plant in enzyme electrophoresis (data not shown) and found that it showed similar enzyme phenotypes as *C. alpinum* for several enzyme systems. Together with the close relationship between *C. alpinum* and *C. arvense* found with cpDNA and AFLP markers, this suggests that these two species have a polyploidization history with intricate connections. Interestingly, alpine forms of *C. arvense* in the central Alps have been reported to have 2n = 36 (Söllner, 1954) while northern populations of *C. arvense* are considered as having 2n = 72 (Jonsell, 2001). Sequencing of non-coding regions of the RNA polymerase genes is now being used to identify ancestor lineages and the most recently suggested maternal candidate is the tetraploid *C. semidecandrum* (2n = 36), while several tetraploids in central Europe are potential paternal lineages for *C. alpinum* (Brysting, personal communication).

Conclusions and future perspectives

In conclusion, I have shown that the polyploid *C. alpinum* colonized Fennoscandia from two directions now seen in an eastern and a western lineage that meet in a hybrid zone in northern Scandinavia. The ability to grow in natural toxic serpentine soils, characterized by high concentrations of metals like Mg and Ni, is not a constitutive trait within *C. alpinum*. Instead, local adaptations to specific soil conditions have occurred in parallel in the two immigration lineages.

The ancestral species of *C. alpinum* are still unknown. It will indeed be interesting to see what sequences of nuclear genes have to say about the polyploidization history of *C. alpinum*. I think, however, that sequence anonymous AFLP markers still can be used to resolve much of the postglacial immigrations, given that the sampling covers all important areas (both in the Arctic and in the central and southern European Mountain Ranges).

It would be very interesting to study whether the same genes are involved in the metal tolerance in the two independent immigration lineages. One approach would be to screen many individuals from each population for many independent loci (for example by using many AFLP primer combinations). If I would find markers that are associated with serpentine (and not with geographic origin), I may conclude that this suggests that the same genes are involved. I think, however, that the best way would be to know what genes are involved in the tolerance and then analyze these further – because markers without known function (anonymous AFLP markers for example) only provide indirect evidence. One way of doing this would be to apply a QTL (quantitative trait loci) approach. I would then make crosses between serpentine and non-serpentine plants within each lineage have a F1 and then an F2 population. This F2 population would be used for phenotypic as well as genetic characterization, which would enable the analysis of correlations. This approach involves first the development of polymorphic markers evenly distributed along the chromosomes. This will of course be a challenge in a polyploid plant. In fact, a QTL approach is at present used to study the genetic basis of serpentine adaptation in the related *S. vulgaris* (Bratteler *et al.*, 2002). A

third way of attacking the problem would be to use gene expression tools such as microarrays. If the same genes were active in the tolerant plants from both in the east and the west but not in the non-tolerant ones, I would interpret it as evidence that the same genes are involved in the tolerance. Much is already known about metal tolerance in *Arabidopsis*. Given mRNAs active there one could see if they have counterparts involved in the metal tolerance here.

No one has measured the uptake of different elements in *C. alpinum* under controlled growth conditions. If I would do this, I would make efforts to have the plants grown under different steady state conditions to be able to separate the effects of stress from different metals from that of nutrient shortages.

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