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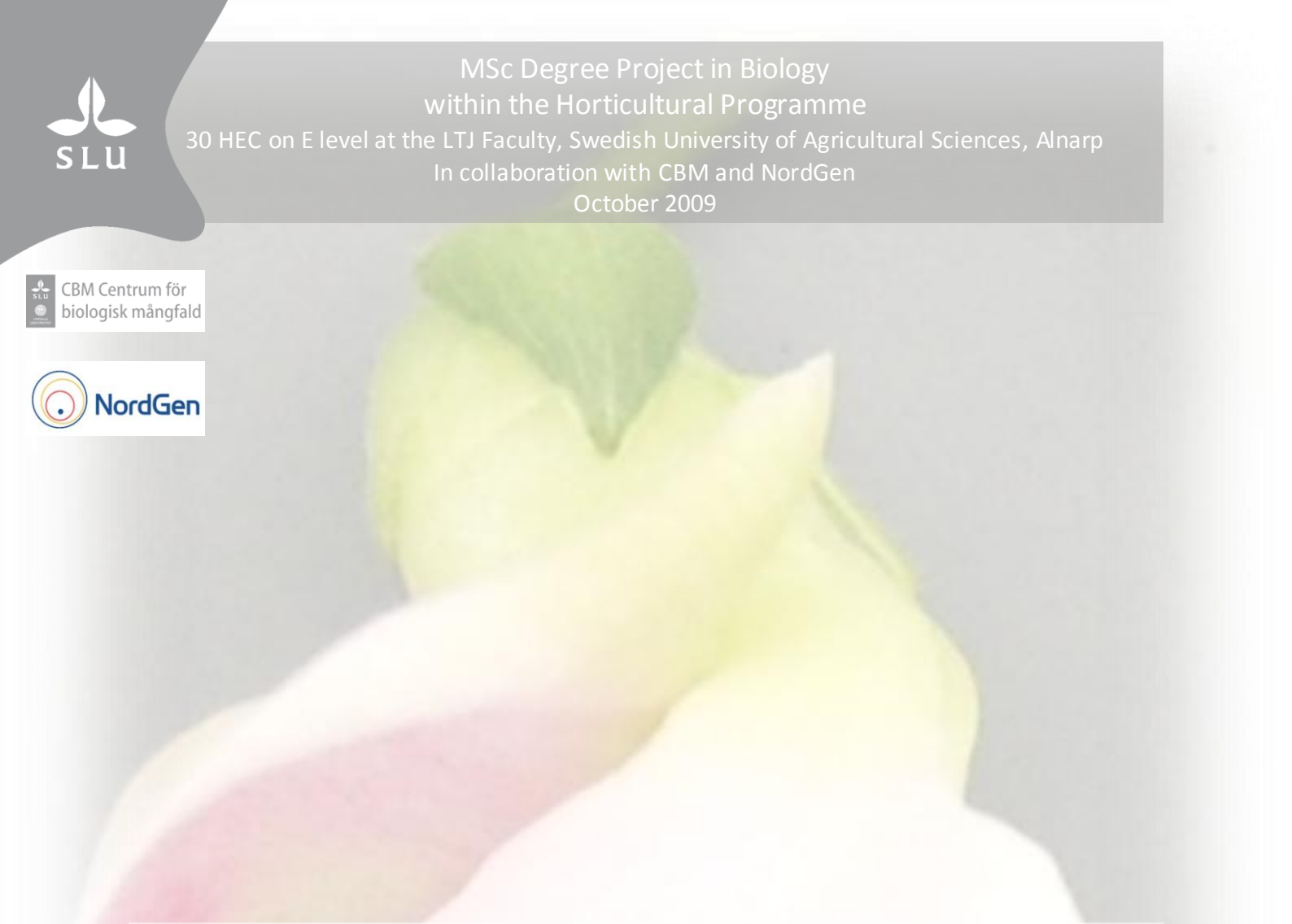

Analysis of Swedish Accessions of *Phaseolus vulgaris* L. Using SSR Markers

Analys av Svenska Accessioner av *Phaseolus vulgaris* L. med SSR-markörer

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In August 2004 I enrolled at the Horticultural Programme here at SLU, Alnarp, not quite certain of what to expect. What does a horticulturist do? Where? Why? How? Today, thousands of study hours, hundreds of classes, and many exams and new friends later, and so close to finishing my MSc thesis, I still don't have all the answers. There's so much within this field yet to explore. But, for now at least, having spent twenty weeks with this project and learning so much, I can put a check mark in the applied plant genetics box!

My warmest and most thanks go to CBM and **Jens Weibull** whom I contacted on the issue of finding a project for my thesis and who introduced me to this particular garden bean project. Many thanks to my examinator **Björn Salomon**, also course responsible. Many thanks also to my supportive and kind supervisor **Li-Hua Zhu** who gave me useful advice during the planning and writing of this thesis; to my assistant supervisor **Agnese Kolodinska Brantestam** who helped to plan my laboratory work and had great therotical knowledge input; to **Alfia Khairullina** and **Pia Ohlsson** who safely guided me in the lab and through my laboratory work, and occasionally also saved my b*ttom; to **Svein Oivind Solberg**, **Eva Johnsson** and **Jerker Niss** who kept my precious plants alive; to **Malin Dörre**, **Fredrik Ottosson**, **Lena Dafgård** and **Simon Jeppson** who all gave me pieces of advice and input on everything from photographing to plant supports; and to everyone else at **NordGen** who locked and unlocked doors when I was locked out. I would also like to thank my fellow horticulturist students and friends **Anna Holmkvist**, **Anna Thynell**, **Jennifer Sandström**, **Carin Emanuelson** and **Madeleine Kylin** without whom I would have succumbed to the oh-so-powerful force of navel-gazing. Thank you my far away but ever supportive love **Jon Brunk**. To all my **other friends** and **family** – thank you for being there – and everyone else who feel they deserve an epic thank you, **THANK YOU**! You know I couldn't have done it without you.

Elin

Abstract

The genetic diversity in cultural plants is considered to be threatened due to global climate change and antropogenic influence. Unknowing of future conditions for cultivation, gene banks all over the world are now trying to build up costly safety nets of species and varieties that may become highly valuable owing to their specific traits and genetic heredity. Hence, in 2002 there was a national inventory, aimed at the Swedish public, asking for seeds believed to be lost. Among the resulting collected seeds were some accessions of garden bean, *Phaseolus vulgaris*. The objective of this particular study was to assemble a collection of unique varieties and discard possible duplicates. With scarcity of their background information, the 35 chosen accessions were fingerprinted and compared with eight reference varieties using seven microsatellite (SSR, simple sequence repeats) loci and corresponding species-specific primers. The reference varieties were known to have been grown in Sweden during the 1850s until early 1900s when garden bean cultivation was strikingly popular. Four morphological markers were also recorded: growth habit, terminal leaflet shape and colours of the flower standard and wings. The results from the SSR data alone revealed 15 accessions as unique. A combination of the SSR-data and the morphological characters detected an additional nine accessions as unique, while eleven accessions in four groups remained uncertain where potential duplicates could be present. None of the 35 accessions were duplicate to any of the eight reference varieties. Since the preservation of genetic material in gene banks implies high costs, the remaining accessions with potential duplicates should be examined further for clarity and the unique accessions found should be preserved at NordGen to facilitate the future utilisation of them as genetic resources.

Sammanfattning

Den genetiska mångfalden hos våra kulturväxter anses vara hotad på grund av globala klimatförändringar och mänsklig påverkan. Då vi nu står inför en framtid med ovisshet om hur förutsättningarna för odling kan komma att förändras så håller genbanker världen över på att bygga upp kostsamma depålager av arter och sorter som kan visa sig vara värdefulla tack vare sina egenskaper och genetiska arv. År 2002 riktades därför ett nationellt upprop till svenska folket, där man eftersökte frön som man inte längre trodde fanns. I det inkomna materialet fanns bland annat en del accessioner av trädgårdsböna, *Phaseolus vulgaris*. Syftet med den här specifika studien var att upprätta en samling av unika sorter, samt att avlägsna eventuella duplikat. Med mycket begränsad bakgrundsinformation kartlades de 35 utvalda accessionerna och jämfördes med åtta referenssorter med hjälp av sju mikrosatellitlokus (SSR, simple sequence repeats) och tillhörande artspecifika primerpar. Referenssorterna vet man odlades i Sverige mellan 1850- och början på 1900-talet då det var otroligt populärt att odla trädgårdsbönor. Dessutom registrerades fyra morfologiska markörer: växtsätt, bladform samt färg på blommans segel och vingar. Resultaten från enbart SSR-data utpekade 15 accessioner som unika. En kombination av SSR-data och de morfologiska markörerna påvisade ytterligare nio accessioner som unika medan elva accessioner i fyra olika grupper kvarstod där potentiella duplikat kan finnas. Ingen av de 35 accessionerna var duplikat till någon av de åtta referenssorterna. Eftersom bevarandet av genetiskt material i genbanker är förenat med höga omkostander, bör de återstående accessionerna där potentiella duplikat finns, undersökas ytterligare för att bringa klarhet i frågan och de unika accessioner som påträffades bör bevaras vid NordGen för att möjliggöra framtida användning av dem som genetiska resurser.

Contents

Introduction

The importance of plant genetic resource conservation

Not many decades ago the green revolution had its break through. What was then thought to be the solution to world starvation was a combination of new biocides and the breeding of higher yielding and resistant cultivars. Our fields turned greener and more productive in a way never before imagined. But the green revolution also had a darker side. The higher yielding cultivars generating generous profits to the providers soon outcompeted many of the old cultivars. Voices concerned with genetic erosion and loss of biological diversity were beginning to speak up, and slowly but surely gaining ground.

Finally, the long term global food supply was seriously questioned. This lead to the ratification, signing and adoption of the Convention on Biological Diversity (CBD) by the United Nations (UN) and most countries worldwide. It all took place at the Earth Summit held in Rio de Janeiro in 1992. The conclusion was that our uncertainty of what resources the world population might need tomorrow implies that every country has a responsibility to preserve its native species (POM, 2008). The CBD was the very first legally binding treaty on preservation of genetic assets to ensure sustainable development worldwide (CDB, 2000).

For more than 30 years the Nordic countries have collaborated on preservation of plant genetic resources in the form of The Nordic Gene Bank which was established in 1979. In 2008 they merged with two other institutions and was renamed the Nordic Genetic Resource Center (NordGen). Primarily NordGen's mission is to assure genetic diversity and sustainable utilisation of food- and agricultural genetic resources. Since all Nordic countries have national CBD programs, NordGen also coordinates the Nordic countries' work in pursuing them (NordGen, 2009).

In an effort to take responsibility for and preserve the Swedish plant genetic resources the Swedish Board of Agriculture developed in 1998 a national Programme for Diversity of Cultivated Plants (POM). The first inventory of cultural plants through POM was an inventory for seeds believed to be lost, initiated in 2002. Among many others, samples of *Phaseolus vulgaris* accessions were received. All POM seeds are stored at NordGen (POM, 2008). Their high value springs from them as genetic resources for having adapted to the

Nordic climate – some for millennia. They may possess desirable traits that are not present in the varieties available on the market today. These seeds and plants are collected they are currently being evaluated and compared to known varieties to seek uniqueness and eliminate duplicates (POM, 2002; Nordgen 2009).

P. vulgaris botany

With its origin in the area around Central or South America (Figure 1), *P. vulgaris* is now a worldwide crop (Gaitán-Solís *et al.*, 2002; Davis, 1997). To Europe it was introduced to the Iberian peninsula and the first record from Western Europe is from 1538 in England (Gepts and Bliss, 1988). Illustrations show how an array of bean varieties were grown in the mid 1500s and due to their diverse seed appearance, taxonomists were kept puzzled for 200 years (Saucer, 1993). The diversity is compiled within its 22 chromosomes (Cimpeanu *et al.*, 2005). This herbaceous twining and vine-like legume is commonly called "garden bean", "snap bean", "French bean" or "common bean". It belongs to the family Fabaceae and is a dicotyledonous annual. Its compound leaf is pinnately trifoliate and the leaves are alternately arranged on the stem (Figure 2 d). Determinately growing 60 cm tall as a bush variety, or climbing indeterminately (Figure 2 b) up to 3 m, it takes between 60 and 80 days under favourable conditions from germination until pods and seeds are ripe. The flowers are usually white, pink, or purple (Figure 2 a and c), while seed pods turn green, blueish green, purple, yellow, or multi-coloured and range from 8–20 cm in length. *P. vulgaris* beans grow optimally at a temperature around 20–25°C and require warmth also in the rhizosphere. A slightly acid soil with a pH range of 6.0–6.5 is needed for germination and proper root development. Usually the root system is shallow but in easily penetrable soils the taproot can reach 1 m down (Rubatzky and Yamaguchi, 1997).

Figure 1. The Central- or South American origin and introduction to the Iberian peninsula in Europe of *P. vulgaris* during the 1500s.

Figure 2. *P. vulgaris* plants and flowers. a) Pink coloured flowers. b) Climbing varieties on pole supports in the greenhouse. c) White flower and flower bud. d) Young bush specimen showing the trifoliate compound leaf. *Photo: © Elin Bengtsson.*

They will thrive in well-drained sand or clay soil provided there is a microorganism community of nitrogen fixating *Rhizobium* bacteria which induce nodules on the roots for nitrogen fixation. These bacteria are responsible for the universally known effect of reduced soil nitrogen leakage and application from pulse cultivation (Kraft, 1983).

Local history of cultivation

Today leguminous crops are among the most economically important ones worldwide. In Scandinavia they have been cultivated since 3000 B.C. when the first farm settlements were established here. During the Viking age the garden cultivations were finally separated from the field cultivations and the first kitchen gardens appeared close to the housing. Beans of *Vicia* type were still field grown. Local, and later national, laws from the Middle Ages manifested punishments for theft of peas, beans, hemp and flax from the field.

Advancements in gardening techniques were lead by the monasteries with their good connections to sister monasteries in Europe from where a lot of the imported seeds were obtained. Monastic rules also decreed monks and nuns to be self-supporting of garden produce. During the 1600s the nobility was generously rewarded for their war efforts and so their positions were strengthened at the expense of the state. With tax revenues from local farmers, noblemen could now afford an extravagant way of living. Architecturally, large enclosed allotments became popular for fruits, flowers, medicinal herbs, and kitchen plants, now including *Phaseolus* beans. The surplus was often sold at town markets. In the 17th century king Karl XI regained half of the land the nobility had had at their disposal and it

became more difficult for them to sustain their gardens. Madam at the Årsta mansion was Märta Helena Reenstierna. In her diary from the late 1790s, she wrote on April 29 in 1796

"4 cans of beans were sown on the seed plot. Ditto 3 cans on the plot below the wall and on the skirts of the seed plot."

And 2 years later on June 24, she wrote

"All the oxen had broken into the garden and there ruined the beans and cabbage plots."
(Flink, 1994).

As a result of hereditary tradition, farmers' pieces of land had been broken up into many land strips, but in the mid 1900s there was a reform to shift these back into one single piece of land, as it had once been. There was a fantastic opportunity to grow a garden, but seeds and plants were scarce and difficult to get hold of (Israelsson, 1996). Gardening and self-sufficiency of utility plants became supported by the state (Flinck, 1994) and in 1842 a law was even enforced for schools to teach it (Israelsson, 1996). The main interest for gardening was taken over by priests and town citizens who invested in land and laid out gardens, often located just outside the town borders. However, they were heavily criticised by farmers who felt the competition on the grain market. Instead, the priests and town citizens were encouraged to grow garden products for direct sales on town markets. Potatoes, tobacco, beans, carrots and parsnip are a few examples. This type of production was during the second half of the 19th century taken over by specialised and professional gardeners.

The occupation of gardening kept rising throughout the 19th century and never before had so many different varieties of vegetables been cultivated. Especially towards the end of the century the number of varieties available per species was increased by the many seed companies. For instance, there were yellow and black dwarf beans, extra large dwarf beans, early white pole beans, Turkish beans, and so on. In the countryside the harvest consisted of the entire stalk which was hung up at the roof-base to dry (Flinck, 1994). A direct comparison between an old and a more recent seed catalogue illustrates the difference in the number of available bean varieties. In 1895 there were 43 varieties available – in 1995, sadly, only nine varieties (Israelsson, 1996).

Well appreciated, the first colony of garden plots was established for the working class in 1888. Many affordable houses with small gardens were also built outside town centres for the less affluent. This type of gardening was still popular and utilised during the world wars when the demand for them increased. However, the arrival of functionalism changed the view of the garden in favour for it as an extension of the home and a place for resting and relaxation – an outdoor living room. The better availability of vegetables at the grocer's made vegetable growing into a hobby rather than a necessity. At the same time plant nurseries shifted to a narrower assortment and the number of varieties offered was drastically reduced. This is still much the case today, but thanks to a rising demand springing from hobby gardeners the assortment is yet again on the rise (Flinck, 1996).

Types of markers and their applications

Different markers have been used for more than a century as tools for understanding e.g. inheritance and relationships between individuals and populations. The "first" geneticist was the now legendary 19th century scientist Gregor Mendel. By looking at traits on peas such as the frequency of green vs. yellow seed colour and smooth vs. creased seed surface he used these as morphological markers to study the patterns of inheritance. As a tribute to his work, the expression "Mendelian inheritance" was coined – a term for how traits segregate when they are passed on from parent to offspring.

During the last three decades' development in biochemistry and molecular biology many new marker systems have emerged. Scientists have been eager to find markers that are independent of the developmental stage of an organism. Traditional morphological markers have the limitations of exhibiting relatively few traits and a low degree of polymorphism. They can also depend on the expression of unlinked genes, as well as be highly variable depending on the environment (Shulman, 2007). The new biochemical markers and in particular allozymes became popular at the time, for their accessibility and the ease of separation and scoring by electrophoresis. They were discernable at an earlier developmental stage and easier to score due to better stability through morphological mutation than other biochemical markers, for example nutritional ones (Ganapathy and Scandalios, 1973). However, it was not until the DNA markers came along that the truly stable markers regarding an organism's developmental stage became available. With the advent of the genetic markers and marker systems, scientist now have a array of markers to study variability

and diversity, when constructing linkage maps, and in diagnosing individuals or lines that carry specific linked genes. In essence a DNA marker is a specific nucleotide sequence that corresponds to a location, a locus, in the genome. The molecular markers are coarsely divided into methods detecting target sequences in the genome and ones that use general primers to amplify anonymous sequences (Shulman, 2007).

Among the most commonly used marker methods are AFLP (amplified fragment length polymorphisms), RFLP (restriction fragment length polymorphisms), RAPD (random amplified polymorphic DNA), SSR (simple sequence repeats, also known as microsatellites), and SNP (single nucleotide polymorphisms) (Agarwal *et al.*, 2008). These DNA markers can be broadly categorised into two groups: non-specific and specific markers. To the former group belong AFLP, RFLP, RAPD, and ISSR (inter simple sequence repeats) techniques, while SSR and SNP techniques belong to the latter since they require DNA sequence information beforehand, which the former DNA markers do not. RAPDs have been used in plant breeding programs and genetics (Waugh and Powell, 1992; Powell *et al.*, 1996a), SSRs have been developed for a wide range of plant species (Powell *et al.*, 1996a) and also for *P. vulgaris* (Blair *et al.*, 2008; Masi *et al.*, 2003; Métais *et al.*, 2002). Other *P. vulgaris* studies have combined AFLP and ISSR markers (Svetleva *et al.*, 2006), SSR and RAPD (da Silva *et al.*, 2003), SSR and AFLP (Masi *et al.*, 2009), or SSR, ISSR and CpSSR (chloroplast simple sequence repeats) (Sicard *et al.*, 2005).

Motives for choosing SSR markers

Within human and mammalian research SSRs revolutionised the field. SSRs have also proven to have a significant impact even in plant science (Powell *et al.*, 1996b) because of their high degree of length polymorphism and their high abundance in the genome. They are useful in many fields, for example population biology studies (Franzén *et al.*, 2006), for creating genetic linkage maps (Mayerhofer *et al.* 2008), and for identifying hybrid purity (LiAng *et al.*, 2009). SSR loci are particularly appropriate for inferring recent events such as dispersal or mate choice. Multi-locus markers, on the other hand, facilitate the fast and concurrent screening of several loci (Freeland, 2005). For instance, AFLPs may provide more information thanks to the large number of bands obtained in a single experiment (Weising *et al.*, 2005). However, multiplexing is also possible with SSRs, albeit more laborious (Masi *et*

al., 2003). SSRs have been used previously at Nordgen for this type of study on peas collected via POM (Kolodinska Brantestam *et al.*, 2006).

In plants the predominant SSRs consist of AT/TA repeats (Powell *et al.*, 1996b). There are several reasons why locus specific SSRs are believed to be superior for cultivar identification, at least to RAPD, but also to AFLP and ISSR. First, genotypic data can be obtained based on allele information. Second, primer sequences are easily allocated to various laboratories, and compared to RAPD, SSR experiments are more easily reproduced. Third, their higher degree of length polymorphism provides better resolution at the individual level. Additionally, their codominant nature facilitates distinction between hetero- and homozygotes (Weising *et al.*, 2005). Since this is a PCR based marker method, a great advantage is also that such a small amount of DNA template is needed (Masi *et al.*, 2003).

Motives for additional morphological markers

Some morphological markers were chosen as secondary but complementary to the SSR markers. Some studies undertake the use of a combination of genetic and morphological markers, because it can improve the characterisation of accessions (Chiorato *et al.*, 2006). The set of morphological markers were chosen from the UPOV guiedlines for characterisation of *P. vulgaris* (UPOV, 2005). The first chosen marker was growth type – bush or climbing – because it was suitable and applicable at the time considering the plants' growth stage. Terminal leaflet shape was chosen as a second morphological marker based on the fact that there were good reference illustrations at hand, as we lacked the recommended reference varietes. At the time of the decision for recording morphological data, the plats were close to flowering, which is why also colour of the flower standard and of the flower wings were chosen as morphological markers. Both growth type and colour of the standard "have been agreed as useful grouping characteristics", among other characteristics (UPOV, 2005).

Objectives of the study

The purpose of this study was to analyse and determine the genetic variation within and between *P. vulgaris* accessions received during the inventory for seeds initiated by POM. This was in order to exclude duplicates in the collection and screen for unique varieties believed to have vanished over the years of meagre bean cultivation due to lack of interest by private growers as well as the limited assortment provided by seed companies.

The questions posed for this study were

- Are there duplicate accessions among the samples received by POM?
- Are any of the POM accessions duplicates of the reference varieties that have been grown here from the late 1800s to the early 1900s?
- Which accessions are unique?

Material

Plant material

A total of 43 accessions (Table 1) of *P. vulgaris* Stored at NordGen were in this study. Of these, 35 were collected via POM. Two of them were collected in Denmark, one in Norway and the remaining 32 in Sweden. An additional eight accessions and characterised varieties were retrieved from the Leibnitz Institute of Plant Genetics and Plant Crop Research, (IPK) in Gatersleben Germany, serving as reference material. The reference varieties were ones that were often imported to Sweden, highly popular and cultivated during the time between the mid 1800s and beginning of 1900s. In this context it is important to understand that an accession is a seed sample. Therefore, due to possible a mix-up of several varieties, it could not be excluded that the examined accessions from POM might exhibit variation within an accession.

Table 1. List of the *P. vulgaris* accessions examined in this study which includes 35 accessions collected by POM and stored at the NordGen gene bank, and eight IPK reference varieties (the last eight varieties in the table to the right).

Accession number	*Name*	*Country of origin*
NGB2578	BRUN BÖNA, GOTLAND (CARIOCA)	SE
NGB4150	BRUN BÖNA, "EBBES MORS"	SE
NGB5023	BRUN BÖNA	SE
NGB5026	BRUN BÖNA	SE
NGB6795	GOTLANDSBÖNA	SE
NGB7791	OLSOK	NO
NGB9298	HUNDREDE FOR EEN	DK
NGB11554	GOTLANDSBÖNA	SE
NGB11570	STELLA	SE
NGB11761	GOTLÄNDSK SVART BÖNA	SE
NGB11762	GOTLÄNDSK BRUN BÖNA	SE
NGB11763	GOTLÄNDSK VIT BÖNA	SE
NGB13763	SWEDISH BROWN	SE
NGB13764	SWEDISH ONE DOT	SE
NGB13782	BRUN BÖNA ÖSTERGÖTLÄNDSK LANTSORT	SE
NGB13858	BRUN BÖNA FRÅN ORNAKÄRR	SE
NGB17801	HALLAND	SE
NGB17803	SLOALYCKE	SE
NGB17805	KRISTIN	SE
NGB17807	HARPLINGE	SE
NGB17808	RYSK KEJSARBÖNA	SE
NGB17809	BERNADINA	SE
NGB17810	PETTERSONS BÖNA	SE
NGB17812	STÅSTORP	SE
NGB17813	HANAS STRIMMIGA	SE
NGB17815	SANDA	SE
NGB17816	GULLSPÅNG	SE
NGB17821	FISKEBY	SE
NGB17823	SIGRID	SE
NGB17824	KULLA	SE
NGB17825	SIGNE	SE
NGB17827	Extra-Hatif de Juillet	SE
NGB18054	Gulböna från Östergarn	SE
NGB18186	Prinsesse	DK
NGB18675	BÅSTAD	SE
PHA 211	*Juli*	
PHA 44	*Ideal*	
N 477	*Flageolet*	
PHA 49	*Weibulls Express*	*SE*
PHA 1357	*Nordstem*	*DE*
N 408	*Chevriert Vert*	*FR*
PHA 119	*Konserva*	*DE*
PHA 474	*Mont d'Or*	*FR*

Methods

DNA extraction

Six seeds per accession were sown in the NordGen greenhouse. After three weeks, two randomly selected seedlings within each accession were transplanted into 3 L black plastic pots. Leaf tissue samples of 1.5–2 cm^2 were taken from young leaves (first, second, or third true leaf pair) and put into 2 mL Eppendorf tubes on ice. The material was then frozen in liquid nitrogen before being put into a Heto-Holten® LyoLab 3000 freeze vacuum dryer for freeze-drying at -57^oC for 65 h.

Genomic DNA was extracted using a modified CTAB method protocol, described as follows. Samples were homogenized in Eppendorf tubes using a Retsch® shaker machine, shaking at 20 rps for 2 min. To bind cell wall membrane lipids, 700 µL CTAB surfactant buffer (0.1 M Tris pH 8.0, 0.01 M EDTA, 0.7 M NaCl, 1g CTAB and 1 mL mercaptoethanol per 100 mL final solution) was added to each sample tube. The homogenized samples were incubated in an Eppendorf® Thermomixer Comfort machine at 60^oC and 650 rpm for 1 h. Non nucleic acid molecules and proteins were denatured and precipitated by the addition of 700 µL chloroform-isoamylalcohol (24:1). After centrifugation at 13 200 rpm for 20 min, the DNA-containing water phase was transferred to a new Eppendorf tube, containing 5 µL of 1 mg/mL RNAse. Once again, tubes were put on the Eppendorf® Thermomixer Comfort machine, this time at 37^oC and 600 rpm for 30 min. The DNA was precipitated by adding 500 µL ice-cold isopropanol and centrifuged for 10 min at 13 200 rpm. The DNA pellet was then washed with 600 µL washing solution (76% ethanol and 0.2 M sodium acetate) for 20 min, centrifuged again for 5 min at the same rpm, then washed again with 600 µL of rinsing solution (76% ethanol and 0.01 M ammonium acetate) and centrifuged for 5 min at 13 200 rpm. After pouring off the supernatant, the tubes were left open over night. The DNA pellet was then dissolved in 50 µL TE buffer (0.01 Tris and 1mM EDTA, both pH 8). The quality and quantity of DNA was measured on an Eppendorf® BioPhotometer, and the DNA was diluted to a concentration of 10 µg/mL for PCR reactions.

SSR markers

P. vulgaris species-specific primers of AT-rich markers, developed by Blair *et al.* (2008) were selected for multiplex SSR analysis (Figure 3). One marker was chosen for each of the eleven

linkage groups (chromosomes), except for linkage group B11 for which two markers were chosen. Also, linkage groups B7 and B10 had one marker in common (ATA150 a and b).

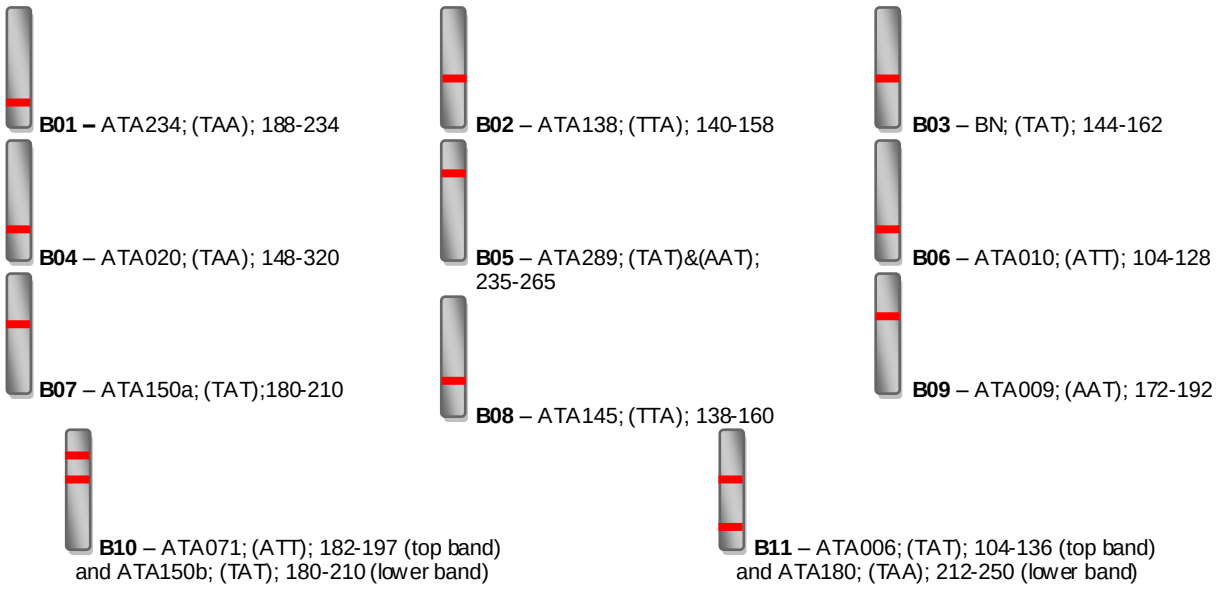

Figure 3. Illustration of chromosome (in fact; linkage group) designations B01-B11, and the corresponding SSR loci developed by Blair *et al.* (2008). Within brackets are the repeat sequences, and behind are the expected repeat lengths. Adapted from Blair *et al.* (2008).

PCR

The PCR reaction was carried out in a volume of 25 µL containing 2.5 µL Thermobuffer, 0.5 µL 10 mM dNTP, and 0.75 µL 10 pmol/µL each of forward and fluorescently labelled reverse primers (produced by New England Biolabs Inc.), 18.35 µL millipore water, and finally 2 µL of 10 µg/mL DNA.

Primers were initially tested using the program according to Blair *et al.* (2008) beginning with 3 min at 92°C, followed by 30 cycles of 92°C for 30 s for denaturing, annealing at 60°C for 30 s and extension at 72°C for 45 s with final extension for 5 min. Out of twelve primer pairs only six resulted in bands with this PCR program. To further test the remaining six primer pairs, a touchdown profile step with nine cycles was added in the beginning after the initial hot start at 92°C. The annealing temperature was decreased by 1°C per cycle from 60°C to 52°C. This was followed by 35 cycles following the same program as previously used. Once nine markers were successfully amplified, a last try to amplify the final three markers was done using a ThermoWhite Taq DNA polymerase (Saveen & Werner) and supplied 10x

buffer Y (containing 200 mM Tris-HCl, 160 mM $(NH_4)_2SO_4$, 0.1% Tween 20 and 20 mM $MgCl_2$) (Franzén *et al.*, 2006). Eventually ten amplified markers out of the original twelve proceeded for fragment size analysis. The final PCR programs for each of the ten markers and primer pairs are presented in Appendix 1.

Fragment analysis

PCR product quality was confirmed by separating amplicons via polyacrylamide gel electrophoresis using CleanGels using 10%- and 52-sample-gels from ETC Elekrophorese-Technik. Gels were loaded with 7 µL PCR product plus 5 µL loading buffer and run with an initial current of 75 mA and voltage of 300 V until samples had exited wells, after which the voltage was raised to 500 V.

A 2.5-hour-long staining procedure called "Fast and sensitive silver staining" was conducted to fix the DNA fragments within the gel. The procedure included bathing the gel in a tray containing 200 mL fixing solution (0.6% benzene sulphonic acid and 24% ethanol) on a rotation board, slowly rippling for 40 min. The gel was then washed in 200 mL of washing solution (0.07% benzene sulphonic acid) 3 times for 10 min. Thereafter the gel was stained in 200 mL of staining solution (0.2% $AgNO_3$, 0.07% benzene sulphonic acid, 0.05% formaldehyde, and freshly added 0.002% Na-thiosulphate) for 40 min, then rinsed in millipore water for 2 min in preparation for the 5–6 minutes long development process where a 200 mL solution containing Na_2CO_3, 0.05% formaldehyde, and 0.002% Na-thiosulphate was used. The staining was finally stopped and preserved in 215 mL of a 10% acetic acid and 10% glycerol solution for 3 x 10 min.

PCR products that were generated with the SSR markers and detected on gel (Appendix 1) were forwarded to the Clinical Chemistry DNA laboratory at the University and Regional Laboratories Region Skåne, in Malmö for further fragment analyses. They used 1µL PCR product and 9µL HiDi formamide along with a ROX Internal Lane Standard to precisely determine the DNA fragment sizes. The analysis was run using a Applied Biosystems 3130 Genetic Analyzer.

Morphological characterisation

As additional morphological marker information, photographs were taken at the time of flowering of the entire plants and flowers. Growth habit and terminal leaflet shape were also recorded with the help of two guidelines – the first one in text describing different morphological characters of *P. vulgaris*, produced by the International Union for the Protection of New Varieteies of Plants, UPOV, located in Geneve, Switzerland (UPOV, 2005) and the second one comprising colour scales and pictures of growing type, flower colour and terminal leaflet shape, produced by Dobroudja Agricultural Institue, General Toshevo, Bulgaria (Genchev and Kiryakov, 2005). Growth habit and flower colour were easily judged, while terminal leaflet shape proved more difficult. Even though one of the lower located terminal leaflets was suggested for judgement, a glance up the stem for a leflet in proximity could exhibit a slightly different shape.

Flower colour (Figure 4) was estimated separately for the standard of the flower and the flower wings on a scale of 1–4 (1=white, 2=light pink, 3=pink, and 4=violet). Growth habit was recorded as either climbing or bush type (Figure 2, b and d, respectively). Finally, terminal leaflet shape (Figure 5) was judged on a scale from 1–5 (1=triangular, 2=triangular to circular, 3=circular, 4=circular to quadrangular, and 5=quadrangular). Raw data are presented in Appendix 2.

Figure 4. *P. vulgaris* flowers. a) Variety Nordstern scored 1 for white. b) NGB17815.2 from Sanda scored 2 for light pink. c) NGB11761.4 black bean from Gotland scored 3 for pink. d) NGB17807.2 from Harplinge scored 4 for violet. *Photo: © Elin Bengtsson.*

Figure 5. *P. vulgaris* terminal leflet shape. The leaf is a pinnately trifoliate compound leaf where one of the characteristics that can be judged for variety determination is the shape of the terminal leaflet. a) Triangular leaf, scoring 1. b) Circular leaf, scoring 3. c) Quadrangular leaf, scoring 5. Intermediates scoring 2 for triangular–circular, and 4 for circular–quadrangular are not shown. *Photo: © Elin Bengtsson.*

Accession data

Some additional information such as location and bean colour was retrieved from the gene bank documentation system SESTO on NordGens homepage. All accessions are registered into this database upon arrival.

Data analysis

The multiplex genetic marker data were analysed using Peak Scanner™ Software, v1.0, Copyright 2006, Applied Biosystems. Peak size data was transferred manually to Microsoft Excel 2007 and WordPad into a format suitable for NTSYSpc 2.10x. A dendrogram (Appendix 3) was inferred using Modified Rogers' Distance (d_W) (Wright, 1978).

$$d_W = \frac{1}{\sqrt{2m}} \sqrt{\sum_{i=1}^{m} \sum_{j=1}^{n_i} (p_{ij} - q_{ij})^2}$$

The unweighted pairgroup method with arithmetic mean, (UPGMA), was applied for cluster analysis along with the SAHN clustering algorithm. The data was transformed for output using principle coordinate analysis so that dot plot matrix diagrams could be constructed. The method was chosen for having euclidean properties (Reif *et al.*, 2005) and it ignores the assumption that there would be an infinite number of alleles and makes sense out of the data for producing dot plot matrices.

Results and discussion

SSR data

The seven final primer pairs, from which sufficient data could be obtained, detected a total of 25 alleles (Table 2). Primer pair ATA138 revealed only one allele i.e. was monomorphic. The most polymorphic primer pair was ATA071 which detected six alleles. The allelic composition for each accession is described in table 3.

Table 2. The number of alleles found for each of the seven final primers generating sufficient SSR data.

SSR primer pairs	*Gene diversity h*	*Alleles found*
ATA138	0.000	1
ATA009	0.622	3
ATA289	0.689	4
ATA145	0.354	4
BN	0.487	3
ATA010	0.420	4
ATA071	0.210	6

Table 3. SSR allele fragment size (base pair). Allelic composition for all accessions.

SSR primers and allele sizes							*All accessions*	
ATA138	ATA009	ATA289	ATA145	BN	ATA010	ATA071	Name	Accession number
164	195	246	147	155	102	193	BRUN BÖNA, GOTLAND (CARIOCA)	NGB2576
164	183	265	147	155	102	193	BRUN BÖNA "EBBES MORS"	NGB4150
164	183	246	147	145		193	BRUN BÖNA	NGB5023
164	192/183				102	193	BRUN BÖNA	NGB5026
164	183/192	265	147	155	102	193	GOTLANDSBÖNA	NGB6795
164	183	246	157	142		190	OLSOK	NGB7791
164	192	243	150	142	123	193	HUNDREDE FOR EEN	NGB9298
164	183/195	265	147	155	102	193	GOTLANDSBÖNA	NGB11554
164	183		147		102	193/186+194	STELLA	NGB11570
164	183				102	193	GOTLÄNDSK SVART BÖNA	NGB11761
164	183				99	193	GOTLÄNDSK BRUN BÖNA	NGB11762
164	183				102	193	GOTLÄNDSK VIT BÖNA	NGB11763
164	183				102	193	SWEDISH BROWN	NGB13763
164	183				102	193	SWEDISH ONE DOT	NGB13764
164	192	246	147	155	102	193	BRUN BÖNA ÖSTERGÖTLÄNDSK LANTSORT	NGB13782
164	183				102	193	BRUN BÖNA FRÅN ORNAKÄRR	NGB13858
164	192				102	193	HALLAND	NGB17801
164	195	246	147	155	102	193	SLOALYCKE	NGB17803
164	195				102	193	KRISTIN	NGB17805
164	195	265	147	155	99	193	HARPLINGE	NGB17807
164	195				102	193	RYSK KEJSARBÖNA	NGB17808
164	195		147	155	102	193	BERNADINA	NGB17809
164	192	246	147	155	99	193	PETTERSONS BÖNA	NGB17810

164	192		147	155	102	193/189+192	STÅSTORP	NGB17812
164	195				102	193	HANAS STRIMMIGA	NGB17813
164	195	265	157	155	102	193	SANDA	NGB17815
164	192	246	147	155	99	193	GULLSPÅNG	NGB17816
164	195				99	193	FISKEBY	NGB17821
164	183	243	157	145		193	SIGRID	NGB17823
164	183	265	147	155		193	KULLA	NGB17824
164	183				102	193	SIGNE	NGB17825
164	183				104	190	Extra-Hatif de Juillet	NGB17827
164	183	265	147	155		193	Gulböna från Östergarn	NGB18054
164	195	240	154	142	99	193	Prinsesse	NGB18186
164	183	246	147		102	193	BÅSTAD	NGB18675
164	183	243	147	142		193	Chevriert Vert	N 408
164	183	240	147	145		190	Flageolet	N 477
164	183	240	147	145		193	Juli	PHA 211
164	195	243	147	155		193	Ideal	PHA 44
164	195	265	150	155		193	Mont d'Or	PHA 474
164	192	246	147	155		193	Konserva	PHA119
164	183	246	147	145		190	Nordstern	PHA1357
164	192	265	147	155		193	Weibulls Express	PHA49

Morphological data

All morphological data are presented in table 4. Based on growth habit, accessions were divided into bush type (35) or climbing type (12). "Swedish one dot" (NGB13764) was recorded in both types. A total of 20 different groups were found when all three morphological markers – growth habit, terminal leaflet shape, and flower colour – were combined. The group sizes ranged from one to seven in number of accessions. Noteworthy is that three of the other accessions diverged into separate groups: "Gotlandsböna" (NGB11554), "Swedish one dot" (NGB13764), "Bernadina" (NGB17809), and "Gullspång" (NGB17816). This was also anticipated from the start. Some accessions that were sent in may have included a couple of varieties, due to variety mix-up over the years of cultivation.

Screening for duplicates

The dendrogram inferred from the SSR data (Appendix 3), revealed eight groups where duplicates potentially were present. Combining the SSR data and morphological characters (Table 5) pointed out four groups of accessions that could contain possible duplicates. Meanwhile, the majority of accessions diverged from one another, showing individual property combination and indicating uniqueness.

Table 4. *P. vulgaris* morphological markers: growth habit, shape of the terminal leaflet and and flower standard and wing colour. Grouped accessions will have a similar appearance with respect to the traits presented. Characters were described with guidance of UPOV (2005).

Legend for table 4.

Score:	Colour	Term. leaflet shape
1	white	triangular
2	light pink	triangular to circular
3	pink	circular
4	violet	circular to quadrangular
5	-	quadrangular

Term. leaflet shape	Standard colour	Wing colour	Name	Accession number
Bush accessions				
1	2	2	BRUN BÖNA "EBBES MORS"	NGB4150
1	2	2	GOTLANDSBÖNA *	NGB11554
1	2	2	BERNADINA *	NGB17809
1	2	2	SIGNE	NGB17825
1	2	2	Gulböna från Östergarn	NGB18054
2	1	1	GOTLANDSBÖNA	NGB6795
2	1	1	STELLA	NGB11570
2	1	1	SWEDISH ONE DOT*	NGB13764
2	1	1	BÅSTAD	NGB18675
2	1	1	Konserva	PHA119
2	2	2	BRUN BÖNA	NGB5023
2	2	2	GOTLANDSBÖNA*	NGB11554
2	2	2	HALLAND	NGB17801
2	2	2	RYSK KEJSARBÖNA	NGB17808
2	2	2	BERNADINA*	NGB17809
2	2	2	HANAS STRIMMIGA	NGB17813
2	2	2	KULLA	NGB17824
2	3	2	SLOALYCKE	NGB17803
2	3	3	STÅSTORP	NGB17812
2	4	4	FISKEBY	NGB17821
3	1	1	HUNDREDE FOR EEN	NGB9298
3	1	1	BRUN BÖNA FRÅN ORNAKÄRR	NGB13858
3	1	1	Nordstern	PHA1357
3	1	1	Chevriert Vert	N 408
4	1	1	KRISTIN	NGB17805
4	1	1	Ideal	PHA 44
4	2	2	BRUN BÖNA, GOTLAND (CARIOCA)	NGB2578
4	2	2	BRUN BÖNA ÖSTERGÖTLÄNDSK LANTSORT	NGB13782
4	2	2	SANDA	NGB17815
4	2	2	Weibulls Express	PHA49
4	3	3	GOTLÄNDSK BRUN BÖNA	NGB11762
4	4	3	PETTERSONS BÖNA	NGB17810
4	4	4	HARPLINGE	NGB17807
4	4	4	GULLSPÅNG*	NGB17816
5	4	4	GULLSPÅNG*	NGB17816
Climbing accessions				
-	3	3	GOTLÄNDSK SVART BÖNA	NGB11761
1	1	1	Extra-Hatif de Juillet	NGB17827
1	2	2	BRUN BÖNA	NGB5026
2	1	1	OLSOK	NGB7791
2	1	1	SWEDISH ONE DOT *	NGB13764
2	1	1	SIGRID	NGB17823
2	1	1	Juli	PHA 211
3	1	1	Flageolet	N 477
4	1	1	GOTLÄNDSK VIT BÖNA	NGB11763
4	1	1	SWEDISH BROWN	NGB13763
5	3	3	Prinsesse	NGB18186
5	3	3	Mont d'Or	PHA 474

Accessions "Rysk Kejsarböna" (NGB17808), "Hanas strimmiga" (NGB17813) and "Bernadina" (NGB17809) had four alleles in common including the uninformative ATA138. Allele similarity of the remaining three loci could not be determined due to missing SSR data. The morphology was uniform; bush type with a circular to triangular terminal leaflet and light pink flower, apart from a slight tendency towards a more triangular terminal leaflet shape in "Bernadina" (NGB17809). According to SESTO (2009) the collection sites for "Bernadina"

(NGB17809), which had yellow seeds, and "Rysk Kejsarböna" (NGB17808) were Lindesberg and Mjölby, respectively – a distance of about 170 km. "Hanas strimmiga" (NGB17813) had yellow and red mottled seeds.

Accession "Brun böna, 'Ebbes mors'" (NGB4150) had complete allelic information and was grouped with "Gulböna från Östergarn" (NGB18054) and "Kulla" (NGB17824) which both lacked data from the one and same SSR locus. They all exhibited the same morphology apart from "Kulla" (NGB17824) which had a more triangular to circular terminal leaflet shape, as opposed to the triangular shape. SSR data were missing from an additional three loci in the accession "Signe" (NGB17825), but having the same morphology as "Brun böna, 'Ebbes mors'" (NGB4150) and "Gulböna från Östergarn" (NGB)18054, this accession could presumably also be a duplicate. None of the collection sites were registered in SESTO, however both "Kulla" (NGB17824) and "Signe" (NGB17825) had brown seeds (SESTO, 2009).

SSR data could not separate accessions "Gotländsk vit böna" (NGB11763) and "Swedish brown" (NGB13763) which also shared all morphological characters; climbing with a circular to quadrangular terminal leaflet and an entirely white flower. These could potentially be duplicate accessions. However, a look in SESTO revealed that the first accession indeed had white seeds, as according to the name, while the latter one had brown seeds and was sent in from the Waldoboro Highschool somewhere in the USA (SESTO, 2009).

The two most probable duplicates are "Pettersons böna" (NGB17810) and "Gullspång" (NGB17816) despite the latter's segregation into two different morpholgical groups based on different terminal leaflet shape within the accession. They shared all seven alleles and exhibited very similar morphology; bush type with circular to quadrangular terminal leaflet (or quadrangular for one of the two individuals in "Gullspång" (NGB17816)), and a violet flower, apart from the more pink wing color of "Gullspång" (NGB17816). Both accessions had black seeds (SESTO, 2009).

Table 5. Screening for duplicates. The *Dendrogram grouping* column shows the accession numbers as grouped in the dendrogram (singleton accessions not shown). In the *Morphological score* column, the 1st, 2nd, 3rd and 4th digits represent growth habit, terminal leaflet shape, colour of flower standard and colour of flower wing, respectively, as described in the table 4 legend. Missing values refer to the lack of fragment size data i.e. allelic data of a particular locus.

Dendrogram grouping	*Morphological score*	*Comment*	*Conclusion*
2578	1422	All 7 alleles alike. Diverse morphology.	
17803	1411		
17805	1232		
17809	1222/1122	1 value missing from above.	Duplicate?
17808	1222	2 additional values missing. Same morphology as above.	Duplicate?
17813	1222		Duplicate?
Mont d'Or	2533	1 missing value.	
17821	1244	3 other SSR values missing. Other morphology than above.	
4150	1122	Information on all 7 alleles.	Duplicate?
18054	1122	1 value missing. Slightly diverse leaflet shapes.	Duplicate?
17824	1222		Duplicate?
17825	1122	3 other values missing. Morphology as 18054.	Duplicate?
11761	2-33	3 missing values, same as above. Diverse morphology, except for 11763 and 13763.	
11763	2411		Duplicate?
13763	2411		Duplicate?
13764	1211/2211		
13858	1311		
6795	1211	Information on 7 alleles.	
5026	2122	3 missing values. Different morphology.	
5023	1222	1 value missing.	
11762	1433	3 values missing. Leaflet and flower colour different.	
13782	1422	Information on all 7 alleles. Flower light pink.	
Konserva	1211	1 value missing. Different leaflet & flower color.	
17801	1222	3 values missing. Different leaflet from 13782.	
17810	1443	Information on all 7 alleles. Only slightly diverse flower wing colour.	Duplicate?
17816	1444/1544		Duplicate?
7791	2211	1 value missing.	
17827	2111	3 other values missing. Slightly different leaflet.	

Genetic diversity

Singleton accessions "Hundrede for een" (NGB9298) and "Prinsesse" (NGB18186) were the two most distal accessions in the dendrogram, showing their dissimilarity with the other accessions. These were both collected in Denmark (SESTO, 2009). All eight reference varieties were evenly distributed in the dendrogram revealing their relevance as reference material in this study. Accessions "Stella" (NGB11570) and "Ståstorp" (NGB17812) both

exhibited diversity within the accessions due to heterozygosity in one of the two examined individuals.

As illustrated in the dot plot diagrams (Figure 6), none of the phenotypic traits growth habit, terminal leaflet shape, colour of the standard or colour of the wing, seems to correlate with the SSR data. It indicates that these phenotypic traits are not closely linked to the SSR sequences. Several studies also state that there are more than one gene involved in the regulation and expression of the phenotypic traits in question. In *P. vulgaris* the *P* gene determines whether or not there will be colour expression of flower and seed. The recessive *p* gene supresses colour expression (Erdmann *et al.*, 2002). The grey-white seedcoat colour of a Florida dry bean line with violet flowers was reported to result partly from the recessive p^{gri} allele (Bassett, 1994). In combination with the V or v^{lae} allele the p^{gri} allele results in a pale blue flower (Bassett 1992). The v^{lae} allele along with the *T* and *P* genes gave a pink flower color and rose stem (Lamprecht, 1935). A scarlet flower resulted from the combination of at least four genes, and at least three different genes were listed to generate different leaflet morphologies. Although not mentioned whether or how they interact, some were reported as mutants (Bassett, 1996). This shows that the retrieved additional phenotypic information indeed was useful for the segregation of unique accessions within the germplasm collection.

Evaluation of results

To evaluate the accuracy of the results it is important to look at the number of markers used. Mariette *et al.* (2002) showed that even a low number of SSR markers could be sufficient to predict the overall genome diversity provided the genomic heterozygosity was low through generations. Because *P. vulgaris* flowers are capable of self-fertilisation they are often homozygous and seldom exhibit heterozygosity (Ferreira *et al.*, 2007). Looking at the results obtained with as little information as from three to seven SSR markers, the number of accessions with potential duplicates was substantially reduced to 28 accessions separated into eight groups. Despite only sampling two individuals per accession, it was intersting also to see variation within some accessions with respect to homo- and heterozygosity.

There is also good reason to consider a combination of different types of markers when characterising accessions. The results from an identification study at a germplasm gene bank of *P. vulgaris* accessions where several duplicates were found, showed that the combination

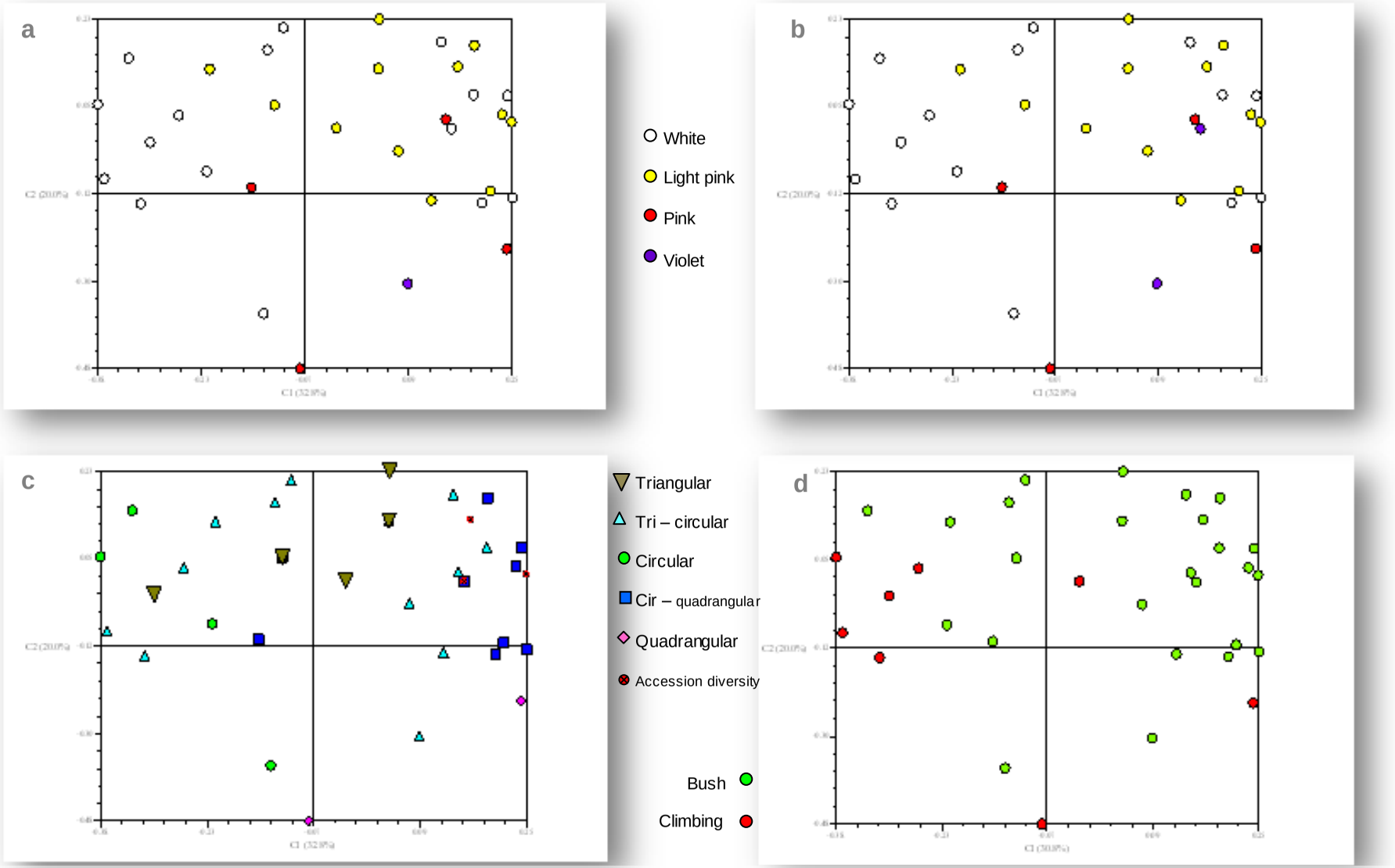

Figure 6. Dot plot diagrams inferred from SSR data using Modified Rogers' Distance (d_W) (Wright, 1978). The 1st , 2nd, 3rd and 4th dimensions explain 32.8%, 20.0%, 18.1%, and 14.0% of the genetic distance respectively. a) Colour of flower wing. b) Colour of flower standard. c) Terminal leaflet shape. d) Growth habit.

of RAPD DNA markers and phenotypic data allowed for improved characterisation (Chiorato *et al.*, 2006). Although labour-intense, a collection of 112 landraces were assessed based solely on 24 morphological characters in a Spanish study aiming at more efficient conservation and breeding (Flores *et al.*. 2003). However, the large number of landraces (and the fact that morphological characters are subject to qualitative rather than quantitative assessment and therefore can be difficult to judge) had to be compensated for by the larger number of morphological. In the present study, combining the SSR marker data with information on the morphological characters, the number of groups with possible duplicates was further reduced from eight to four groups including a total of eleven accessions.

Conclusions

Based on a combination of seven SSR and four morphological markers, this study revealed eleven accessions in four separate groups where potential duplicates are present. SSR and morphological data were not correlated but complementary according to data analyses. Additional data retrieved from SESTO confirmed the suspicion of duplicates based on seed colour in at least two cases. The eight reference varieties also proved relevant to the study because of their even distribution within the dendrogram. None of the four final groups with possible duplicates included any of the reference varieties, which still may be cultivated and circulating in private gardens.

Since keeping duplicates in germplasm collections is costly, further examination using additional markers and/or those five SSR markers that failed to amplify during PCR, is suggested in order to exclude potential duplicates. The remaining 24 accessions confirmed as unique should be preserved at the NordGen gene bank for facilitating future utilisation of these genetic resources.

If there would have been more time available within this course, I would have liked to continue this work to establish whether there are any duplicates or not. It would also have been interesting to further study all 35 accessions to see how they may have adapted to the Nordic or Swedish climate over the decades of cultivation here. Finally, I wish CBM and NordGen good luck in their future work with these *P. vulgaris* accessions.

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Appendices

Appendix 1 – Abbreviations

AFLP	Amplified Fragment Length Polymorphisms
CBD	Convention on Biological Diversity
CBM	Centrum för Biologisk Mångfald
CpSSR	ChloroPlast Simple Sequence Repats
IPK	Leibnitz Institute of Plant Genetics and Crop Plant Research
ISSR	Inter Simple Sequence Repeats
NGB	Nordiska Genbanken
NordGen	Nordic Genetic Resource Center
PCR	Polymerase Chain Reaction
POM	Programmet för Odlad Mångfald
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorhisms
SAHN	Sequential, Agglomerative, Hierarchical and Nonoverlapping
SSR	Simple Sequence Repeats
UN	the United Nations
UPGMA	Unweighted Pair Group Method with Arithmetic mean

Appendix 2 – Primer pairs and PCR programs

Presented below are the PCR programs that were used when amplified products were sent away for further fragment analysis of the original 10 loci at the Clinical Chemistry DNA laboratory at the University and Regional Laboratories Region Skåne, in Malmö.

Program	exp003	exp004	exp005	exp006/008	exp007
Primer pairs	ATA289 ATA009 ATA071 ATA006 BN	ATA145	ATA020	ATA138 ATA150	ATA010

Program									
exp003	92°C	92°C	x 30	72°C	72°C				
	3min	30s	60°C	45s	5min	4°C			
			30s			∞			
exp004	92°C	92°C	x 8	72°C	92°C	x 36	72°C	72°C	
	3min	30s	55→48°C	45s	30s	48°C	45s	5min	4°C
			60s			30s			∞
exp005	92°C	92°C	x 3	72°C	92°C	x 38	72°C	72°C	
	3min	30s	62→60°C	45s	30s	60°C	45s	5min	4°C
			30s			30s			∞
exp006/008	92°C	92°C	x 9	72°C	92°C	x 21	72°C	72°C	
	3min	30s	60→52°C	45s	30s	60°C	45s	5min	4°C
			30s			30s			∞
exp007	92°C	92°C	x 8	72°C	92°C	x 22	72°C	72°C	
	3min	30s	55→48°C	45s	30s	48°C	45s	5min	4°C
			60s			30 s			∞

Appendix 3 – Dendrogram

Dendrogram inferred from SRR data using Modified Roger's Distance (d_W) (Wright, 1978).