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The Faculty of landscape Planning, Horticulture and Agriculture Science
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Genetic diversity of Roseroot (*Rhodiola rosea* L.) from Sweden, Greenland and Faroe Islands

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Abstract

Roseroot (*R. rosea* L.) has gained more attention the last years, mainly because of its phytopharmacologic properties. Even though many reports in roseroot have been published, only a few articles are dealt with the genetic diversity.
The Nordic Genetic Resource Center has collected Roseroot material from Sweden, Greenland and Faroe Islands under different expeditions. The samples were analysed to study the genetic diversity study. Such studies would facilitate The Nordic Genetic Resource Center (NordGen) in conservation of roseroot in its gene bank.

In this study, the roseroot materials collected in the NGB were analysed using two different DNA marker methods, microsatellites (SSR) and inter simple sequence repeats (ISSR) for evaluating the genetic diversity. PCR products were separated on polyacrylamide gels and developed with fast and sensitive silver staining. Gels were read manually with replications. Co-dominant and dominant marker data were analysed separately using POPGENE 1.32; and NTSYSpc 2.11.

Four ISSR primers and four SSR primers were studied and resulted in 37 and 12 polymorphic bands, respectively. Percentages of polymorphic bands for ISSR markers were for Sweden 83.78%, Greenland 94.59% and Faroe Islands 48.65%. Only two samples appear to be clonal material, the rest 89 samples are unique. No gender-specific or population-specific primers were found.

Key words: roseroot, *rhodiola rosea*, genetic diversity, microsatellites (SSR), inter-simple sequence repeat (ISSR)

Sammanfattning

Rosenrot (*R.rosea* L.) är en växt som fått mer intresse om sig de senaste åren, mestadels för dess fytofarmakologiska egenskaper. Även om många artiklar om rosenrot har blivit publicerade, så är det endast ett fåtal som handlar om dess genetiska diversitet.
Nordiskt Genresurscenter har samlat in material från rosenrot ifrån Sverige, Grönland och Färöarna under olika expeditioner. Proven blev analyserade för att studera den genetiska diversiteten. Resultat från en genetisk diversitetsstudie kan hjälpa Nordiskt Genresurscenter i deras bevarandesyfte för sin genbank.

I den här studien har insamlat material från NordGen blivit analyserade av två olika DNA markör metoder, mikrosatelliter (SSR) och inter simple sequence repeats (ISSR) för att utvärdera den genetiska diversiten. PCR produkter blev separerade på en polyacrylamidgel och framkallades med en silvermetod. Gelerna blev avlästa manuellt med replikationer. Co-dominant och dominant markör data blev separat analyserade med hjälp av POPGENE 1.32; och NTSYSpc 2.11.

Fyra ISSR-primers och fyra SSR-primers blev studerade och resulterade i 37 (ISSR) och 12 polymorfiska band (SSR). Procent av polymorfiska band för ISSR markörer var för Sverige 83.78%, Grönland 94.59% och Färöarna 48.65%. Bara två prover visade sig vara klonmaterial, de resterande 89 proverna var unika. Inga könsspecifika eller populationsspecifika primers hittades.

Nyckelord: rosenrot, *Rhodiola rosea*, genetisk diversitet, mikrosatelliter (SSR), inter-simple sequence repeat (ISSR), Nordiskt Genresurscenter, NordGen,

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Introduction

1.1 Botany

Rhodiola rosea, belonging to the Crassulaceae family, is a succulent and herbaceous perennial plant. The plant ranges from 3 to 30 cm in height, (Mossberg & Stenberg 2003) but is generally around 20-30 cm (Dragland 2005).

The root is spindle-shaped and become thicker at the base, developing into short rhizomes (Hegi 1963). During autumn, leaves and stems wither, leaving buds on rhizomes ready to burst in early spring (Dragland 2005). The scale-leaf structure (Lagerberg, 1947, Tutin 1964, Flora of China 2010) on the thick short rhizome emits, when wounded, a rosaceous odour (Hegi 1963, Lindman 1964, Nyman 1980, Krok & Almquist 1994, Mossberg & Stenberg 2003, Ljungkvist 2006).

Stalk is erect, thick, and round (Hegi 1963) ranging from 5-35 cm x 2-6 mm (Tutin 1964) with alternate placed leaves (Tutin 1964, Nyman 1980, Lid 1985).

Leaves are fleshy (Lindman 1964, Nyman 1980, Sandberg & Göthberg 1998, Mossberg & Stenberg 2003), 0.7-3.5 x 0.5-1.8 cm (Flora of China 2010), orbicular-ovate to linear-oblong, dentate, without hair or almost waxy (Lagerberg 1947, Tutin 1964) with a green-bluish colour (Nyman 1980, Mossberg & Stenberg 2003). Leaf-shape is variable depending on geographical location, where observed leaves in the south are long and narrow but short and broad in the north (Tutin 1964).

Inflorescence is arranged in terminal cymes (Tutin 1964) with flowers usually being 4-merous (Tutin 1964, Krok & Almquist 1994, Mossberg & Stenberg 2003) or sometimes 3- or 5-merous (Hegi 1963). The plant is dioecious with male and female flowers located on different plants, and rarely hermaphroditic (Tutin 1964, Krok & Almquist 1994, Mossberg & Stenberg 2003) with various features. The male flowers are larger and prominent during the flowering period (Dragland 2005), June to August (Hegi 1963, Mossberg & Stenberg 2003). Male flowers

have a yellow colour with sometimes reddish nuance (Hegi 1963, Lindman 1964, Sandberg & Göthberg 1998) and a pleasant scent (Mossberg & Stenberg 2003). Male plants are larger and heavier then female plants (Galambosi et al 2009).

As soon as the female flowers have developed their erect follicles, the attention is no longer on the male flowers. Each female flower has four red follicles (Lid 1985, Mossberg & Stenberg 2003, Dragland 2005), 6-12 mm long (Hegi 1963). Seeds are brown with a lanceolate shape (Hegi 1963, Flora of China 2010), 1-1.5 mm (Hegi 1963) to 2 mm long, having wings at one end. Fruits mature in July-September (Flora of China 2010). Chromosome number is 2n=22 (Lid 1985).

1.2 Distribution

Occurrence of roseroot is usually in mountainous areas at rock ledges, precipices, tundra, borders of brooks and river banks (Mossberg & Stenberg 2003). In Europe the plant is distributed in northern Europe and can be found in mountains of central Europe, the Pyrenees, Central Italy and Bulgaria (Tutin 1964). According to GBIF (2010) roseroot occurs in 28 countries in the northern hemisphere including Italy, Bulgaria, Andorra, Austria, Bulgaria, Denmark, Faroe Islands, Finland, France, Germany, Greenland, Iceland, Ireland, Italy, Norway including Svalbard and Jan Mayen, Poland, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland and United Kingdom (GBIF 2010). It is also found in Bosnia-Herzegovina and Czech Republic (Galambosi et al 2007). In North America it occurs in United States and Canada and in Asia in China, Japan, Mongolia and Russian Federation (GBIF 2010).

1.3 Utilisation

1.3.1 Traditional use

As traditional medicinal herb, roseroot has been used for several different purposes, such as to increase physical endurance, work productivity, longevity, resistance to high altitude sickness, and to treat fatigue, depression, anaemia, impotence, gastrotestinal ailments, infections and nervous system disorders. It was also

used to improve fertility in Georgia, cure tuberculosis and cancer in Mongolia (Brown et al 2002). In Norway it has been used to prevent hair loss, heal burns, scurvy, pneumonia and was seen as a diuretic drug (Dragland 2001).

Swedish botanist and physician Carl von Linné stated in his *Flora Svecica* in 1755 that roseroot was good against headache and had a very pleasant odour. It was included in the first Swedish Pharmacopeia in 1755, but sources witness of an earlier use, the Vikings used it to enhance their physical strength and endurance (Brown et al 2002).

R.rosea has been used for many purposes by arctic people (Källman 1997, Ljungkvist 2006) mainly as a medicinal plant, but has also been used in domestic work, as giving wool a greenish colour when cooked together with alum (Nyman 1980).

Norwegians realised early the broad spectra which the plant can be used for. Roseroot rhizomes was served to the Danish-Norwegian king Kristian IV when he visited Norway 1599 (Dragland 2001), but no indications have shown that roseroot was used as food in Norwegian eating habits (Alm 2004). Roseroot was recommended from the Norwegian government in year 1762 to be planted on peat-moss roofs as a fire protection (Sandberg & Göthberg 1998, Hanelt 2001, Alm 2004).

Norwegians also have a great word treasure concerning roseroot indicated on many sectors of applications. Many of the names for roseroot have calf in it, e.g. *kalvedans* and *kalvegror* (Alm 2004). These names could derive from the use of roseroot as forage to calves in early spring when stored winter supply was finished (Dragland 2001) or as Alm (2004) indicates that the word calf (anatomy) comes from roseroot.

In other part of the world, roseroot has been used as food. Both rhizomes and leaves have been used in cooking. The native population of Greenland, the Inuits, used it as food, as well as the Eskimos of North America and Alaska natives (Alm 2004). Children ate leaves raw and grounded leaves were mixed with the bread dough (Dragland 2001, Alm 2004) in harsh times (Alm 2004). On south Greenland roseroot can only be observed where sheep cannot get access to it, since it is highly appreciated by sheep (Wedelsbäck Bladh et al 2006).

Among ethnic groups in Alaska and Siberia, roseroot was one of the 20 most used plants. Here, parts of roseroot have been soured, something similar to sauerkraut (Källman 1997).

1.3.2 Medicinal use

Pharmacological effects

R. rosea is classified as an adaptogen, meaning a plant which is able to adapt its effect to the body's needs (Hedman 2000, Brown et al 2002). The pharmacological effects have been studied by Soviets Union since year 1965, and were given to athletics and cosmonauts (Hedman 2000). It is classified as a medicine in Russia (Brown et al 2002). Pharmacological effects are said to be antifatigue, anti-stress, antihypoxic, anticancer, antioxidant, immune enhancing, and sexual stimulating effects. Roseroot has very low toxicity level and is reported to have few side effects (Brown et al 2002).

Phytochemistry

Chemical compounds in *R.rosea* are divided into six groups (Brown et al 2002):

- Phenylpropanoids: rosavin, rosin, rosarin
- Phenylethanol derivates: salidroside (rhodioloside), tyrosol
- Flavanoids: rodiolin, rodionin, rodiosin, acetylrodalgin, tricin
- Monoterpernes: rosiridol, rosaridin
- Triterpenes: daucosterol, beta-sitosterol
- Phenolic acids: chlorogenic and hydroxycinnamic, gallic acids

Interesting chemical constituents for medicinal purposes are the group phenylethanol derivates and phenylpropanoids (Brown et al 2002) and are mostly found in rhizomes and roots, therefore are the root parts mostly used for medicinal production (Dragland 2001). In a study, natural and cultivated roseroot roots in Finland were compared. Cultivated material was concluded to be better then raw material from wild populations, even though cultivated material contained lower percentage of active substances. Advantages weighing over for using cultivated plant material was that it had less dead root parts and did not have the low frequency of collectable plants in the wild (Galambosi et al 2007). Concentration of active substances, rosavins and salidroside, from wild growing roseroot, seems to differ from different regions in Norway. Collected wild plants were planted in fields so the growing conditions would be the same. Variations were also visible regarding the morphological appearance (Dragland & Mordal 2006).

It is not only *R. rosea* that contains salidroside in genus of *Rhodiola*, something that has lead to using other Rhodiola species as raw material substitute in the 1970s. Since then, scientific investigations have discovered that *R. rosea*'s chemical composition differs from other genus in *Rhodiola* and therefore cannot be replaced or substituted by other Rhodiola species. Specific chemical constituents only found in *R.rosea* are the group phenylpropanoids, also called *rosavins*, containing rosavin, rosin and rosarin, and they are nowadays used as chemical marker for identifying *R.rosea* raw material. A standardisation used in many human studies of roseroot substance is a minimum of 3% rosavins and 0.8-1% salidrose (Brown et al 2002). For further information concerning studies recently made of pharmacological effects, see *Rhodiola rosea*: A phytomedicinal overview by Brown et al 2002.

Unsatisfied pharmacological studies

Blomkvist et al (2009a & 2009b) proved that many of the recent medicinal studies concerning roseroot substances show considerable inadequacy in the statistical part. Blomkvist et al (2009) could only find few reports of the considered studies where good statistical methods had been applied and where the method had been fully described. They conclude after evaluating the literature of roseroot that a potential adaptogenic effect is yet to be sufficiently documented in a scientifically satisfying way. Criticism was given to the different paper editors that published many of the unsatisfying studies in a Swedish newspaper (Blomkvist et al 2009b) where the criticised scientists replied, without giving any clarification about the weak methods used in the studies (Larhammar, personal communication, January 21, 2010).

1.3.3 Market

Today, the interest for the plant is great and this can be proved by the amount of published papers about the medicinal properties (see Appendix III). The market for roseroot is presumed to reach as high as the market for Ginkgo (*Ginkgo biloba*) and Ginseng (*Panax* spp) and while many scientist claims that roseroot is a better supplement, there are reasons to believe that the demand of roseroot will increase more. Perfume industry is interested in Norwegian roseroot (Dragland 2001) and

the demand is increasing for wild Norwegian roseroot (Dragland 2005). 46 companies and 30 suppliers use *R.rosea* in their production worldwide (Ampong-Nyarko 2005 cited in Galambosi et al 2007). Products made from the plant are available as either pills, pure alcohol extracts and as combination with other medicinal plants (Galambosi 2006).

1.4 Rhodiola, an endangered genus

The largest population of *R.rosea* in the world is found in Altai, south Siberia (Galambosi 2006). Species of *Rhodiola* has been used in traditional Tibetan medicine for more then 1000 years, but only in small extent by local people. Nowadays more people are aware of the many beneficial substances found in *Rhodiola* species, which has lead to a higher demand and harvest ratio of natural *Rhodiola* populations (Xia et al 2007). Up to now, most of the raw material for production of roseroot is taken from the natural populations, causing, in many countries, that the natural populations of roseroot are endangered. Lei et al (2006) report of an accelerated and uncontrolled use of *Rhodiola* species during the 1980s in southwestern China to the extent that they are now listed as endangered in whole China (Lei et al 2006). One example of an endangered species in China is *R. sachalinensis*, where heavy disturbances by human activities are the main reason for its status as in danger of extinction (Yan et al 2003). Other factors reported to threaten *Rhodiola* is a deforestation of natural habitat and excessive grazing (Xia et al 2007). Galambosi (2006) confide the same pattern is shown in several countries, among them Russia, where roseroot is nowadays on the Russian Red List and collection is restricted. Despite red listed, estimated quantity of *Rhodiola* roots exported from Russia was around 20-30 tons/year (Galambosi 2006). If this quantity comes from natural populations is unclear. In Europe *R.rosea* is endangered in Czech Republic, Bosnia-Herzegovina and vulnerable in Slovakia (Galambosi et al 2007).

Roseroot, as being the only *Rhodiola* species found in Scandinavia including Greenland, Faroe Islands and Svalbard (with a few exceptions of *R.kirilowii* found in botanical gardens in Sweden) (GBIF 2010), is today *not* red listed in any of the Scandinavian countries, except in Västra Götaland County (Länstyrelsen Västra Götalands län 2010) and Göteborg and Bohuslän counties in Sweden (Mossberg &

Rydberg 1995). This might probably be because of the high labour cost of collection natural populations and difficulties of transport in such an inaccessible area.

The main reason for overexploitation of natural roseroot resources in the alpine areas is believed to derive from the severe alpine climate it grows in, which leads to a slow development of the plant (Galambosi et al 2007, Węglarz et al 2008). Simple solution to this would be cultivating roseroot to produce raw material in sufficient quantities for industrial purposes (Galambosi et al 2007).

1.4.1 Genetic diversity studies of Rhodiola and *R.rosea*

Very limited information about the genetic diversity of *R. rosea* is available (Elameen et al 2008) and few studies have been carried out concerning genetic research of the genus *Rhodiola* (Yan et al 1999, 2003, Xia et al 2005, Lei et al 2006, Yanabaev et al 2007, Xia et al 2007, Elameen et al 2008).

Elameen et al (2008) studied the genetic diversity on *R. rosea.* This study showed a wide range of genetic variability in the germplasm collected in Norway. 82.3% of polymorphic bands were detected among the 97 clones and the genetic similarity ranged from 0.440 to 0.950 with a mean of 0.631 showing no close genetic similarity among clones related to their original growing county. Further the study showed a significantly greater variation within regions (92.03%) than among regions (7.97%) and a low level of genetic differentiation (F_{ST}=0.043). There was no finding of a gender-specific marker in the study and the level of genetic diversity of male, female and hermaphroditic clones was very similar (Elameen et al 2008).

The SSR primers for solely *R.rosea* were developed to be used for future genetic diversity studies in Italy (Zini et al 2007).

In China, the research has been designated to study the genetic diversity of other species within the genus *Rhodiola*. In the case of *R. chrysanthemifolia* growing in the Qinghai-Tibet Plateau, Xia et al (2007) found a substantial genetic diversity at species level (89.7% polymorphic bands). At the population level the percentage of polymorphic bands (PPB) ranged from 21.97% to 48.8%, and genetic variation was mainly among populations (77.3%) and only 22.7% occurred among individuals within populations. This study showed as the one made by Elmeen et al (2008) that there was no close genetic similarity among clones related to their original growing location. For *R. crenulata* from Henduan Mountains Region (China) the opposite was shown, genetic differentiation is correlated to its geo-

graphical distribution. Comparing the genetic diversity within and between populations was found to be almost equal (Lei et al 2006). In *R. angusta* the genetic variability for four populations increased as the altitude was raised (Yan et al 1999).

For *R. alsia* originating from Tibetan Plateau there was a pronounced genetic variation at the species level (80%). Genetic diversity was mainly found among populations (70.3%), and within populations the variation was 29.7% (Xia et al 2005). *R. sachalinensis* showed similar pattern, genetic differentiation was higher among populations, followed by within populations (Yan et al 2003). A Russian study of *R.iremelica* originating from southern Urals also showed a high genetic variation at the species level (Yanbaev et al 2007) as for *R. alsia* and *R. chrysanthemifolia* (Xia et al 2005, 2007).

Variation between populations has been proved by studying the essential oils from the rhizomes of *R. crenulata* from Tibet and Yunnan in China. Differences in the two regions were seen by two chemotypes, the first chemotype characterized by the presence of geraniol as the major compound, while the other having both *n*-octanol and geraniol as major compounds (Lei et al 2004).

1.5 DNA Markers

A DNA marker is a sequence of DNA or a gene, which is situated on a chromosome (Collard et al 2005, Schulmann 2007). Markers make it possible to detect differences between individuals by showing polymorphism, and therefore detecting genetic differences between individual organisms or species (Collard et al 2005).

DNA markers are used in many different areas, such as genetic mapping, finding mutant genes which are connected to hereditary diseases, paternal tests, individual identification, epidemiology and food safety, population history, population studies, etc (Hartl & Jones 2005).

The practice in agriculture has since the introduction of DNA makers in the 1980s broaden and simplified both for scientific and commercial uses to reveal new information about crops, as e.g. disease resistance (Collard et al 2005). Today many plant breeders use DNA markers to trace and identify desirable traits for

crops and other important plants. For some plant researchers, DNA markers are used to study genetic diversity and construct linkage maps (Schulmann 2007).

There are many advantages of DNA markers compared with morphological and biochemical markers. For instance, when comparing morphological markers with DNA markers, the environmental influence can be disregarded with the latter one. But, if still comparing the two marker groups, the advantage of morphological markers have over DNA markers is that it does not require a lot of expensive and often time consuming laboratory work, which is necessary when working with DNA markers (de Vicente & Fulton 2003).

1.5.1 Types of DNA markers

There are different types of DNA markers available and new methods are continually being developed. Today, no method is ideal for all applications so scientist teams must weigh both pros and cons of methods when starting a new project (Harlt & Jones 2005).

DNA markers are grouped after their different abilities of showing homozygosity (dominant marker) and/or heterozygosity (co-dominant marker) (Hartl 1988, Hartl & Jones 2005). The most commonly used *dominant* DNA marker for genetic diversity in plants are: Random Amplified Polymorphic DNA (RAPD); DNA amplification fingerprinting (DAF); Arbitrarily primed polymerase chain reaction (AP-PCR), Inter-simple sequence repeat (ISSR) and Amplified Fragment Length Polymorphisms (AFLP), whereas the most used *co-dominant* markers are: Restriction Fragment Length Polymorphisms (RFLP); Microsatellites (SSR); Sequence-characterised amplified region (SCAR); Cleaved amplified polymorphic sequence (CAPS); Expressed sequence tag (EST) and Single-Nucleotide Polymorphism (SNP) (de Vicente & Fulton 2003). Both dominant and co-dominant markers can be use to detect DNA polymorphism, which further is used to assess the level of genetic variation in diverse populations, and can indicate for instance population history, patterns of migration, and breeding structure (Hartl & Jones 2005).

1.5.2 Microsatellite markers

Microsatellites are short nucleotide tandem repeats of a motif, usually one to six bases. They are presence in bacterial, fungal, plant, animal and human genomes,

and are often referred to simple sequence repeats (SSR). Microsatellites are easy to amplify and are highly abundant and evenly distributed throughout genome (Weising et al 2005b), which makes the method highly polymorphic and specific (Bornet & Branchard 2001). Studies have shown that the most abundant repeated motifs in plants are $(A)_n$, $(AT)_n$, $(GA)_n$, and $(GAA)_n$ (Weising et al 2005a). As being a co-dominant marker, both homo- and heterozogotes can be detected (Weising et al 2005b). Nowadays microsatellites are one of the most important genetic marker for population studies, since they show extensive variation between individuals and within populations (Coates & Byrne 2005).

The broad spectrum of application for SSR is one of its advantages. SSR are used for plant breeding, conservation biology and population genetics as forensics, paternity analysis and gene mapping (Coates & Byrne 2005). The methods require little amount of DNA, which does not have to be of high quality. Another advantage is the simple interpretation of results (de Vicente & Fulton 2003).

The main disadvantage of SSRs is the requirement of a known sequence to be amplified (Weising et al 2005b). Developing new microsatellites are time consuming and notably expensive (Coates & Byrne 2005). Another disadvantage is the phenomena null-alleles, which are nonamplifying alleles and appears frequently. Null-alleles leave no bands when having homozygousity, but when having heterozygousity it leaves one band visual. This will interfere and complicate reading of data, since it will be registered as a homozygote individual when actually being a heterozygote. To reduce error due to null alleles, population studies should contain many diverse SSR primers so that different multiple microsatellite loci are investigated (Weising et al 2005b).

1.5.3 Inter-simple sequence repeats

Inter-simple sequence repeats (ISSRs) are regions found between microsatellite repeats (Ziętkiewicz et al 1994, Godwin et al 1997, Bornet & Branchard 2001, de Vincente & Futon 2003). They were developed to overcome the problem of having the prior knowledge of genetic sequence, as for SSR (Godwin et al 1997). This is done by having a primer with a repeat, as $(CA)_n$ or $(AGC)_n$ with a 3'-achor, resulting in $(CA)_8$RG or $(ACG)_6$TY. Sequence amplification will occur in between two SSRs (Godwin et al 1997), producing multiple loci (Ziętkiewicz et al 1994, Bornet & Branchard 2001, de Vincente & Futon 2003).

ISSR have been used since 1994 for a wide range of organisms (Ziętkiewicz et al 1994) in DNA fingerprinting, diversity analysis and genome mapping (Godwin et al 1997, Bornet & Branchard 2001, de Vincente et al 2004). The advantages of using ISSR is that it is quick, easy to apply, highly reproducible and polymorphous and with the most important advantage that no prior information about genomic sequence is required (Bornet & Branchard 2001).

1.5.4 Nordic Genetic Resource Center and its roseroot collection

The Nordic Genetic Resource Center (NordGen) is responsible for the conservation and sustainable use of genetic resources of farm animals and cultural plants within agriculture, horticulture and forestry. The overall collection on NordGen is today at about 30 000 accessions, the majority being cereals, but also consisting of accessions of forage, oil, vegetable, and medicinal crops (Ottosson, personal communications, May 15, 2010).

Current collection of roseroot in NordGen is 108 accessions, originating from Sweden (23 accessions), Greenland (46 accessions), Finland (13 accessions), Faroe Islands (21 accessions), and Island (3 accessions). Available is also accessions from Germany (1 accession) and Austria (1 accession).

Collected material from Greenland was made during 2006 under the expedition ARKPRO, *Insamling, odling och nyttjande av Grönländska gräs och medicinalväxter för grönländskt bruk*, a part project of the climate study, Artic Climate Impact Assessment (ACIA).

Swedish plant material was collected in the project *Spice and medicinal plants in the Nordic and Baltic countries* (SPIMED). The Greenlandic material is only available for research at NordGen, according to agreements with Greenlandic authorities.

1.6 Objectives

The purpose of this study was to evaluate whether the two different genetic marker methods, ISSR and SSR, are applicable for analysing the genetic diversity of the collected materials of *R.rosea* and to evaluate the genetic diversity of the roseroot materials collected in NordGen . The results from this study will serve as a base

for further genetic diversity studies on *R.rosea* from Scandinavian countries and facilitate NordGen to decide which accessions should be kept to maintain sufficient genetic diversity of roseroot in NordGen.

Questions addressed for this study were:

- Which SSR and ISSR primers can be applied to *R.rosea* collected from Sweden, Greenland and Faroe Islands?
- What is the degree of the genetic diversity of analysed *R. rosea*?
- Are there any gender or population-specific primers for *R. rosea*?
- Are there any clones in the investigated material?

2 Material and Methods

Plant material was assembled after their originating country, and each country was analysed as one population. This decision was based on the way the samples were collected, and the goal with the different collection expeditions was not to use the material in a genetic study. In genetics the definition of a population is a group of individuals who share a common genepool and have the potential to interbreed (de Vicente et al 2004).

In this project both SSRs and ISSRs markers were chosen. ISSR markers for *R. rosea* have been pre-examined in the NordGen laboratory in earlier projects (Khairullina & Ohlsson, personal communication, January 18, 2010), where different ISSR markers have given polymorphic results. The ISSR markers used have also shown good results in other reports concerning different species in the genus of *Rhodiola* (Xia et al 2005, Lei et al 2006, Xia et al 2007).

Regarding the SSR markers the selected primers specific for *R.rosea* have been based on the report by Zini et al (2009).

Both methods have been used considerably in the laboratory of NordGen on other plant species, e.g. Paeonia (Khairullina & Ohlsson, personal communication, January 18, 2010).

2.1 Plant Material

2.1.1 Swedish plant material

Of total 91 samples, 24 samples from 20 accessions came from Jämtland County in Sweden. All Swedish samples came from wild growing plants, except for five individual samples, which was cross-bred with five parents coming from previous

declared county at Alnarp (see appendix IV- Dataset for *R.rosea*). Swedish samples were all combined into one population. Collection sites are presented in Appendix V and all collection sites (including Greenland and Faroe Island collection sites) are available as a Google map for close-up maps.

2.1.2 Greenlandic plant material

Plant material from Greenland contained 64 samples from 20 accessions. Material was collected during a field trip to Greenland. The collecting sites were divided into three areas, South Greenland (Narsaq, Narsarsuaq, Qaqortoq, Brattahlíð), West Greenland (Nuuk) and East Greenland (Tasiilaq). Collecting sites on South Greenland was dominated by grassy land, Nuuk was dominated by peat land and when arriving to Tasiilaq, the snow had already fallen, but could still collect roseroot. Accessions were collected inside a square metre and material was shipped to Sweden, Alnarp, where it was cultivated and plant material was prepared for DNA analysing (Wedelsbäck Bladh et al 2006). Greenlandic samples were all combined into one population Collection sites are presented in Appendix VI and on Google maps.

2.1.3 Faroese plant material

In the study three samples from Faroe Islands were included. Chosen samples were collected sporadically around Faroe Island which is visual in Appendix VII, and on Google maps.

2.2 DNA extraction

Genomic DNA was previously extracted from leaves year 2007 using a protocol previously described by Doyle & Doyle (1990) with some modifications (see appendix VIII- Protocol for DNA Extraction).

2.3 ISSR

In a preliminary test, six inter-specific sequence repeats (ISSR) were evaluated and tested, 834; 841; 888; 890; G05; G11. Five of these resulted in visual bands and four of these (834; 841; G05; G11) were further used for analysing all the samples and more details are listed in Table 1.

Table 1. The six ISSR markers used in this study.

Oligo name	Primer sequence	Annelling temperature (ºC)
UBC 834 (G07)	5'-AGAGAGAGAGAGAGAG YT-3'	54.5
UBC 841 (G08)	5'-GAGAGAGAGAGAGAG AYC-3'	53
UBC 888	5'-BDBCACACACACACACA-3'	52
UBC 890	5'-VHVGTGTGTGTGTGTGT-3'	54
G05	5'-CAGAGGGGCACCTGG-3'	53
G11	5'-GCTCTGGCGCACCGA-3'	55.5

Note B=C/G/T; D=A/G/T; H=A/C/T; Y=C/T.

2.3.1 ISSR-PCR

For PCR reaction, the following master mix was prepared: 19.1 µl Milli-Q H_2O; 2.5 µl 10x Thermo Pol Buffer; 0.5 µl 10 mM dNTP; 0.75 10 pmol/µl primer; 0.15 µl Taq polymerase, and 2 µl of 20 µg/µl of sample DNA, giving a volume of 25 µl in each PCR well.

For PCR amplification, a touch-down thermal profile for each ISSR primer developed from earlier tests in NordGen (Khairullina & Ohlsson, personal communication, January 18, 2010) using a 2720 Thermal Cycler from Applied Biosystems. Initial step was 94ºC for 3 minutes and continued with 20 cycles with 94ºC for 0:30 seconds, 55-57.5ºC) for 0:50 minutes with decreasing -0.1ºC per cycle, and last step at 72ºC for 0:50 minutes. Next 25 cycles started with 94ºC for 0:30 minutes, 53-55.5ºC for 0:50 minutes and 72ºC for 0:50 minutes. A last step with two holds at 72ºC for 3 minutes completed the PCR program.

2.4 SSR

Four out of eight SSR markers developed for *R.rosea* by Zini et al (2009) were selected, with the following locus names: RRC10; RRD6; RRE3 and RRE9. Three markers have 63ºC annealing temperature (T_a), whereas RRE9 has 64ºC T_a. The products of all amplications range within 143-186 bp. Stock solution for primers was dissolved in $T_{10}E_{01}$ according to enclosed information from producer TAG Copenhagen A/S. Four working solution was prepared with 90 µl $T_{10}E_{01}$ and 10 µl of primer stock solution.

2.4.1 SSR-PCR

For SSR PCR analysis, all 91 *R. rosea* DNA samples were prepared as follows: 18.35 µl Milli-Q water; 2.5 µl 10x Thermo Pol Buffer; 0.5 µl 10 mM dNTP; 0.75 10 pmol/µl forward primer; 0.75 pmol/µl reverse primer; 0.15 µl Taq polymerase, and 2 µl of 20 µg/µl of sample DNA, giving a volume of 25 µl in each PCR well.

PCR amplification followed the touch-down thermal profile developed by Zini et al (2009). Initial step starting at 95ºC for 5 minutes and further 5 cycles was pursued at 95ºC for 30 seconds, 65-60ºC where 1º was decreased per cycle, and 72ºC for 1 minute. PCR profile ended with 25 cycles at 95ºC for 30 seconds, 60ºC and 72ºC for 1 minute, respectively. The PCR amplification was performed in a 2720 Thermal Cycler from Applied Biosystems.

Table 2. The four SSR markers used according to Zini et al (2009).

Oligo name	Primer sequence (5'-3')	Annelling temperature (ºC)	Repeat motif
RRC10 (EU407480)	F: FAM-GACTTGGATTTCAATTTCATGG	63	$(GT)_7$
	R: CTGAAACACACAATCCCACCT		
RRD6 (EU407481)	F: FAM-TCAGGCTTGCTAAAGTTCCA	63	$(GT)_9$
	R: CTGAAACACACAATCCCACCT		
RRE3 (EU407483)	F: FAM-CCTGAGTAACATTATTTCCCAGAA	63	$(CT)_8$
	R: ATCATTCCCGGTAACTGGTG		
RRE9 (EU407485)	F: FAM-CCAACTTTGTAGAGCGAACCA	64	$(CAA)_8$
	R: CTCTGCCATGAAACCAGTCA		

2.5 PAGE analysis of PCR products

PCR products from the different markers were separated on polyacrylamide gels (CleanGel 10% 52S from ETC Elektrophorese-Technik GmbH) to visualize polymorphism. Every sample from the PCR was prepared by adding 5 µl loading buffer (20% sucrose, 10% Ficoll, 0.05% Bromephenol blue, 5 M urea and 1 mM EDTA), centrifuged and followed by mixing for 2 minutes to receive uniformity with the loading buffer and the samples in the PCR wells. Each well on the gel was loaded with 7 µl of the dyed samples, leaving one wells on each side which was loaded with 5 µl of the ladder DNA Molecular Weight Marker V from Roche. To verify the previous mentioned ladder, an additional DNA ladder from Fermentas (GeneRuler 50bp) was used.

The gels were run for 30 minutes with 310V and increased to 510 V until the blue dye reached the anode wick (1 hour).

2.6 Developing polyacrylamide gels with fast and sensitive silver staining

For developing gels, fast and sensitive silver staining was used. After electrophoresis, the gels were transferred into a vessel with 200 ml 0.6% Benzene sulfonic acid and 24% Ethanol. After 40 minutes the gels was washed with 3x10 minutes in 0.07% Benzene sulfonic acid. The gels were then silvered for 30 minutes in 0.2% AgNO3, 0.07% Benzene sulfonic acid, 0.05% formalaldehyde and 0.002% Na-thiosulfate and another 2 minutes in milli-Q water to clean the gels from silver. For developing, 1-2 minutes appeared to be adequate for PCR products with SSR-markers, while 3 minutes with an ISSR-marker. Last step was stopping and preserving with 10% acetic acid and 10% glycerol for 30 minutes (see Appendix IX). This step was repeated and left over night before the gels were dried and analysed.

2.7 Analysing gels and data

2.7.1 Analysing the gels

Analysis of the polyacrylamide gels was made manually for both ISSR and SSR markers, with two replications. Bands on gels made with ISSR markers were scored as presence (1) or absence (0). Gels with SSR primers were scored for different alleles, including transforming them into presence (1) or absence (0) to be able to combine the results from the two methods for statistical calculations. The combined resulting data was calculated as dominant marker data (diploid) for all statistical analysis.

2.7.2 Data analysis

NTSYSpc 2.11

Numerical Taxonomy System (NTSYSpc), version 2.11, from Applied Biostatics Inc. (2002), was used to analyse the results obtained from scoring. For measuring similarity between sample sets, (similarity for qualitative data), Jaccard similarity coefficient was used (Jaccard 1908).

Cluster analysis was performed with Sequential agglomerative hierarical nested cluster analysis (SAHN) with unweighted pairgroup method with arithmetic mean (UPGMA). The output, a dendrogram, from cluster analysis is presented in appendix X - Dendrogram.

POPGENE 1.32

Population Genetic Analysis (POPGENE) version 1.32 (1997) was used to estimate expected (H_E) and observed (H_O) heterozygosity, deviation from Hardy Weinberg equilibrium (H_{WE}) and Shannon's Information Index (*I*) for co-dominant data marker (SSR). For dominant marker data (ISSR), POPGENE was used to estimate number of polymorphic bands, percentage of polymorphic bands, Nei's gene diversity (*h**) and Shannon's Information Index (*I*).

3 Results and discussion

3.1 Methods

3.1.1 ISSR

Out of the six ISSR primers studied on *R.rosea*, only primer 890 did not show any bands at all. The other tested primers (834; 841; 888; G05; G11) all gave rise to polymorphic bands. Primers 834, 841, G05 and G11 were further studied and the results are presented in Tables 3 and 4. Average number of bands per primer was 9.25, with primer 841 produced the highest number of polymorphic bands. The lowest number of bands was produced using the primer G11.

Table 3. Number of polymorphic bands, with total and average number of bands per ISSR primer.

ISSR Primer	Number of polymorphic bands
834	10
841	12
G05	9
G11	6
Average	9.25
Total	37

The percentage of polymorphic bands ranged from 48.65% for population Faroe Islands to 94.59% for population Greenland (see table 4) with a mean of about 75.67%. Nei's gene diversity ranged from 0.1814 for population Faroe Islands to 0.2848 for population Greenland, Estimates of Shannon's index (I) ranged from 0.2706 for population Faroe Islands to 0.4368 for population Greenland with an average of 0.3534, and at species level, I was 0.4498.

Table 4. The total number and percentage of polymorphic loci resulting from ISSR analyses of rose-root from the three countries.

Population	Sample size	No. of polymorphic bands	% of polymorphic bands	h* (St. Dev.)	*I** (St. Dev.)
Sweden	24	31	83.78	0.2276 (0.1868)	0.3529 (0.2520)
Greenland	61	35	94.59	0.2848 (0.1657)	0.4368 (0.2147)
Faroe Islands	3	18	48.65	0.1814 (0.1998)	0.2706 (0.2905)
Average				0.2313	0.3534
Species total	88	37	100	0.2917 (0.1550)	0.4498 (0.1949)

* h = Nei's (1973) gene diversity

* I = Shannon's Information index [Lewontin (1972)]

3.1.2 SSR

The numbers of alleles per locus are presented in Table 5, with a total of 12 alleles, ranging from 4 for primer RRC10 and RRD6 to 2 for primer RRE3 and RRE9. Average number of alleles per loci was 3. For comparison, the results from Zini et al (2009) are presented as well in Table 5. This shows a difference for all loci, except for RRC10. Different methods in visualising the results in the two studies can have played a role. The original report used a DNA sequencer instead of manually reading polyacrylamide gels, which sometimes were hard to analyse, probably due to little separation visually for the naked eye. Results can also suggest a difference between populations from Italy and Nordic sited *R.rosea*.

Table 5. Comparing the number of alleles per loci found in this study and in Zini's report

SSR Primer	Number of alleles found in this study	Number of alleles found by Zini et al (2009)
RRC10	4	4
RRD6	4	5
RRE3	2	3
RRE9	2	3
Average	3	3.75
Total	12	15

Observed heterozygosity (H0) ranged from 0.094 to 1.00, whereas expected heterozygosity (He) (genetic diversity) ranged from 0.198 for Sweden at RRE3 to 0.733 for Faroe Islands at RRC10 (see table 6). Lowest and highest observed heterozygosity for the same loci analysed by Zini et al (2009) ranged from 0.091 at RRE3 to 0.696 at RRE9, and lowest value for expected heterozygosity was achieved at RRE3 (0.165) and highest value was at RRC10 (0.553) (Zini et al 2009).

Table 6. Genetic parameters for the three countries (populations) of R.rosea L.

	Sweden *(N=24)*			Greenland *(N=64)*			Faroe Islands *(N=3)*		
Locus name	H_0	H_e	P_{HW}	H_0	H_e	P_{HW}	H_0	H_e	P_{HW}
RRC10	0.261	0.587	0.000529	0.262	0.498	0.000003	1.000	0.733	0.572407
RRD6	0.261	0.634	0.000023	0.094	0.585	0.000000	0.500	0.500	1.000000
RRE3	0.217	0.198	0.604479	0.258	0.227	0.260354	0.333	0.333	1.000000
RRE9	Mono-morphic	-	-	0.000	0.502	0.000000	0.000	0.533	0.020921

* Expected heterozygosity were computed using Levene (1949)

** P_{HW} probabilities associated with Hardy Weinberg test

Zini et al (2009) studied 8 SSR primers for *R.rosea* from Val di Sole (Trentino region) in Italy and all showed polymorphism. Results gained from SSR method by Zini et al (2009) are being further used to examine genetic structure of *R. rosea* populations from Italy. However, it is uncertain if those primers would work for *R. rosea* of Nordic origin. In this study, we have shown that all four selected SSR primers (RRC10, RRD6, RRE3 and RRE9) used by Zini et al worked well for Nordic roseroot. Our results further proved that SSR markers work fine for analysing genetic diversity in natural *R. rosea* populations.

This study shows that both SSR and ISSR methods work well for analysing the genetic diversity of *R. rosea*. Both methods could be used for detecting and analysing genetic diversity. Genetic studies of Roseroot using AFLP gave also good results (Elameen et al 2008). Evaluating which method that could be better suitable for R.rosea is not possible, because the low amount of analysed loci (4) as the case with microsatellites.

Up to now, there are no published reports concerning ISSR method for analysing the genetic diversity of *R. rosea*. However, ISSR markers have been tested in *R.crenulata* (Lei et al 2006), *R.chrysanthemifolia* (Xia et al 2007), and *R.alsia* (Xia et al 2005) and showed polymorphism. In this study, primers 834, 841, G05, and G11 gave polymorphic bands. Tested primers for *R.crenulata*, *R.chrysanthemifolia* and *R.alsia* could be of interest for further genetic studies of *R. rosea*.

Manual scoring of gels in this study may affect the accurate judgement of bands since the band density was influenced by many elements, such as developing time of gels, optimising PCR programme, loading uneven on gels, time for loading gels, tired eyes when scoring gels, etc,. It would be interesting to confirm these results by performing sequencing.

3.2 Unique primers for *R.rosea*

3.2.1 Gender

Eight studied samples were labelled after its gender, four female and four male. However, studies on gels did not show any specific bands due to gender, implicating no gender-specific primers for ISSR and SSR were found in this study. The negative outcome was also reported by Elameen et al (2008) using AFLP method. They did not find gender-specific primers, and UPGMA cluster analysis did not group the clones into gender-based clusters. The amount of active substances in roots from female and male plants was also found to be similar, even if the root of female plants had a higher dry weight (Galambosi et al 2009). Finding a gender-specific primer could therefore be rather marginal for industry regarding extract from Roseroot, but such a finding is still important for breeding purposes and scientific studies. Hence, the search for gender-specific primers should proceed.

3.2.2 Population

There was no marker present in one population but absent in other, indication that no population-specific primers were found with the tested ISSR primers.

3.3 Clone material

Even when many samples came from the same accession, there could not be seen any indications of clonal material in the investigated material, except within the accession 18411 (samples 18411_3 and 18411_4) from Greenland. No clone material was found among the 20 Swedish and 3 Faroese accessions, indicating that no material should be discarded. These results are visualised in the dendrogram (appendix X - dendrogram) and are based on both dominate data from ISSR and converted SSR results.

3.4 Future investigations

Suggestions for future investigations considering *R.rosea* from Scandinavia countries could be a combined project where genetic diversity, gender, geographical location and active substances would be evaluated together. Efforts of finding gender- and population specific primers should continue. A careful collecting process could also be on the agenda.

4 Conclusion

This study shows that both SSR and ISSR methods work well for analysing the genetic diversity of *R. rosea*. The ISSR primers 834, 841, G05 and G11 gave good result for *R. rosea*. Percentages of polymorphic bands were for Sweden 83.78%, Greenland 94.59% and Faroe Islands 48.65%. SSR primers RRC10, RRD6, RRE3, and RRE9 worked as well on *R. rosea* samples from Sweden, Greenland and Faroe Islands. No gender-specific primer was found, nor a population-specific primer. There was no clone material revealed, except for one accession from Greenland.

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Appendices

Appendix I – Abbreviations

ACIA	Artic Climate Impact Assessment
AFLP	Amplified Fragment Length Polymorphisms
AP-PCR	Arbitrarily Primed Polymerase Chain Reaction
bp	Base Pair
CAPS	Cleaved Amplified Polymorphic Sequence
DAF	DNA amplification fingerprinting
EST	Expressed Sequence Tag
GBIF	Global Biodiveristy Information Facility
ISSR	Inter Simple Sequence Repeats
NGB	Nordic Gene Bank
NordGen	Nordic Genetic Resource Center
NTSYSpc	Numerical Taxonomy SYStem
PCR	Polymerase Chain Reaction
PPB	Percentage of Polymorphic Bands
RADP	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphisms
SAHN	Sequential Agglomerative Hierarical Nested cluster analysis
SCAR	Sequence-Characterised Amplified Region
SLU	Swedish University of Agricultural Sciences
SNP	Single-Nucleotide Polymorphism
SPIMED	Spice and medicinal plants in the Nordic and Baltic countries
SSR	Simple Sequence Repeats (Microsatellites)
STS	Sequence-Tagged Sites
T_a	Annealing Temperature
UPGMA	Unweighted Pair Group Method with Arithmetic mean

Appendix II – Taxonomy and Nomenclature

Classification:
Kingdom: Plantae
Phylum: Magnoliophyta
Class: Magnoliopsida
Order: Rosales
Family: Crassulaceae
Genus: *Rhodiola*
Species: *Rhodiola rosea* L. (1753).

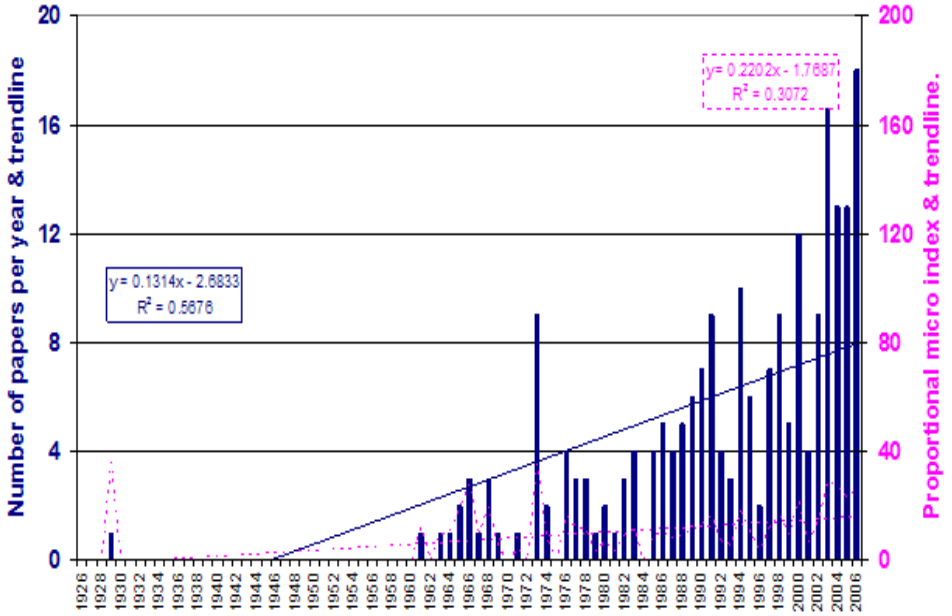
Synonyms:
Sedum rosea (L.) Scop. (1771).
Sedum rhodiola DC.
Rhodiola roanensis,
Sedum rosea var. roanense

Subspecies:
Rhodiola rosea subsp. arctica
Rhodiola rosea subsp. atropurpurea
Rhodiola rosea subsp. borealis
Rhodiola rosea subsp. elongata
Rhodiola rosea subsp. integrifolia
Rhodiola rosea subsp. krivochizhinii
Rhodiola rosea subsp. neomexicana
Rhodiola rosea subsp. polygama
Rhodiola rosea subsp. roanensis
Rhodiola rosea subsp. rosea
Rhodiola rosea subsp. sachalinensis
Rhodiola rosea subsp. tachiroi

Common names:
English: Roseroot, artic root, golden root, roseroot stonecrop

German: Rosenwurz
Swedish: Rosenrot
Norwegian: Rosenrot
Samí: Galbberáhta
Danish: Rosenrod
Finnish: Ruusujuuri
Icelandic: Burnirót, Svæfla
Russian: Zolotoj koren, rodiola rozovaja
Japanese: Iwa-benkei

Appendix III – Increased amount of published papers of *R.rosea*



Figur 1. Popularity of *R.rosea* in relation to published papers per year taken from the Australian government statutory authority Rural Industries Research and Development Corporation (RIRDC) 2010. This shows a clear trend of increased interest for the plant. Available at http://www.newcrops.uq.edu.au/listing/species_pages_R/Rhodiola_rosea.htm. [Accessed 14 January 2010].

Appendix VI – Dataset for *R.rosea*

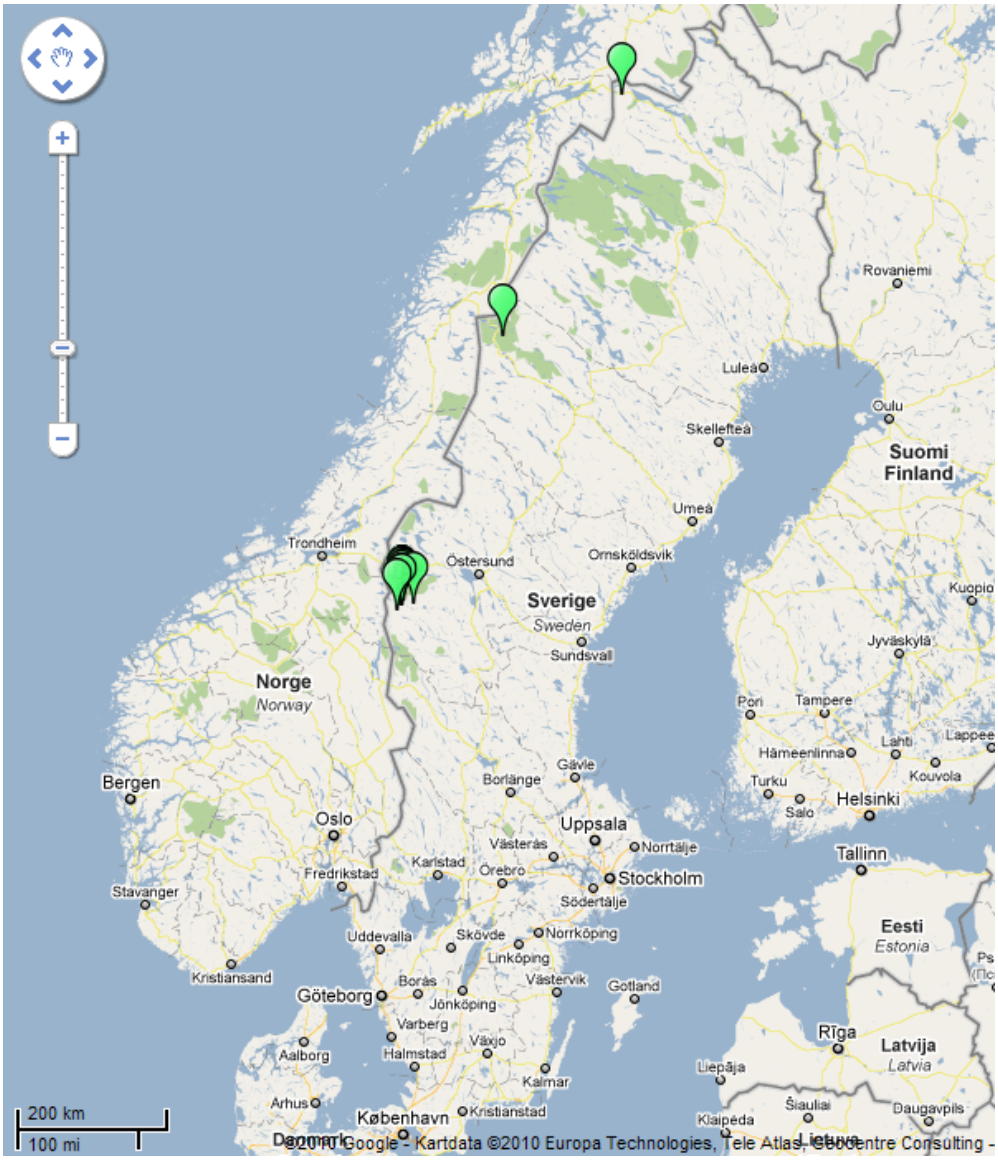
Scientific taxon name	Accession number	Accession name	Gender	Origin country	Collecting date	Latitude	Longitude	Elevation
Rhodiola rosea	NGB16428_1	Svaletjåkke	*	Sweden	20020729	6246--N	01222--E	1020
Rhodiola rosea	NGB16429_1	Helagsstugan 6	F	Sweden	20020730	6255--N	01230--E	1040
Rhodiola rosea	NGB16430_1	Helagsstugan sjön	*	Sweden	20020730	6255--N	01230--E	1060
Rhodiola rosea	NGB16431_1	Helagsstugan stigen	F	Sweden	20020730	6256--N	01230--E	1060
Rhodiola rosea	NGB16432_1	Storuman I	*	Sweden	20020807	655541N	0150850E	825
Rhodiola rosea	NGB16681_1	Fältjägarstugan I	*	Sweden	20020729	6250--N	01227--E	1030
Rhodiola rosea	NGB16682_1	Fältjägarstugan II	*	Sweden	20020729	6250--N	01228--E	1000
Rhodiola rosea	NGB16683_1	Helagsstugorna 4	*	Sweden	20020729	6253--N	01230--E	1020
Rhodiola rosea	NGB16684_1	Helagsstugorna 5	*	Sweden	20020729	6254--N	01231--E	1100
Rhodiola rosea	NGB16685_1	Helagsstugan 6	M	Sweden	20020730	6255--N	01230--E	1040
Rhodiola rosea	NGB16686_1	Helagsstugan stigen	M	Sweden	20020730	6256--N	01230--E	1060
Rhodiola rosea	NGB16688_1	Helagstoppen	M	Sweden	20020731	6255--N	01229--E	1080
Rhodiola rosea	NGB16690_1	Helags glaciärbäcken I	*	Sweden	20020731	6255--N	01229--E	1100
Rhodiola rosea	NGB16692_1	Helags bäckfåran	*	Sweden	20020731	6255--N	01229--E	1170
Rhodiola rosea	NGB16693_1	Helagsstugan stigen 11	*	Sweden	20020731	6255--N	01229--E	1240
Rhodiola rosea	NGB16693_2	Helagsstugan stigen 11	*	Sweden	20020731	6255--N	01229--E	1240
Rhodiola rosea	NGB16693_3	Helagsstugan stigen 11	*	Sweden	20020731	6255--N	01229--E	1240
Rhodiola rosea	NGB16694_1	Björnskallen	*	Sweden	20020718	6251--N	01248--E	830

Rhodiola rosea	NGB16696_1	Vassijaure	F	Sweden	1947----	6825--N	01819--E	850
Rhodiola rosea	NGB2985_1	NGB16429+NGB16685	*	Sweden	*	*	*	*
Rhodiola rosea	NGB2985_2	NGB16429+NGB16685	*	Sweden	*	*	*	*
Rhodiola rosea	NGB8631_1	NGB16686+NGB16431	*	Sweden	*	*	*	*
Rhodiola rosea	NGB8631_2	NGB16686+NGB16431	*	Sweden	*	*	*	*
Rhodiola rosea	NGB8632_1	NGB16686+NGB16432	*	Sweden	*	*	*	*
Rhodiola rosea	NGB17983_1	Narsaq Dyrenäs	*	Greenland	20060823	605626N	0460426W	93
Rhodiola rosea	NGB17983_2	Narsaq Dyrenäs	*	Greenland	20060823	605626N	0460426W	93
Rhodiola rosea	NGB17983_3	Narsaq Dyrenäs	*	Greenland	20060823	605626N	0460426W	93
Rhodiola rosea	NGB17984_1	Narsaq Qaqqarsuaq	*	Greenland	20060725	605435N	0455934W	16
Rhodiola rosea	NGB17984_2	Narsaq Qaqqarsuaq	*	Greenland	20060725	605435N	0455934W	16
Rhodiola rosea	NGB17984_3	Narsaq Qaqqarsuaq	*	Greenland	20060725	605435N	0455934W	16
Rhodiola rosea	NGB17985_1	Narsaq Qaqqarsuaq	*	Greenland	20060825	605437N	0455934W	102
Rhodiola rosea	NGB17985_2	Narsaq Qaqqarsuaq	*	Greenland	20060825	605437N	0455934W	102
Rhodiola rosea	NGB17985_3	Narsaq Qaqqarsuaq	*	Greenland	20060825	605437N	0455934W	102
Rhodiola rosea	NGB17985_4	Narsaq Qaqqarsuaq	*	Greenland	20060825	605437N	0455934W	102
Rhodiola rosea	NGB17985_5	Narsaq Qaqqarsuaq	*	Greenland	20060825	605437N	0455934W	102
Rhodiola rosea	NGB17985_6	Narsaq Qaqqarsuaq	*	Greenland	20060825	605437N	0455934W	102
Rhodiola rosea	NGB17989_1	Quaqortoq Tasersuuaqq	*	Greenland	20060827	604458N	0460429W	
Rhodiola rosea	NGB17989_2	Quaqortoq Tasersuuaqq	*	Greenland	20060827	604458N	0460429W	
Rhodiola rosea	NGB17989_3	Quaqortoq Tasersuuaqq	*	Greenland	20060827	604458N	0460429W	
Rhodiola rosea	NGB17989_4	Quaqortoq Tasersuuaqq	*	Greenland	20060827	604458N	0460429W	
Rhodiola rosea	NGB17989_5	Quaqortoq Tasersuuaqq	*	Greenland	20060827	604458N	0460429W	
Rhodiola rosea	NGB17994_1	Nuuk	M	Greenland	20060831	6410--N	05139--W	10
Rhodiola rosea	NGB17994_2	Nuuk	F	Greenland	20060831	6410--N	05139--W	10

Rhodiola rosea	NGB17994_3	Nuuk	*	Greenland	20060831	6410--N	05139--W	10
Rhodiola rosea	NGB17998_1	Quaqortoq Havefjeld	*	Greenland	20060826	604345N	0460244W	40
Rhodiola rosea	NGB17998_2	Quaqortoq Havefjeld	*	Greenland	20060826	604345N	0460244W	40
Rhodiola rosea	NGB17998_3	Quaqortoq Havefjeld	*	Greenland	20060826	604345N	0460244W	40
Rhodiola rosea	NGB18001_1	Nuuk Stora Marlene	*	Greenland	20060831	641005N	0513935W	49
Rhodiola rosea	NGB18001_2	Nuuk Stora Marlene	*	Greenland	20060831	641005N	0513935W	49
Rhodiola rosea	NGB18001_3	Nuuk Stora Marlene	*	Greenland	20060831	641005N	0513935W	49
Rhodiola rosea	NGB18001_4	Nuuk Stora Marlene	*	Greenland	20060831	641005N	0513935W	49
Rhodiola rosea	NGB18002_1	Nuuk Lilla Marlene	*	Greenland	20060831	641041N	0513954W	143
Rhodiola rosea	NGB18004_1	Narsaq Dyrenäs,	*	Greenland	20060824	605619N	0460403W	71
Rhodiola rosea	NGB18004_2	Narsaq Dyrenäs,	*	Greenland	20060824	605619N	0460403W	71
Rhodiola rosea	NGB18005_1	Narsaq Fjeld	*	Greenland	20060824	695641N	0460442W	190
Rhodiola rosea	NGB18005_2	Narsaq Fjeld	*	Greenland	20060824	695641N	0460442W	190
Rhodiola rosea	NGB18005_3	Narsaq Fjeld	*	Greenland	20060824	695641N	0460442W	190
Rhodiola rosea	NGB18005_4	Narsaq Fjeld	*	Greenland	20060824	695641N	0460442W	190
Rhodiola rosea	NGB18006_1	Narsaq Kvanefjeld	*	Greenland	20060821	605838N	0455840W	473
Rhodiola rosea	NGB18006_2	Narsaq Kvanefjeld	*	Greenland	20060821	605838N	0455840W	473
Rhodiola rosea	NGB18007_1	Narsaq Kvanefjeld	*	Greenland	20060825	605753N	0455914W	287
Rhodiola rosea	NGB18007_2	Narsaq Kvanefjeld	*	Greenland	20060825	605753N	0455914W	287
Rhodiola rosea	NGB18007_3	Narsaq Kvanefjeld	*	Greenland	20060825	605753N	0455914W	287
Rhodiola rosea	NGB18007_4	Narsaq Kvanefjeld	*	Greenland	20060825	605753N	0455914W	287
Rhodiola rosea	NGB18009_1	Quaqortoq Havefjeld	*	Greenland	*	*	*	*
Rhodiola rosea	NGB18009_2	Quaqortoq Havefjeld	*	Greenland	*	*	*	*
Rhodiola rosea	NGB18010_1	Quaqortoq Havefjeld	*	Greenland	20060826	604343N	0460232W	134
Rhodiola rosea	NGB18010_2	Quaqortoq Havefjeld	*	Greenland	20060826	604343N	0460232W	134

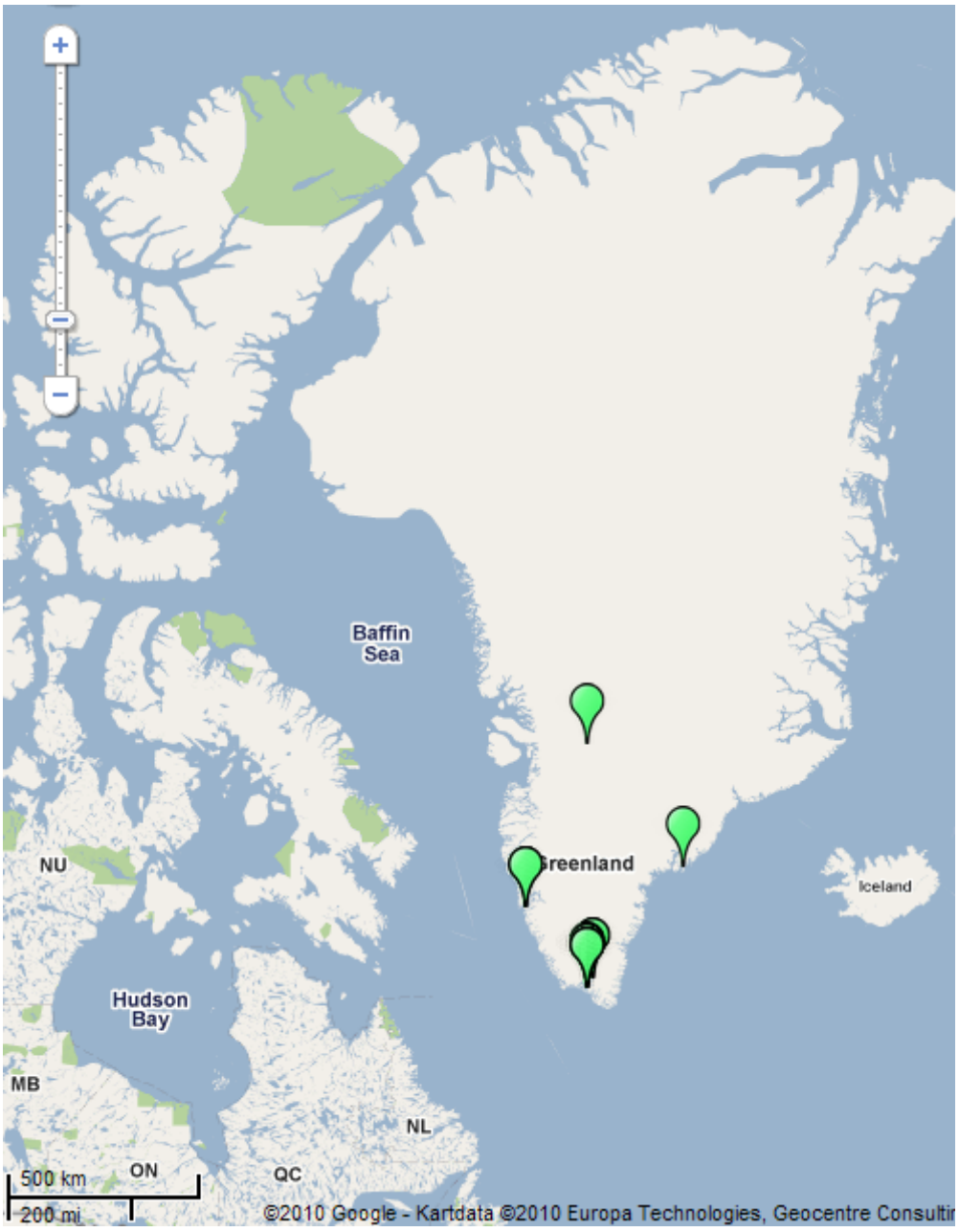
Rhodiola rosea	NGB18010_3	Quaqortoq Havefjeld	*	Greenland	20060826	604343N	0460232W	134
Rhodiola rosea	NGB18010_4	Quaqortoq Havefjeld	*	Greenland	20060826	604343N	0460232W	134
Rhodiola rosea	NGB18012_1	Quaqortoq Saarlussuaq	*	Greenland	20060827	604343N	0460602W	115
Rhodiola rosea	NGB18012_2	Quaqortoq Saarlussuaq	*	Greenland	20060827	604343N	0460602W	115
Rhodiola rosea	NGB18012_3	Quaqortoq Saarlussuaq	*	Greenland	20060827	604343N	0460602W	115
Rhodiola rosea	NGB18012_4	Quaqortoq Saarlussuaq	*	Greenland	20060827	604343N	0460602W	115
Rhodiola rosea	NGB18014_1	Qassiarsuk Bratthalid	*	Greenland	20060829	610916N	0453155W	112
Rhodiola rosea	NGB18014_2	Qassiarsuk Bratthalid	*	Greenland	20060829	610916N	0453155W	112
Rhodiola rosea	NGB18014_3	Qassiarsuk Bratthalid	*	Greenland	20060829	610916N	0453155W	112
Rhodiola rosea	NGB18015_1	Qassiarsuk Bratthalid	*	Greenland	20060829	610853N	0453234W	157
Rhodiola rosea	NGB18015_2	Qassiarsuk Bratthalid	*	Greenland	20060829	610853N	0453234W	157
Rhodiola rosea	NGB18015_3	Qassiarsuk Bratthalid	*	Greenland	20060829	610853N	0453234W	157
Rhodiola rosea	NGB18015_4	Qassiarsuk Bratthalid	*	Greenland	20060829	610853N	0453234W	157
Rhodiola rosea	NGB18015_5	Qassiarsuk Bratthalid	*	Greenland	20060829	610853N	0453234W	157
Rhodiola rosea	NGB18410_1	Innertivik LB0206	*	Greenland	*	*	*	*
Rhodiola rosea	NGB18411_1	No information available	*	Greenland	*	*	*	*
Rhodiola rosea	NGB18411_2	No information available	*	Greenland	*	*	*	*
Rhodiola rosea	NGB18411_3	No information available	*	Greenland	*	*	*	*
Rhodiola rosea	NGB18411_4	No information available	*	Greenland	*	*	*	*
Rhodiola rosea	NGB18413_1	No information available	*	Greenland	*	*	*	*
Rhodiola rosea	NGB16698_1	Torshavn	*	Faroe Islands	1986----	6202--N	00647--W	
Rhodiola rosea	NGB18665_1	Goger	*	Faroe Islands	20070920	621923N	0065602W	98
Rhodiola rosea	NGB21650_1	Eidi	*	Faroe Islands	20070919	621512N	0070288W	33

Appendix V – Maps: Collection sites in Sweden



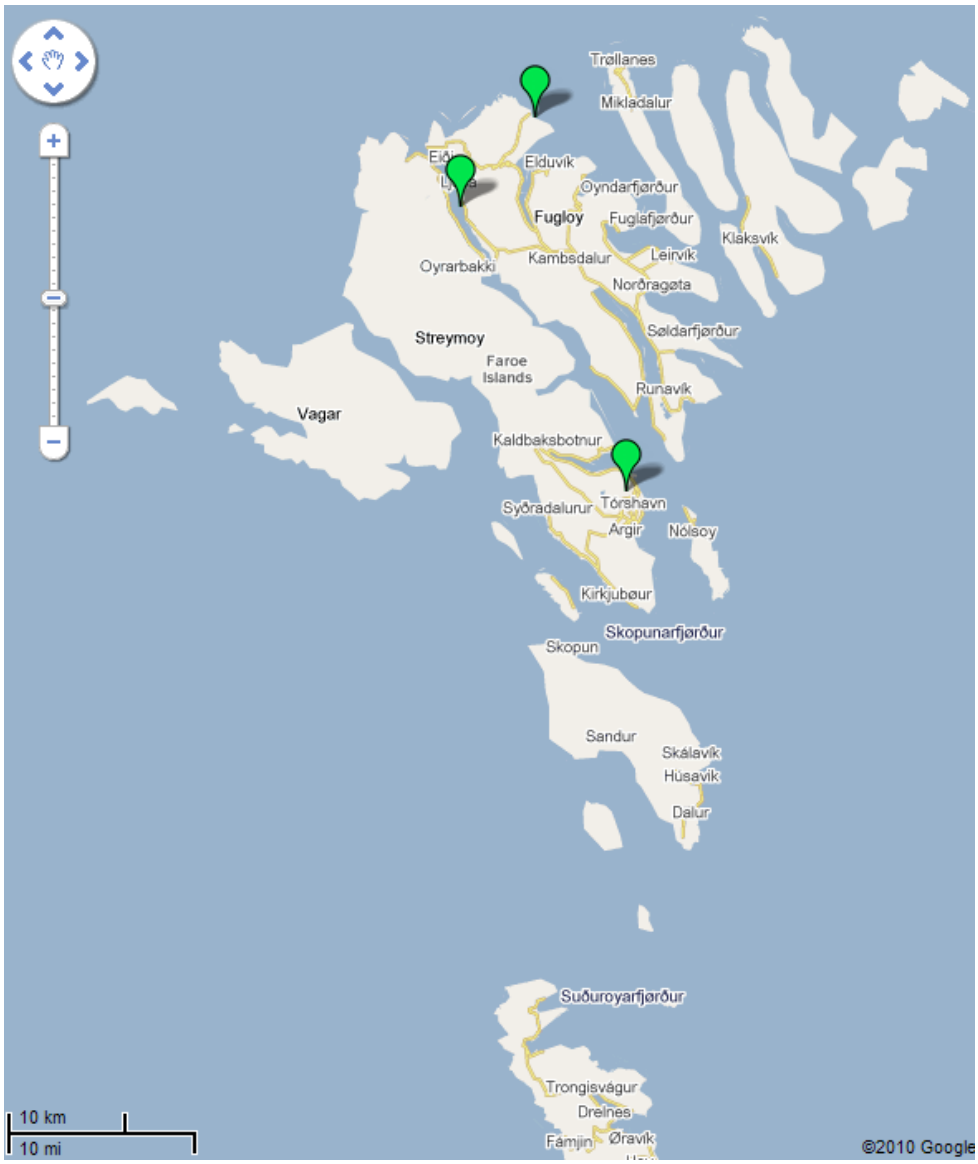
Figur 2. For a closer look at the collection sites please visit: http://maps.google.se/maps/ms?ie=UTF8&hl=sv&msa=0&msid=111223212872530040450.0004883444557f929216b&z=3

Appendix VI – Maps: Collecting sites on Greenland



Figur 3. For a closer look at the collection sites please visit: http://maps.google.se/maps/ms?ie=UTF8&hl=sv&msa=0&msid=111223212872530040450.0004883444557f929216b&z=3

Appendix VII – Maps: Collecting sites on Faroe Islands



Figur 4. For a closer look at the collection sites please visit: http://maps.google.se/maps/ms?ie=UTF8&hl=sv&msa=0&msid=111223212872530040450.0004883444557f929216b&z=3

Appendix VIII - Protocol for DNA Extraction

After Doyle & Doyle, with unpublished modifications from NordGen

1. Grind plant material with Retsch mixer mill.
2. Add 600 µl CTAB-buffer and incubate in Eppendorf Thermomixer in +60°C for 1 h, 600 rpm.
3. Extract with 1 volume (600 µl) chloroform isoamyl alcohol (24:1). Shake carefully.
4. Centrifuge for 20 minutes, 13 200 rpm.
5. Carefully remove the water phase and transfer it to new Eppendorf tubes and add 5 µl RNase (1 mg/ml). Put the tubes into Thermomixer for 30 minutes, +37°C, 600 rpm.
6. Add 0.8 volume ice-cold isopropanol (500 µl water phase => 400 µl isopropanol) and carefully mix 16 times.
7. Centrifuge for 10 minutes, 13 20 rpm.
8. Remove the supernatant by spilling, and save the pellet.
9. Add 500 µl washing solution, mix together and let rest for 20 minutes in room temperature.
10. Centrifuge for 5 minutes, 13 200 rpm.
11. Spill the supernatant and save the pellet.
12. Add 500 µl rinse solution and mix it carefully.
13. Centrifuge for 5 minutes, 13 200 rpm.
14. Remove the supernatant, save the pellet and let it dry.
15. Add 50 µl TE to the pellet and let it dissolve over night.
16. Save the samples in refrigerator.

Solutions

CTAB-Buffer	(0.1M Tris pH 8.0; 0.01M EDTA; 0.7M NaCl; 1% CTAB; 1% mercapto-ethanol)
Washing solution	(76% Ethanol; 0.2M Sodium acetate)
Rinse solution	(76% Ethanol; 0.01M Ammonium acetate)
TE	(0.01M Tris pH 8.0; 1mM EDTA pH 8.0)

Appendix IX – Protocol for fast and sensitive silver staining

	Time	Final solution	Protocol
Fixing	40 min	40 ml Fixing concentrate; 160 ml Fixing Diluter	0.6% Benzene sulfonic acid; 24% Ethanol
Washing	3x10 min	100 ml Washing concentration; 500 ml dist. H20	0.07% Benzene sulfonic acid
Silvering	30 min	40 ml silvering concentrate; 160 ml Milli-Q H20; 260 µl Formalaldehyde	0.2% AgNO3; 0.07% Benzene sulfonic acid; 0.05% Formalaldehyde; 0.002% Na-Thiosulfate
Water	2 min	200 ml Milli-Q H20	
Developing	2 min SSR; 3 min ISSR	40 ml Developing concentrate; 160 ml Milli-Q H20; 260 µl Formalaldehyde; 200 µl Na-Thiosulfate Solution	2.5% Na2CO3; 0.05% Formalaldehyde; 0.002% Sodium thiosulfate
Stopping and preserving	30 min + over night	75 ml Acetic acid; 75 ml Glycerol	10% Acetic acid; 10% Glycerol

Appendix X- Dendrogram