Biodiversity in Nordic Horseradish

(*Armoracia rusticana*)

Studies with Respect to Conservation and Utilization

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Abstract

The biodiversity of horseradish was studied in accessions collected from old gardens in Denmark, Finland, Norway and Sweden. In total 176 accessions were collected.

Horseradish, *Armoracia rusticana* (P. Gaertn., B. Mey. & Scherb.), has been used by man during long time and it was already mentioned in "De material medicia libri quinque" written year 100 AD. The plant, which has its origin in Southeast Europe, was brought to the Nordic countries by monks during the thirteenth century. In the Nordic countries, horseradish was used both as a bitter spice and as a medicinal plant to treat headaches, digestive disorders, high blood pressure and gout.

The root has a high content of vitamin C and was often used to prevent scurvy on long sailing tours and as a component in preserving food. Horseradish also contains high levels of sulfur-containing glycosides, so called glucosinolates. Glucosinolates (GLS), or their breakdown products are responsible for the pungent taste and claimed medicinal effects. The dominate GLS in horseradish is sinigrin (>80%) followed by gluconasturtiin and glucobrassicin. Although if the traditional use of horseradish has decreased new areas has developed, as a natural preservative, in medical treatments, in laboratories and in industrial production.

Studying the genotypic diversity with AFLP, the results showed a significant genetic diversity among Nordic horseradish. The accessions clustered in accordance with their country of origin with the highest diversity among the Finnish accessions, followed by the Danish accessions.

The result from the phenotypic analyses with morphological characters and glucosinolate content showed a high diversity among the studied accessions. In the morphological studies the accessions showed all types of leaf- and root shapes and divided in different clusters. Also a high diversity in the glucosinolate content with both high and low levels of sinigrin and gluconasturtiin were found.

No correlation between genotype and phenotypic characters in the Nordic horseradish was revealed. Despite that horseradish is a mainly vegetatively propagated plant the results show a high diversity among the collected accessions.

Keywords: AFLP, condiment to food, core collection, glucosinolates, medicinal plant, molecular markers, morphological characterization, vegetative propagated crops,

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Dedication

To my beloved family: Mikael, Eleonora and Sophia

”*By perseverance the snail reached the ark*”
Charles Spurgeon (1834-1892)

The Delphic oracle told the god Apollo that “the radish is worth its weight in lead, the beet its weight in silver and the horseradish its weight in gold”

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Wedelsbäck Bladh K., Olsson K (2011). Introduction and Use of Horseradish (Armoracia rusticana) as Food and Medicine from Antiquity to the Present: Emphasis on the Nordic Countries. Journal of Herbs, Spices & Medicinal Plants 17(3), 197-213.

II Wedelsbäck Bladh K., Olsson K. M., Yndgaard F. (2013). Evaluation of glucosinolates in Nordic Horseradish (Armoracia Rusticana) Bothanica Lithuanica 19: (1) 48-56.

III Wedelsbäck Bladh K., Liljeroth E., Poulsen G., Yndgaard F., Kolodinska Brantestam A. (2013) Genetic diversity in Nordic horseradish Armoracia rusticana as revealed by AFLP markers. Genetic Resources and Crop Evolution 60(6), 1-12.

IV Wedelsbäck Bladh K., Yndgaard F., Liljeroth E., Asdal Å., Suojala-Ahlfors T., Kjeldsen Bjørn G., Kolodinska Brantestam A. Morphological characterization of Nordic Horseradish (Armoracia rusticana). Manuscript

Papers I-III are reproduced with the permission of the publishers.

The contribution of Katarina Wedelsbäck Bladh to the papers included in this thesis was as follows: participated in the collecting of horseradish in Sweden and maintained and the Swedish collection in the field.

I Collected information and made a literature review on new and older publications on horseradish together with co-author.

II Participated in the molecular analysis together with the staff at the laboratory. The results were analysed together with the co-authors.

III Participated in the analysis of the glucosinolates and analysed the data together with the co-authors.

IV Performed the morphological characterisations together with the participants in the working group for vegetables at NordGen. Analysed the data and wrote the manuscript together with the co-authors.

Abbreviations

AFLP	Amplified fragment length polymorphism
AITC	Allyl isothiocyanate
AMOVA	Analysis of Molecular Variance
CBM	Centrum för biologiskt mångfald (Swedish biodiverse centre)
DIAS	Danish Institute of Agricultural Sciences
DM	Dry Matter
DNA	Deoxyribonucleic acid
FW	Fresh weight
GLS	Glucosinolates
HPR	Horseradish peroxidase
IRD	Internal root discoloration
MTT	Agrifood Research Finland
NGB	Nordic Gene Bank
NordGen	Nordisk Genresurscenter (Nordic Genetic Resource Center)
PEITC	2-phenylethyl isothiocyanate
POM	Programmet för odlad mångfald (Programme for the Diversity of Cultivated Plants)
SLU	Sveriges Lantbruksuniversitet (Swedish Agricultural University)
SSR	Simple Sequence Repeat
TuMV	Turnip mosaic virus
UPOV	Union for the Protection of New Varieties of Plant

1 Introduction

1.1 Taxonomy and genetics

Horseradish *Armoracia rusticana* (P. Gaertn., B. Mey. & Scherb.) is a hardy perennial which has been mentioned by various names over the years. One of the earliest names are *Sinapi perscium* or *Persicum sinapi* which the greek physican Pedanius Dioscorides (AD 40-90) called horseradish (Beck, 2005; Courter & Rhodes, 1969). During the 16th and 17th century names as *Raphanus vulgaris*, *Raphanus rusticanus* and *Raphanus sylvestris* were used. Later in the 18th century Carl von Linneus named it *Cochlearia armoracia* (Courter & Rhodes, 1969). The name *Armoracia rusticana* was given by the German botanist Joseph Gaertner (1732-1791) in the end of the 18th century (Courter & Rhodes, 1969).

The genus *Armoracia* belongs to the *Brassicaceae* family which contains nearly 340 genera and more than 3350 species (Johnston *et al.*, 2004), about 40 of these genera and around 100 species are found in Sweden (Anderberg & Anderberg, 2010; Johnston *et al.*, 2004). This family contains many economically important species such as *Brassica rapa* ssp. *oleifera* (DC.) Metzg and *Brassica napus* L. for oil production and root crops and cabbages of *B. oleracea* L. for direct consumption (Anderberg & Anderberg, 2010). In the genus *Armoracia,* the three species *A. macrocarpa* (Waldst. & Kit.) Baumg.*, A. rusticana* (horseradish), and *A. sisymbrioides* (DC) Cajand are found. *Armoracia sisymbrioides,* which is native to the Siberian parts of Russia, is distinguished from horseradish by its whitish, glaucous leaves and auriculate leaves on the stem (Sampliner & Miller, 2009). *A. macrocarpa* differs from horseradish in flowers, fruits and number of seeds. *A. macrocarpa* has larger flowers and a more abundant seed production (Sampliner & Miller, 2009). It is only found in the wild (Sampliner & Miller, 2009*)* but has decreased due to drainage of wet meadows and are now regarded as endangered on the IUCN red list of threatened species in the south Eastern Europe and Balkan region (Stevanović, 2013).

The genetic diversity center for horseradish is located in eastern Europe and south-western parts of Russia (Mossberg & Stenberg, 2003; Courter & Rhodes, 1969). However, recent attempts to find natural populations of horseradish have failed and it may today only exist in cultivation (Sampliner & Miller, 2009). Horseradish is a tetraploid with a somatic chromosome number of 32 (2n=4x=32) (Simmonds, 1976).

Figure 1. Horseradish plant New Kreüterbuch by Leonhart Fuchs, 1543. Source: Université de Strasbourg, Service Commun de la Documentation (France).

1.2 Morphology

Horseradish flowers in early summer with white flowers and have long, white to light yellow fleshed, strong tasting roots (Anderberg & Anderberg, 2010). The basal leaves are lancet to heart shaped with toothed margins and long stalks. The small white flowers are gathered in branched clusters. It seldom sets viable seeds (Anderberg & Anderberg, 2010; Mossberg & Stenberg, 2003).

The morphology varies throughout the growing season, where the plant produces entire laminate leaves in the summer, turning to more pinnate leaves in the autumn. The plants start to produce the pinnate leaves when they prepare for winter dormancy, probably induced by the colder temperature in the autumn (Shehata *et al.*, 2008).

Figure 2. Different leaf shapes in horseradish accessions a. narrow elliptic shape b. broad elliptic to ovate shape Photo: K Wedelsbäck Bladh.

1.3 Production and cultivation

In the Nordic countries the commercial production has been concentrated to a few locations. The largest production sites have been in Sindal in Denmark, Enköping and Fjärrås in Sweden. Until the end of the 19th century the production center in Sweden was in Enköping (middle Sweden) which was called the horseradish town. In 1920 the production decreased and moved to Halland in the southern part of Sweden (Enköpings-museum, 2007). Today only very few producers are left in the Nordic countries.

Production of horseradish is found in all parts of the world. In Europe the main production take place in Hungary with a production on 1200 ha, but also Austria, Germany and Poland have a commercially production of horseradish. A large amount is produced in North America, in both Canada and USA (Shehata *et al.*, 2009). The commercial production of horseradish in the USA started in the 1890s by German settlements (Rhodes, 1977; Courter & Rhodes, 1969). USA is now the largest producer of horseradish in the world with an area on 1600 ha, with Illinois as the main production center (Walters & Wahle, 2010; Shehata *et al.*, 2009). In China, production has increased during the last years (Shehata *et al.*, 2009; Henriksen & Bjørn, 2004).

Figure 3. Root sets from the collected horseradish accessions. Photo: K Wedelsbäck Bladh.

Horseradish thrives in deep, well cultivated light clay soil. Many of the known production sites are found on earlier seafloors, as in Enköping and Halland in Sweden and Sindal in Denmark. In Sweden the cultivation starts in May when tiny roots, (sets) approximately 30-40 cm, are planted. The roots are laid horizontally with 60 cm distance between the plants and 70 cm between the rows. During middle of June the upper part of the roots are lifted and small roots are removed. The main root is then replaced back into the soil. This practice ensures the development of commercially favorable thick even roots. The mature roots are harvested in April where the central root is distributed to the consumers and the sets are stored for a month until they are replanted for next year production[1].

Figure 4. Horseradish production in Enköping Sweden (Source: Enköpings museum).

[1] Personal communication, Mikael Jonsson 2014.

Figure 5, Horseradish in Eastern part of Enköping (Source: Enköpings museum).

1.4 Diseases

Horseradish is susceptible to a number of diseases and insect pests. The most common insects attaching horseradish are the leaf beetle hopper (*Circulifer tenellus*), which is the vector for brittle root diseases (*Spiroplasma citri*), and the horseradish flea beetle *Epitrix* spp. which causes damage on the shoots after planting. *Cercospora* leaf spot, caused by *Cercospora armoraciae,* is a common fungal disease. White rust, caused by the fungus *Albugo candida* LEV, is common in Europe but rare in USA and gives the leaves and stems a whitish layer. Turnip mosaic virus (TuMV) causes yellow rings on the horseradish leaves and streaming on the petioles (Yu, 2010; Bratsch, 2006). The most severe economic losses is caused by internal root discoloration (IRD), where up to 100% loss of yield has been reported (Yu, 2010). It was first described in Europe during the 1920s and in the USA in the beginning of the 1930s (Yu, 2010). Three fungal pathogens, *Verticillium dahliae* (Kleb), *V. longisporum* (Stark) and *Fusarium solani* (Mart.) Sacc, have been identified to be the casual agents of IRD. The disease is spread through symptom-less but infected sets and is therefore difficult to control. By using *in vitro* propagation the plants can stay healthy and maintain high yield (Uchanski, 2007). Another method against this disease is to treat the sets at 47ºC for 20-30 min. This method can control the set-borne inoculum of the pathogenic fungi and also result in a higher germination rate of the sets (Babadoost Kondri, 2009).

1.5 Historical use and origin

Horseradish is associated with interesting traditions and diverse use, both as a medicinal herb and a condiment to food (Agneta *et al.*, 2013; Wedelsbäck Bladh & Olsson, 2011; Courter & Rhodes, 1969). In more modern time it has also been used in various industrial processes like production of horseradish peroxidase and production of isothiocyanate products for different uses.

It is believed that horseradish was known by the ancient Greeks but few evidences have been found to prove this. Since horseradish is mentioned in literature with different names it is sometimes difficult to find out if horseradish or other plants has been described. In the 'De Agri Cultura' (circa 160 BC) written by the warrior Marcus Porcius Cato (234–149 BC) practical instructions for farmers are given. He described how to sow and cultivate "radish," which was used for food or medicine by the Roman people. Although some researchers mean that he described cultivation of horseradish it is doubtful in view of the poor seed setting in horseradish. Gaius Plinius Secundus (23-73 AD) suggested that anyone who ate too much should finish with grated horseradish. If someone suffered from asthma, honey roasted radish seeds would help according to Plinius (Courter & Rhodes, 1969). These statements indicate that the Romans were familiar with horseradish. In the paintings of Pompeii dating back to 79 AD pictures with long white roots have been depict and interpreted as horseradish (Feemster & Meyer, 2002) although no certain evidence for that has been presented.

In the "De Materia Medica" by the Greek physician Pedanius Dioscorides (AD 40–90) another early record indicating the use of horseradish plants. Dioscorides named the plant Sinapi perscium or wild radish and described the thin and long root as diuretic and very hot. Both roots and leaves could be eaten boiled as potherbs (Beck, 2005). Another early record is Pliny the Elders "Naturalis Historia" published around 77–79 AD. In this book he mentioned Persicum napy to be consumed freshly grated for the digestion after a heavy meal (Dalby, 1998; Bostock, 1855).

There are different theories about how horseradish was introduced from Russia to the north (Scandinavia) by the Vikings, as they sailed on the Russian rivers to these areas. However, no reliable sources to confirm this have been found (Wedelsbäck Bladh & Olsson, 2011; Campbell, 2001). In Russia the tradition of using horseradish is probably the oldest in the world since the genetic diversity center is located in the south east of Europe and in the southern part of Russia. According to Hellie (1999) horseradish was mentioned along with mustard in Kievian times. Further on it is noted in the Novogrod postal record books 1586-1631 that beets, onions, garlic, horseradish and radishes were prepared for the tsars table. Although documents for buying horseradish have been found in the Expenses book of Nikon, Metopolitan of Novogrod and Velikie Luki (year 1651-52) where the expenses for horseradish was documented to a price between 2 and 15 kopek (Hellie R., 1999). This could be compared with the cost to send a letter between Moscov and Riga in 1663 which costed 10 kopek (Johnsson & 1917).

1.5.1 Medical use

Horseradish was described by the abbess Hildegard from Bingen, Germany, as a medicinal plant in the 12th century. She mentioned horseradish as a treatment for lung and heart diseases (Schweiger & Kammerer, 1998). It is believed that it was introduced by humans to the central parts of Europe during the Middle Ages and later to the northern parts of Europe during the 13th century (Schweiger & Kammerer, 1998). The first written record about this plant in Scandinavia is by the Danish canon Henrik Harpestreng (1164-1244), (Lange, 1999). Henrik Harpestreng described several ways of using horseradish as medicine in his 'Liber Herbarum'. He also described the following odd ways of using the plant: "If you rub your hands with horseradish you will handle snakes without getting bites " and "Horseradish gives heat and is good against deafness" (Klemming, 1886).

Horseradish was used as a medicinal plant in the monasteries, which is confirmed in the ‘Nådendals klosterbok’. The book was written by monks at Vadstena monastery in Sweden in 1440 and sent to their sister monastery at Nådendal in Finland (Löfgren, 2009).

Some of the uses mentioned in the literature include that horseradish can heal wounds, that it is good for fever and pain, relieves headaches, is good for high blood pressure and gout, and helps against digestive problems. It was also used for treating urinary tract infections and bronchitis and its bactericidal properties (Wedelsbäck Bladh & Olsson, 2011). However horseradish is best known as a cure for scurvy. John Woodall who was a military surgeon in Lord Willoughby 's regiment in the late 1590s and the first Surgeon General to the East India Company wrote in his book Surgeons mate published in 1639 that citrus and horseradish were good for scurvy (Woodall, 1639). Both roots and leaves could be stored a long time on the ships but also grown in sandboxes (Villner, 1986; Gréen, 1976). At this time they were not aware of that the effect was due to the high levels of vitamin C. There is a considerable variation between different clones but on average the content of vitamin C is about twice that of an orange (National Food Administration, 2010).

1.5.2 Condiment

During the 1500s, horseradish was used as a complement to food. It is believed that this custom began in the East and Central Europe and later came to England and Scandinavia. In 1571 the Italian physician and botanist Matthiolus (1501-1577) wrote: "The Germans call horseradish ‘cren’, they and the Hungarian and Polish people eat this root as a condiment to their meals" (Becker-Dillingen, 1938).

Even the English herbalist John Parkinson (1567-1650) wrote in his book 'Theatrum Botanicum' (1640) that horseradish sauce could be served to country people and strong laboring men, but it was too strong for sensitive stomachs (Grieve, 1979). In Austria and south east Europe such as Russia, Bulgaria and Romania it is still used both as a condiment and as a medicinal plant (Sampliner & Miller, 2009).

The traditional consumption of horseradish has decreased in the Nordic countries during later years since it is no longer so common to cook traditional food. Horseradish was also an important ingredient in preservatives where the horseradish not just contributed to the taste, but also had a bacteriostatic effect. New areas and products are developed in which horseradish is commonly used as a cheaper replacement for wasabi.

Figure 6. Horseradish roots Photo: Kerstin Olsson.

1.6 Chemical content in horseradish

1.6.1 Glucosinolates

The species in the *Brassicaceae*–family are rich in sulphur containing glycosides, so called glucosinolates (GLS) (Kushad *et al.*, 1999; van Doorn *et al.*, 1998). In horseradish sinigrin is the major glucosinolate which is also responsible for the pungent taste. About 120 structurally different GLSs have been described and about 20 of them are present in *Brassica* vegetables (Fahey *et al.*, 2001). The relation between the different GLSs varies between the leaves and the roots in horseradish (Agneta *et al.*, 2014a; Agneta *et al.*, 2014b; Agneta *et al.*, 2012; Li & Kushad, 2004). Harvest time, use of fertilizers and maturity of the plants influence the GLS concentrations as well (Sarıkamış *et al.*, 2009; Rosa *et al.*, 1996; Zhao *et al.*, 1993; Nebel, 1987). Also environmental factors such as climate and soil affect the GLS levels in the plants (Kosson & Horbowicz, 2008; Ciska *et al.*, 2000). Agneta et al. (2012) identified 12 GLSs in the roots and 16 GLSs in the sprouts of horseradish. Sinigrin and gluconasturtiin were most abundant and that explains the strong and bitter taste. When plant tissues are damaged, isothiocyanates are formed from the GLS with help of the enzyme myrosinase. Sinigrin and gluconasturtiin turn into allyl isothiocyanate (AITC, mustard oil) and 2-phenylethyl isothiocyanate (PEITC), respectively.

1.6.2 Vitamin C

Already in the 16th century lemon juice was found to be effective against scurvy. As citrus fruits were not grown in northern Europe alternative antiscorbutic plants were looked for. One of these was horseradish, which was recommended both by the English surgeon John Woodall and the American herbalist William Coles in the middle of the 17th century (Sumner, 2004; Woodall, 1639). The active compound, unknown at the time, was vitamin C (ascorbic acid). It was not until the beginning of the 20th century that the structure and benefit of vitamin C became known (Szent-Györgyi A., 1933).

Vitamin C is essential for humans who are unable to synthesize it. The daily need for grown up humans is about 45 milligrams (World Health Organization, 2004). Fruits and vegetables are our main sources, the concentration varies between different crops with high levels in for example black currant, lemon, strawberries, peppers, potatoes, cabbages and horseradish (National Food Administration, 2012; Lee & Kader, 2000). In addition to genotype, factors like climate, fertilizer, light, and maturity affect the concentration of Vitamin C in plants (Ciska *et al.*, 2000; Carlsson *et al.*, 1981). The content can also vary depending on harvest time and year as well as between different clones (Nebel, 1987).

1.7 New areas for using horseradish

An important product is horseradish peroxidase (HRP). The enzyme in HRP has applications in detoxifying polluted industrial wastewaters (Wagner & Nicell, 2002) and to reduce the odor from swine manure for several days (Ye *et al.*, 2009). It has also many applications in laboratory and medical industry (BBI-enzymes, 2009; Bickerstaff, 1987).

Another modern use of horseradish is as a substitute for wasabi, *Wasabai japonica* Matsum. Wasabi, which is commonly used in the Japanese cuisine, also belongs to the *Brassicaceae* family. Both in wasabi and in horseradish the pungent taste comes from sinigrin which is the major glucosinolate (Agneta *et al.*, 2014a; Wedelsbäck Bladh *et al.*, 2013b; Kushad *et al.*, 1999). Since horseradish is cheaper to produce it is often used as a substitute for wasabi (Oregon State University, 2002).

An increasing interest for natural ingredients that inhibit various bacteria could increase the demand of horseradish products for this market. Different studies have shown that allyl isothiocyanate strongly inhibits the growth of *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Serratia grimesii* and *Staphylococcus aureus* (Lin *et al.*, 2000; Ward *et al.*, 1998). The allyl isothiocyanate has shown to be effective against the fish-infecting oomycete *Saprolegnia parasitica* which causes problems in the fish industry (Khomvilai *et al.*, 2006).

Horseradish has also been shown to be effective in treating different kinds of nasal and sinus dysfunctions. Several drugs including sinigrin or allyl isothiocyanate have been patented for this use (Iherb, 2014; Friedman, 1995).

Consumption of *Brassica* vegetables has been shown to protect the body from various forms of cancer (Nilsson *et al.*, 2006). Horseradish is one of the plants with increased interest for anti-cancer substances (Weil *et al.*, 2005). Studies have shown that the allyl isothiocyanate which is released when the plant cells in horseradish are crushed could benefit health and inhibit different kinds of cancer such as human prostate cancer (Srivastava *et al.*, 2003; Xiao *et al.*, 2003) as well as induction of lung cancer and development of tumors in liver and fore stomach (Kosson & Horbowicz, 2008). The antimicrobial and antioxidant effects of horseradish is also used by the cosmetics industry as strengthening and bleaching substances (Ilcsi Beautifying Herbs Ltd., 2014).

Figure 7. Horseradish with flowers Photo: K Wedelsbäck Bladh.

1.8 Conservation methods

1.8.1 *Ex situ* and *in situ* conservation

Both *in situ* and *ex situ* conservation should be considered for horseradish. It is mainly a vegetatively propagated crop, which is not suitable for conservation in seed gene banks, since less than 3% of the plants produce viable seeds (Stokes, 1955). The seed production is used only for breeding purposes, why other methods for conservation have to be considered.

To conserve the accessions in their natural habitat *in situ* is the best way to keep the gene-pool maintained. However, the accessions are distributed over large area and the sites are not protected, which can lead to losses of many accessions. For particularly important or threatened accessions, the *ex situ* conservation is necessary.

Systems with field gene banks are used for example in the Nordic countries. Since the plants are easily spread over large areas if not controlled problems with keeping the accessions separated could be solved by keeping a large distance between them. Putting many different accessions together also increase the risk of diseases in the collection, although the Nordic collections have been successfully conserved in the different sites.

To investigate alternative conservation methods a study with four different horseradish accessions for preservation *in vitro* were carried out at the NordGen (Nordic Genetic Resource Center). The same method as described by (Corecka, 1987) was used. Plant material from flower buds, leaf veins, meristem from leaf axils and root tip were tested for growing on Linsmaier-Skoog medium (Corecka, 1987).

In general, *in vitro* cultivation of horseradish worked well. The accessions and the different parts of the plants responded differently, however. Analysis of more accessions is needed to select the optimal procedures (Wedelsbäck Bladh *et al.*, 2006). Not only different clones respond different to *in vitro* cultivation, but also different leaf types. A study made by Shehata et al. (2008) showed that the laminate leaves regenerated 77% more shoots compared to pinnate leaves.

Field collections are the most economic way to conserve Nordic horseradish accessions. However, back up collections are necessary for a safe conservation of important accessions.

Figure 8. Field collection of Nordic horse radish in Alnarp. Photo: K Wedelsbäck Bladh.

Figure 9. In vitro cultivation of horseradish. Figure 10. Seed pods of horseradish
Photos: K Wedelsbäck Bladh.

2 Objectives of the study

The idea for starting this project arose within the working group for vegetables at the NGB (Nordic Gene Bank), now called NordGen, Alnarp. We discovered a gap of knowledge about the diversity in Nordic horseradish and a lack of established collections of horseradish accessions. Accessions from different parts of the Nordic countries, i.e. Denmark, Finland, Norway and Sweden were collected in order to map the diversity and select representative material for conservation. The hope is that this work will be practically useful and inspire future collections and conservation of other minor crops.

The results from this study can be used as a basis for selecting Nordic horseradish clones with the widest possible diversity and preserve them in a core collection of Nordic horseradish.

More specifically the objectives were to

Increase the knowledge about diversity in Nordic horseradish populations

Determine the relationships between various phenotypic characters used in horseradish characterization

Determine the relationships between genetic and phenotypic diversity in Nordic horseradish

Provide information for the establishment of a Nordic core collection of horseradish

3 Material and Methods

3.1 Plant material

The total numbers of accessions included in this study were 176 but some of them were not used in all studies. The major part, 149 accessions, were collected from Denmark, Finland, Norway and Sweden during 2002 and planted in clone archives in their home countries for ex situ conservation. Most of the accessions had been grown at specific site, e.g. a home garden for more than 50 years but their origins are unknown. Another 27 accessions of different European types grown in Årslev Denmark since the 1970s, the "old" Danish collection, were also included in the studies.

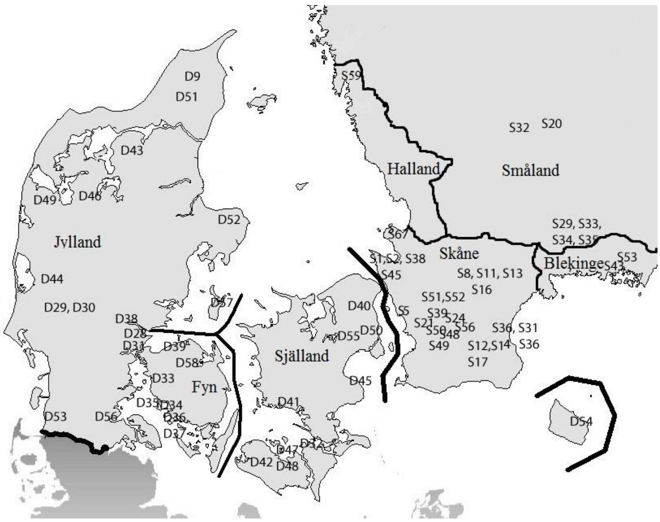

Figure 11. Collecting sites for the horseradish accessions in Denmark and Southern parts of Sweden figure 2. Paper II (Wedelsbäck Bladh et al., 2013a).

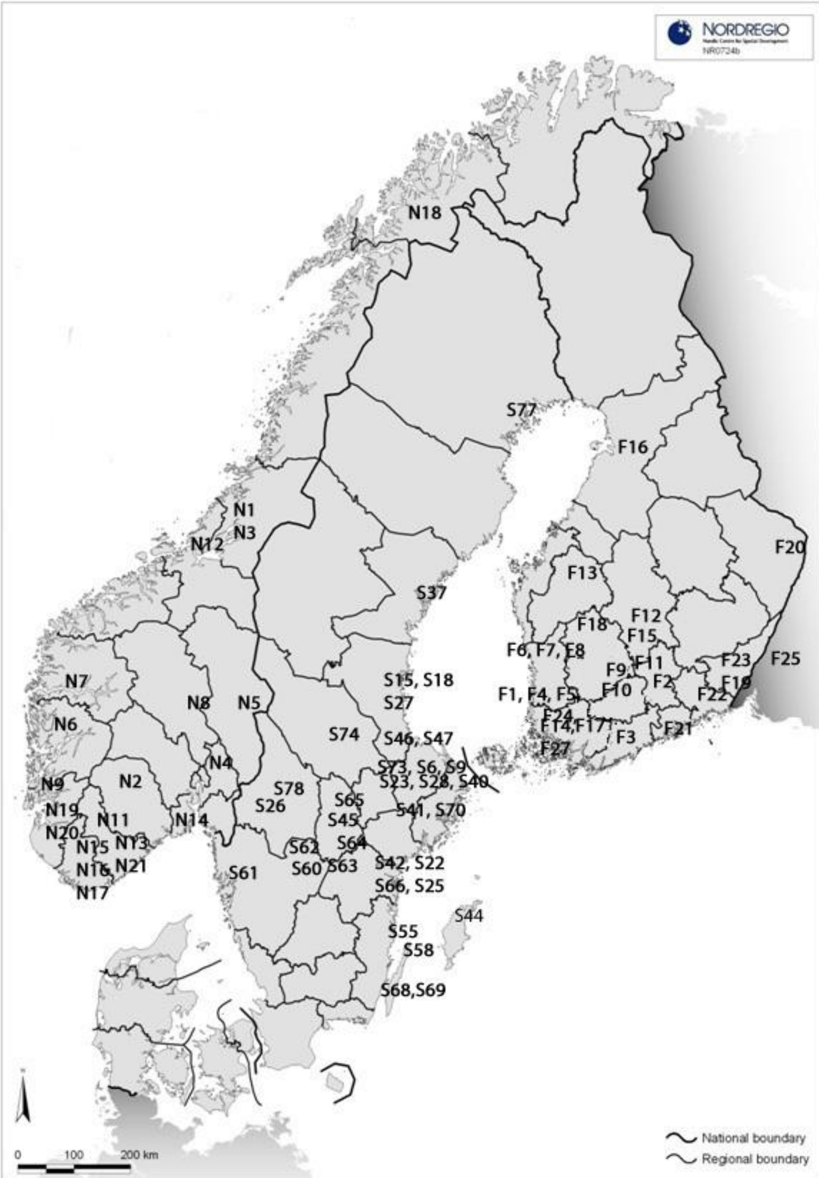

Figure 12. Collecting sites for the horseradish accessions in Norway, Finland and in the central and northern parts of Sweden figure 1. paper II (Wedelsbäck Bladh et al., 2013a).

3.2 Methods

Three different methods were used to study the diversity among the horseradish plants. At DNA level the AFLP method was used and for characterisation of the morphological diversity the UPOV system was followed. The studies of the chemical content focused on glucosinolate concentrations in the roots, analyzed by high-performance liquid chromatography (HPLC). Accessions from Finland, Norway and the new collection in Denmark (collected in gardens) were also analysed for the Vitamin C content with the HPLC method.

3.2.1 AFLP analysis

The AFLP analysis was carried out according to the method described by Vos *et al.*, (Vos P, 1995). Three primer combinations for AFLP fragment amplification were used. The reaction products were separated with polyacrylamide electrophoresis and visualized with silver staining (Bassam *et al.*, 1991). Further information is found in paper II, (Wedelsbäck Bladh *et al.*, 2013a).

3.2.2 Glucosinolate and Vitamin C analysis

Glucosinolate analysis

After maceration each sample was boiled in ethanol to hinder degradation of GLS by myrosinase. Glucotropaeolin and hot ethanol was added, the sample was homogenized and extraction by boiling continued. After cooling and centrifugation, the supernatant was applied on a DEAE Sephadex A-25 column and treated according to Heaney and Fenwick (1980). The sample was washed with water; sulfatase was added and left over-night. The desulphoglucosinolates were eluted and analyzed by HPLC according to the method described in paper III, (Wedelsbäck Bladh *et al.*, 2013b).

Vitanim C analysis

The vitamin C analysis was performed with HPLC. Each sample (5 g) was mixed with 30 ml 3% metaphosphoric acid (Merck) and homogenized (Ultra-Turrax TP 18/10) for two minutes. After homogenization the sample was diluted with 3% metaphosphoric acid (Merck) up to 100 ml and filtered through F1, 15 cm (Munktell Filter AB). The solution was filtered a second time through a 0.2 µm membrane filter (Minisart, Sartorius AG, Göttingen, Germany). The analysis was performed using a VARIAN 9012 gradient evaluation pump (Walnut Creek , California USA), with auto sampler VARIAN 9100, UV-VIS detector VARIAN 9050 and a steal column: Waters Nova Pak C-18, 3.9 x 300 mm, particle size 5 µm (Millipore, USA). The mobile phase was 100% 0.05 M K_2HPO_4 (p.a., waterfree, Merck) dissolved in water (Milli-Q quality) and adjusted to pH 2.6 with ortophosphoric acid (p.a., Merck). The flow rate was 0.80 mL/min, with a 40 µL injection volume; the detection wavelength was 254 nm. At this wavelength metaphosphoric acid and ascorbic acid were detected as two separate peaks. The method was developed in a thesis at LTH School of Engineering at Campus Helsingborg, (Matulaniec, 2002).

3.2.3 Morphological characterization

The plants were first characterized in their country of origin, i.e. in Denmark at The Danish Institute of Agricultural Sciences (DIAS) in Årslev (55°30’N, 10°48’E), in Finland at MTT (Agrifood Research Finland) in Pikkiiö (60°23’N, 22°30’ E), in Norway at Bioforsk in Landvik (58°20’N, 08° 31’E) and in Sweden at Svalöf Weibull AB, Landskrona (55°52’N, 12°49’E) in 2004. Later all of the accessions were replanted and characterized two times, 2012 and 2013, at NordGen, Alnarp in southern Sweden, (55 39' N, 13 05' E).

The characterization followed the UPOV guidelines TG/191/2-2001 (UPOV The International Union for the Protection of New Varieties of Plants, 2001) where 19 of the 29 morphological descriptors were studied. The studied characters are found in paper IV, Wedelsbäck Bladh *et al.* (2014).

3.3 Statistical analysis

The statistical system R, (R Core Team, 2013) was used for the analyses except for the AMOVA which used GENALEX 6 (Peakall & Smouse, 2006), details are given in the individual papers.

4 Summary of the results

The work consists of four parts where Nordic horseradish samples were studied from different perspectives; the historical use and future potential, the genetic diversity in the Nordic samples, the glucosinolate content in the roots and morphological characters in the plants.

4.1 Genotypic diversity

In total 176 accessions from the four Nordic countries Denmark, Finland, Norway and Sweden were analysed with the AFLP method. The results were presented in a dendrogram in which the accessions were divided in 17 clusters. The accessions mainly separated by country where the Finnish accessions appeared to be the most diverse. The Principal Coordinate Analysis showed a clear separation of accessions depending on their country of origin. The Norwegian accessions divided in two groups although some of the accessions from the new Danish collection grouped together with some of the Finnish and Norwegian accessions. The Swedish accessions and the accessions from the old Danish collection separated in two different groups (paper II), (Wedelsbäck Bladh *et al.*, 2013a). The results are shown in table 1.

4.2 Phenotypic diversity

4.2.1 Morphology

The result from the morphological study showed a high diversity in the studied accessions. The variation caused by different trial years contributed less to the general variation compared to the diversity between accessions, showing that the main part of the characters were stable over the years. The studied accessions that included all types of leaf shapes and root shapes divided in different clusters. The type with long leaves and a high quota for the leaf index was most common but also types with elliptic and heart shaped leaves were found. Correlations between the studied characters were also found where the characters separated in five different clusters. In two clusters only characters for the leaves were found; Leaf shape and Leaf width in the first cluster and Leaf length, Leaf index (length/width) and petiole length in the second. The characters for the root divided in three different cluster with two cluster only containing root character and one with a combination of leaf and root characters (paper IV) (Wedelsbäck *et al.*, 2014).

4.2.2 Chemical content

There was a high variation in glucosinolate levels among the investigated accessions and the concentration also differed between different parts of the roots. Two glucosinolates, i.e. sinigrin and gluconasturtiin, were found at much higher concentrations than the other glucosinolates. In the collections from the different countries various combinations with low or high sinigrin content combined with low or high content of gluconasturtiin could be found (paper III) (Wedelsbäck Bladh *et al.*, 2013b). In the study with Vitamin C content the results also indicate a high variation between accessions - from 57 to 182 mg /100g FW.

4.3 Correlation between genotypic and phenotypic diversity

The different studies are presented in table 1 as an overview of how the accessions clustered in the molecular and morphological studies. These results were combined with the outcome from the chemical analysis. The table could be used to get an overview of the diversity in the collections and to select potentially relevant accessions for more tests.

A low correlation between genotypic and phenotypic diversity was found. In table 1 the accessions in the molecular clusters are not a morphologically homogeneous group. In the morphological study the accessions in the major molecular clusters divided in at least three different clusters showing different morphological characters. The accessions in the morphological clusters belong to different molecular clusters showing that plants with similar morphological characters may not have the similar genotype.

The analyses of chemical content in the accessions are represented with different colors and accessions with high Vitamin C content over 125 mg/100

FW are highlighted in yellow (Table 1). The accessions were divided in three groups for the content of sinigrin, low content (up to 23.99 (μmol/g DM) marked with blue, medium in orange (24.00-29.99 μmol/g DM) and high content marked with red color (over 30.00 μmol/g DM). There is a variation in the sinigrin content both within each molecular cluster as well as within each morphological cluster. The Vitamin C content was tested in the accessions from Denmark (new collection), Finland and Norway and according to this study no correlation between the sinigrin content and Vitamin C were found.

Table 1. Clustering of horseradish accessions from morphological and AFLP analyses in combination with sinigrin content and high Vitamin C levels.

Clustering from molecular analyses (AFLP)		**Clusters based on morphological studies**							
		1	**2**	**3**	**4**	**5**	**6**	**7**	**Accessions not included in morphological clustering**
	1	**N16**		**N8, N20**				**N2**	**N3, N10, N11, N14, N19, N7, N12, N21**
	2				**S37**	**S62**	**S46, S49**		
	3				**S8, S22, S24, S68=S71, S25, S32, S33,S34,S35, S42, S12, S15, S16, S26, S29, S36**	**S11, S19, S20, S41, S21, S27,S30,S53,S59, S63,S65,S72=S69, S6, S54, S55**	**S1, S18, S56, S66, S31, S39, S40, S4, S5, S10, S17, S23, S48, S50, S51, S52, S57, S38, S9, S13, S45, S47, S58, S67,S70, S2, S5, S64**		**S43, S44, S14, S28, S60, YougS, S61, S73**
	4	**D1 D7, D4, D14 D24 D25**	**D22**	**D21**		**D10, D12, D19, D26, D5, D8, D11,**		**D15**	**D2,D3, D6, D9, D13, D16, D17, D18, D20, D23, D27, F27 S74, S75**
	5			**N9**				**N13**	**N4, N6, D32, D34, D37, D43, D44, D46, D31, D33, D39, D40, D42, D51, D54, D55, D57, D58 N1, D28, D29, D30, D35, D36, D38, D41, D45, D47, D48, D49, D50, D52, D53, D56**

Clustering from molecular analyses (AFLP)	Clusters based on morphological studies							
	1	2	3	4	5	6	7	Accessions not included in morphological clustering
6.								N5, N15, N17, N18
7.								F13, F16, F25
8		F18, F22						F1
9		F10, F19			F2, F21, F17	F24, F23		F9, F12, F14, F15,
10								F7
11								F11
12		F4						
13					F3			
14		F20						
15								F6
16						F8		
17					F5			

Sinigrin content: High=Red, Medium=Orange, Low=Blue, Black= Not analysed, Yellow=High content of Vitamin C. The numbers are corresponding to the numbers in figure 11 and 12 and to the numbers in appendix paper II.

4.4 Discussion

Vegetatively propagated crops have limited frequency of recombination and may therefore be expected to have a low genotypic and phenotypic diversity among accessions (Curtis, 1983). Horseradish is mainly a vegetatively propagated crop with low seed setting and a low rate of seed germination (Sampliner & Miller, 2009; Stokes, 1955). In contrast to the expectations the results in this study shows high diversity among accessions on basis of AFLP and morphological traits and the content of glucosinolates in the roots. Recently, a study including morphological, molecular AFLP studies and glucosinolate content on Italian horseradish accessions was performed that showed a similar level of diversity (Agneta *et al.*, 2014a; Agneta *et al.*, 2014b). In evolutionary ecology studies of crops biologist have often neglected clonally propagated crops (McKey et al., 2010) However, the few studies that are available have indicated that clonally propagated crops may be more diverse than earlier believed. Many of the clone propagated food crops shows diversity with respect to phylogenetics, morphology and ecology and these crops needs to be studied in more detail (McKey *et al.*, 2010).

My results can be compared with studies in old clones of Swedish hop (*Humulus lupulus* L.) where some similarities were found. Hop has been grown in Sweden at least since the 13th century and between the 15th and 19th century the cultivation was stated by law. In inventory of plants from the previous hop gardens, marked in historical maps, was recently carried out by Karlsson Strese *et al.*, (2014). Hop is a dioecious species, with separate male and female plants. In traditional cultivation the female plants are kept for production of cones while the male plants are removed. The plant is normally vegetatively propagated through cuttings to keep the selected clone in the cultivation (Karlsson Strese *et al.*, 2014). This is similar to the propagation and selection of horseradish accessions. The results from Karlsson Strese *et al.* (2014) show that sexual reproduction of hop appears to be rare in Sweden since the observed differentiation between the populations are strict. However, a considerably variation in the chemical content of the cones and a high variation in the morphological traits were found among the studied hop clones (Karlsson-Strese, 2008). The hop collection was further studied for genetic diversity with SSR. The results indicated high genetic diversity among Swedish clones and between Swedish and European clones (Karlsson Strese *et al.*, 2014). This pattern is rather similar to the findings in this thesis on horseradish where also a separation between accessions with different country of origin was found.

The most likely explanation to the high diversity in horseradish could be the introduction to Nordic cloisters and subsequent distribution to different parts of the countries at many different occasions. Several monastery orders were established in the Nordic region from the 11th century and onwards. Only in Skåne (southern part of Sweden) Cistercian, Benedictine, Augustinian, Premonstratensians, Dominicans, Franciscans, Carmelites and Helgeands cloisters were founded. The cloisters also had many farms connected to them. For example, about 450 farms spread over Skåne belonged to ‘Dalby cloister’ (outside Lund) in 1530 (Carelli, 2010). The connections between the farms and the cloisters have probably facilitated the distribution and a more common use of the plant. Different cloister orders settled in different locations in the Nordic region, for example, the Cistercian order was the dominant one in Sweden with exception from Skåne where the Premonstratensians were more common (Carelli, 2010). The different cloister orders probably had several sources for their horseradish plants since they recruited people from different geographical regions of Europe, which may have contributed to the diversity of horseradish.

As horseradish was introduced to the Nordic countries in the 13th century and later spread to different parts of the country from the cloisters some of the accessions could be residues from this time. However, few studies on the age of vegetatively propagated accessions have been performed and no available records have been found that date horseradish accessions back to this time.

During the time from the introduction until now there has also been an important trade with horseradish. The import and export of roots to the Nordic countries has probably added new genotypes which has affected the diversity of horseradish.

Another factor contributing to the diversity in horseradish could be seed setting despite the very low germination rate of about 3% that has been reported (Winiarczyk & Bednara, 2008; Stokes, 1955). There are different reasons to the poor seed setting.

Horseradish is a cross pollinated plant where the seed development follows the normal fertilization process in cruciferous plant. The fertilization occurs 18-30 hours after the pollination (Shehata *et al.*, 2009) and the fruits mature in three to four weeks (Weber, 1949). The ovary contains 16-20 ovules (Weber, 1949) with a seed number between 1-6 in the pods (Stokes, 1955). Stokes (1955) reported that fertilization occurs only in one-third of the ovules and failure of the endosperm is the most frequent cause of seed sterility.

Differences in pollen quality in the two major types of horseradish (‘Bohemian’ and ‘Common’) have also been observed. ‘Bohemian’ types have some functional pollen while the ‘Common’ type fail to produce functional pollen (Stokes, 1955). There are reported differences in the flowers where the ‘Common’ type is smaller and more often fails to develop. The flowers abort before opening to a higher degree compared to the ‘Bohemian’ and ‘Big Top Western’ types (Shehata *et al.*, 2009).

According to Stokes (1955) the failure to set seeds following fertilization is primarily due to endosperm-maternal tissue incompatibility and embryo abortion. Aneuploidy and variation in chromosome number in meiotic stages of pollen and mother cells in different plants, n=14 and n=16, respectively were reported (Shehata *et al.*, 2009).

Horseradish is also reported to be a possible allotetraploid species since some homology occurs between the two genomes and some viable seeds are produced (Shehata *et al.*, 2009).

Despite the problems in developing functional seeds low production of new seed plants have probably occurred during the 800 years that horseradish has been grown in the Nordic countries. Even a low number of functional seeds produced from a cross-pollinated plant could make an important contribution to the diversity.

Problems have been reported in seed production for breeding purposes. A successful method to overcome spontaneous embryo abortion is the use of plant tissue culture. Embryos derived from horseradish crosses and self pollinations were rescued with plant tissue culture and resulted in a better germination rate compared to conventional germination in soil (Ozgur *et al.*, 2004).

Few studies have been made to map the wild relatives to horseradish. Differences between horseradish and the wild relatives *Armoracia macrocarpa* and *Armoracia sisymbrioides* are found, for example, in seed setting. The two wild relatives produce a rich amount of seeds. Natural populations are harvested but in some areas *A. sisymbrioides* is also cultivated. *A. sisymbrioides* is used for the same purposes as horseradish (Sampliner & Miller, 2009) and could be an important gene source for the future.

The most dominating glucosinolates in horseradish are sinigrin, gluconasturtiin and glucobrassicin. However, rather few studies on variation of glucosinolate content in different horseradish accessions have been performed. Li and Kushad (2004) studied the glucosinolate content of leaves and roots and found large variation between the accessions. In this study the sinigrin content varied between 2 to 258 μmol/g DM for unpeeled horseradish roots and the variation between accessions from the same country could be up to 30-fold (Li & Kushad, 2004). Recently, Italian accessions were analysed for the glucosinolate content and the results showed that sinigrin was predominating followed by gluconasturtiin and glucobrassicin (Agneta *et al.*, 2014a; Agneta *et al.*, 2014b). Li and Kushad (2004) reported that the average proportion of sinigrin of the total glycosinolate content was 83%, while the investigated Italian accessions varied between 53% and 87%. In the Nordic accessions this value varied between 82-90%.

Conservation is of particular interest since this species most probably only exists in cultivation and no wild populations remain. The analysis performed on horseradish in this thesis, also revealing accession with unique characters, and could be a base for the selection of interesting accessions for future conservation. The high diversity among the horseradish accessions suggests that genotypes suitable for different end-uses can be selected. The conservation could be arranged in different ways in private gardens or in common Nordic collections. Most important is to keep the accessions separated, healthy and promote its utilization in breeding for different areas. An effective way could be to create a Nordic core collection with back-up collections in the different Nordic countries.

5 Conclusions

Horseradish is a vegetative propagated plant with low frequency of seed sets. The Nordic horseradish populations could therefore be expected to have a low level of diversity. Based on the genotypic and phenotypic studies on horseradish accessions collected in Denmark, Finland, Norway and Sweden the following conclusions can be drawn:

- Results from the molecular study showed a relative high level of diversity among the studied accessions that separated into 17 different clusters and grouping of accession was mainly according to country of origin.

- The Finnish accessions showed the highest diversity compared to the other countries.

- The results from the chemical analysis of the glucosinolate content showed high variation among the studied accessions. No correlation was found between the chemical content and molecular analysis.

- The most dominant glucosinolates found in Nordic horseradish were sinigrin followed by gluconasturtiin and glucobrassicin.

- A broad diversity in morphology, in both leaf and rhizome characters, was found on basis of the studied UPOV characters.

- A clustering of the morphological characters were also observed where correlations between the different characters were detected, indicating that fewer characters could be used in future morphological studies of horseradish for a rational and cost efficient characterization.

6 Future developments

The relationships between the Nordic horseradish accessions were studied together with some accessions from Europe. To increase the knowledge of the world diversity and to learn more about the introduction history of horseradish to different areas, more accessions from different part of the world needs to be studied.

Morphological characters have been an important factor for selection of horseradish accessions for commercial production. With an increased use in industrial production the morphological characters will be less important in favor for the content of chemical compounds. Further research should be performed to find accessions with different chemical content for various uses.

Today the supply of horseradish roots in the shops are limited and only one cultivar, without additional information such as strong or weak taste, is offered. A diversification to more products and information on the health aspect could increase the production and consumption.

Establishing a Nordic core collection of horseradish could be based on the results presented in the thesis. However, the diversity in diseases resistance should also be studied before a final selection of the accessions is done.

Wild relatives to cultivated plants are important genetic recourses in plant breeding. For example, important genes for disease resistance may be found in wild relatives. The wild relative to horseradish *Armoracia macrocarpa* is on the red list of endangered plants. Only a few seed accessions are stored in the gene banks and conserved for the future. Therefore, a collecting mission of *Armoracia macrocarpa* would be of great value. It is likely that this species could contribute to future development of horseradish.

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