Towards Marker Assisted Breeding in garden roses: from marker development to QTL detection

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Contents

Chapter 1

General introduction

Roses: taxonomy and horticultural classification

Garden roses are woody perennials belonging to the genus Rosa (family *Rosaceae*, subfamily *Rosidae*) that are from an economical perspective the most important ornamentals. According to latest taxonomical reports between 100 and 250 rose species exist (Gudin, 2000). Genus Rosa is divided into 4 subgenera: *Hesperhodos*, *Hulthemia*, *Platyrhodon*, and *Rosa*. Within subgenus Rosa 10 sections have been allocated: *Pimpinellifoliae*, *Carolinae*, *Cinnamomeae*, *Synstylae*, *Caninae*, *Gallicanae*, *Indicae*, *Banksiae*, *Laevigatae*, and *Bracteatae*. In sharp contrast to this multitude of species only 10 to 20 species have been involved in modern rose breeding, belonging mainly to 5 sections: *Synstylae* (*R. moschata*, *R. wichurana*, and *R. multiflora*), *Gallicanae* (*R. gallica*), *Indicae* (*R. chinensis* and *R. gigantea*), and *Pimpinellifoliae* (*R. foetida*). Additionally, *R. spinosissima* (sect. *Pimpinellifoliae*), *R. damascena* (sect. *Gallicaneae*), *R. cinnamomea* and *R. rugosa* (both from the section *Cinnamomeae*) contributed to the development of modern rose cultivars (Gudin, 2000; Wissemann, 2003; Smulders et al., 2011).

First records on rose cultivation date back to 5000 years ago from China, western Asia, and northern Africa. In ancient civilizations of Crete, Greece, Mesopotamia, Persia, Egypt, and Rome roses had been planted mainly because of their fragrant petals and edible hips. In Europe roses have been grown during the Middle Ages as a food source. Thanks to their scent, medicinal and culinary attributes rose usage expanded to industry. For instance, during World War II in Great Britain rose hips were harvested and used as a source of vitamin C (Gudin, 2010). Rose breeding for ornamental use already experienced swift expansion in the 1860s and since then rose breeding has been growing continuously, which is reflected in numerous cultivars. The fact that in 2007 about 20% (723 million €) of all ornamentals exported from the European Union into other countries were roses (Heinrichs, 2008) underlines the importance of the rose industry.

The exact number of rose cultivars is difficult to estimate, as it is not known if some old cultivars still exist and some cultivars have different (more than one) names: an official (code) and a commercial one, but cultivars are also grown under a local name (the sale of some cultivars would not be efficient in some countries if they would have their original trade name) or a fake name (to avoid paying license). According to Helpmefind, a specialized website for rose breeders and growers, roses are represented with more than 45.000 cultivars (http://www.helpmefind.com/rose; accessed July 13, 2014). Among all these numerous cultivars it is not an easy task to group cultivars and apply simple, uniform classification. In principle, rose cultivars differ in ploidy level, growth type, disease resistance, hardiness to stress factors, and phenotype characteristics. Most common is grouping based on usage or breeding purpose like cut, garden, and rootstock roses (Figure 1; Shepherd 1954; Gudin 2000). Rootstock roses are often used for hybrid tea rose grafting. They originate mainly from *R. canina*, *R. multiflora*, and *R. indica*. Rootstock breeding is mainly focused on characteristics of rooting system, resistance to nematodes, winter hardiness, and grafting capacity. Cut roses belong to the Hybrid Tea roses. The most important characteristics of cut roses are: flower shape and color, length and strength of the stem, fragrance, duration of the

vase-life, leaf quality, number of prickles, resistances and vigor (www.schreurs.nl/ch/news/886/Cut-Rose-Cultivation-Manual).

Garden roses represent the widest variation among rose groups. Besides use in gardens, garden roses are also used for hedging, landscape design, hip production and production of components for the food and cosmetic industry. Among such a large number of phenotypically different cultivars it is extremely difficult to implement a simple classification system. Horticultural classification of garden roses is therefore primarily based on botanical characters. All garden rose cultivars are classified into one of the three main groups: wild, old garden or modern garden roses. While wild roses, which include natural species and their hybrids, are characterized by low-maintenance shrubby and once flowering phenotypes that are tolerant to poor soil and shade, the old garden rose group represents a wider and more variable class of roses. In practice, an old garden rose is defined as any rose which existed before the introduction of "La France", the first modern rose cultivar. Old garden rose breeding is divided into two periods: initial (classical) and new. Flower colors of old garden roses from the initial period differ in shades from white to pink and red and they are notably disease resistant. The new era of old garden roses started with an introgression of East Asia and China and Tea roses at the beginning of the 18th century, which led to the introduction of new subclasses of old garden cultivars that were recurrent blooming. The current old garden rose group can be divided into 15 subclasses: Alba, Gallica, Damask, Centifolia or Provence, Moss, Portland, China, Tea, Burbon, Noisette, Hybrid Perpetual, Hybrid Musk, Hybrid Rugosa, Bermuda "Mystery" Rose and Miscellaneous (Thomas, 2004; Hessayon, 2004; Richer et al., 2000).

The French breeder Jean-Baptiste André Guillot introduced the first hybrid tea rose ("La France") in 1867, which is considered the beginning of the modern roses era (Roberts et al. 2003). Since then, different kind of roses have been grown and bred in gardens all over the world and acquired a huge adaptability to the range of environments through natural and controlled pollination and mutations. Generally, modern roses are woody once-blooming fragrant shrubs, European or Mediterranean by origin. The Modern Garden Roses were initially created by hybridizing Hybrid Perpetuals with Tea roses (Thomas 2004; Hessayon 2004; Richer et al, 2000).

Even though in general crosses with wild species increase diversity, only few accessions have been used as gene donors for specific traits, and acted as founders for all cultivars within a group, so these share a similar gene pool. Additionally, strong selection for traits of interest has reduced genetic diversity. The reduction of diversity is also noticed after roses have been divided into garden and cut roses. In principle, cut and garden roses have been crossed within their own group, which led to narrowing down of genetic variation as well. Also clear differences between different garden rose types became less clear due to choosing limited number of progenitors with peculiar characteristics (flower colour, growth type, fragrance) in breeding for different types (Gudin, 2000).

Figure 1. Representatives of three main rose groups.

A Garden rose

B Cut rose

C Rootstock

Classification of Modern Garden Roses is quite confusing as many modern roses have in their ancestry old garden roses. Furthermore, appearances and characteristics among modern garden roses differ a lot. The most notable division, used by breeders, is into: Hybrid Tea, Pernetiana, Polyantha, Floribunda, Grandiflora, Miniature, Climbers, Shrubs, Modern English Rose/David Austin Type (MOE), Canadian Hardy, Landscape (Ground Cover) and Patio (Thomas 2004, Hessayon 2004, Richer et al. 2000).

Rose genetics

In spite of the economic importance of roses, the knowledge of rose genetics is limited. One of the reasons why roses are not genetically well studied (characterized) is their complicated polyploid nature and fact that, as in many crops, private companies mainly deal with breeding. As a consequence applied genetic knowledge is kept as a business secret and remains unpublished (De Vries & Dubois, 1996; Gudin, 2010).

Cytogenetic studies indicate that roses have a small basic chromosome number (n=7). The ploidy level among roses varies from diploid (2n) to octaploid (8n; Wiessemann & Hellwig, 1997). Most commercial garden rose cultivars are tetraploid. The DNA content in roses is small, ranging from 0.78 pg/2C in diploids to 3.99 pg/2C in octaploids (Yokoja et al., 2000).

Based on meiotic behavior polyploids can be classified into two categories: autopolyploid and allopolyploid. In autopolyploids, also named multivalent polyploids, the genome originated from duplication of diploid genomes within a species. In an autopolyploid meiosis the chromosomes pair randomly among homologous copies (tetrasomic inheritance), and as a consequence double reduction can occur. Double reduction is the phenomenon that two sister chromatides sort into the same gamete (Bever & Felber, 1992; Butruille & Boiteux 2000). In contrast, allopolyploids originate from the fusion of the genomes of different species. Under such circumstances, only homologues chromosomes pair among each other and thus preferential pairing occurs (e.g., *Fragaria*). This type of inheritance is disomic (Sybenga, 1994; Ronfort et al., 19998). In some polyploids it was detected that homologous chromosomes to some degree have the ability to pair among each other. In this case chromosome pairing is between disomic and tetrasomic (Sybenga, 1996). Basically, looking through the lens of time, polyploidization and evolution of genomes, caused by polyploidization is a dynamic process, as was shown by Soltis & Soltis (1995) and Song et al. (1995) by the changes in the nuclear genome of synthetic polyploids, in each of the first five generations. It can therefore be expected that the genome of roses may be allopolyploid by origin, as generated by fusion of genomes of two species, and thus following disomic inheritance. However, after several generations of crossing within groups and strong selection on traits of interest the whole or part of the rose genome may have become more similar and the inheritance may switch to tetrasomic (Sybenga, 1996). In cut roses, Koning-Boucoiran et al. (2012) tentatively found tetrasomic inheritance, while the intermediate mode of inheritance could not be excluded.

As a predominantly autotetraploid crop, roses have four sets of homologous chromosomes. The application of molecular tools in breeding of polyploid species has been limited. This is largely due to the complexities of segregation and recombination during meiosis. For better understanding of rose genetics it is necessary to be able to follow inheritance with molecular markers, preferably in the form of a dense genetic map with full genome coverage.

Co-dominant markers provide much more information compared to dominant markers. According to Luo et al. (2001) estimation of recombination frequencies based on multiallelic markers are up to four times as informative as the best estimates from dominant markers.The presence of sets of highly similar chromosomes in polyploids means that identical marker alleles can occur simultaneously on different homologous chromosomes, so it is very important to be able to score dosage. This is a complication for dominant (bi-allelic) markers. In the case of co-dominant markers multiple alleles at the same locus in a single plant can be mapped to duplicated linkage groups.

The most suitable co-dominant marker types given information content and efficiency are:

SSR markers

SSRs (Single Sequence Repeats) are PCR-based, co-dominant, multi-allelic, and highly polymorphic markers that have been widely used in plant genome analysis (Song et al., 2011). SSR markers are the only marker system in which mapping of multiple alleles to the different homologous linkage groups in polyploids can be easily achieved. The importance of SSR is reflected by the many recent studies in a wide number of genera, for instance: *Cucurbita* (Berzegar et al., 2013), *Medicago* (Zitouna et al., 2013), *Pinu*s (Iwaizumi et al., 2013), Triticum (Ansari et al., 2013), *Vitis* (Doulati-Baneh et al., 2013), *Portunus* (Guo et al., 2013). One of the drawbacks of SSRs is that they need to be developed and screened for polymorphism which is time consuming. The development of new strategies to screen for highly polymorphic SSR markers would be valuable. Recently, many experiments have been conducted on SSR development using expressed sequences (Durand et al., 2010; Park et al., 2010; Duran et al., 2013, Blair & Hurtado, 2013). Although SSR markers have characteristics that make them very suitable for mapping studies in polyploids and screening methods for the identification of highly polymorphic SSR markers have become possible with NGS sequencing technology, their application in genotyping is time consuming and costly. In order to overcome this pitfall, high density maps may be generated in combination with other marker types that can provide a mapping backbone, such as SNPs.

SNP markers

SNPs (Single Nucleotide Polymorphism) represent single base differences among DNA sequences. SNPs are the most commonly present DNA variations in genomes and thus

present a rich source of markers for genome screening/diagnostics, high density genetic map construction, phylogeny and diversity approaches (Rafalski, 2002; Trick et al., 2009). The main pitfalls of wider SNP application from the past, the costly development and difficult analysis and not very high throughput, have been overcome with decreased sequencing costs, and improvement in SNP genotyping technologies toward high throughput methods. All these together led to increased SNP marker use in genetic analysis. In comparison to SSRs, SNPs are bi-allelic and thus less polymorphic (Rafalski, 2002). Hence, the combination of SNP and SSR markers may improve map coverage in and genetic knowledge on polyploids.

Current knowledge on genetic basis of traits in rose

Until now few genetic maps have been developed for diploid (Rajapakse et al., 2001; Yan et al., 2005; Zhang et al., 2006; Hibrand Saint Oyant et al., 2008; Spiller et al., 2011) and tetraploid rose populations (Gar et al., 2011; Koning-Boucoiran et al., 2012), with relative low marker coverage. QTLs for some traits of interest, such as flowering time and inflorescence architecture (Dugo et al., 2005; Hibrand-Saint Oyant et al., 2008; Kawamura et al., 2011), stem and leaf characteristics (Dugo et al., 2005; Yan et al., 2007), as well as resistance to powdery mildew and black spot (Dugo et al., 2005, Linde et al., 2004; Linde et al., 2006; Whitaker et al., 2010; Moghaddam et al., 2012) have been mapped.

Rose breeding

Even though roses are economically the most important ornamental crop, rose breeding is still mostly dependent on empiricism. Breeding in garden roses is a long process, from the initial step of making a cross to the introduction of a cultivar to the consumer takes up to 8 years. Garden rose breeding consists of two phases: selection among a large number of seedlings in the greenhouse (in first and second year) and performance testing and further selection on the field (from year 3 to 8; Noak, 2003). The most important reasons why conventional breeding is not replaced with marker-assisted selection (MAS) are: roses are highly heterozygous outcrossing plants, most commercial roses are tetraploid which makes inheritance complicated, and the most important traits for success of a cultivar (flower color, flower shape, plant posture) can be selected by eye. The latter is no longer true, as also other characteristics have become important, such as disease resistances and fragrance. This calls for research into the genetic basis of these traits. Most research in this area has been done in crosses of wild, diploid rose species. Further efforts on detecting markers linked to important traits in rose may make revolutionary changes in rose breeding towards the inclusion of marker-assisted breeding in the breeding process.

In the growing market for garden roses in Eastern and Central Europe there are yet other characteristics that need to be combined. The continental climate in these regions limits the growth of rose, because plants freeze during cold winters or stop recurrent blooming in hot summers. Breeders would like to develop material that is adapted to these continental

growing conditions: plants that are winter hardy and continuously blooming in hot summers. These characteristics are only partly present in the current garden rose varieties and breeding material. North-American cultivar collections have been bred for winter hardiness, but their range of colors is very limited (pink), their flower characteristics are not attractive for consumers (small flowers), and their growth habit resembles too much that of wild roses (very large bushes).

Resistance to low temperature

Low temperature is, besides high temperature, drought, and salinity, one of the most important abiotic factors limiting growth, productivity and geographical distribution of agricultural crops (Schröter et al., 2005; Xue et al. 2008). Low temperature impairs seed germination, reduces seedling vigor, weakens photosynthetic ability by inducing leaf discoloration, reduces plant height, and can cause degeneration of reproductive organs. In general, stress induced by low temperature can be classified as chilling (<20°C) and freezing (<0°C) stress. Temperate plants have evolved a repertoire of adaptive mechanisms such as seed and bud dormancy, photoperiod sensitivity, vernalization, super cooling (prevention of ice formation in xylem parenchyma cells up to homogenous ice nucleation temperature, −40◦C), and cold acclimation (Tantau et al. 2004; Jung and Muller, 2009). The extent of adaptation is typically dependent on a combination of the minimum temperature experienced and the length of exposure to cold stress. Variation in cold tolerance can be genetically determined, but it is also affected by the developmental stage and the physiological status at the time of exposure. Plant survival over the winter period – termed winter hardiness – can be broken down into a number of simpler components, one of the most important of which is frost tolerance (Tondelli at al., 2011).

General plant strategies to combat low temperature

Plant species from most latitudes and climates are exposed to low temperature and, on the base of timing, level and duration of cold period, they follow diurnal (tropical species) or annual cycles (temperate species). The two distinct strategies taken by plants to combat low temperature stress are avoidance and tolerance. Avoidance means that plants avoid cold damage by dormancy; they postpone the reproductive phase (germination, reproduction, and senescence) until the temperature is stable in spring. Seeds of spring annual plants germinate only when a minimal temperature threshold is reached. On the other hand, seeds of winter annuals are characterized by vernalization. Namely, winter annuals germinate in fall and winter is spent in vegetative state, but flowering is programmed for spring. The plant's ability to flower is acquired by exposure to low temperature in winter (Hemming & Trevaskis, 2011; Preston & Sandve, 2013). In temperate herbaceous perennials, the reproductive phase is shifted to the warm season, and additional, a secondary round of vegetative growth from dormant underground meristems is possible. Trees from temperate zones are characterized by

an endodormancy-adaptive strategy to cope with low temperatures (Howe et al., 2003, Preston & Sandve, 2013).

Stress avoidance means preventing the freezing of sensitive tissues. Avoidance strategies range from survival in the form of seeds or dormant organs (many annual herbs) to more complex avoidance involving super cooling. Extremely winter hardy species can generate an extremely viscous solution, 'liquid glass', that prevents ice nucleation even at -196 °C. Their cells become osmotically, thermally and mechanically de-sensitized to the presence of external ice (Li et al., 2008).

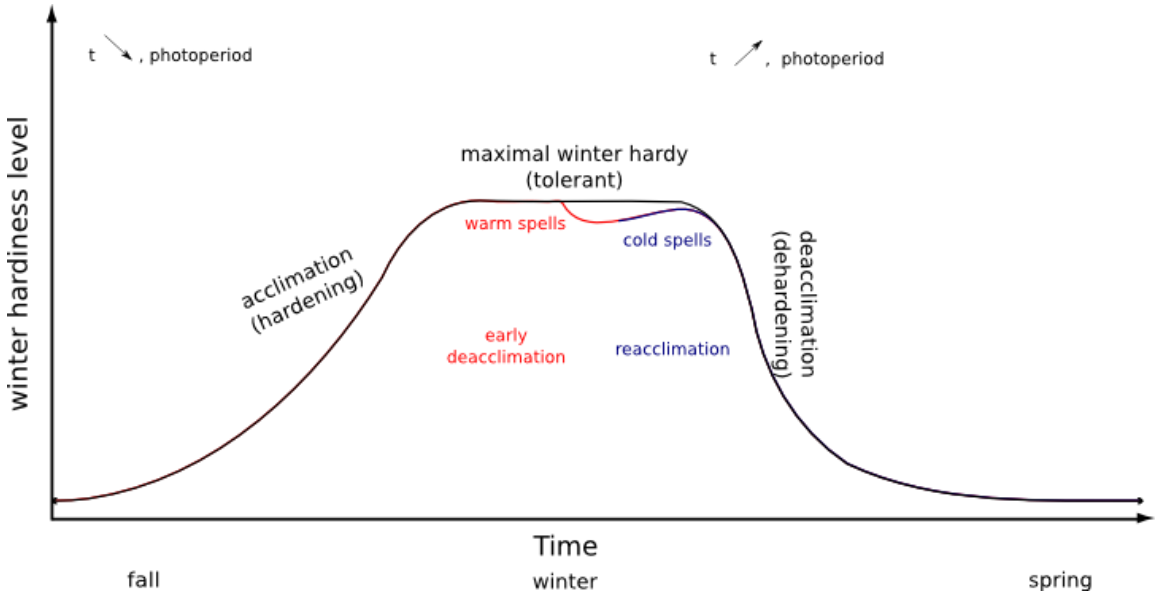

Figure 2. Cold hardiness level.

Cold hardiness consists of three phases: acclimation, winter hardiness, and de-acclimation. In fall a decrease of temperature (t) and a shortening of the photoperiod (including light intensity) initiate acclimation. All changes in plants caused by acclimation induce a spectrum of physiological and biochemical changes (changes in membrane lipid composition and accumulation of low weight antifreeze (cryoprotective) compounds such as proteins). During winter hardiness the maximal degree of tolerance is achieved. De-acclimation is a process of losing winter hardiness mainly as a response to temperature increasing. De-acclimation it is a relatively fast process (magnitude of days to weeks). Warm spells during the middle of winter may cause early de-acclimation and plants may become vulnerable to low temperature. If warm spells are followed by cold spells plants may increase in hardiness again through the slower process of re-acclimation but during this period they are vulnerable to freezing damage.

Tolerance means that plants (from boreal and temperate climate zones) have evolved the ability to acclimatize and develop cold hardiness. Cold hardiness is a plants' ability to survive low temperatures. It is a seasonal phenomenon and consists of three phases: acclimation, winter hardiness, and de-acclimation (Figure 2). Each of the three steps in cold

hardiness are the result of complex processes and lead to increasing and decreasing plant tolerance to freezing winter temperatures based on physiological processes initiated by outside cues like light intensity, photo period and temperature. In principle, changes in environmental conditions that are perceived through signal transduction pathways in the plant have influence on gene expression patterns, which regulate a plant's response to stress (Zuzak et al., 1997; Haidarvand & Amiri, 2010; Pagter & Arora, 2013, Visioni et al., 2013). Finally, during plant adaptation to low temperatures modulation of plant physiological pathways have influence on lowering the freezing point of cells (Daly et al., 2012).

Cold tolerance

Cold acclimation, known also as hardening, is a process initiated by low positive, sub-optimal temperatures. During the process of cold acclimation plants become tolerant to low temperatures. Cold acclimation is a slow process, taking several weeks to months to reach the maximal level of winter hardiness. The process is triggered by environmental factors, mainly decreasing photoperiod, decreasing light intensity and temperature decline. All changes in plants caused by acclimation induce a spectrum of physiological and biochemical changes (changes in membrane lipid composition, accumulation of low weight antifreeze (cryoprotective) compounds, such as proteins), which finally results in winter hardening (Zuzek et al., 1997, Kalberer et al., 2006; Li et al. 2008; Heidarvand & Amiri, 2010).

The second stage is winter hardiness, during which the full degree of tolerance is achieved. Winter hardiness represents the lowest temperature a plant can withstand after acclimation without causing injury. It requires a period of exposure to sub-zero temperatures. Winter hardiness level varies from year to year for the same species or cultivar and it is caused by general plant condition (health, growth stage) and annual temperature fluctuation (Zuzek et al., 1997; Li et al. 2008). When temperature drops below the maximal winter hardiness level plant will be vulnerable to low temperature.

The final stage is deacclimation (Li et al. 2008). Deacclimation is a process of losing winter hardiness mainly as a response to temperature increase. Deacclimation it is relatively fast process (magnitude of days to weeks) and as such can also happen in late fall or early spring (Zuzek et al., 1997; Kalberer et al., 2006; Pagter & Arora, 2013). The temperature at which deacclimation is induced is species-specific and once this temperature is reached the rate of deacclimation increases with temperature (Jørgensen et al., 2010).

Damage induced by low temperature may occur at three stages: in late fall, midwinter and late winter/early spring. In late fall, when plants begin to harden and the maximal level of winter hardiness is not yet reached, a strong decrease of temperature to lower absolute values cause injury. During midwinter, if the lowest temperature drops below the minimal temperature that plants can withstand, plants will be damaged. Finally, in late winter and/or early spring warm spells may be inducing a signal for de-hardening. Return of cold temperatures under such circumstances, may cause damage. Plants may suffer to different degrees of cold injury in such cases depending on their rates of acclimation and

deacclimation, although they might have similar levels of winter hardiness. In summary, the suitability of a particular species or cultivar to specific climate zones depends on both maximal winter hardiness levels and characteristics of acclimation and deacclimation (timing and rates, Larcher, 2005; Hokanson & McNamara, 2013). Cold hardiness is commonly indicated by a LT50 value. LT50 is defined as the temperature that kills 50% of the plants (Dami et al., 2012).

Under conditions of unstable weather during winter with a tendency to increasing and decreasing temperatures, frost damage also depends on a plants ability to re-acclimate. Under such circumstances deacclimation triggered by increased temperature may be annulled (cancelled) with re-acclimation. Positive effects of re-acclimation on frost injury have been reported for some trees, such as poplar (Cox & Stushnoff, 2001) and red spruce (Strimbeck et al., 1995). Snow cover plays a role as natural insulator and in many studies the level of plant damage (in cm) corresponds with the snow height (Zuzek et al., 1997).

Genes involved in plant response to low temperature

Functional genomics studies suggested several genes to be involved in a plant's response to low temperature stress. These genes are involved in different steps of plant response: low temperature perception, signal transduction and transcriptional regulation (Yamaguchi-Shinozaki & Shinozaki, 2006; Heidarvand & Amiri, 2010; Tondelli et al., 2011).

First insights into cold hardiness response came from the model plant *Arabidopsis thaliana* (Novillo et al., 2007). The CBF (C-repeat/drought-responsive element Binding Factor) gene family has a critical role in plant cold-induced responses (Novillo et al., 2007). CBF genes encode for transcription factors that by controlling the level of cold-regulated (COR) expression, regulate plant response (Visioni et al., 2013).

A very important role in cold acclimation in various plant species is played by the ICE1–CBF transcriptional cascade (Kacperska 1999; Jung and Muller, 2009; Novillo et al., 2007; Miura et al., 2007). A cold signal in plants activates CBF-dependent and CBF-independent transcriptional pathways. Arabidopsis encodes three cold-inducible CBF genes CBF1, CBF2, and CBF3 also referred to as DREB1b, DREB1c, and DREB1a, respectively. Induction of the CBF genes occurs within 15 min of transferring plants to low temperature (4°C), followed by induction of the CBF target genes about 2 to 3 h later. Arabidopsis plants with constitutively overexpressed CBF1 genes are characterized by slow growth, dwarf stature and postponement of flowering. Plants that overexpress CBF1 have reduced levels of biologically active GAs because of the increased expression of two genes encoding GA 2-oxidases. The decrease in active gibberellins results in an increase in DELLA proteins, which in turn causes the dwarf and delayed flowering phenotypes. Interestingly, constitutive overexpression of CBF1 did not result in dwarf and delayed flowering phenotypes in plants that carried the gai-t6 and rga-24 mutations that result in the inactivation of the two major DELLA proteins, GAI and RGA respectively. Novillo et al. (2007) identified seven QTLs,

one of which, FTQ4, mapped to the CBF locus and accounted for about 20% of the variation in freezing tolerance.

USDA Plant Hardiness Zone Map

In order to help growers and gardeners to determine which plants are adapted to a specific location, The United States Department of Agriculture developed the Plant Hardiness Zone Map (PHZM; http://planthardiness.ars.usda.gov/PHZMWeb/). PHZM (Figure 3) visualizes geographic patterns of low temperature severity by mapping climatological variables correlated with patterns of plant survival. The first plant hardiness zone map has been developed in 1927 by Rehder and since then it has been updated a few times. The latest PHZM was developed in 2004 based on the average of annual extreme lowest temperature over a period of 30 years (1976-2005). The map consists of 13 "full" zones with a temperature range of 5.6°C (10°F). Each full zone is subdivided into 2 subzones (a and b) of 2.8°C (5°F (Daly et al., 2012; Widrlechner et al., 2012)).

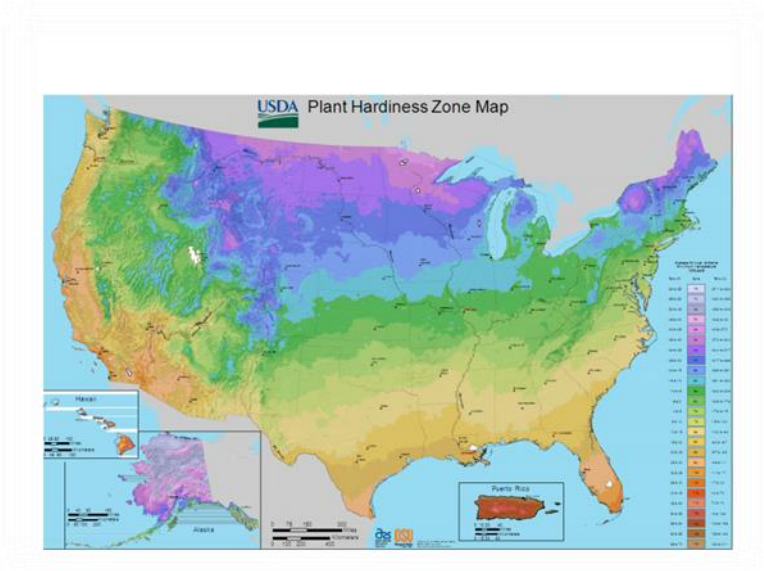

Figure 3 USDA Plant Hardiness Zone Map (Source: http://planthardiness.ars.usda.gov/PHZMWeb/)

Winter hardiness experimental design

Due to low temperature negative effect on plant growth and geographical distribution, a lot of effort has been invested in understanding the biological response to cold stress. Different possibilities for experimental designs have been considered that all have theoretical and practical advantages and disadvantages. In principle experiments on low temperature effects can be conducted under uncontrolled open field trials or under controlled laboratory conditions. Open field experiments have the advantage that large amounts of plants can be

included, whereas they have the disadvantage of uncertainty due to temperature variations from year to year. Therefore, experiments may have to be repeated for a number of years until the optimal low temperature (not too low and not too high) is reached and distinction between genotypes becomes visible. Additionally, in such multi-year uncontrolled field trials it is impossible to separate low temperature effects from other abiotic and biotic stresses. Hence, field experiments provide data about cold tolerance in a given location and year. In order to overcome insecurity of climate conditions attention has been paid to creating laboratory freezing (cold chambers) experiments. In cold chambers, so-called controlled environment, the effect of the most important stress (cold) can be singled out and is evaluated. Of course, it is impossible to simulate effects and interactions of all stress factors presented in field situations. For instance, wind, snow cover, moisture, day length and daily temperature fluctuations have effects on plant responses to suboptimal temperature as well as plant interaction in the field (sowing density) (Heidarvand & Amiri, 2010; Li et al., 2011). Therefore to really be able to predict plant responses in the field from year to year many other aspects and stress factors need to be studied as well. Additionally, laboratory freezing tests are limited by space. Thus cold chamber observations should not be taken as absolute predictors, but rather indicators of potential field performance (Hokanson & McNamara, 2013). This can be a pitfall because QTL mapping under controlled and uncontrolled conditions may detect different QTLs due to the fact that different genes have been involved in response to low temperature. A solution to avoid this trap may be to conduct both open field and laboratory experiments and distinguish and compare QTLs and genes involved in low temperature and general stress resilience.

During the plant's response to suboptimal temperature many changes at biochemical and physiological levels occur, leading to the final outcome – a level of damage/injury (Table 1). The level of damage caused by low temperature may be estimated at the plant level, but also, as exposure to low temperature has an effect on plant physiology and biochemical compound levels, plant injury may be indirectly estimated from biochemical and physiological effects (Table 1). In recent years many indicators have been developed to measure for direct (damage on phenotypical level) and indirect (phenotypic traits like recovery and/or regrowth, biochemical compound content, physiological changes) effects of low temperature on plants and the damage that may occur because of that (Khodakovskaya et al., 2005; Morin et al., 2007; Burbulis et al., 2011; Fernández-Escobar et al., 2011; Moran et al., 2011; Taulavuori et al., 2011; Dami et al., 2012; Davarynejad et al., 2012; Kirchhoff et al., 2012; Koehler & Randall, 2012; Talanova et al., 2012; Turhan et al., 2012; Zhang & Dami, 2012; Livingston III et al., 2013 Schreiber et al., 2013a; Schreiber et al., 2013b, Szymajda et al., 2013).

Table 1. Techniques for estimating winter hardiness in plants.

Phenotype	• Survival rate (% of survived plants) • Stem survival (dissection and measuring) • Root damage (dissection and measuring) • Bud dissection (dissection and measuring) • Trunk damage (browning) • Crown dissection • Periderm formation (counting shoot internodes that changed colour) • Buds, flower buds • Vascular injury (phloem and xylem browning) • Stem recovery • Recovery (indicator flowering and fruit production)
Physiology	• Electrolyte leakage • Electrolytic conductivity • Chlorophyll fluorescence • Cytoplasm coagulation • Osmotic concentration • Chloroplast destruction • Palisade cell damage • Water content • Amount of native xylem embolism • Timing of leaf senescence • Timing of bud break
Biochemistry	• Soluble sugars • Proteins • Proline • Fatty acids • Lipids • Apoplastic enzyme activity • Total carotenoid content • Anthocyanin content • Cysteine proteinases activity • Malondialdehyde (MDA) content (indicator of lipid peroxidation)

Winter hardiness in rose

Early Canadian studies have indicated that winter hardiness in garden rose depends on a few major loci (Svejda, 1974). Winter hardiness of rose probably is the result of a combination of several physiological processes, including early growth of buds, frost tolerance itself, and a delay in bud break in spring (so that damage due to late spells of frost can be avoided). This would mean that cultivars that flower later in spring may more often be winter-hardy. During the past 50 years a large set of Canadian cultivars has been produced,

some of which can withstand up to -45 ºC. Depending on the parental hardiness level very hardy offspring can be obtained in one to three generations of breeding, which suggests that winter hardiness in roses is controlled by a few major genes or closely linked genetic factors. Additionally, the lack of variability in hardiness levels among offspring from different hardy parents at the diploid and tetraploid level supports this hypothesis (Svejda, 1979).

Two sets of cultivars with different backgrounds, the so-called Parkland and Explorer types, have been developed through selective breeding of hardy roses by the Agriculture and Agri-Food of Canada (AAFC). Over 16 unique and winter-hardy Parkland roses have been developed from the late 1960's until the 1990's. The Parkland Series cultivars are especially developed with the aim to withstand the extreme low temperatures of around -35 °C during Canadian winters at the Morden Research Station in Manitoba. Not only can they survive low temperatures, they are also adapted to warm and humid summers. Additionally, Parkland roses possess desired characteristics, such as recurrent blooming and disease resistance. Interestingly, they grow on their own root, so even when they die back to the ground the plants can survive and resprout in spring (*http://www.midwestgardentips.com/parkland_roses.html*;http://www.stargazerperennials.com/Parkland_Series_Roses.php). The pedigree of the cultivars of the Parkland series shows that 2 wild species (*Rosa arkansana* Potter and *Rosa kordesii* Hort) and 18 garden rose cultivars have been involved in the crosses.

Although very hardy, the Parkland Series cultivars are not quite so cold tolerant and disease resistant as the Canadian Explorer Series cultivars. The Explorer series is a set of the most popular cold-hardy rose cultivars. All Explorer Series roses are named after Canadian explorers, and they were developed at the Ottawa research station. They are hardy down to -35 ºC with only snow as protection, have disease resistance to blackspot and powdery mildew and are characterized by recurrent blooming throughout the summer. The Explorer rose series involves 18 cultivars: (http://www.simplegiftsfarm.com/explorerroses.html#ixzz1FuagMhMF; http://www3.sympatico.ca/galetta/tables/explorerroses.html; http://www.hortico.com/roses/series.asp?cid=3). In developing the Explorer series 13 cultivars and 8 accessions of 7 wild species (*Kora kordesii* Hort (2x), *Rosa acicularis* Lindl, *Rosa amblyotis* C.A.Mey, *Rosa laxa* Retzius, *Rosa spinossisima* L., *Rosa rugosa var.* Kamschatca Regal and *Rosa rugosa* var. plena Regel) have been involved. In general, the breeding strategy has been to cross cultivars and wild roses in the first step and then to backcross progeny with the wild parent or cross with other wild species. As a result, this developed germplasm has been enriched with a substantial amount of wild germplasm. As a result the habitus of these newly developed cultivars resembles wild roses and the level of winter hardiness and disease resistance is higher.

Outline of the thesis

Because the classification of roses into different groups is mainly based on usage, little is known about the genetic relationships among the different rose groups and among the different garden rose types. In **Chapter 2**, a study is presented in which genetic diversity among rose groups (garden, cut, and rootstocks) has been estimated. Furthermore, the genetic relatedness among representatives of different garden roses types was studied in more detail.

As for many microsatellite markers the number of different alleles is lower than the number of homologous chromosomes that are present in polyploid roses, quantification of allele dosages is critical for adequate genotyping in mapping experiments. Using quantitative scoring it is possible to extract more information, map more markers, but also to map more accurately. In **Chapter 3**, results are presented on quantitative scoring of microsatellite markers in the garden rose population (Red New Dawn x Morden Centennial), which represents a cross between a Canadian and European garden rose cultivar. In this chapter is demonstrated how quantification of allele dosage enables us to resolve the tetraploid genotype of the progeny.

The most desired SSR markers are characterized by a high effective number of alleles. In the past development of SSR markers has been time consuming and costly due to the fact that screening for polymorphic markers had to be done manually. In **Chapter 4**, a strategy for the development of highly polymorphic SSRs is described that utilizes the fact that in Next Generation Sequencing large numbers of sequences can be generated with multiple reads for each homologous region, which can subsequently be screened for length differences in simple sequence repeats.

The development of a high density genetic map is a crucial step in finding QTLs and linking genetic markers to traits of interest in polyploids. In **Chapter 5**, a study on generating a high density map for garden roses using SNPs is presented. Additionally, QTLs for traits of interest for breeders have been detected.

As winter hardiness is one of the limiting factors for plant distribution, it is important to look into plant response to low temperature and detect QTLs linked to genes involved in this response to cold. In **Chapter 6**, a study on rose response to low temperature is presented combining results from open field and cold chamber experiments.

In the General Discussion I will elaborate how the availability of markers can be used in rose breeding in general and in obtaining dedicated winter hardy roses.

Chapter 2

Genetic diversity and differentiation in roses: A garden rose perspective

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Genetic diversity and differentiation in roses: A garden rose perspective

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Abstract

For the first time genetic diversity among modern garden rose cultivars has been evaluated using a set of 24 microsatellite markers covering most chromosomes. A total of 518 different alleles were obtained in the set of 138 rose cultivars and this led to the conclusion that in terms of genetic diversity cut roses can be considered as a subgroup of the garden roses.

Genetic differentiation among types of garden roses (Fst=0.022) was four times that among cut roses, and similar in magnitude to the differentiation among breeders, due to the fact that horticultural groups and breeders overlap largely in classification. Winter hardy Svejda's cultivars (Canadian Explorer roses) showed the least similarities to European roses, and introgression from wild species for winter hardiness was clearly visible. Roses of Harkness and Olesen shared a similar genepool. Comparison of the differentiation among linkage groups indicated that linkage group 5 is potentially a region containing important QTLs for winter hardiness. Linkage group 6 contains the largest amount of genetic diversity, while linkage group 2 is the most differentiated among types of garden roses.

Introduction

The genus *Rosa* consists of over 100 species, mostly from Asia but some native to North America, Europe and northwest Africa. Many of these species are thought to have arisen by hybridization, often accompanied by polyploidization, either naturally or during cultivation (De Riek et al. 2013; Zhang et al. 2013). The wild ancestors of domesticated

ornamental roses are found mainly in the sections (sect.) *Synstylae* (*R. moschata*, *R. wichurana* and *R. multiflora*), *Gallicanae* (*R. gallica*), *Indicae* (*R. chinensis* and *R. gigantea*) and *Pimpinellifoliae* (*R. foetida*) (Wylie 1954). Smaller contributions are from *R. spinosissima* in section *Pimpinellifoliae* and *R. cinnamomea* and *R. rugosa* in section *Cinnamomeae* (Smulders et al., 2011). This subset of wild species has enabled the enormous diversity of roses in shape, colour, and fragrance.

Variability of species and intraspecific hybridizations make genetic relationships within the genus *Rosa* complicated (Koopman et al., 2008), especially for cultivars. The most common grouping of ornamental roses is on the basis of usage into cut roses, garden roses and rootstocks (Shepherd 1954; Gudin 2000; Debener and Linde 2009). Rootstock roses are wild or semi-wild genotypes, mostly *R. canina* (sect. *Caninae*, dogroses), which are pentaploid, and *R. laxa* (sect. *Cinnamomae*), which is tetraploid. Cut and garden roses belong to the hybrid tea roses; they are mostly tetraploid. Cut roses are under strict selection criteria such as absence of stem bending, production (high number of stems per m^2), thornlessness, and long vase life. At the same time various ornamental traits, including flower colour and shape, are bred to be quite diverse. In contrast garden roses are a varied group, as they are not bred and valued only for flowers, but also as potted plants, for hedging, for landscaping, for hip production and even for the production of components for food and cosmetic industry. In such a wide spectrum of cultivar uses it is not possible to implement a simple classification system. Traditionally garden rose cultivars are placed in one of three main groups: wild, old garden and modern garden roses (Table 1).

Hybridisation with and introgression from wild species is more common in garden rose breeding than it is in cut rose breeding. Specific traits, such as winter hardiness, are introduced from wild relatives (*R. rugosa*, *R. arkansana*, etc.). Each breeder uses a source for a trait of interest from wild species or cultivars with the preferred trait. In general, breeders are specialized for one or a few rose types and want to be recognisable by their cultivars so they use a set of germplasm that is different from other breeders. As a consequence it is possible to distinguish breeders on the basis of cultivar phenotype (e.g., Paulsen, Harkness, Austin, and Noack). At the same time, the sources for other traits, such as winter hardiness, thornlessness, recurrent blooming, and patio growth type are limited, so breeders may use the same or similar germplasm and gene donors.

Table 1**.** Rose classification, morphological characteristics and origin of rose types.

Group	Circumscription	Cultivar group	Morphology	Information on ancestry
Wild	Natural species and hybrids		Low-maintenance shrubby, once flowering phenotypes tolerant to poor soil and shade.	-
Old Garden Roses	All roses that existed before the introduction of La France, first modern rose in 1867.	**Alba**	Strong growing shrubs with well-scented white to pale pink flowers and few thorns. Foliage and stems tend to be greyish.	An ancient groups of roses derived from *R.canina* and *R. gallica*, probably introduced by the Romans.
		Gallica	*R. gallica* is a species native to southern and central Europe eastwards to Turkey and the Caucasus. Cultivars of this species and hybrids close to appearance are considered as a cultivar group. It is an ancient group of short, compact shrubs with most commonly double or semi double once blooming flowers. The flower colour range from white (rare) to pink to the darkest purple.	The exact ancestry is unknown and other species may be involved.
		Damask	Once-blooming, thorny shrubs with intensely fragrant white to pink flowers. They are especially valued for their natural oils.	DNA analysis showed that damask roses evolved as a result of natural double crossing of *R. gallica* with *R. moschata* crossed again with *R. fedtschenkoana*. This hybridisation probably happened in Central Asia
		Centifolia or Provence	Known also as Cabbage rose thanks to the large number of petals. They are fragrant and extremely hardy roses with white or pink flowers.	It is a complex hybrid mainly derived from Gallica and Alba or Damask roses.
		Moss	The main characteristic of this rose group is mossy growth of sepals, calyx and stems. They can be once- or repeat-blooming cultivars.	Appeared as a mutation of Centifolia roses in 18th century. Later more compact and repeat-flowering hybrids evolved from the Damask roses.
		Portland	Small group of shorter, more compact shrubs with ability to repeat bloom in autumn. The flower colour range from white to pink and red.	It is a small group of hybrids derived from a rose named after plant collecting of Portland around 1780. DNA analysis showed that they are hybrids of Gallica and Damask roses.
		China	This is the class upon which modern roses are built. China roses are characterized with moderate fragrance and small blooms carried over twigs. They bloom repeatedly through summer and late autumn	The China roses, based on *R.chinensis,* have been cultivated in East Asia for centuries. From 18th century they have been cultivated in Western Europe.

Table 1 (*continued*)**.** Rose classification, morphological characteristics and origin of rose types.

Group	Circumscription	Cultivar group	Morphology	Information on ancestry
		Tea	Tea roses are introduced in 19th century. They are class of repeat-blooming roses, named for their scent reminds on Chinese black tea. The colour range includes pastel shades of white, pink and yellow apricot. They have individual flowers with petals that tend to roll back at the edge.	The Tea-scented China roses are hybrids of *R. chinensis* and *R. gigantea*.
		Burbon	This group originated from Bourbon on the coast of Madagascar. They are vigorous shrubs with glossy foliage that bloom repeatedly .	Probably they developed as a result of a cross between Damask and Old Blush China roses.
		Noisette	The first Noisettes were small-blossomed, winter-hardy climbers, but later introgression of Tea rose genes created a Tea-Noisette subclass with larger flowers, smaller clusters, and considerably reduced winterhardiness.	The first Noisette rose was bred by John Champneys as a seedling of China roses and *R. moschata*.
		Hybrid Perpetual (HP)	They are repeat- or once-blooming cultivars with tendency for massive spring blooming. The flower colour palette is limited to white, pink and red.	Represents a group of roses derived from Asian and European cultivars (Chinas, Bourbons, Noisette).
		Hybrid Musk	They arose when the era of Old Garden Roses was finished; still they are classed with them as their growth type is similar to Old Garden Roses. Hybrid musks are disease resistant cultivars characterized with repeat-blooming and clustered flowers. They are recognized by strong musk scent.	This group was mainly developed by Joseph Pemberton. *R. multiflora* is confirmed as a parent and R. moschata also figures in Hybrid Musk pedigrees.
		Hybrid Rugosa (HRG)	This is a group of vigorous, extremely disease resistant and fragrant cultivars characterized with recurrent blooming and double flat flowers.	Hybrid musk derived from R. rugosa from Japan and Korea in 1880s.
		Bermuda Mystery Rose	This group is discovered in Bermuda. The roses of this group have value and interest for breeders in tropical and semi-tropical regions, since they are highly resistant to nematodes and fungal disease. Additionally, they are capable to bloom during hot and humid seasons.	The parentage is unknown.

Table 1 (*continued*)**.** Rose classification, morphological characteristics and origin of rose types.

Group	Circumscription	Cultivar group	Morphology	Information on ancestry
		Miscellaneous	This group includes miscellaneous climbing and shrub forms.	The parentage is unknown.
Modern Garden Rose	Once-blooming fragrant shrubs, European or Mediterranean by origin.	**Hybrid Tea (HT)**	Exhibit traits midway between both parents: hardier than Teas, but less hardy compared to Hybrid Perpetuals and more recurrent blooming than the Hybrid Perpetuals, but less so than Teas. This group of roses is characterized with large, well-formed flowers. The flowering stalk terminates in a single bloom.	Initially created by hybridising Hybrid Perpetuals with Tea roses.
		Pernetiana	Contain a new range of flower colours with shades from apricot, yellow, copper and orange to scarlet. Flower colour was introgressed together with disease susceptibility and scentless.	Initiated by Joseph Pernet-Ducher in 1900, included genes from *R. foetida*, also known as the old Austrian briar rose.
		Polyantha	Disease-resistant garden roses covered with tiny red, pink or white flowers of 2.5 cm in diameter on average. Polyanthas are the rose group characterised with prolific bloom from spring till late fall.	Developed in the late 19th century in France. Polyanthas were originally derived from crosses between *R. chinenses* and *R. multiflora.*
		Floribunda (F)	Roses characterized with blooming with Polyantha profusion and Hybrid Tea floral colour range and shape.	In 1907 Danish breeder Dines Poulsen introduced Floribunda roses as a result of crosses between Polyantha's and Hybrid Tea.
		Grandiflora	Grandifloras are typically larger than Hybrid Teas and Floribundas with flowers clustered in small groups of three to five.	In the mid-20th century a new rose group Grandiflora was introduced in order to designate back-crosses between Floribundas and Hybrid Tea roses.
		Miniature (Min)	They represent a group of twiggy, repeat-blooming shrubs ranging from 15-92 cm in height with almost 30-61 cm height range.	They are result of crosses between miniature Old Garden Roses and repeat-blooming Asian species to produce ever blooming miniature roses.
		Climbers (LCL)	Most climbing roses grow 20-56 cm in height. They are characterized with continuously blooming.	In many cases they are result of spontaneous mutations.

Table 1 (*continued*)**.** Rose classification, morphological characteristics and origin of rose types.

Group	Circumscription	Cultivar group	Morphology	Information on ancestry
		Shrubs (S)	This is not precisely defined as a rose class, but is commonly used in books and catalogues. Roses of this class tend to be robust, what makes them suitable for borders or hedging.	As this class is defined on the base on their growth type their pedigree is not simple and unique.
		Modern English Rose (MOE)	The MOE group of roses that featured blooms with old-fashioned shapes and fragrances, evocative of classic *gallica*, *alba* and damask roses, with repeat-blooming characteristics and the larger colour range as well.	The MOE group was developed in 1960 by David Austin. His idea was to combine flower shape and fragrance of Old Garden roses, mainly from *R. gallica*, *R. alba* and *R. damascena* with new flower colour range and recurrent flowering of Floribundas and Hybrid Teas.
		Canadian Hardy (Can)	These cultivars are extremely tolerant to low temperature and can withstand temperature of -35°C. Additionally, all Canadian roses share similar growing type: they are bushy, scentless cultivars that remind a lot on wild species. Flowers are simple with poor colour range, mostly shades of pink.	As a response to extreme weather conditions in Canada at the Morden Research Station in Morden and Experimental Farm in Ottawa were created rose cultivars from Explorer (CE) and Parkland (CP) Series. Canadian roses derived mostly from crosses of wild species *R. rugosa* and *R. arkansana* with other species or cultivars.
		Landscape (Ground Cover)	This class is developed mainly for mass amenity planting. They are susceptible to pests and diseases. They are characterized with repeat flowering, lower growing habit, usually under 61 cm. Interestingly, they are grown on their own roots.	In the late 20th century they are involved in market. Their pedigrees are not known.
		Patio (PATIO)	Since 1970s attention of many breeders has been focused on compact rose development. This group of roses is suitable for small gardens and terraces, combines characteristics of miniature roses and Floribundas. The class of shrubs is not precisely defined garden rose class. It includes some single. and repeat flowering cultivars which tend to be robust, making them recommended for use as shrub borders or hedging.	As this class is defined on the base on their growth type their pedigree is not simple and unique.

Table 1 (*continued*)**.** Rose classification, morphological characteristics and origin of rose types.

Group	Circumscription	Cultivar group	Morphology	Information on ancestry
		Renaissance (Ren)	Renaissance rose is a group of large flower and extremely scented cultivars created by Danish breeder Poulsen. This class is often marked as a class of Hybrid Tea roses. Renaissance roses remind a lot on MOE roses. They are characterized with recurrent blooming and disease resistance.	The little data are available for the Renaissance rose pedigrees. According to the literature, in their pedigrees are involved Floribundas (Avignon, Radox Bouquet, Evening Star). They are crossed with other cultivars or seedlings from Poulsen breeding program. Interestingly, in many pedigrees of Renaissances is involved Claire Renaissance. Additionally, climbers such as Jazz and Shrubs (Queen Margaret) are involved in their pedigree.

Sources: Kruissmann, 1981; Hessayon, 2004; Thomas, 2004; Encyclopedia Britannica, 2012; http://historicroses.org, accessed 17 April 2013; http://www.oldroses.co.uk, accessed 17 April 2013; www.wikipedia.com, accessed 17 April 2013.

In a number of studies the genetic diversity between different horticultural groups of roses has been studied. Esselink et al. (2003) concluded that rootstock roses were clearly distinguished from the Hybrid Tea varieties using 24 microsatellites markers. Scariot et al. (2006) used 6 microsatellite markers to analyse differences between wild species and old garden roses, and produced a classification similar to that based on morphology. Differentiation among modern rose cultivars mostly has been evaluated on the basis of morphological traits. Smulders et al. (2009) studied genetic differentiation among cut rose cultivars and found that the genetic differentiation among 17 breeding companies was less than 1%, which indicated that all companies basically used the same cut rose gene pool.

Only few studies have compared garden rose cultivars, and these studies included only a small set of cultivars (Vainstein et al., 1993; Ben-Meir and Vainstein, 1994; Debener et al., 1996). Debener et al. (1996) found that cultivars did not cluster according to the groups to which their parents belong to and, similarly as previously had been reported (Vainstein et al. 1993), that the Hybrid Tea and the Floribunda groups share the highest genetic similarity.

Ben-Meir and Vainstein (1994) also observed that Hybrid Tea and Floribunda cultivars shared least similarity with Miniature roses.

In this study we have determined the genetic differentiation among eleven types of European garden roses and two Canadian garden rose programs, and thus also among breeders, using a large set of 110 cultivars. For comparison we have also included a small set of cut rose cultivars and rootstocks (28 in total). In order to be able to identify the footprint of introgression from specific wild species into certain types of modern cultivars, which would increase the diversity in certain areas of the genome, and that of selection, which may decrease diversity locally, we employed a set of microsatellite markers that tagged most of the chromosome arms. As an example of functional trait introgression we used winter hardiness.

Materials and Methods

Plant materials and DNA extraction

A set of 94 European and 16 Canadian garden rose cultivars was studied. For comparison we also included 19 cut rose cultivars and 9 rootstock roses (Table 2). Genomic DNA was extracted from freeze-dried young leaves using the DNeasy Plant Mini Kit (Westburg, The Netherlands) following the protocol of Esselink et al. (2003).

Twenty plants of population 97/7 (95/13-39 × 82/78-1; Linde et al., 2006; Spiller et al., 2011) were used to determine the linkage group (LG) of 13 previously unmapped microsatellite markers (RA044b, RA023b, RMS082, RMS080, RMS017, RMS097, RMS034, RMS008, Rog9, Rog18, Rog27, Rog3, and Rog5) in JoinMap 4 (Van Ooijen, 2006).

Table 2. Description of Rose material and origin.

Code	Name	Type	Breeder	Code	Name	Type	Breeder	Code	Name	Type	Breeder	Code	Name	Type	Breeder	Code	Name	Type
E-1	AbrahamDarby	MOE	Austin	E-20	Cygne noir			E-39	James Galway	MOE	Austin	E-58	Papagena	HT	McGready	E-77	Snowdon	HRG
E-2	Alan Titchmarsh	MOE	Austin	E-21	Desinger sunset	F	Pearce	E-40	Kings Mac	HT	Fryer	E-59	Pat Austin	MOE	Austin	E-78	Songs of praise	F
E-3	Amber Queen	F	Harkness	E-22	Diamond border	S	Olesen	E-41	L'aimant	MOE	Harkness	E-60	Patricia Kent	MOE	Harkness	E-79	St. Alban	MOE
E-4	Amelia renassaince	REN	Olesen	E-23	Double terrazza	Patio	De Ruite	E-42	Lavander dream	S	Austin	E-61	Pink terrazza	Patio	De Ruiter	E-80	Summer song	MOE
E-5	Anna Purna	HT	Dorieux	E-24	Eglantyne	MOE	Austin	E-43	LD Braithwaite	MOE	Austin	E-62	Pearl ambudance	F	Harkness	E-81	Sun hit	S
E-6	Apple blossom	HRG	Noack	E-25	Escopade	F	Harknes	E-44	Lemon coture	S	Pearce	E-63	Penny Lane	LCL	Harkness	E-82	Sunset buolevard	F
E-7	Astrid Lingren	F	Olesen	E-26	Evelyn	MOE	Austin	E-45	Leonardo da Vinci	F	Meilland	E-64	Perception	HT	Harkness	E-83	Sweet dreams	S
E-8	Betty Harkness	F	Harkness	E-27	Ferdinand Pitchard	HP	Tanne	E-46	Lilian Baylis	MOE	Harkness	E-65	Perpetually yours	LCL	Harkness	E-84	Teasing Georgia	MOE
E-9	Buttercup	MOE	Austin	E-28	FP/1	Patio	De Ruite	E-47	Madrigal	MOE	Harkness	E-66	Peter Cottrel	F	Harkness	E-85	Tivoli	HT
E-10	Caribia	HT	Wheatcro	E-29	FP/2	Patio	De Ruite	E-48	Margareth Merril	F	Harkness	E-67	Piccolo	F	Tantau	E-86	Velvet fragnance	HT
E-11	Charles Darwin	MOE	Austin	E-30	FP/3	Patio	De Ruite	E-49	Majroire Marshall	MOE	Harkness	E-68	Pink tiare	S	Perace	E-87	Violet parfume	HT
E-12	Charlotte	MOE	Austin	E-31	Gentle Hermione	MOE	Austin	E-50	Mary rose	MOE	Austin	E-69	Princess Alexandra	MOE	Olesen	E-88	White lace	S
E-13	Christopher Marlowe	MOE	Austin	E-32	CE-Gertrude Jackyll	MOE	Austin	E-51	Mayflower	MOE	Austin	E-70	Princess of Wales	F	Austin	E-89	Wild Edric	HRG
E-14	City of London	F	Harkness	E-33	Glowing pink	S	Pearce	E-52	Mullard jubilee	HT	McGready	E-71	Queen of Sweden	MOE	Austin	E-90	Wildeve	MOE
E-15	Claire rose	MOE	Austin	E-34	Graciously pink	S	Unknow	E-53	Nadia renessaince	REN	Olesen	E-72	Samaritian	MOE	Harkness	E-91	Winchester chatedrale	MOE
E-16	Climbing Bonica	LCL	Unknown	E-35	Graham Thomas	MOE	Austin	E-54	Nipper	MIN	Harkness	E-73	Sharifa Asma	MOE	Austin			
E-17	Compassion	LCL	Harkness	E-36	Helene renaissance	REN	Olesen	E-55	Nostalgie	HT	Tantau	E-74	Shephardess	MOE	Austin			
E-18	Cream ambudance	F	Harkness	E-37	Heritage	MOE	Austin	E-56	Orange terrazza	Patio	De Ruiter	E-75	Shorpshire lass	MOE	Austin			
E-19	Crown princess Margareta	MOE	Austin	E-38	Irish hope	MOE	Harknes	E-57	Othello	MOE	Austin	E-76	Snow goose	HRG	Austin			
CP-1	Adelaide Hoodless	CanP	Marhall	CP-2	Cuthbert Grant	CanP	Marshal	CP-3	Hope for humanity	CanP	Collicutt	CP-4	Morden amaretto	CanP	Marshall	CP-5	Morden centerial	CanP
CP-6	Winnipeg parks	CanP	Marhall															

*CP, Canadian garden roses Parkland Group

**CE, Canadian garden roses Explorer Group

Rose types: MOE Modern English roses; F Floribunda; REN Renaissance; HT Hybrid Tea; HRG Hybrid Rugosa; LCL Climbing roses; S Shrubs; Patio Patio roses; MIN Miniature roses; CanE Canadian Explorer roses; CanP Canadian Parkland roses

Table 2 (*continued*). Description of Rose material and origin.

Group	Code	Name	Type	Breeder	Code	Name	Type	Breeder	Code	Name	Type	Breeder	Code	Name	Type	Breeder	Code	Name	Type	Breeder
CP*	CP-1	Adelaide Hoodless	CanP	Marhall	CP-2	Cuthbert Grant	CanP	Marshall	CP-3	Hope for Humanity	CanP	Collicutt	CP-4	Morden Amorette	CanP	Marshall	CP-5	Morden Centenn	CanP	Marshall
	CP-6	Winnipeg Parks	CanP	Marhall																
CE**	CE-1	Alexander McKenzie	CanE	Svejda	CE-2	David Thompson	CanE	Svejda	CE-3	Henry Kelsey	CanE	Svejda	CE-4	Jens Munck	CanE	Svejda	CE-5	Johan Franklin	CanE	Svejda
	CE-6	John Cabot	CanE	Svejda	CE-7	John Davis	CanE	Svejda	CE-8	JP Connel	CanE	Svejda	CE-9	Therese Bugnet	CanE	Bugnet	CE-10	William Baffin	CanE	Svejda
Cut rose	Cut-1	Lexmei/Dolce Vita+			Cut-2	Olijredsp/El Toro			Cut-3	Meivildo/Yves Piaget			Cut-4	Pekcoujenny/First Red			Cut-5	Tanotika/Akito		
	Cut-6	Ruiy 5451/Wow			Cut-7	Seliron/Bull's Eye			Cut-8	Korflapei/Frisco			Cut-9	Predesplen/Splend id Surprise			Cut-10	Selaurum/Grand Prix		
	Cut-11	Ruirovingt/Prophyta			Cut-12	Schrazuid/Limonc hello !			Cut-13	Avalanche+			Cut-14	Schremma/Femma			Cut-15	Presur/Surprise		
	Cut-16	Briroro/Valentino			Cut-17	Interlis/Lydia			Cut-18	Korcilmo/Escimo			Cut-19	Brigold/Helio						
Rootst ock rose	R-1	Drora			R-2	Moerex/1001			R-3	Heinsohn'S Rekord			R-4	Ivtamar/1568			R-5	R.Inermis 2		
	R-6	R.Rubiginosa			R-7	Kiese			R-8	Smit'S Stekelloze			R-9	R.Rubrifolia Glauca						

*CP, Canadian garden roses Parkland Group

**CE, Canadian garden roses Explorer Group

Rose types: MOE Modern English roses; F Floribunda; REN Renaissance; HT Hybrid Tea; HRG Hybrid Rugosa; LCL Climbing roses; S Shrubs; Patio Patio roses; MIN Miniature roses; CanE Canadian Explorer roses; CanP Canadian Parkland roses

Microsatellite marker genotyping.

Microsatellite markers were chosen on the basis of the level of polymorphism they revealed. In total, 25 microsatellite markers, covering most linkage groups except LG3, were used to genotype all cultivars (Table 3). Genotyping was performed on an ABI 3730 DNA analyser (Applied Biosystems, Foster City, California) or a Li-Cor 4300 analyser (Li-Cor Biosciences, Lincoln, NE, USA). Amplification reactions used for ABI were performed in 10µl containing 8 ng DNA, 5µl multiplex kit (QIAGEN, Germany) and 4 pmol of each forward (labelled) and reverse primer. Amplification was under the following condition: an initial denaturation at 95°C for 15 min. following with 30 cycles of 94°C for 30 sec, ramp 1°C/s to 50°C, 50°C for 30 sec, ramp 1°C/s to 72°C, 72°C for 120 sec and final extension at 72°C for 10min. One µl of 100x diluted PCR product was mixed with Hi-Di formamide (Applied Biosystems) containing GeneScan-500 LIZ size standard (Applied Biosystems) and run on an ABI 3730 DNA analyser. Output from the ABI platform was analysed with Genemapper 4.0 software (Applied Biosystems).

The microsatellite reaction mixtures used for Li-Cor contained 10 ng genomic DNA, 2 µl 10x Tag PCR buffer, 0.2 mM of dNTP, 10 pmol of each (labelled) forward and reverse primer, 0.5 U of Tag polymerase, in a final volume of 20 µl. PCR conditions were initial denaturation at 94°C for 180s, then 35 cycles of 94°C for 30s, ramp to 55-58°C (1°C/s), 55-58°C for 30s, ramp to 72°C (1°C/s), 72°C for 60s and final extension at 72°C for 7 min. The 20x diluted amplification products were analysed on a Li-Cor 4200 or 4300 analyser.

Data analysis

Even though there are methods to score SSRs co-dominantly, such as MAC-PR (Esselink et al., 2004), obtaining reliable results in sets of unrelated genotypes is often not possible for the majority of tested markers. We therefore scored presence or absence of individual alleles for each microsatellite locus (dominant scoring). The data were recorded into a binary data matrix (1 for present and 0 for absent) and for each locus the "allelic phenotype" was taken (Esselink et al. 2003; Becher et al. 2000; Park et al. 2010). To assess and visualize genetic relationships among genotypes, we used NTSYS version 2.10 to

perform a principal coordinate (PCO) analysis. A PCO can visualise data from various ploidy level data (De Riek et al. 2007). For the diversity estimation we used fixation index (Fst) and expected heterozygosity (H_e). Fst is a measure of population differentiation (genetic distance) based on allele frequency differences among populations (Holsinger and Weir, 2009). H_e, also referred to as gene diversity, is the probability that two randomly chosen alleles at a locus within a set of genotypes will be different under Hardy-Weinberg equilibrium (i.e., assuming random mating). For the genetic differentiation (Fst) and expected heterozygosity (H_e) we used SPAGeDi 1.3, which also can analyse various ploidy level data (Hardy and Vekemans 2002).

Results

The microsatellite markers

A total of 25 microsatellite markers, which produced clear alleles and showed a high degree of polymorphism, were selected for this study (Table 3). Markers Rog 9 and Rog 10 (Meng et al., 2009) gave identical genetic results. Comparison of primer sequences showed that the forward primer of Rog 9 (TCCTGAAAACGAAGCCTCC) is largely the same (underlined) as the reverse primer of Rog 10 (TTCCTGAAAACGAAGCCT) but a few bp shifted. As some alleles of Rog 10 showed weaker amplification, only Rog 9 was used for further analysis, hence we used the data of 24 microsatellite markers.

Table3. Characteristics of the microsatellite markers used in study.

marker name	Repeat sequence	LG	A			G			C			R		
			(n=138)			(n=110)			(n=19)			(n=9)		
			A	AP	He	A	AP	He	A	AP	He	A	AP	He
RMS015[a]	GA	1	32	94	0.89	29	80	0.89	7	11	0.76	15	9	0.95
RMS047[a]	GA	1	18	55	0.80	17	47	0.80	3	3	0.62	12	9	0.91
RhD201[b]	(TCT)33	1	15	45	0.75	22	38	0.75	4	6	0.71	12	8	0.83
RMS062[a]	GA&GT	2	24	93	0.89	24	75	0.88	9	13	0.83	13	9	0.93
RhB303[b]	(GA)11	2	20	58	0.86	19	37	0.81	6	15	0.81	9	8	0.82
RhO506[b]	(CAG)6(CAA)18–7(CAG)6	2	20	76	0.88	17	61	0.86	5	6	0.68	12	9	0.90
RMS082[a]	2xGA	2[e]	17	39	0.74	13	31	0.73	3	5	0.56	12	9	0.90
RMS080[a]	GT	4[e]	18	45	0.78	17	39	0.77	3	3	0.67	9	7	0.85
RhAB40[b]	(TC)14(AC)11-1	4	35	89	0.90	32	72	0.89	8	10	0.79	11	9	0.94
RhD221[b]	(TCT)21–1	4	27	52	0.77	14	38	0.76	5	12	0.76	7	6	0.80
RMS029[a]	GA	5	19	43	0.76	16	33	0.75	6	7	0.65	11	9	0.92
RA044b	(AG)14	5[e]	22	43	0.76	17	36	0.76	3	4	0.53	11	9	0.92
RA023b	(GA)20	5[e]	16	81	0.85	14	65	0.85	7	11	0.83	9	9	0.86
RMS017[a]	AT&GT	6e	32	103	0.90	30	91	0.90	8	13	0.82	7	5	0.71
RMS097[a]	GA&GT	6[e]	13	20	0.62	9	15	0.61	2	3	0.50	7	7	0.69
RhE2b[b]	(TGT)26	6	20	62	0.87	18	53	0.86	5	6	0.66	7	8	0.82
Rog9[c]	(AG)13	6[e]	17	58	0.84	15	43	0.82	9	14	0.85	9	6	0.84
Rog18[c]	(AG) 17	6[e]	14	78	0.87	14	62	0.86	9	16	0.85	8	6	0.85
RMS003[a]	GA	7	24	70	0.87	20	59	0.86	6	9	0.75	10	6	0.92
RMS008[a]	GA	*[f]	21	50	0.79	17	40	0.77	4	5	0.70	10	9	0.82
Rog3[c]	(CT)8	*[f]	23	70	0.87	18	57	0.85	7	12	0.81	13	8	0.91
Rog5[c]	(GA)10	*[f]	21	55	0.82	20	45	0.81	5	7	0.71	13	9	0.94
RMS034[a]	GA	*[g]	29	66	0.83	22	58	0.83	4	5	0.68	15	9	0.93
Rog27[c]	(TG)10	*[g]	21	70	0.86	19	55	0.85	8	11	0.80	14	8	0.91
Average			21.6	58.6	0.8	18.9	65.4	0.8	5.7	8.6	0.7	10.7	8.0	0.9

LG, Linkage Group, A, All rose samples; G, Garden roses; C, Cut roses; R, Rootstock roses; EG, European garden roses; CG, Canadian garden roses; CP, Canadian garden roses Parkland group; CE, Canadian garden roses Explore group , n, number of rose samples; A, number of alleles; AP, number of allelic phenotypes; He, expected heterozygosity; a, Microsatellite markers for genetic analyses and the differentation of roses; b, Esselink et al, 2003; c, Meng et al, 2009; d, Kimura et al, 2006; e, Mapped in 97/7 population; f, could not be mapped in 97/7 population; g, Not polymorphic in 97.7 population

Table 3 (*continued*). Characteristics of the microsatellite markers used in study.

marker name	Repeat sequence	LG	EG (n=94)			CG (n=16)			CP (n=6)			CE (n=10)		
			A	AP	He	A	AP	He	A	AP	He	A	AP	He
RMS015[a]	GA	1	26	71	0.89	12	15	0.890	11	6	0.904	10	10	0.891
RMS047[a]	GA	1	15	40	0.78	10	14	0.857	6	5	0.808	10	10	0.887
RhD201[b]	(TCT)33	1	20	30	0.73	12	12	0.857	6	5	0.900	10	8	0.826
RMS062[a]	GA&GT	2	18	64	0.88	18	14	0.915	7	5	0.858	15	10	0.931
RhB303[b]	(GA)11	2	19	34	0.83	7	10	0.709	5	4	0.664	5	7	0.728
RhO506[b]	(CAG)6(CAA)18–7(CAG)6	2	14	53	0.84	13	13	0.881	11	6	0.923	9	8	0.869
RMS082[a]	2xGA	2[e]	13	31	0.74	6	5	0.569	4	4	0.586	4	3	0.570
RMS080[a]	GT	4[e]	12	30	0.75	11	12	0.878	6	5	0.821	10	8	0.899
RhAB40[b]	(TC)14(AC)11-1	4	28	64	0.89	19	14	0.910	7	5	0.904	15	10	0.927
RhD221[b]	(TCT)21–1	4	14	31	0.74	6	11	0.784	6	6	0.795	6	9	0.798
RMS029[a]	GA	5	11	24	0.71	13	14	0.892	6	6	0.768	11	9	0.912
RA044b	(AG)14	5[e]	10	26	0.72	15	14	0.920	8	6	0.876	15	9	0.948
RA023b	(GA)20	5[e]	13	57	0.85	10	14	0.813	9	6	0.894	7	9	0.753
RMS017[a]	AT&GT	6e	26	80	0.89	20	16	0.943	10	6	0.905	18	10	0.958
RMS097[a]	GA&GT	6[e]	5	9	0.57	8	12	0.815	3	3	0.547	8	9	0.832
RhE2b[b]	(TGT)26	6	17	46	0.85	10	14	0.882	7	5	0.851	9	10	0.886
Rog9[c]	(AG)13	6[e]	14	40	0.82	9	10	0.831	5	5	0.727	9	6	0.879
Rog18[c]	(AG) 17	6[e]	13	52	0.85	9	14	0.852	6	5	0.814	9	10	0.878
RMS003[a]	GA	7	19	48	0.85	14	14	0.900	11	6	0.906	11	10	0.899
RMS008[a]	GA	*[f]	15	33	0.76	9	13	0.815	6	5	0.825	7	9	0.809
Rog3[c]	(CT)8	*[f]	14	47	0.84	13	14	0.882	7	5	0.777	12	9	0.875
Rog5[c]	(GA)10	*[f]	15	35	0.79	13	14	0.886	7	5	0.807	11	9	0.903
RMS034[a]	GA	*[g]	18	47	0.81	17	16	0.914	9	6	0.865	16	10	0.945
Rog27[c]	(TG)10	*[g]	16	43	0.83	15	14	0.910	8	6	0.891	13	10	0.916
Average			16.0	43.7	0.8	12.0	13.0	0.9	7.1	5.3	0.8	10.4	8.9	0.9

LG, Linkage Group, A, All rose samples; G, Garden roses; C, Cut roses; R, Rootstock roses; EG, European garden roses; CG, Canadian garden roses; CP, Canadian garden roses Parkland group; CE, Canadian garden roses Explore group , n, number of rose samples; A, number of alleles; AP, number of allelic phenotypes; He, expected heterozygosity; a, Microsatellite markers for genetic analyses and the differentation of roses; b, Esselink et al, 2003; c, Meng et al, 2009; d, Kimura et al, 2006; e, Mapped in 97/7 population; f, could not be mapped in 97/7 population; g, Not polymorphic in 97.7 population

Some markers used in the study had not been mapped previously. Using the 97/7 population, marker RMS082 was mapped on linkage group (LG) 2, RMS080 and RA044b were mapped on LG4, and RA023b was mapped on LG5 together with Rog 9, Rog18, RMS017 and RMS097. Markers Rog 27 and RMS034 were not polymorphic in the 97/7 population, and thus they could not be mapped. Although Rog 3, Rog 5 and RMS008 were polymorphic in the 97/7 population, they remained unmapped using only 20 plants.

A total of 518 different alleles were observed across the 24 markers (Table 3), with an average of 21.6 alleles per marker. RhAB40 had the highest number of alleles (35 alleles), while RMS097 had the lowest (13 alleles). In total, 1515 allelic phenotypes (Esselink et al. 2003) were identified among the rose samples. The most discriminating locus was RMS017 with 103 different allelic phenotypes in the 138 genotypes analysed, i.e., 75% of the genotypes could be distinguished using this locus alone.

Gene diversity (H_e) ranged from 0.618 to 0.902. Markers with fewer alleles generally had lower H_e values except Rog18, which had 14 alleles but a H_e value of 0.867. This H_e value is comparable to values of markers with much higher numbers of alleles. An exception was also marker Rhd201, it had 27 alleles but the H_e value was with 0.753 relatively low. In the garden rose group, cut rose group and rootstock rose group the H_e value ranged from 0.611 to 0.902, 0.503 to 0.852, and 0.693 to 0.947 respectively. On the basis of a Mann-Whitney test (Supplementary table 1), H_e value differences between rootstocks and garden roses and between rootstocks and cut rose cultivars were highly significant ($P<0.001$, two-tailed test), while the difference between garden roses and rootstocks was significant ($P<0.001$, two-tailed test).

Distinction of cut roses, rootstocks and garden roses

The PCO analysis showed that cut roses and rootstock roses were clearly separated from garden roses (Fig. 1). Genetic differentiation among cut, garden and rootstock roses was moderate (Fst=0.052; Supplementary Table 2). Cut rose and rootstock rose were the most distinct groups (Fst=0.132). Garden roses showed more similarity with cut roses (Fst=0.042), while their differentiation from rootstocks was higher (Fst=0.081).

The genetic differentiation between rose groups varied among different linkage groups (LGs). LG2 showed the highest differentiation (Fst=0.074) and three of the four markers on this linkage group had the highest genetic distance in certain pairwise comparisons. Genetic differentiation between cut roses and rootstocks were similar for all LGs, ranging from 0.137 for LG2 to 0.120 for LG6. Garden roses showed the highest differentiation from cut roses for LG2 (0.080). The highest differentiation between garden roses and rootstocks was found for LG6 (Fst=0.106).

Private alleles

We defined private alleles as those that were characteristic for one group or set of cultivars and did not appear in other groups. Private alleles are indicative for larger genetic variation. European garden roses had private alleles for each microsatellite marker. Similarly, rootstock roses had private alleles for all microsatellite markers except Rog18 and RMS062. Cut roses did not have any private alleles (Supplementary Table 3). The complete absence of private alleles in the set of cut roses cannot be ascribed to the small size of this group (only 19 cultivars). Although they had a lower number of alleles for all loci compared to garden roses, they still revealed 103 unique allelic phenotypes. In addition, there were only 9 rootstock roses, and these had as many as 63 private alleles (on average 7 per cultivar). Rather, the absence of private alleles in the cut roses may be an indication that they contain a subset of the variation present in the garden roses.

Partly owing to the large number of samples, European garden roses had the largest number of private alleles (97; on average 0.94 private alleles per cultivar). Of the two Canadians garden rose groups, the Explorer group had more private alleles (33, on average 3.3 alleles per cultivar) than the Parkland group (10, on average 1.67 alleles per cultivar). Some private alleles in the rootstock rose group, the European garden rose group and in the Canadian Explorer group occurred in more than one plant, but only the European garden rose group included samples with more than one private allele in the same cultivar.

Comparing linkage groups it was notable that rootstocks had the largest number of private alleles on LG5 (10), while for European garden roses this was on LG6 (21). For Canadian Explorer roses the same number of private alleles (6) was found on LG2 and LG6. Canadian Parkland roses had most on LG1 (3). Cultivars of cut roses, European and Canadian Explorer garden roses also showed the largest number of unique allelic phenotypes on LG6.

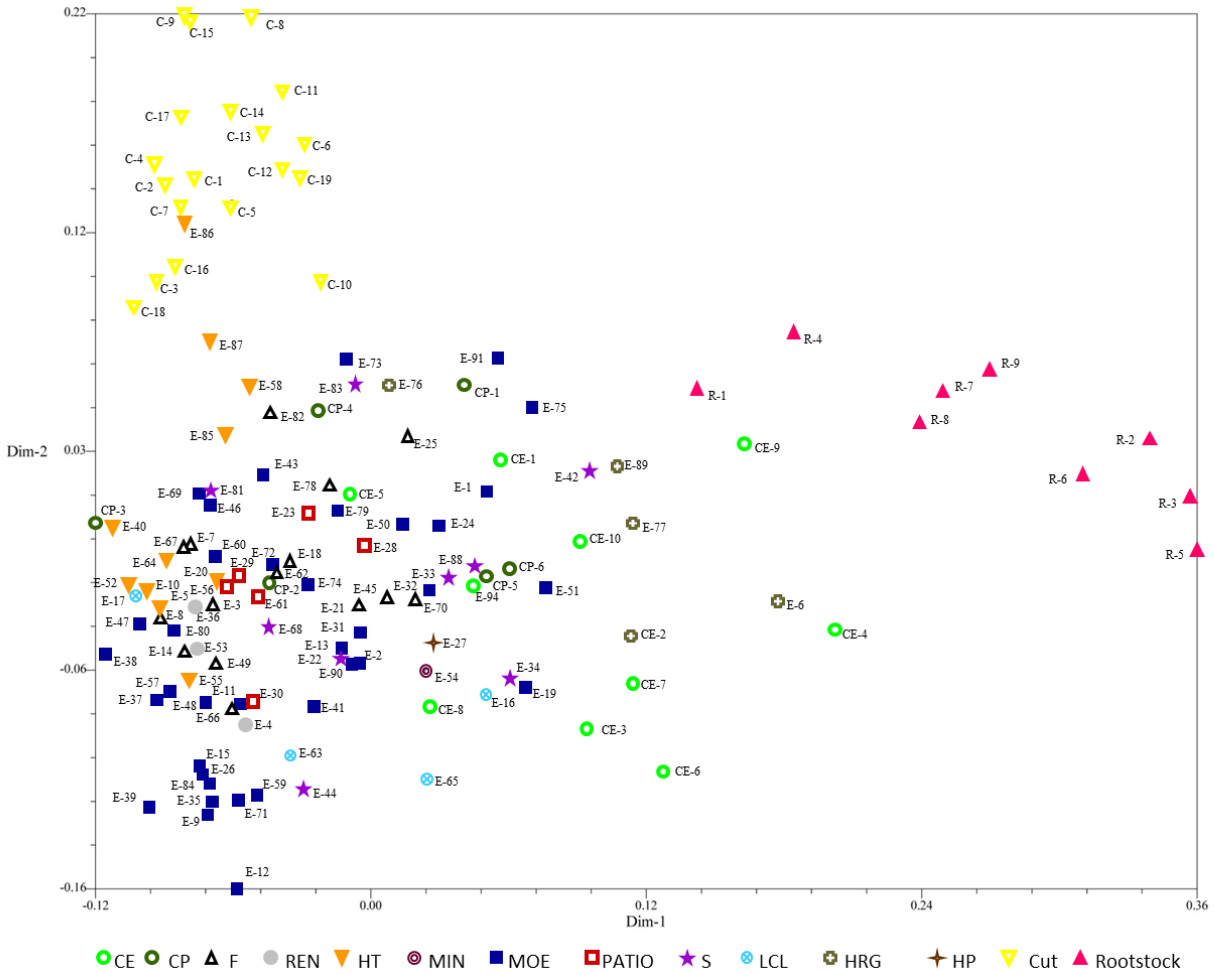

Fig.1 PCO plot based on genetic distances among rose cultivars. PCO axes 1 and 2 explain 7.0% and 4.85% of the variation.

CE Canadian Explorer, CP Canadian Parkland, F Floribunda, REN Renaissance, HT Hybrid Tea, MIN Miniature roses, MOE Modern English roses, PATIO Patio roses, S Shrubs, LCL Climbers, HRG Hybrid Rugosa, HP Hybrid Perpetual, Cut Cut roses

Rootstock roses contained the largest number of unique allelic phenotypes on LG2 and Canadian Explorer roses on LG5 (Supplementary Table 3).

Distinction of different garden rose cultivars groups

For each garden rose type there are specific breeding goals. Usually, sources for those characteristics of interest are wild species or commonly used cultivars, which leads to the hypothesis that cultivars groups are also genetically differentiated from each other. The 110 garden rose cultivars used in this study belonged to seven different types: Canadian (CAN), Floribunda (F), Hybrid Tea (HT), Renaissance (REN), Hybrid Rugosa (HRG), English Modern Rose (MOE), and Shrubs (S). Cultivars of two Canadian breeding programs, Canadian Explorer (CE) and Canadian Parkland (CP), are phenotypically similar and can be clearly distinguished from European garden roses on the basis of their pedigrees and characteristics. The main characteristic of Canadian roses is winter hardiness; phenotypically Canadian roses are similar to wild species. Due to the small number of cultivars, four groups of garden roses (Climbers (LCL), Miniature (MIN), Hybrid Perpetual (HP), and PATIO) have been excluded from the analysis per type (Fig. 2).

In the PCO analysis, cultivars of each garden rose type (MOE, CAN, HRG, F, and S) clearly grouped together, but types largely overlapped (Fig. 1). Interestingly, 'Velvet Fragrance', one of the European garden roses cultivars, was positioned in the cut rose group, while the other European cultivars were distant from these. The Hybrid Tea's were the garden rose group that was closest to the Cut roses. The Canadian Parkland group overlapped with European cultivars, while the Canadian Explorer group was positioned close to the Rootstock roses, together with the Hybrid Rugosa's.

Genetic differentiation among garden roses

The differentiation among types of garden roses (Fst=0.022, Table 4) was lower compared to the differentiation among cut, garden roses, and rootstocks. The largest Fst value (0.055) among garden rose types was found between Canadian Explorer and Hybrid Tea cultivars. In general, the Canadian Explorer group was the most differentiated from the rest of the groups, which is in agreement with the PCO. According to Fst values Renaissance roses fully overlap with Floribunda (Fst=0.000), Modern English (Fst=0.003), and Parkland roses (Fst=0.007).

Figure 2. Representatives of garden rose types used in study displaying variation in flower (colour, shape, number of petals, architecture) and leaf (number, shape, colour) characteristics, and growth type.

REN Renaissance rose, LCL Climbers, CE Canadian Explorer, MIN Miniature rose, MOE Modern English rose, HT Hybrid Tea, F Floribunda, Patio Patio rose, S Shrub, HRG Hybrid Rugosa, CP Canadian Parkland.

Comparative analysis of the two Canadian programs (Table 4) showed that LG6 is most differentiated (Fst=0.031). Overall, Fst values between Rootstock roses and each of the Canadian programs was similar, except for LG5 and LG6, where Canadian Explorer showed respectively larger genetic differentiation from Rootstocks, while Rootstock rose and Canadian Parkland group had the highest differentiation for LG6 (Fst = 0.116). Interestingly, LG5 showed the lowest differentiation between Canadian Explorer and Rootstocks (Fst=0.006). Canadian Explorer cultivars showed the most differentiation from European cultivars (Floribunda, Modern English roses, Hybrid Rugosasa, Renaissance, and Shrubs) for LG5 (Fst=0.045-0.074), while LG4 had the lowest Fst value for comparisons between Parkland roses and Hybrid Rugosas (-0.0027), Modern English roses (0.007), and Floribundas (0.009). The LG1 of Modern English roses showed most differentiation among

Table 4. Genetic differentiation (Fst) among garden rose types.

Locus	LG	All*	Pairwise Fst values									
			CE-HT	CE-CP	CE-F	CE-MOE	CE-HRG	CE-REN	CE-S	CP-HT	CP-F	CP-MOE
RhD201	1	0.004	-0.007	0.005	0.024	0.013	-0.022	-0.057	-0.008	0.041	0.061	0.055
RMS015	1	0.031	0.033	-0.008	0.016	0.012	0.017	-0.022	0.048	0.011	-0.004	0.013
RMS047	1	0.018	0.056	-0.006	0.023	0.010	-0.014	-0.026	0.033	0.026	0.003	0.006
Average		0.018	0.027	-0.003	0.021	0.011	-0.006	-0.035	0.024	0.026	0.020	0.025
RMS082	2	0.017	0.023	0.048	0.063	0.065	0.020	0.065	-0.014	-0.022	-0.015	-0.006
RhB303	2	0.027	0.043	0.067	0.050	0.075	0.076	0.045	0.013	0.008	-0.001	0.043
RhO506	2	0.028	0.030	-0.023	0.026	0.044	0.008	0.030	0.050	0.051	0.037	0.054
RMS062	2	0.018	0.044	0.035	0.012	0.010	-0.004	0.009	-0.003	-0.003	0.028	0.008
Average		0.023	0.035	0.031	0.038	0.049	0.025	0.037	0.012	0.008	0.012	0.025
RhD221	4	0.017	0.003	-0.028	0.052	0.040	-0.009	0.054	0.035	-0.002	0.052	0.032
RMS080	4	0.021	0.056	0.012	0.042	0.043	0.019	0.031	0.029	0.026	0.009	0.011
RhAB40	4	-0.006	-0.022	-0.020	-0.001	0.000	0.009	0.011	0.001	-0.018	-0.035	-0.023
Average		0.011	0.013	-0.012	0.031	0.028	0.007	0.032	0.022	0.002	0.009	0.007
RA044b	5	0.028	0.093	-0.009	0.046	0.073	-0.011	0.026	0.075	0.040	-0.001	0.026
RA023b	5	0.021	0.077	0.015	0.041	0.029	0.102	0.101	0.066	0.044	0.017	0.019
RMS029	5	0.043	0.180	0.061	0.112	0.068	0.043	0.095	0.078	0.049	-0.003	-0.012
Average		0.031	0.117	0.022	0.066	0.057	0.045	0.074	0.073	0.045	0.004	0.011
Rog9	6	0.020	0.029	0.035	0.016	0.022	0.034	0.075	0.021	-0.002	0.041	0.026
RMS097	6	0.048	0.163	0.081	0.180	0.112	-0.012	0.030	0.117	-0.005	-0.012	-0.007
Rog18	6	0.031	0.054	0.016	0.024	0.038	0.041	0.059	0.032	0.070	0.051	0.065
RMS017	6	0.005	0.033	-0.009	0.023	0.017	0.002	-0.013	0.003	0.000	0.004	-0.002
RhE2b	6	0.011	0.002	0.033	0.016	0.023	0.048	-0.018	-0.015	0.020	0.019	-0.001
Average		0.023	0.056	0.031	0.052	0.042	0.023	0.027	0.031	0.017	0.021	0.016
RMS003	7	0.021	0.069	0.002	0.039	0.056	0.018	0.027	0.019	0.036	0.018	0.034
RMS008	-	0.051	0.073	0.021	0.060	0.029	-0.007	0.038	0.139	0.006	0.012	0.005
Rog3	-	0.024	0.106	0.012	0.059	0.041	0.012	0.065	0.013	0.015	-0.018	-0.010
Rog5	-	0.014	0.063	0.137	0.040	0.039	-0.016	0.031	0.014	0.019	-0.004	0.015
RMS034	-	0.022	0.050	-0.005	0.034	0.043	-0.006	0.021	0.037	0.010	-0.009	0.010
Rog27	-	0.018	0.047	0.031	0.033	0.023	-0.005	0.040	0.024	0.028	-0.005	0.010
ALL LOCI		0.022	0.055	0.015	0.042	0.038	0.011	0.0302	0.0341	0.0193	0.0109	0.016
Jackknifed estimators (over loci)												
Mean		0.022	0.055	0.015	0.042	0.038	0.011	0.030	0.034	0.019	0.011	0.016
SE		0.003	0.010	0.006	0.007	0.005	0.005	0.008	0.008	0.005	0.00515	0.005

* All: all garden rose types; CE Canadian Explorer, CP Canadian Parkland, HT Hybrid Tea, F Floribunda, MOE Modern English Roses, HRG Hybrid Rugosa, REN Renaissance Roses, S Shrubs

Table 4 (*continued*). Genetic differentiation (Fst) among garden rose types.

Locus	LG	All*	Pairwise Fst values									
			CP-HRG	CP-REN	CP-S	F-HT	F-REN	F-MOE	F-HRG	F-S	HT-REN	HT-HRG
RhD201	1	0.004	-0.017	-0.097	-0.005	-0.001	-0.039	-0.005	0.087	-0.009	-0.047	0.0404
RMS015	1	0.031	-0.004	-0.014	0.025	0.005	0.016	0.023	0.035	0.044	0.030	0.079
RMS047	1	0.018	-0.019	-0.001	0.018	0.01	0.017	0.008	0.039	0.004	0.073	0.064
Average		0.018	-0.014	-0.037	0.013	0.005	-0.002	0.009	0.054	0.013	0.019	0.061
RMS082	2	0.017	-0.002	0.016	0.022	0.011	-0.019	-0.004	0.037	0.042	0.023	0.064
RhB303	2	0.027	0.196	-0.100	0.043	-0.023	-0.008	0.012	0.051	0.013	0.001	0.019
RhO506	2	0.028	-0.037	0.040	0.022	0.001	-0.032	-0.002	0.032	0.048	-0.024	0.073
RMS062	2	0.018	0.028	0.025	0.066	0.015	-0.001	0.018	0.011	-0.005	-0.005	0.050
Average		0.023	0.046	-0.005	0.038	0.001	-0.017	0.006	0.033	0.025	-0.001	0.051
RhD221	4	0.017	-0.032	0.068	0.035	0.014	0.000	0.005	0.051	0.019	0.0001	-0.020
RMS080	4	0.021	-0.010	0.015	0.015	0.004	-0.012	0.004	0.028	0.024	0.0127	0.061
RhAB40	4	-0.006	-0.038	0.024	-0.018	-0.010	0.023	-0.010	0.002	0.006	0.004	-0.001
Average		0.011	-0.027	0.036	0.011	0.003	0.004	0.000	0.027	0.016	0.006	0.013
RA044b	5	0.028	0.039	0.033	0.020	-0.006	0.038	0.003	0.054	0.010	0.128	0.122
RA023b	5	0.021	-0.037	0.023	0.019	-0.002	-0.035	0.008	-0.015	0.023	-0.058	-0.030
RMS029	5	0.043	0.021	-0.029	-0.017	0.009	-0.035	0.014	0.019	0.000	-0.025	0.085
Average		0.031	0.008	0.009	0.006	0.000	-0.011	0.008	0.019	0.011	0.015	0.059
Rog9	6	0.020	0.132	0.142	0.042	0.022	0.029	0.006	0.031	0.031	0.123	0.096
RMS097	6	0.048	-0.056	-0.109	0	-0.010	-0.057	0.011	0.038	0.026	-0.034	0.033
Rog18	6	0.031	0.091	0.068	0.119	0.008	0.062	0.008	0.052	0.032	0.064	0.067
RMS017	6	0.005	0.002	-0.008	-0.017	-0.000	0.008	0.009	0.037	-0.005	0.035	0.033
RhE2b	6	0.011	0.011	-0.015	-0.001	0.026	-0.012	0.003	0.055	0.001	-0.017	0.019
Average		0.023	0.036	0.016	0.029	0.009	0.006	0.008	0.043	0.017	0.034	0.050
RMS003	7	0.021	0.046	0.004	0.011	0.002	-0.020	-0.006	0.050	0.001	0.007	0.089
RMS008	-	0.051	0.022	-0.014	0.079	0.019	0.000	0.040	0.037	0.052	-0.013	0.052
Rog3	-	0.024	0.022	-0.003	0.002	0.038	0.013	0.005	0.057	-0.003	0.040	0.073
Rog5	-	0.014	0.037	-0.019	-0.013	0.002	-0.027	0.006	0.029	-0.002	0.009	0.044
RMS034	-	0.022	-0.007	0.039	0.008	-0.007	0.051	0.000	0.032	0.033	0.066	0.046
Rog27	-	0.018	0.025	0.002	0.040	0.016	0.002	0.001	0.023	0.019	-0.020	0.060
ALL LOCI		0.022	0.022	0.007	0.022	0.006	0.000	0.007	0.036	0.017	0.017	0.050
Jackknifed estimators (over loci)												
Mean		0.022	0.022	0.007	0.022	0.006	0.000	0.007	0.036	0.017	0.017	0.050
SE		0.003	0.013	0.010	0.007	0.003	0.006	0.002	0.004	0.004	0.010	0.007

Table 4 (*continued*). Genetic differentiation (Fst) among garden rose types.

Locus	LG	All*	Pairwise Fst values							
			HT-MOE	HT-S	REN-HRG	REN-MOE	REN-S	HRG-MOE	HRG-S	MOE-S
RhD201	1	0.004	-0.002	-0.014	-0.023	-0.038	-0.090	0.066	0.025	-0.007
RMS015	1	0.031	0.043	0.067	0.021	0.016	0.033	0.020	0.053	0.070
RMS047	1	0.018	0.037	0.034	-0.008	-0.021	0.019	0.029	0.034	0.021
Average		0.018	0.026	0.029	-0.003	-0.014	-0.01	0.039	0.037	0.028
RMS082	2	0.017	0.009	0.008	-0.031	-0.028	0.033	0.029	0.055	0.043
RhB303	2	0.027	0.004	0.002	0.020	0.026	0.026	-0.019	0.033	0.033
RhO506	2	0.028	0.011	0.100	0.025	-0.025	0.049	0.034	-0.010	0.052
RMS062	2	0.018	0.017	0.060	0.035	0.007	0.011	0.000	-0.005	0.029
Average		0.023	0.010	0.042	0.012	-0.005	0.030	0.011	0.018	0.039
RhD221	4	0.017	0.007	0.006	0.072	-0.006	0.022	0.044	0.016	0.003
RMS080	4	0.021	0.010	0.048	0.040	-0.013	0.013	0.037	0.040	0.023
RhAB40	4	-0.006	-0.008	-0.010	0.060	0.021	-0.026	0.006	0.009	0.008
Average		0.011	0.003	0.015	0.058	0.001	0.003	0.029	0.022	0.011
RA044b	5	0.028	0.004	-0.018	0.102	0.054	0.112	0.105	0.117	-0.006
RA023b	5	0.021	0.024	0.020	-0.076	0.015	-0.004	-0.018	-0.020	0.012
RMS029	5	0.043	0.037	0.061	-0.019	-0.025	-0.020	0.015	-0.004	0.012
Average		0.031	0.021	0.021	0.002	0.015	0.029	0.034	0.031	0.006
Rog9	6	0.020	0.032	0.048	0.229	0.011	0.005	0.056	0.127	0.006
RMS097	6	0.048	0.014	0.057	-0.117	-0.072	-0.083	-0.036	-0.014	0.005
Rog18	6	0.031	0.014	0.068	0.096	0.014	0.082	0.033	0.079	0.022
RMS017	6	0.005	0.000	-0.006	0.006	0.026	-0.010	0.024	0.008	-0.004
RhE2b	6	0.011	0.026	-0.019	0.106	-0.008	-0.040	0.047	0.040	0.001
Average		0.023	0.017	0.030	0.064	-0.006	-0.009	0.025	0.048	0.006
RMS003	7	0.021	0.004	0.033	0.016	-0.005	-0.021	0.069	0.017	0.023
RMS008	-	0.051	0.009	0.081	0.016	-0.009	0.078	0.023	0.099	0.113
Rog3	-	0.024	0.025	0.046	-0.054	0.004	0.029	0.020	0.019	0.011
Rog5	-	0.014	-0.004	0.005	0.025	0.005	-0.011	0.036	-0.007	0.001
RMS034	-	0.022	-0.000	0.053	0.072	0.070	0.042	0.034	0.039	0.058
Rog27	-	0.018	0.023	-0.004	0.033	0.016	-0.001	0.003	0.020	0.017
ALL LOCI		0.022	0.014	0.031	0.032	0.003	0.012	0.028	0.033	0.231
Jackknifed estimators (over loci)										
Mean		0.022	0.014	0.031	0.032	0.003	0.012	0.028	0.033	0.023
SE		0.003	0.003	0.007	0.014	0.006	0.009	0.006	0.008	0.006

* All: all garden rose types; CE Canadian Explorer, CP Canadian Parkland, HT Hybrid Tea, F Floribunda, MOE Modern English Roses, HRG Hybrid Rugosa, REN Renaissance Roses, S Shrubs

linkage groups in comparison with Canadian Parkland (Fst=0.025), Floribunda (Fst=0.009), Hybrid Tea (Fst=0.026), and Hybrid Rugosa (Fst=0.039), while Shrubs, with an exception of Canadian Parkland cultivars, which showed the most differentiation for LG2.

Genetic differentiation (Fst) between breeders

Most breeders are specialized in breeding of roses with specific characteristics. Usually they use a specific gene pool as donor of a specific trait, which may include wild species, existing cultivars and seedlings from their breeding programs. As a result, cultivars from different breeders are well distinguished in morphology. We have quantified the genetic differentiation among breeders using 73 cultivars from the breeding programs of Austin (A), Harkness (H), Olesen (O) and Svejda (S). Cultivars from the Austin breeding program mostly included Modern English roses, while cultivars from Svejda involved Canadian roses of the Explorer series. Most of Harkness' roses belong to Modern English and Floribunda types, while roses from the Olesen breeding programme are of the Shrub, Renaissance, Modern English, and Hybrid Tea types. Only few cultivars in this study were from Marshall (M), Noack (N), and Pearce (P) and these were not included in this analysis.

Genetic differentiation among cultivars of different breeders was moderate with an overall Fst value of 0.022 (Table 5), which is the same value as the differentiation among types. The set of Svejda cultivars showed the least similarities with Austin roses (Fst=0.035), while the level of differentiation between Svejda and Harkness (Fst=0.05) and Svejda and Olesen roses (0.04) was at the same level. The largest differentiation among European cultivars (Fst = 0.014 between Harkness and Austin roses) was much lower than that between any of them and the Canadian' Svejda roses. Differentiation between Harkness' and Olesen's cultivars (Fst=0.006) was almost zero, indicating that a similar gene pool was used for breeding. Comparing linkage groups, among all breeders by far the largest differentiation was present on LG5 (Fst=0.034). Comparison of pairs of breeders showed that between Austin and Harkness cultivars LG2 was most differentiated (Fst=0.024), while Olesen's cultivars are most differentiated from Austin's and Harkness's for LG5 (Fst=0.035 and Fst=0.011 respectively).

Table 5. Genetic differentiation (Fst) between breeders.

Locus	LG	among all (Austin, Harkness, Svejda, Olesen)	among Austin, Harkness, Olesen	between Austin and Harkness	between Austin and Olesen	between Harkness and Olesen
RhD201	1	0.018	-0.002	0.003	-0.010	-0.021
RMS015	1	0.017	0.024	0.025	0.036	-0.003
RMS047	1	0.007	0.007	0.010	0.003	0.000
Average		0.014	0.010	0.013	0.010	-0.008
RMS082	2	0.017	0.006	0.011	-0.002	-0.010
RhB303	2	0.051	0.042	0.041	0.054	0.026
RhO506	2	0.024	0.002	0.005	-0.005	-0.007
RMS062	2	0.026	0.033	0.039	0.035	0.001
Average		0.029	0.021	0.024	0.020	0.003
RhD221	4	0.026	0.016	0.021	0.000	0.023
RMS080	4	0.016	0.006	0.009	0.001	0.000
RhAB40	4	-0.003	-0.004	-0.002	-0.012	0.000
Average		0.013	0.006	0.009	-0.004	0.008
RA044b	5	0.039	0.025	0.006	0.063	0.040
RA023b	5	0.023	0.009	0.009	0.014	0.003
RMS029	5	0.041	0.022	0.024	0.029	-0.010
Average		0.034	0.018	0.013	0.035	0.011
Rog9	6	0.020	0.005	-0.002	0.010	0.025
RMS097	6	0.062	0.031	0.041	0.006	0.018
Rog18	6	0.019	0.006	0.006	0.000	0.010
RMS017	6	0.008	0.007	0.010	0.009	-0.005
RhE2b	6	0.010	0.009	0.013	0.007	-0.005
Average		0.024	0.011	0.014	0.006	0.009
RMS003	7	0.023	0.005	0.008	-0.003	0.004
RMS008	-	0.018	0.015	0.022	0.010	-0.002
Rog3	-	0.033	0.024	0.020	0.021	0.050
Rog5	-	0.012	-0.001	-0.001	0.002	-0.004
RMS034	-	0.013	0.006	0.003	0.016	0.001
Rog27	-	0.016	0.013	0.015	0.013	-0.002
ALL LOCI		0.022	0.013	0.014	0.013	0.006
Jackknifed estimators (over loci)						
Mean		0.022	0.013	0.014	0.013	0.006
SE		0.003	0.003	0.003	0.004	0.004

*, All, Austin, Svejda, Harkness, Olesen

Discussion

Genetic Diversity

In this study we have compared the genetic diversity in various types of garden roses with that of cut roses and rootstocks as outgroups. Of these three groups, the rootstocks showed the highest value of expected heterozygosity (or gene diversity) (H_e=0.86), while it was somewhat lower in garden roses (H_e=0.82) and considerably lower in cut roses (H_e= 0.73). Nybom (2004) showed that levels of heterozygosity can be compared across taxa, provided the markers are equally polymorphic and scored in the same way (dominantly or co-dominantly). We can add that the taxa should have the same ploidy level. This precludes a comparison of our study with studies on cultivated varieties of diploid Rosaceae, such as peach commercial varieties (H_e=0.46; Aranzana et al., 2010), almond commercial varieties (H_e=0.67; Rigoldi et al., 2011), and sweet cherry cultivars (H_e=0.55; Marti et al., 2012). Peach is partly selfing, but the lower values found in almond and sweet cherry may be due to the lower ploidy level. We can compare with Esselink et al. (2003) who used 24 microsatellites to study the diversity among rootstock and cut roses and also found that rootstocks had a significantly higher gene diversity than cut roses.

Consistent with lower gene diversity, cut roses had the smallest number of alleles across all loci. Importantly, they contained only few alleles (6 out of 147) that were also not present in garden roses. This suggests that cut rose germplasm is a subset of the germplasm present in garden roses, even though as a group they are differentiated, and in the PCO plot (Fig. 1) they are clearly distinct from garden roses. The rootstock roses had many unique alleles, which indicates that they form a separate gene pool. Indeed, it is known that their progenitors have not been involved in garden rose and cut rose breeding (Phillips and Rix, 2004).

Genetic differentiation of garden rose types

Based on our set of cultivars we found the highest similarity between Renaissance, Modern English, Floribunda, and the Canadian Parkland cultivars. These findings are in agreement with what is known about the pedigrees of these types of roses and confirms that

the same genepool was used in breeding. For example, the small group of Renaissance roses is positioned in the PCO between Floribunda and Hybrid Tea roses. This position is not surprising as both Floribunda and Renaissance contain Hybrid Tea roses in their ancestry (Phillips and Rix, 2004). In only few studies cultivars from different rose types have been compared. The genetic similarity of Hybrid Tea and Floribunda had also been observed by Vainstein et al. (1993), Ben-Meir and Vainstein (1994), and Debener et al. (1996). They also observed that the Miniature roses were genetically most distant, but we did not include a sufficient number of miniature roses to be able to confirm this.

Our data showed that Hybrid Tea roses are close to cut rose cultivars. If we look more carefully to their pedigrees, Hybrid Tea roses were derived from crosses between Tea and Hybrid Perpetuals. The Hybrid Perpetuals combined Old European and Asian wild species and cultivars such as: Hybrid Chinas, Hybrid Bourbons, Hybrid Noisettes, *R. alba, R. centifolia, R. gallica*, and *R. chinenses* (Thomas, 2004). Modern cut roses were obtained by crossing Chinese roses with Bourbon and Hybrid Perpetuals (Zlesak, 2007). Thus, cut rose and Hybrid Tea varieties share a largely similar gene pool. Indeed, some Hybrid Tea roses are phenotypically close to cut roses and also used as cut roses. In our study this is exemplified by 'Velvet fragrance', a Hybrid Tea cultivar that in the PCO has a position among the Cut Roses. This cultivar was used both for cut flower production and in gardens.

We included cultivars from two different Canadian breeding programs, both bred with the purpose of creating cultivars that were very winter hardy. The Canadian Explorer group used introgressions of germplasm from various wild species. It indeed had the largest gene diversity value (0.876) of all garden rose types, and it had many unique alleles (on average more than 3 per cultivar). The Parkland group cultivars were made using European founders, mostly Pernet-Ducher cultivars. Compared to European garden roses, Parkland roses had 15 unique alleles (out of 181); these alleles can present a species contribution/introgression. Indeed, the Fst between Parkland and the European garden rose types was small (0.010 overall) while that of the Explorer roses was 0.036. In the PCO the Explorer roses and Rugosa types are found closest to the Rootstocks, which may reflect the introgression of rootstocks (*R. arkansana*) and *R. rugosa* into the Explorer roses.

Differentiation among breeders

The results showed that the genetic differentiation among breeders is the same as that among garden rose types (Fst = 0.022). This is not unexpected if we keep in mind that each breeder is specialised in breeding of a specific type of roses with one or a few specific traits. Even if a breeder brings different rose types to the market he probably still used the same parents/genepool. Hence, basically, in garden roses horticultural groups and breeders overlap. The level of genetic differentiation is fourfold the value among breeders of cut roses (Fst = 0.0056, Smulders et al. 2009).

According to PCO plot Austin's Modern English cultivars represent the modern rose type characterized by the largest genepool. The set of Austin's cultivars may be divided into five subgroups based on their origin. The English old rose hybrids were the original English roses and they are characterized by pink, crimson or purple flower colour and strong fragrance, such as 'Eglantyne', 'Gentle Hermione', and 'Sharifa Asma'. In the pedigrees of the English Leander roses Old rose hybrids and Tea roses are involved, which enabled flower colour range improvement, while the fragrance is still strong (e.g., 'Alan Titchmarsh', 'James Galway', 'Pat Austin'). The English musk hybrids are the result of crosses between Old rose hybrids and Noisettes roses and they are characterized by soft fragrance and flower colour ('Heritage', 'Wildeve', 'Graham Thomas'). The English alba hybrids originated from crosses between English and Alba roses and their phenotype reminds of Wild rose growth type ('Shropshire lass', 'The Alexandra rose'). Finally, there is a group of Modern English cultivars that do not fit in some of the earlier mentioned subgroups, such as 'Princess Anne' and 'Wild Edric' (Austin, 2012). To sum up, mainly, there are five strategies in developing Modern English roses and for this purpose four sources of donors have been used. The selection for specific phenotype characteristics (flower architecture, fragrance, etc) led to the similarity in phenotype of Modern English cultivars.

Evidence of introgression / functional variation

Twenty four microsatellite markers were used in this study in order to be able to determine genetic diversity and differentiation for separate linkage groups. We were interested in this, as introgression would be expected to increase the genetic diversity in terms of number of alleles, while selection for specific phenotypes would be expected to reduce the number of alleles and increase the differentiation between garden rose types. Thus,

differences between LGs may reflect introgression events and selection pressure during breeding.

The highest diversity (most alleles and most allelic phenotypes) for cut roses and garden roses were found on linkage group (LG) 6. So far it has been observed that on LG6 several QTLs are located for days to flowering (Dugo et al., 2005), leaf colour and growth rate (Yan et al., 2007). These are traits of general interest, and can be found in wide germplasm, not just in a single source. Additionally, breeders of pot roses are focused on developing rose genotypes characterized by earlier blooming. Until now breeders have been working on reducing numbers of days to flowering by combining different rootstocks during a process of budding and they succeeded to reduce the period to two weeks.

Overall, LG2 was by far the most differentiated among types of garden roses, which may indicate that during breeding selection for several traits has affected loci on this linkage group. QTLs for flower size, leaf size (Dugo et al., 2005), vigour and leaf colour (Yan et al., 2007), and inflorescence architecture (Kawamura et al., 2011) are all located on LG2. Indeed, beside fragrance, rose breeders are mainly focused on flower (size and shape/architecture) and leaf (looking for big, shiny, dark green leaf) characteristics.

The highest differentiation between the Canadian programs and European garden roses that are different in winter hardiness was found on LG5. Interestingly, the Canadian Explorer series was most similar to the rootstock roses, which are also winter hardy roses, on LG5. Furthermore, Olesen is a breeder whose set of cultivars includes the few European winter hardy genotypes. The largest differentiation between Olesen's and the European cultivars of Austin's and Harkness, which are quite susceptible to low temperatures, was also found on LG5. These observations would suggest that LG5 may contain an important QTL for winter hardiness.

The largest differentiation between Austin's and Harkness' cultivars is also noted on LG5. This LG is also the location of various QTLs mainly related to plant vigour (flower and leaf size, number of shoots, nodes and internodes, shoot and internode length, leaf and stem dry weight and grow rate; Dugo et al., 2005; Yan et al., 2007; Kawamura et al., 2011). The large differentiation observed for this LG would suggest that there are differences in growth vigour between cultivars of these two breeders. Indeed, Austin roses are shrubby genotypes and some are even climbers. In sharp contrast, cultivars of Harkness are shorter and more compact, with a few exceptions such as 'Madrigal' and 'Penny Lane'.

Conclusion

Genetic differentiation among all types of garden roses was four times that among cut roses, and similar in magnitude to the differentiation among breeders, due to the fact that horticultural groups and breeders overlap largely in classification. Our results indicate that, in terms of neutral genetic diversity, cut roses represent a subset of garden roses. Our study employed a larger number of markers (24) covering most linkage groups, and using this strategy we could assess that the differentiation varies between linkage groups. This leads us to suggest that LG5 is an important linkage group containing possible QTLs for winter hardiness. LG6 contains the largest amount of genetic diversity, while LG2 is the most differentiated among the garden rose types, which may be indicative of introgression from wild species and selection by breeders. We expect that future studies using denser marker maps or next generation sequencing will uncover more differences among linkage groups within the garden rose germplasm.

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Supplementary table 1. Significance of He value differences among rootstocks, garden roses, and cut rose.

G/R					G/C					C/R				
n_1	**n_2**	**U**	**P (two-tailed)**	**P (one-tailed)**	**n_1**	**n_2**	**U**	**P (two-tailed)**	**P (one-tailed)**	**n_1**	**n_2**	**U**	**P (two-tailed)**	**P (one-tailed)**
24	24	428	0.003*	0.002*	24	24	452.5	0.000408*	0.000204*	24	24	522	2e-06*	1e-06*
normal approx z = 2.9			0.004*	0.002*	normal approx z = 3.4			0.0007*	0.0003*	normal approx z = 4.4			8.9e-06*	4.5e-06*

*These values are approximate.

G/R, Garden roses and Rootstocks; G/C,Garden roses and Cut roses; C/R, Cut roses and Rootstocks

Supplementary table 2. Genetic differentiation (Fst) of different groups cultivars.

Locus	LG	All samples	C-R*	C-G**	R-G	R-CE	R-CP	R-EG	EG-CG***	EG-CE	EG-CP
RhD201	1	0.029	0.050	0.014	0.043	0.011	0.013	0.051	0.014	0.013	0.053
RMS015	1	0.026	0.136	0.029	0.041	0.034	0.039	0.041	0.003	0.005	-0.006
RMS047	1	0.051	0.214	0.040	0.090	0.041	0.060	0.099	0.011	0.017	0.005
Average		0.035	0.133	0.028	0.058	0.029	0.037	0.064	0.009	0.012	0.017
RMS082	2	0.068	0.226	0.009	0.146	0.153	0.175	0.140	0.013	0.035	-0.005
RhB303	2	0.105	0.089	0.124	0.119	0.184	0.136	0.114	0.019	0.049	-0.007
RhO506	2	0.096	0.172	0.163	0.032	0.038	0.020	0.037	0.036	0.032	0.041
RMS062	2	0.025	0.061	0.024	0.049	0.044	0.058	0.050	0.001	0.007	0.007
Average		0.074	0.137	0.080	0.086	0.105	0.097	0.085	0.017	0.031	0.009
RhD221	4	0.047	0.122	0.038	0.077	0.002	0.012	0.091	0.029	0.032	0.012
RMS080	4	0.050	0.207	0.009	0.132	0.074	0.093	0.144	0.020	0.039	0.012
RhAB40	4	0.024	0.081	0.035	0.034	0.021	0.008	0.035	-0.005	0.000	-0.020
Average		0.040	0.136	0.027	0.081	0.033	0.038	0.090	0.014	0.024	0.001
RA044b	5	0.067	0.186	0.060	0.067	0.005	0.040	0.086	0.046	0.066	0.023
RA023b	5	0.025	0.051	0.019	0.009	0.000	0.010	0.015	0.024	0.035	0.022
RMS029	5	0.070	0.177	0.025	0.092	0.014	0.069	0.116	0.043	0.096	0.002
Average		0.054	0.138	0.035	0.056	0.006	0.040	0.072	0.038	0.066	0.015
Rog9	6	0.042	0.062	0.025	0.082	0.070	0.127	0.084	0.014	0.019	0.036
RMS097	6	0.095	0.232	0.009	0.165	0.002	0.189	0.193	0.054	0.143	-0.004
Rog18	6	0.040	0.104	0.002	0.098	0.060	0.095	0.109	0.031	0.028	0.040
RMS017	6	0.031	0.145	0.023	0.099	0.063	0.108	0.104	0.006	0.016	-0.003
RhE2b	6	0.077	0.058	0.132	0.070	0.072	0.061	0.072	0.001	0.015	-0.005
Average		0.057	0.120	0.038	0.103	0.053	0.116	0.112	0.021	0.044	0.013
RMS003	7	0.044	0.097	0.039	0.051	0.059	0.018	0.059	0.034	0.043	0.019
RMS008	-	0.069	0.225	0.012	0.162	0.152	0.159	0.167	0.020	0.040	0.002
Rog3	-	0.050	0.045	0.065	0.036	0.030	0.046	0.041	0.022	0.042	-0.001
Rog5	-	0.045	0.150	0.013	0.087	0.043	0.065	0.097	0.019	0.041	0.001
RMS034	-	0.049	0.177	0.023	0.091	0.043	0.069	0.101	0.017	0.034	-0.001
Rog27	-	0.035	0.097	0.018	0.076	0.025	0.069	0.087	0.012	0.029	0.005
ALL LOCI		0.052	0.132	0.042	0.081	0.053	0.073	0.088	0.020	0.036	0.010

*C, Cut rose; R, Rootstock rose

**C, Cut rose; G,Garden rose

***EG, European garden rose; CG, Canadian garden rose

****CE,Canadian garden rose Explorer Group;CP,Canadian garden rose Parkland Group

Supplementary table 3. Unique allele, unique allelic phenotypes and characteristics of unique allele in each group

Primer name	LG	C*		R*				EG*				CE*			CP*	
		Pa	Uap	Pa	NU	NS	Uap	Pa	NU	NS	Uap	Pa	NU	Uap	Pa	Uap
RhD201	1	-	-	5	2	2	8	6	1	1	19	1	-	5	1	2
RMS015	1	-	6	3	-	-	8	5	3	1	60		-	5	2	4
RMS047	1	-	-	1	-	-	8	4	1	-	30	1	-	5	-	2
Total in LG 1		0	6	9	2	2	24	15	5	2	109	2	0	15	3	8
RMS082	2	-	-	4	-	1	8	3	2	-	22	-	-	-	-	-
RhB303	2	-	3	1	-	-	7	7	1	1	29	-	-	2	-	2
RhO506	2	-	5	2	1	1	8	2	2	-	48	2	1	3	1	4
RMS062	2	-	9	-	-	-	9	1	-	-	59	4	-	7	1	4
Total in LG 2		0	17	7	1	2	32	13	5	1	158	6	1	12	2	10
RhD221	4	-	10	1	1	-	4	5	2	1	25	-	-	4	-	2
RMS080	4	-	-	1	-	-	7	4	1	-	24	4	-	7	-	2
RhAB40	4	-	9	3	1	1	8	11	4	2	54	1	-	5	-	3
Total in LG 4		0	19	5	2	1	19	20	7	3	103	5	0	16		7
RA044b	5	-	-	5	-	1	7	2	2	-	18	2	-	6	-	6
RA023b	5	-	7	2	-	-	8	2	2	-	47	-	-	5	-	5
RMS029	5	-	1	3	2	-	9	2	2	-	14	3	-	7	1	1
Total in LG 5		0	8	10	2	1	24	6	6	0	79	5	0	18	1	12
Rog9	6	-	10	1	1	-	5	3	1	-	30	1	-	2	-	1
RMS097	6	-	-	4	1	-	5	1	-	-	4	2	-	6	-	-
Rog18	6	-	10	-	-	-	6	2	2	-	44	1	1	7	-	4
RMS017	6	-	7	2	-	-	5	9	5	3	69	2	-	6	-	4
RhE2b	6	-	3	2	1	-	6	6	2	3	41	-	-	5	-	1
Total in LG 6		0	30	9	3		27	21	10	6	188	6	1	26		10
RMS003	7	-	5	4	3	2	6	5	2	2	46	-	-	7	1	4
RMS008	-	-	1	4	2	3	9	5	2	1	37	1	-	4	1	2
Rog3	-	-	7	5	-	1	6	5	2	-	38	3	-	7	-	3
Rog5	-	-	2	1	1	-	8	1	1	-	27	3	1	7	1	1
RMS034	-	-	-	7	3	5	8	5	2	-	37	2	1	8	1	2
Rog27	-	-	8	2	-	-	7	1	-	-	34	-	-	7	-	4
Total		0	103	63	0.8	0.7	170	97	1.7	0.6	856	33	0.2	127	10	63

*C, Cut rose; R, Rootstock rose; EG, European garden rose;CE,Canadian garden rose Explorer Group; CP,Canadian garden rose Parkland Group; LG, Linkage group; **Pa, Private allele; Uap, Number of unique allelic phenotypes; NP, Number of Private allele has more than one sample; NS, Number of Sample has more than one private allele

Chapter 3

Quantification of Allele Dosage in Tetraploid Roses

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Quantification of Allele Dosage in Tetraploid Roses

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Summary

Many important crops (wheat, potato, strawberry, rose, etc.) are polyploid. This complicates genetic analyses, as the same locus can be present on multiple homologous chromosomes. SSR markers are suitable for mapping in segregating populations of polyploids as they are multi-allelic, making it possible to detect different alleles of the same locus on all homologous chromosomes. If a SSR primer pair gives fewer alleles than the ploidy level, quantification of allele dosages increases information content. We show the power of this approach for the generation of a genetic map in a tetraploid garden rose population. Alleles were scored quantitatively using the area under the peaks in ABI electropherograms, and allele dosages were inferred based the ratios between the peak areas for two alleles in reference cases in which these two alleles occurred together. We resolved the full progeny genotypes, generated more data and mapped markers more accurately, including "null" alleles. The maps will be used for locating QTLs for winterhardiness in tetraploid roses.

Introduction

The frequent occurrence and widespread distribution of polyploids suggest that they play an important role in evolution. Roughly 50% of angiosperms and 44-95% of ferns and fern allies have a polyploid origin (Luo et al., 2006). Polyploidization has played a major role in a plant evolution by increasing gene redundancy and morphological complexity. As a result, polypoids are often more adaptable and show increased tolerance to environmental conditions (Xie and Schizhong, 1999; Gar et al., 2011). Basically there are two classes of polyploids: autopolyploids and allopolyploids. Allopolyploids or bivalent polyploids originated from at least two different species. Preferential pairing of homologous chromosomes during meiosis leads to the disomic inheritance. Multivalent polyploids or autopolyploids may be derived from a single ancestral species, mostly through duplication of the genome. In autopolyploids

chromosomes pair randomly in meiosis, leading to polysomic inheritance. Sometimes even more than two homologous chromosomes pair with each other, forming tetravalents, which may lead to ‘double reduction’. In reality, the type of pairing may also vary among chromosomes, which makes genetic analysis more complex (Li et al., 2010, Stift et al, 2008).

Garden and cut roses are tetraploids with with small chromosome number and genome size. Despite roses being the most important ornamental, and the huge development of genomics, little is known of rose genetics, largely due to varying ploidy level among species, high degree of heterozygosity and specific sexual reproduction. Rose genetics influences breeding success, especially if we keep in mind low seed germination rate (Gudin, 2000; Yan et al, 2005).

In the era of genomics marker-assisted breeding is rapidly becoming an important tool as it may shorten the breeding period significantly. In major diploid crops it is routinely used for genetic map development and mapping of quantitative and qualitative characteristics . In sharp contrast, the application of molecular tools in breeding of autopolyploid species is still limited. This is largely due to the complexities of gene segregation and recombination during meiosis, namely: multiple allele segregation, double reduction and mixed bivalent and quadrivalent pairing among homologous chromosomes (Luo et al., 2006). This complicates genetic analyses, as the same locus can be present on multiple homologous chromosomes. Genetic segregation in autopolyploids is a reflection of meiosis with a combination of bivalent and multivalent pairing with multiple alleles per locus. Additional complexity presents a specific phenomenon known as double reduction. Under the term of double reduction is assumed multivalent pairing that lead that two chromatids originated from the same chromosome are present in the gamete. Under these circumstances the progeny has exceptional allele composition that is not expected by Mendelian lows (Gar et al., 2011). For better understanding rose genetics is needed full genome coverage, what codominant markers would allow.

Codominant markers such as microsatellites (SSR) provide much more information compared to dominant markers. According to Luo et al. (2001) estimates of recombination frequencies based on multiallelic markers are up to four times as informative as the best estimates from dominant markers. Thanks to their nature SSRs have been widely used for plant genome analysis (Song et al, 2011). SSRs can be applied in polyploid genetic analysis without additional complications, as there can be multiple alleles at the same locus in a single

plant which can be mapped to duplicated linkage groups. Unfortunately, many of the advantages of codominance are lost in the study of polyploids if there are fewer alleles and if the allele dosage cannot be determined (Pfeiffer et al., 2011).

Currently, there is no simple method for quantification of allele dosage. In some cases, due to differences in amplification among alleles, quantitative scoring is not possible for some marker loci. Here we describe a method of codominant scoring, as an extension on MAC-PR (Esselink et al., that allows us to cope with the dose effect, even in case of differences in amplification among alleles at a marker locus. This makes it possible to extract more information and map more markers, including null alleles, in a more accurate way.

Materials and methods

The material consisted of three populations derived from crosses between Morden Centennial, Nipper, Red New Dawn and Winchester Cathedral. Morden Centennial is from a Canadian breeding program for winterhardy garden roses, and it was crossed with European varieties in order to introgress winterhardiness. The smallest population consists of 42 seedlings.

Successful quantification of allele dosage is completely dependent on the quality of the experimental data; the quality must be consistently high, preferably with scorable markers showing no or a little stutter bands (Esselink et al, 2004). A set of 23 SSR markers was selected from the literature (Esselink et al., 2004; Debener et al., 2001; Koning-Boucoiran et al., 2012). SSR were amplified by multiplex- or single PCR according to Esselink et al, 2004. The NED-, HEX- or 6-FAM- labelled products were detected using an ABI Prism 3700 DNA analyser (Perkin Elmer Biosystems, Foster City, California). Fragment sizes and peak areas were automatically determined using GeneMapper.

Results

Procedure. The ABI platform generates electropherograms in which each allele is shows as a peak. As roses are tetraploids the number of expected peaks varies from one to four. Observed electropherograms showed a trend of slightly decreasing peak height with increasing product size. On top of that alleles can have different success of amplification. Alleles amplified using SSRs often have one or few stutter bands. After choosing real alleles and discarding stutter bands, the areas under the peaks are exported to an Excel table.

The first step in quantification of allele dosage is determination of amplification ratio between alleles. Different alleles show differences in amplification ability and precise determination is crucial. Filtering data in Excel table allows identifying all cases in which an offspring plant has four alleles. These are used to determine the allele-specific amplification ratios for single dosage. In the few cases in which the amplification ratios are different between samples, the best solution is to take a range of amplification ratios for single dose. On the base on single dose amplification ratios, the ratios for double or triple doses are calculated.

Second, as a confirmation of these assumptions, it is tested whether observing of ratios within alleles we could separate different categories. In perfect case amplification ratios groups around values for single dose or as double or triple multiplication of these values.

Third, the alleles in the progeny plants are assigned a dosage and the full genotype is established. Even though quantification looks simple, sometimes is very difficult to conclude the allele doses. In theory, amplification ratios are good markers for allele dosage, but they are not constant. In reality, single dose is characterised with a range of amplification coefficient. In a case when coefficients are close to the values for double dose parental genotype helped to conclude the progeny genotype. Namely, on the base of parental genotype is possible to predict alleles that occur in gametes and progeny genotype.

In reality there are alleles that do not show any regularity in amplification success. In this case a valid conclusion is difficult to make and can be done only in a case if the rest of alleles from the samples show orderliness in amplification. In this case according to the allele dose of the rest of alleles, the genotypes were determined.

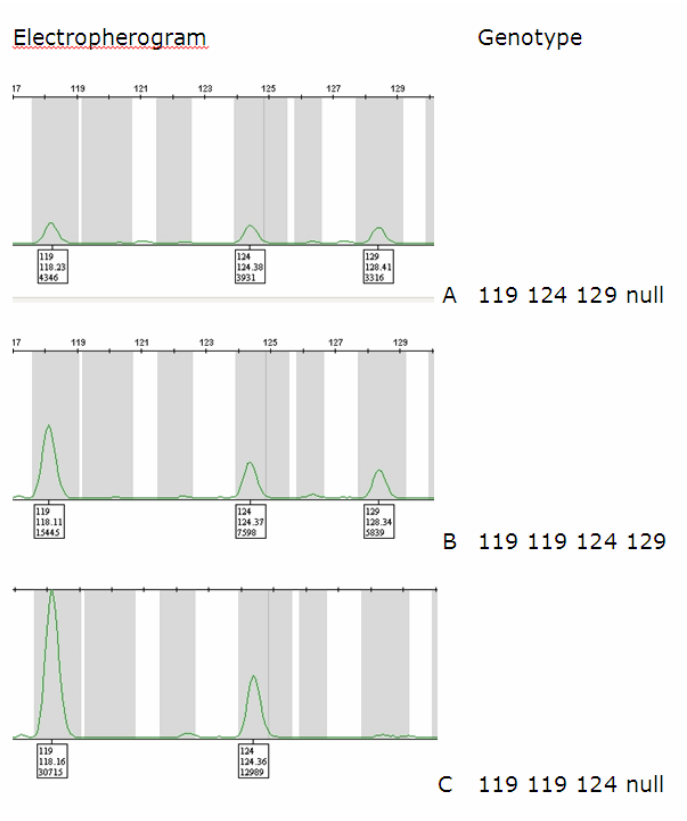

Figure 1. ABI electropherograms for marker RhB303, population Morden Centennial x Red New Dawn.

As an example we show a procedure to score alleles of RhB303 marker (Fig 1). In electropherograms under each peak there is a box with information about given name for the allele (first line), the length of the allele (second line) and peak area (third line). Amplification ratios between alleles in a case when alleles are in simplex are: 1.2 (119/124), 1.3 (119/129), and 1.1 (124/129). In a case A there are three alleles in electropherogram with amplification ratios 1.1 (119/124), 1.3 (119/129) and 1.2 (124/129), what indicates null allele and allele configurations 119 124 129 null. The peak areas are also in agreement with expected trend of peak height decreasing. In a case B there are three alleles with amplification ratios: 2.03 (119/124), 2.6 (119/129), and 1.3 (124/129), what lead to conclusion that genotype of the observed seedling are 119 119 124 129. In a case C there are two alleles 119 and 124 with the amplification ratio of 2.4, what indicate 2:1 ratio and allele configuration 119 119 124 null.

Complicating issues. Bleeding, shifting and stutter bands are drawbacks which make quantitative scoring more complex.

Bleeding is a phenomenon, specific for multiplexed PCR reactions. Bleeding occurs if amplification one or more markers exceed the software intensity threshold. In a case of Genemapper the overloaded peaks are marked with purple band, which width is correlated with the intensity of the luminous signal detected by capillary electrophoresis. When one of markers is overloaded its electropherogram interferes with the electropherograms of other markers. It happens as the fluorescence is not a single wavelength and the filter is not monochromatic. As a result we can see a peak that should not be considered as an allele.

Another common problem that can lead to wrong interpretation is shifting. For some samples is observed that peaks differ a few base pairs to the right or to the left. If all peaks are shifted in the same direction and for the same distance they are not treated as new peaks. The lengths of these peaks are manually changed.

Stutter bands are the phenomenon that the real allele is accompanied by one or more smaller peaks. Stutter bands are fragments one or several repeats shorter or longer than the real allele. They are produced during amplification of SSR markers, especially long dinucleotide SSRs. Bands are marked as stutters if they occur regularly and have constant peak area compared to peak area of real alleles.

Discussion

An understanding of allelic configurations is an essential step of plant genetic studies in polyploids. To early 2000s determination of allele dosage in polyploidy species has been mostly unsuccessful. The bands have been scored and interpreted as phenotypic banding patterns and no attempts have been made to assign precise allele dose. The era of quantitative scoring in polyploids started with pioneer work of Esselink et al in 2004. They succeeded to assign allelic configurations of tetraploid roses in five of six investigated loci.

Using quantitative scoring in populations made of different parents we were able to confirm null allele detection and to resolve allelic configurations for all individuals. In cases when allele amplification ratios indicated null alleles, parental and progeny allele configurations were checked. When null alleles exist their segregation in progeny follow genetic rules. Additionally, in cases when amplification ratios are between two categories (1:1 and 1:2 or 2:1) on the base on parental genotypes and expected segregation ratios can be determined the progeny genotype. In previous investigations a lot of markers which produce

stutter bands have been discarded what influenced the final outcome. Special case when stutter bands and real allele share the same position caused a lot of problems in scoring and making valid conclusion. Correcting peak area for the value of stutter band improved the quantitative scoring and enabled to score neglected markers from the past.

Chapter 4

Efficient development of highly polymorphic microsatellite markers based on polymorphic repeats in transcriptome sequences of multiple individuals

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Efficient development of highly polymorphic microsatellite markers based on polymorphic repeats in transcriptome sequences of multiple individuals

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Abstract

The first hurdle in developing microsatellite markers, cloning, has been overcome by next generation sequencing. The second hurdle is testing to differentiate polymorphic from non-polymorphic loci. The third hurdle, somewhat hidden, is that only polymorphic markers with a large effective number of alleles are sufficiently informative to be deployed in multiple studies. Both steps are laborious and still done manually. We have developed a strategy in which we first screen reads from multiple genotypes for repeats that show the most length variants, and only these are subsequently developed into markers. We validated our strategy in tetraploid garden rose using Illumina paired-end transcriptome sequences of 11 roses. Out of 48 tested two markers failed to amplify but all others were polymorphic. Ten loci amplified more than one locus, indicating duplicated genes or gene families. Completely avoiding duplicated loci will be difficult because the range of numbers of predicted alleles of highly polymorphic single- and multi-locus markers largely overlapped. Of the remainder, half were replicate markers (i.e., multiple primer pairs for one locus), indicating the difficulty of correctly filtering short reads containing repeat sequences. We subsequently refined the approach to eliminate multiple primer sets to the same loci. The remaining 18 markers were all highly polymorphic, amplifying on average 11.7 alleles per marker (range = 6 to 20) in 11 tetraploid roses, exceeding the 8.2 alleles per marker of the 24 most polymorphic markers genotyped previously. This strategy, therefore, represents a major step forward in the development of highly polymorphic microsatellite markers.

Introduction

Thanks to their reproducibility, co-dominant inheritance, and abundance microsatellite (also known as simple sequence repeat - SSR) markers are suitable molecular tools for many applications in genetic analysis and breeding. Additionally, being multi-allelic they are powerful for parentage analysis and haplotyping, particularly for mapping in polyploids as they allow detecting multiple alleles at the same locus on all homologous chromosomes (Vukosavljev et al., 2012). Despite the advent of SNP markers, recent studies in various plant and animal genera, for instance Cucurbita (Berzegar et al., 2013), Euphydryas (Smee et al. 2013), Lilium (Yuan et al. 2013), Medicago (Zitouna et al., 2013), Pinus (Iwaizumi et al., 2013), Portunus (Guo et al., 2013), Scatophagus (Liu et al. 2013), Triticum (Ansari et al., 2013), and Vitis (Doulati-Baneh et al., 2013) indicate that microsatellite markers are still extensively being developed as a molecular tool for various purposes.

Conventional microsatellite development is a long and costly process. Firstly, many microsatellite repeats need to be sequenced. Secondly, often as many as 50-100 primer pairs have to be tested to develop 10 polymorphic markers. Thirdly, for many of these polymorphic markers only few alleles with length differences in the repeat exist in the germplasm. The flanking regions of microsatellite repeats may contain additional SNPs (Xing et al., 2005; Zhang et al., 2013), but to this day these cannot be detected routinely with sufficient precision. Practical usage shows that the best microsatellite markers are multi-allelic and have a high effective number of alleles (Ne) in the germplasm. However, only a small portion of all polymorphic markers published have many alleles and will be widely used.

The development of highly polymorphic microsatellite markers using transcriptomic sequences is an interesting alternative that requires less effort, as sequences are already available or can be generated easily using next generation sequencing (Nybom et al. 2014), and microsatellite repeats can be identified by custom or freely available bioinformatics pipelines, such as PolySSR (Tang et al., 2008) and Pal_Finder (Castou et al., 2012). Indeed, recently several studies reported microsatellite marker development based on expressed sequences from sources such as GenBank or Genome database for Rosaceae (e.g., Durand et al., 2010; Park et al., 2010; Duran et al., 2013) or from custom-made transcriptome sequence libraries (e.g., Blair and Hurtado, 2013). However, from the identification step onwards the process is still slow, as most researchers select random subsets of repeats as a start for marker development (e.g., Liu et al., 2013). Legendre et al. (2007) developed a model, 'SERV', to predict the potential variability of repeats based on number of repeated units, unit length, and purity, which would allow to preselect more promising repeat loci. Tang et al. (2008) developed a pipeline to preselect repeat loci for which sequence reads show polymorphism in repeat length between a few genotypes, to exclude monomorphic repeat loci from the marker-testing step.

Although finding many microsatellite repeats makes it possible to test more markers until a set of high quality markers has been established, it does not speed up the testing process for multi-allelic markers. As one of few new developments for the latter problem, Eschbach and Schöning (2013) screened existing microsatellite markers for within-population polymorphism by scoring differences in sequence reads from a pooled sample of genotypes of the population they studied. Duran et al. (2013) developed a pipeline to extract putatively polymorphic microsatellites from EST data generated by Sanger sequencing and present in Genbank. They saw a relationship between the number of different repeats found in the ESTs and the number of different alleles amplified.

To improve the efficiency of developing multi-allelic microsatellites we have developed a new strategy for these three steps. We first generate transcriptome sequences from multiple genotypes, then screen sequence reads from these genotypes for those repeats that show the most variation in length, and move only these to the testing step. This strategy leads to highly polymorphic markers only. We demonstrate the suitability of this approach by developing highly polymorphic markers for garden roses. Garden roses are tetraploids, and for such a situation microsatellite markers are very appropriate molecular markers. To ensure that the selected markers will have a large effective number of alleles across the garden rose germplasm, we based our marker development on transcriptome sequences from a set of 11 garden roses representing different garden rose cultivar groups (Vukosavljev et al., 2013).

Material and methods

Plant material and RNA extraction

For this study we used a set of 11 tetraploid garden rose cultivars (Table 1), which were bred by different breeders, and belong to different types (Vukosavljev et al., 2013) with a large amount of phenotypic variation (e.g., difference in flower colour, fragrance, number of petals, winter hardiness, growth type, presence/absence of recurrent blooming). From each cultivar flowers in three stages (closed buds, half-way open, and fully open flowers) and young leaves were collected for RNA isolation. Tissues were frozen using liquid nitrogen. Frozen flower material was ground with an IKA mill. Leaf tissue was grinded in a mortar. After grinding, powder of leaf and flowers was pooled in equal amounts. RNA was extracted according the protocol of Cheng et al. (1993). Briefly, 1 to 1.5 g of frozen material was added to a preheated (65°C) CTAB extraction buffer and mixed thoroughly. After two extractions with chloroform, the RNA was precipitated overnight using LiCl. Next, the pellet was dissolved and the RNA purified further by chloroform extraction and EtOH precipitation. RNA integrity, yield and quality were measured on agarose gel and with NanoDrop (Thermo Scientific).

Table 1. Garden roses used in study.

Cultivar	Type *	Breeder	Ploidy	Flower colour	Winter hardiness zone**	Growth type	Fragrance	Number of petals	Blooming
Morden Centennial	CP	Marshall	4n	Pink	3b	Shrubby	Mild	40-45	Recurrent
Red New Dawn	Cl	Robichon	4n	Pink	6b	Rambling climber	Strong	17-25	Prolific, occasionally repeat blooming
Nipper	MIN	Harkness	4n	Red	6b	Ground cover	Strong		Occasionally repeat blooming
Diamond Border	S	Olesen	4n	White	4b	Shrubby	Mild to none	17-25	Recurrent
Princess of Wales	F	Austin	4n	White	6b		Mild to strong	17-25	Recurrent
Graham Thomas	MOE	Austin	4n	Yellow	5b	Shrub	Strong	35	Recurrent
J.P. Connell	CE	Svejda	4n	White	2b	Shrub	Strong	50	Occasionally repeat blooming
City of London	F	Harkness	4n	Light Pink	6b	Shrub	Strong	15-25	Recurrent
Henry Kelsey	CE	Svejda	4n	Pink	2b	Climber	Spicy scent	5-30	Occasionally repeat blooming
Heritage	MOE	Austin	4n	Light pink	5b	Shrub	Strong	40	Recurrent
Adelaide Hoodless	CP	Marshall	3n***	Pink	2b	Shrub	Mild	5-30	Recurrent

* CP Canadian Parkland series, CE Canadian Explorer series, Cl Climber rose, MIN miniature rose, S Shrub, F Floribunda, MOE Modern English Rose

** winter hardiness zone; http://planthardiness.ars.usda.gov (accessed 18 July 2013)

*** according to literature Adelaide Hoodless is triploid rose, but our flow cytometer result indicates tetraploidy (aneuploidy is still possible)

Microsatellite marker prediction

After RNA extraction, cDNA library preparation and Illumina HiSeq sequencing was performed according to manufacturer specifications (Illumina, San Diego, CA, USA) at GATC Biotech (Konstanz, Germany). For each cultivar, around 40 million 100 bp paired-end (PE) reads were obtained (trimmed read lengths 88.9 + 7.1 (S.D.) bp to 89.9 + 4.5 bp, average 89.3 bp), of which after quality checking between 12.1 million and 16.5 million were analysed for marker selection and development (Supplementary Table 1).

Microsatellite repeats were detected by Pal_Finder v0.02.04 (http://sourceforge.net/projects/palfinder) in the raw reads, using a minimum repeat number of 4 for tri- and tetranucleotide repeats, and 3 for penta- and hexanucleotide repeats. Merging of the reads was not necessary, but quality trimming did improve the speed of the process. Detected repeats were mostly located in one of the read pairs, but as they run until the end of the read, the exact length is not known. Primers were designed for tri-, tetra-, penta-, and hexanucleotide repeats by Primer3 (Rosen & Skaletsky, 2000). Dinucleotide repeats were not taken into consideration.

Potential microsatellite markers (‘Potentially Amplifiable Loci’ or PAL) were thus developed for each cultivar separately and the results were ordered (in Excel) by number of different alleles across genotypes, in decreasing order. For the top 100 those markers were excluded that had more than four different length variants per individual tetraploid cultivar. A set of 48

potential markers with ten or more predicted alleles were picked from the top of the list (predicted number of alleles among the 11 cultivars: 24 to 16).

For transcriptome assembly high quality reads were filtered using Prinseq (Schmieder and Edwards 2011). The paired-end reads were merged using FLASH (Fast Length Adjustment of Short Reads to Improve Genome Assemblies; http://www.cbcb.umd.edu/software/flash), producing a read span of 144.6 + 37.6 bp to 162.2 + 53.4 bp, average 152.4 bp. Assembly was done using Trinity (Grabherr et al. 2011). The potential markers were screened for duplicates by blastn of the primers against the transcriptome of one of the genotypes, cultivar Red New Dawn, as well as against the genome sequence of Fragaria vesca. The screening against Red New Dawn identified both duplicate markers that shared forward or reverse primers as well as duplicate markers for which the primer sequences did not overlap.

Validation

Forty eight potentially highly polymorphic microsatellite markers were tested by genotyping the 11 cultivars (Table 2). Amplification reactions were performed in 10 µl containing 8 ng DNA, 5 µl multiplex kit (QIAGEN, Germany) and 4 pmol of each forward (labelled) and reverse primer. Amplification was under the following condition: an initial denaturation at 95°C for 15 min. following with 30 cycles of 94°C for 30 sec, ramp 1°C/s to 50°C, 50°C for 30 sec, ramp 1°C/s to 72°C, 72°C for 120 sec and a final extension at 72°C for 10 min. One µl of 100x diluted PCR product was mixed with Hi-Di formamide (Applied Biosystems) containing GeneScan-500 LIZ size standard (Applied Biosystems) and run on an ABI 3730 DNA analyser. Output from the ABI platform was analysed with Genemapper 4.0 software (Applied Biosystems). For each microsatellite marker presence or absence of individual alleles were scored (dominant scoring).

Multigene markers

A high level of polymorphism may also be associated with multi-locus microsatellites and thus we tested whether an additional step of checking could be implemented. For this we used the predicted protein sequence derived from the cDNA sequence to search protein databases for the likelihood of dealing with a member of a multi-gene protein family by BLASTX (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the closely related strawberry genome.

Results

Microsatellite repeat and motif overview

Microsatellites with tri-, tetra-, penta-, and hexanucleotide repeats were identified among the sequences for each cultivar separately. Dinucleotide repeats were not analysed. The total number of reads with microsatellite repeats per cultivar varied from 259,749 in 'Adelaide Hoodless' to 341,719 in 'Princess of Wales' (Supplementary Table 1). All cultivars showed the same trend in motif frequency distributions; trinucleotide repeats were most abundant (65.1-69.3%), followed by tetranucleotides (16.3-20.5%) and hexanucleotides (9.3-11.6%). Pentanucleotide repeats were the least frequent motif type (4.8-5.4%).

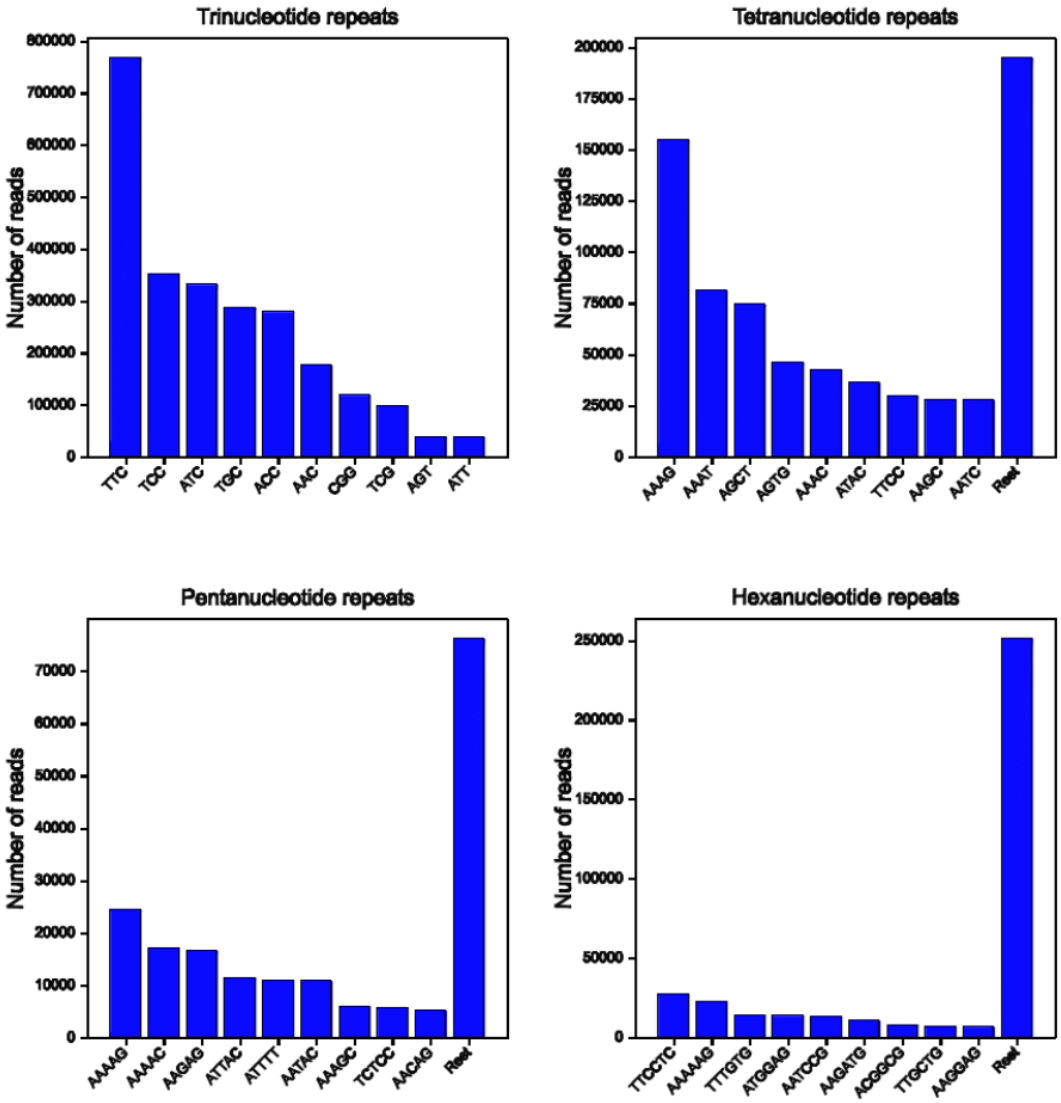

Figure 1. Number of reads for different tri-, tetra-, penta-, and hexanucleotide repeats motifs found in 11 garden rose cultivars.

Microsatellite marker prediction and primer development

With Primer3 we designed primers around each potentially amplifiable microsatellite repeat in each of the sequence reads. As our aim was to develop polymorphic markers, we sorted the read data based on the forward primer of the potential microsatellite marker, and selected primer pairs that corresponded to reads with multiple repeat length variants in each of the eleven cultivars, but not more that four different alleles per tetraploid cultivar. This ordering was a technically simple solution for the problem of identifying multiple alleles of the same locus among paired-end reads in which a relatively large proportion of the sequence information is taken up by simple sequence repeats (but with the risk of not combining all reads of one locus together, see below). Out of a total of 1797 developed markers 48 trinucleotide repeat microsatellite markers were taken from the top of the list.

Polymorphism testing for validation

The selected microsatellite markers were amplified in the 11 cultivars. Two did not give amplification. All other markers were polymorphic and allele presence/absence was scored and compared with the predicted number of alleles. In 10 markers more than four alleles per cultivar were amplified. A careful analysis of the electropherograms of these multi-locus microsatellites showed the occurrence of multiple allele patterns (with and without stutter bands), amplification success (strong and weak amplification), and/or differences in allele length (two groups of alleles that differed one or two repeat units within the group but 20-40 bp between groups, which in theory could be used as a tool for assigning alleles to different loci (not shown).

Thirty-six markers were putative single-locus markers, showing four or less clearly distinguished alleles per genotype. Analysis of their electropherograms did not detect any difference in amplification rate, stutter band pattern, nor shifts in allele lengths, which is consistent with a single-locus marker. They were all polymorphic, but upon close scrutiny ten of them were replicate markers that shared some of the primer sequences, and an additional eight were from replicate loci but did not share any primer sequence (see below). Hence, the net result was a set of 18 unique microsatellite markers, all highly polymorphic as they amplified between 6 and 20 different alleles each in the 11 tetraploid varieties (on average 11.7 different alleles per marker; Table 2). WGR44 has a large allele size range (between 117 and 295 bp). The effective number of alleles in a large set of varieties is unknown, but an approximation, by calculating it for these 11 varieties, gives values from 2 to 17.3.

For evaluation we compared the level of polymorphism with a set of 143 microsatellites, developed from genomic and EST sequences in rose (Rajapakse et al., 2001; Esselink et al., 2003; Yan et al., 2005; Kimura et al., 2006; Zhang et al., 2006; Hibrand Saint Oyant et al., 2008; Meng et al., 2009; Spiller et al., 2010) that were tested on the same set of 11 cultivars. All 143 markers have previously been successfully tested in various rose species and cultivars. After removing microsatellites that did not amplify in our set (10), had low amplification (1), showed no polymorphism (2) and multi-locus ones (23), the 107

polymorphic markers amplified on average 5.1 alleles per marker. The 24 most polymorphic markers of this set of 107 markers (16.8%) were used in the diversity study of Vukosavljev et al. (2013). These amplified on average 8.2 alleles/marker in the 11 cultivars. This comparison shows that our new set of highly polymorphic microsatellites have more alleles per marker.

Possible improvements to the strategy

We initially screened for duplicate markers by comparing the primer sequences of the selected markers in the list. This procedure, which should take into account reverse complement and slightly shifted primer sequences, can be done in Excel but it is not fully conclusive, as duplicate markers may have completely different sets of primer sequences. We found that the most straightforward and conclusive screening for replicate markers was to BLASTx the primer sequences against an assembly of the transcriptome of one of the genotypes. Replicate markers were identified by a hit to the same contig. In our test set of PALs with many alleles, 25 of 48 markers were replicates, of which 8 replicate loci that had no primer sequence in common. In comparison a BLASTn search against the related genome sequence of Fragaria vesca was much less effective. It only discovered eight of the 25 replicates, the others did not have primer sequence matches.

We tested whether we could have predicted which marker is multi-locus based on the number of sequence length variants observed. The prediction of the number of alleles per marker based on observed sequenced length variants was imprecise (Table 2). At a cut-off of three or more reads per length variant to predict an allele, the single-locus markers had 10-24 predicted alleles in the 11 cultivars, while 6-20 were amplified. The multi-locus markers were predicted to have 11-25 alleles, while 11-27 were amplified. Although the average number of amplified alleles of the single locus markers (11.6) was much lower than the average of the multi-locus markers across these cultivars (19), the overlap in the range was so large that a prediction of multi-locus markers based on overall number of length variants did not work. The same was the case when we used the number of length variants per cultivar. Of the eight markers with four or fewer length variants in every cultivar, five were multi-locus and only three were single-locus markers. Only one marker (WGR28) passed the more stringent threshold for a single-locus marker of maximally three predicted alleles in every cultivar. Thus, on basis of the predictive number of alleles no effective distinction can be made between single and multi-locus markers.

We also tested whether we could have distinguished single- from multi-locus microsatellites based on the type of genes in which they resided, using BLASTx against the related Fragaria vesca genome sequence. Some of the multi-locus markers indeed had hits with members of a superfamily or stress-associated proteins. For example, one of the markers that turned out to be multi-locus based on the banding patters, had hits with the R3H-associated superfamily. Additionally, another marker had highly significant hits with two different isoforms of the same protein (stress-associated endoplasmic reticulum protein 2-like

isoform-1 and -2). However, as only 14 (30%) of the repeat-containing contigs we tested had a hit with known genes, this selection criterion may not be very effective.

Discussion

An efficient strategy for polymorphic marker development

The main problem for developing microsatellite markers nowadays is not generating repeat-containing sequences, as next generation sequencing generates more repeat-containing sequences than needed, but it is the testing and selecting of those that are highly polymorphic as a marker, as this is still done manually. We have developed an efficient strategy in which we deploy next generation sequencing of multiple genotypes and select only those repeat loci for marker development that already show a range of different repeat lengths within the set of sequence reads. This selection does not predict the actual number of alleles precisely, but it proved to be very efficient for preselecting highly polymorphic markers (at least 6 and up to 24 alleles in 11 tetraploid garden rose cultivars).

The strategy makes efficient use of the strength of next generation sequencing, namely that sequencing is cheap, and that sequencing multiple genotypes does not require a lot more manual activities. Thus, we save on labour-intensive screening activities by generating sequences from multiple genotypes. For marker development many studies use next generation sequencing of multiple genotypes for SNP retrieval. Although many recent studies have been published on microsatellite marker development in which such sequences are mined (e.g. Cardoso et al., 2013; Lance et al., 2013), most studies do not make use of the full potential of the sequencing data in combination with multiple genotypes to predict the most polymorphic microsatellite markers. To our knowledge, only the recent study by Hoffman and Nichols (2011) utilized a similar approach to our study to identify polymorphic microsatellite markers from 454 sequences of the Antarctic fur seal (Arctocephalus gazella). Their approach rendered promising results (21 polymorphic markers from 50 tested), and had some success in predicting the number of alleles amplified from those found in the reads.

Prediction of allele number and comparison with SNP discovery

The prediction of the number of alleles based on variations in repeat length among our Illumina sequence paired-end reads was very imprecise, as both too many (e.g., WGR04, WGR05 and WGR11) and too few alleles (e.g., WGR31, WGR32) were predicted for some markers. Too many apparent alleles can be the result of mistakes made by the DNA polymerase during PCR amplification prior to next generation sequencing. The frequency depends partly on the repeat type, length, and whether the repeat is perfect or imperfect. This type of mistake is also visible as the relative number and height of stutter bands during detection on an acrylamide gel. One stutter band was present for WGR04 and WGR11, but

not for other markers for which too many alleles were predicted (e.g., WGR11). With regard to predicting too few alleles, two possible reasons can be envisaged. Firstly, only the minimum length of the repeats was known, as the repeats extended up to the end of one of the reads obtained in paired-end sequencing. Only sequencing technologies that produce longer reads can solve this problem. Secondly, our bioinformatics approach was simple and straightforward, but often did not collect all reads of one locus into one contig, as exemplified by the number of replicate markers. Here again longer reads would make it easier to optimise this step. Prediction of the number of alleles based on paired-end short reads is not an easy task. Cao et al. (2014) developed a Bayesian method, STRViper, to predict repeat length variation. Using data from Arabidopsis strains it outperformed all other methods.

Our results indicate that, even though the prediction of exact allele number was imprecise, the strategy for finding a set of polymorphic markers was very efficient, as all unique markers produced here are highly polymorphic (six alleles or more). A random subset of studies using traditional microsatellite marker development in polyploid species produced between 0 and 34% highly polymorphic markers (Supplementary Table 2) irrespective of the use of NGS sequencing. This indicates that it is efficient to sequence more genotypes at lower depth and select those repeats with a large number of predicted alleles for further marker development.

It is interesting to note that the imprecision in allele calling based on Illumina reads appears to be a smaller problem for selecting microsatellite repeats than it is for calling SNPs, where wrong calling usually means that it is a false SNP, and great care has to be taken to avoid them, e.g. by focussing on identifying reliable haplotypes (Tang et al., 2006; Shahin et al., 2012; Nijveen et al., 2013). Nevertheless, some mistakes are better avoided for both types of markers: polymorphisms between paralogs in gene families, and (in polyploids) polymorphisms between subgenomes. Taking all this into account is possible, as e.g. implemented in the IStraw90 90k Axiom array for strawberry, which excludes all SNPs between the four subgenomes of octaploid strawberry (Bassil et al., 2014), but this is time-consuming.

Replicated markers

The single most important screening step in our strategy is identifying replicate markers. More than half of our potential markers with many alleles were replicates. Apparently the sequence information in the short paired-end reads was insufficient to always link the markers of the same locus. Identifying the replicates worked best by BLASTx to a custom-assembled transcriptome. It even enabled identifying 8 replicate markers (32% of the duplicates) that shared no primer information. It was about three times as efficient as a BLASTx to the genome sequence of the related species Fragaria vesca, which did not even identify all replicates with overlapping primers, i.e., it was not better than careful manual screening of primers and reads that have the same repeat (provided one screens all variants in forward and in reverse complement directions). In our Strategy (Fig. 2) we have included the

transcriptome assembly therefore as an option to improve replicate detection. If labs have no possibility to do it, manual screening of replicates will do, as long it is accepted that some replicate markers will end up being tested before being identified from similar genotype patterns.

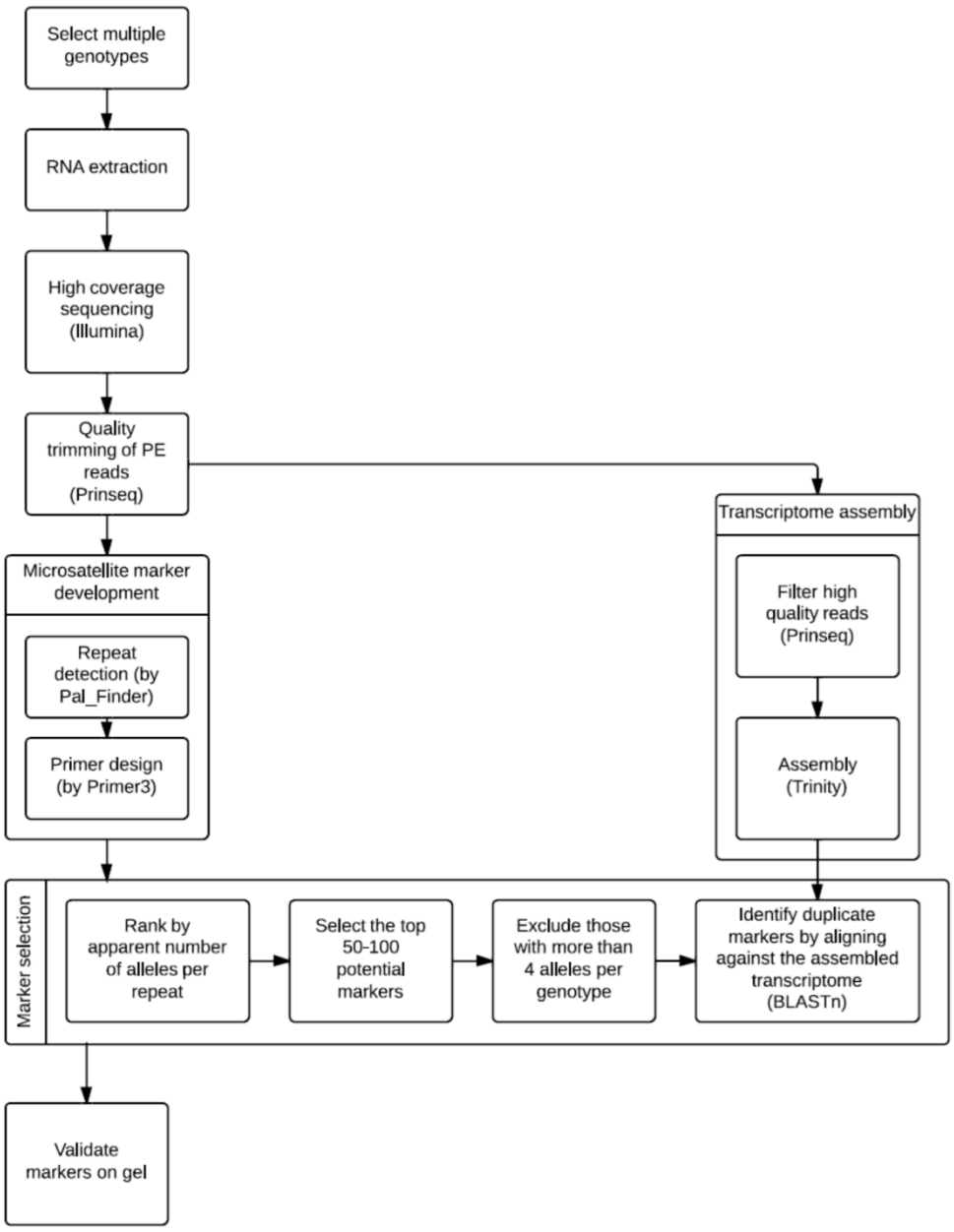

Figure 2. The strategy followed to efficiently develop highly polymorphic microsatellite markers.

Degree of polymorphism for repeats in coding regions

The rate of successful microsatellite amplification (46 of 48; 96%) in our study is higher compared to studies in tetraploid rose that were based on genomic DNA repeats. i.e., mostly located in non-coding DNA (Esselink et al., 2003 (89%); Kimura et al., 2006 (85%); Park et al., 2010 (92%)) or in other tetraploid species, such as cotton (86%; Han et al., 2004) and peanut (87%; Liang et al., 2009). The high level of successful PCR amplification of microsatellites from transcriptome sequences is attributed to their nature: their primers are developed from gene sequences (Saha et al., 2006).

It has been suggested that repeats in coding regions would be less polymorphic than those from random genomic sequences (Dufresnes et al. 2014). It should be noted that such a difference in degree of polymorphism only holds for a random set of repeats. As our strategy was aimed at producing a subset of highly polymorphic markers, one would not expect them to be substantially less polymorphic than a set of highly polymorphic nuclear DNA-based microsatellite markers. Indeed, the 24 most polymorphic markers selected from the range of publications on genomic DNA microsatellite markers in rose, as used by Vukosavljev et al. (2013), amplified on average 8.2 alleles/marker in these 11 cultivars, compared to 11.7 alleles for our set of gene-based markers. As the latter are located in genes and hence their flanking sequences are conserved, such markers are transferrable to related species and therefore form the marker of choice for comparative mapping, and also to tag functional and positional candidate genes to study their co-location with quantitative trait loci (QTLs) (Durand et al. 2010).

Multi-locus markers

In the set of 48 selected microsatellites, 10 amplified more than 1 locus. The presence of multi-locus microsatellites in this study may be attributed to the fact that microsatellites have been chosen on the basis of a maximum number of alleles. We have not tested our strategy on genomic DNA sequences. It may be feasible to use our strategy on genomic DNA in species with small genome size, or with the use of appropriate complexity reduction methods, as are also used for SNP development (Smulders et al. 2012). Note, however, that the degree of amplification of duplicated repeat loci in non-coding sequences is much higher than that of genes families in our RNA-seq approach, and such highly repetitive loci must be excluded. PAL_Finder, which was designed for identifying microsatellites in genomic DNA, counts the occurrence of primer pairs to be able to select against such repeat families (Castoe et al. 2012). We did not employ this counter here, but it may be used in a variant of our strategy.

Conclusion

Highly polymorphic markers can be developed very efficiently by screening transcriptome sequences from multiple genotypes. Such sequence data can be generated on purpose, but often they may be produced for SNP development and highly polymorphic microsatellites can be identified as additional markers. Few studies have used the polymorphism in reads, and we are not aware of any that used RNA-seq reads of multiple genotypes. The microsatellite length data obtained from Illumina paired-end reads are imperfect, but contain sufficient information to make microsatellite development more efficient, notably to develop highly polymorphic microsatellite markers. This strategy can also be used to select markers for specific parental combinations.

Acknowledgements

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Data Accessibility

Sequences in ENA (Hx2000034073).

Author Contributions

MJMS, MV, RGFV, PC and PA conceived the study. PC collected the plant material. MV and WPCVTW extracted RNA and made cDNA. GDE did the bioinformatics analysis. MV and WPCVTW tested the markers. MV and GDE analysed the data. MV, PA and MJMS drafted the manuscript. All authors have read and approved the final version.

Supplementary Table 1. Reads produced and microsatellite motifs found.

	Read length (bp)		Read span after FLASH (bp)		SSR motif				
Cultivar	Mean	S.D.	mean	S.D.	trinucleotide	tetranucleotide	pentanucleotide	Hexanucleotide	Total
Morden Centennial	88.9	7.1	144.6	37.6	206469 (65.4%)	60882 (19.3%)	15730 (5.0%)	32714 (10.4%)	315795
Red New Dawn	89.3	4.9	154.5	50.7	222844 (69.3%)	52368 (16.3%)	16634 (5.2%)	29864 (9.3%)	321710
Nipper	87.9	7.8		47.6	207117 (66.5%)	58867 (18.9%)	15697 (5.1%)	29074 (9.4%)	310755
Diamond Border	89.7	4.8	162.2	53.4	201437 (66.1%)	55135 (18.1%)	15798 (5.2%)	32442 (10.6%)	304812
Princess of Wales	89.7	4.5	158.6	56.9	223188 (65.3%)	62079 (18.2%)	16813 (4.9%)	39639 (11.6%)	341719
Graham Thomas	89.9	4.5	147.9	46.7	227187 (67.2%)	57338 (17.0%)	18133 (5.4%)	35261 (10.4%)	337919
J.P. Connell	89.1	5.4	151.2	42.4	211410 (65.1%)	65758 (20.2%)	15702 (4.9%)	32019 (9.8%)	324889
City of London	89.1	5.5	147.8	43.6	179979 (65.4%)	56373 (20.5%)	13205 (4.8%)	25625 (9.3%)	275182
Henry Kelsey	89.6	4.5	157.1	53.4	221380 (66.4%)	60685 (18.2%)	15927 (4.8%)	35402 (10.6%)	333394
Heritage	89.7	4.1	147.1	40.8	175573 (67.4%)	45352 (17.4%)	13022 (5.0%)	26621 (10.2%)	260568
Adelaide Hoodless	89.4	5.5	152.7	48.8	167451 (64.5%)	53548 (20.6%)	12949 (5.0%)	25801 (9.9%)	259749
Total					2244035 (66.3%)	628385 (18.6%)	169610 (5.0%)	344462 (10.2%)	3386492

Supplementary Table 2. Overview of studies reporting microsatellite development in polyploids.

Species	Initial set of SSR	Amplified no of SSRs	No of polymorphic SSRs	> 5 alleles in test set	Size of test set (individuals)	Reference	Multilocus polymorphic SSrs	Type
Cotton	2937	2273 (77.4%)	~ 30% [1]	Unknown	Unknown	Xiao et al., 2009		Allotetraploid
Rose	35	31 (88.57%)	22 (62.8%)	12 (34.3%)	46	Esselink et al., 2003	7 (20%)	Autotetraploid
Potato	16	12 (75%)	7 (43.7%)	5 (31.2%)	12	Ashkenazi et al., 2001		Autotetraploid
Orchid	15	Unknown	8 (53.3%)	2 (13.3%)	19	Nordström & Hedrén, 2007		
Zoysiagrass	156	Unknown	30 (19.2%)	9 (5.8%)	20	Ma et al., 2007		
Orchid	37	Unknown	8 (21.6%)	4 (10.8%)	30	Swarts et al., 2007		
Cotton	544	468 (86%)	99 (18.2%)	Unknown	2	Han et al., 2004		Allotetraploid
Tobacco	4886	94.6%	892 (18.3%)	57 (1.2%)	8	Tong et al., 2012		
Groundnut	200	Unknown	Unknown	16 (8%)	22	Mace et al., 2006	5 (21.7%)	Allotetraploid
Peanut	251	86.6%	26 (10.3%)	0%	22	Liang et al., 2009		Allotetraploid
Arachis ssp.	251	Unknown	221 (88%)	75 (29.9%)	16	Liang et al., 2009		Allotetraploid
Black locust	10	Unknown	7 (70%)	6 (60%)	18	Lian & Hogetsu, 2002	3 (30%)	
Coffee	18	Unknown	9 (50%)	0 (0%)	45	Baruah et al., 2003		
Rose	287	92%	183 (64%)	Unknown	47	Park et al., 2010		Autotetraploid

Chapter 5

High-density SNP-based genetic maps for tetraploid garden roses with separate homologous linkage groups

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High-density SNP-based genetic maps for tetraploid garden roses with separate homologous linkage groups

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Abstract

Even though garden roses are economically very important ornamentals, breeding is still mostly conventional, mainly due to tetraploidy and the lack of genetic maps and knowledge about the genetic base of important traits. Next-generation sequencing and sophisticated software for genetic analysis at the tetraploid level now enable wide application of SNP markers using genotyping arrays with 10,000s of SNPs. In rose breeding crosses with unintended parents are a regular occurrence and detection of these is not always straightforward, especially when the intended and actual parents are genetically related varieties. Moreover, in polyploids detection of off-type offspring often relies on detecting differences in allele dosage rather than the presence of new alleles. We developed a method to separate progeny into putative populations which share parents, even if one of the parents is unknown, using PCO analysis and sets of markers for which allele dosages are incompatible. Subsequently, SNP linkage maps were generated for a biparental and a self-pollinated population with one parent in common, using the WagRhSNP Axiom rose SNP Array. We confirmed a tetrasomic mode of inheritance for these crosses and created a starting point for QTL analysis for important morphological traits and future implementation of marker-assisted breeding in garden roses.

Introduction

Garden roses are tetraploid woody perennials from the genus *Rosa* (family *Rosaceae*, subfamily *Rosidae*). The cultivation of garden roses started 5000 years ago and since then roses have been bred not only for ornamental purposes but also for food (hips and petals), pharmacy, cosmetic industry, and horticultural purposes (Debener and Linde, 2009; Gudin, 2010; Smulders et al. 2011). The high popularity of garden roses, wide range of usages and intensive breeding has resulted in numerous cultivars and a high genetic diversity compared to cut rose. The latter, in combination with introgression from wild species (Vukosavljev et al., 2013; Zhang et al. 2013) indicate that some garden rose chromosomal regions may be

different (genetically distant) from those in cut rose. Considering this, a garden rose map would be useful for the study of rose genetics and for QTL analysis. A high-density map will enable localization and mapping of genes and QTLs for important traits, as a step towards marker-assisted breeding.

The genetics of tetraploids is complex as each individual has four sets of chromosomes. Under such circumstances one locus may have more than two different alleles in one plant, while any given allele may exist in the plant in up to four copies (the allele 'dosage'). Also the pairing of the four chromosomes may vary. While in autopolyploids sets of chromosomes are identical or highly related due to their origin (they originated from duplication of a single diploid genome), allopolyploids carry divergent sets of chromosomes, as a result of interspecific hybridization (Stift et al., 2008; Li et al., 2011). As a result of their origin and different levels of similarity of their chromosomes the mode of inheritance of tetraploids may be characterized as: disomic (preferential pairing of chromosomes), tetrasomic (random pairing) or intermediate (Jackson and Jackson, 1996). Completely homologous chromosomes of autopolyploids can have random pairing of bivalents or form quadrivalents in meiosis, while allopolyploids show preferential bivalent pairing of pairs of homologous chromosomes, with no pairing between homoeologous chromosomes. When quadrivalents are formed, (parts of) sister chromatids may end up in the same gamete during the second meiotic division. This phenomenon, double reduction, only occurs for a specific orientation of the chromosomes in meiosis and requires both the occurrence of quadrivalents and a crossover between the centromere and the end of the chromosome. Double reduction increases the production of homozygous gametes compared to what is expected under random chromosome segregation. The frequency of double reduction depends on the occurrence of quadrivalents and the probability of a crossover between the centromere and the observed locus, therefore segregation patterns are expected to vary among loci (Ronfort et al. 1998). The maximum frequency of double reduction is 1/7 (with pure random chromatid segregation), and 1/6 (with complete equational segregation) and can be reached under specific circumstances: if quadrivalents are always formed at meiosis, one effective crossover occurs between the locus and its centromere, and the recombined chromosomes migrate to the same pole at anaphase I (Stift et al. 2008).

At any locus, two out of four alleles per parent are transmitted to an offspring individual, six possible two-allele combinations per parent, giving rise to 36 possible combinations of alleles, increasing to 100 possible combinations if double reduction may also occur (Stift et al., 2008). This increases the complexity of genetic analysis of tetraploid progenies considerably, in comparison with diploids.

Depending on the parental dosage, we can distinguish between different segregation types of markers in a tetraploid biparental progeny. Parents may have dosage 0, 1, up to 4 (nulliplex, simplex, duplex, triplex, quadruplex) for a marker allele. In the segregating progeny all possible combinations (simplex x nulliplex, duplex x nulliplex etc.) can occur with many symmetrical types (E.g., for a biallelic marker triplex x quadruplex can also be written as simplex x nulliplex for the alternative allele). For the construction of a genetic map, the following dosages in the parents are most informative: SxN (simplex x nulliplex;

one dose in one parent, allele absent in the other), DxN (duplex x nulliplex; two doses in one parent, absent in the other) and SxS (simplex x simplex; in both parents one copy of a SNP allele is present). Any dosage can be converted into a maximum of 2 by using symmetry argumentation: quadruplex = nulliplex of the other allele, triplex = simplex of the other allele. With such a conversion QxT (quadruplex x triplex; four doses in one parent and three in another), DxQ (duplex x quadruplex; two doses in first and four doses in second parents), and TxT (triplex x triplex; three doses in both parents) markers could also be used for map construction.

TetraploidMap is currently the only software specifically developed for mapping in autotetraploids (Hackett and Luo, 2003) and although it can include Duplex x Nulliplex markers, limitations in the number of markers and the need for manual interaction and visual inspection limit its implementation (Voorrips and Maliepaard, 2012; Koning-Boucoiran et al., 2013). This indicates that development of more sophisticated software for dealing with tetraploids at each stage of genetics/mapping studies is needed. The first steps to improve mapping in polyploids have been made rather recently; fitTetra (Voorrips et al., 2011), software for dosage estimation of SNP markers, enables routine SNP scoring in tetraploids. In addition, recently developed software for simulation of gamete formation in tetraploids, PedigreeSim (Voorrips and Maliepaard, 2012), gives better insight into tetraploid meiosis and was used here for a new concept in mapping in tetraploids.

Genetic maps are built based on molecular markers or sequence reads (resequencing). In tetraploids multi-allelic markers, such as SSRs, may amplify up to four different alleles in a single genotype, while SNP markers are biallelic and can only distinguish two alleles. Thus SSR markers are more informative than SNPs (Yang et al., 2012), provided that they are scored co-dominantly, as dominant scoring provides less information on linkage based on repulsion between homologous chromosomes (Hackett et al., 2013; Hackett et al., 2014). Recently, development of highly polymorphic SSR markers (Vukosavljev et al. 2014) and techniques for determination of allele dosage of SSRs (Esselink et al., 2004; Vukosavljev et al., 2012) were improved, but it is still a laborious and time-consuming analysis. In contrast, SNP detection can be done for thousands of markers in parallel in one hybridisation step, and this compensates for the lower information content of SNPs. Recently the WagRhSNP array was developed for roses (Smulders et al. 2014; Koning-Boucoiran et al., in prep.), and the SNPs of this array were employed here.

Until now, genetic maps in the genus *Rosa* have been generated for several diploid (Debener and Mattiesch, 1999; Crespel et al., 2002; Yan et al., 2005; Dugo et al., 2005; Linde et al., 2006; Hibrand Saint Oyant et al., 2008; Remay et al., 2009; Kawamura et al., 2010; Spiller et al., 2011) and a few tetraploid (Rajapakse et al, 2001; Zhang et al., 2006; Gar et al., 2011; Koning-Boucoiran et al., 2012) cut, garden, and wild and hybrid rose populations. However, the coverage of these maps is relatively low. The average distance between markers is large, except in the consensus map (Spiller et al., 2011), where it was 0.88 cM when combining all information from five populations. Looking into the subset of tetraploid maps the average marker distance is between 2.4 cM (Gar et al., 2011) and 5.3 cM (Rajapakse et al., 2005). Likewise, on the basis of the expected map length for diploid roses

(500 cM; Yan et al., 2005), it is estimated that the length of a tetraploid rose map if the homologs are mapped separately should be around 2000 cM per parent (Koning-Boucoiran et al., 2012). Apparently, still a large part of the genome (38 – 46%) is not mapped in the current maps. In addition to this, the maximum gap size in the tetraploid maps varied from 16 cM (Gar et al., 2011) to 27 cM (Koning-Boucoiran et al., 2012) in a set of tetraploid maps which indicated that it is unclear whether all homologous chromosomes and chromosomal regions were tagged by markers. The use of SNP markers is crucial for improving map coverage and density, and for reducing the efforts and costs involved in producing such maps.

The aim of this study was to generate, for the first time, a high-density genetic map for garden rose and study the mode of inheritance as well as the genetics of a number of morphological traits. The mode of inheritance is a starting point in tetraploid genetics and therefore we studied whether the inheritance in garden roses is disomic, tetrasomic or intermediate. We made use of the recently developed WagRhSNP array for roses (Smulders et al. 2014; Koning-Boucoiran et al., in prep.), and the SNPs of this array were employed here, along with a number of SSR markers. The use of the tetraploid garden rose map is demonstrated by QTL mapping of a number of morphological traits: recurrent blooming and prickle shape. Recurrent blooming was introduced into European cultivars from Chinese roses. Roses with continuous blooming have a short juvenile period (6 to 8 weeks) and determinate growth compared to non-recurrent blooming cultivars (1 to 2 years) and indeterminate type of growth (Foucher et al., 2008). Recurrent blooming was reported as a monogenic trait and was mapped on rose chromosome 3 (Debener, 1999; Rajapakse et al., 2001; Crespel et al., 2002; Shupert and Byrne, 2007; Kawamura et al., 2010, Spiller et al., 2011). Prickles are epidermal outgrowths that have a protective function and in rose they pass through four stages of development (Kellogg et al., 2011). According to the International union for the protection of new varieties of plants (UPOV, 2010) rose prickle shape can be describes as straight, upward or downward curved. Presence/absence of prickels is determined by multiple genes located on chromosomes 2, 3, and 7 (Crespel at al., 2002; Koning-Boucoiran et al., 2012), but prickle shape has not been studied before.

Materials and Methods

Mapping populations

An F1 mapping population was obtained from a cross intended to be between two standard garden rose cultivars “Red New Dawn” (RND, mother) and “Morden Centennial” (MC, pollen donor). Genomic DNA was extracted from freeze-dried young leaves of 224 seedling plants plus the parents using the DNeasy Plant Mini Kit (Westburg, The Netherlands) following the protocol of Esselink et al. (2003). After running a set of 32 SSR markers we noticed that 47 offspring individuals amplified alleles not present in parental genotypes. These were assumed to be outcrosses with another parent and discarded from the population.

Analysis of segregation patterns in the population indicated that the intended parents were not the true parents of the offspring (presented in Results section) and that the initial population consisted of 2 subpopulations with one common parent (RND). Furthermore, the presence (SxN, SxS, DxD) or absence (DxN) of specific marker types indicated that one subpopulation was the result of selfing (RNDxRND), while the pollen donor of the other subpopulation was unknown (hypothetical pollen donor, HP). The plants were grown on their own roots in a greenhouse in Wageningen, The Netherlands, but also, for the purpose of phenotyping and QTL analysis, 85 genotypes of the RNDxRND and 61 genotypes of the RNDxHP population were grafted on *Rosa laxa* rootstocks. In total between 8 and 42 replicates per genotype were grown under standard procedure.

SSR markers

Microsatellite markers were chosen on the basis of their level of polymorphism. In total 144 SSR markers (Rajapakse et al., 2001; Liebhard et al., 2002; Esselink et al., 2003; Yan et al., 2005; Kimura et al., 2006; Zhang et al., 2006; Hibrand Saint Oyant et al., 2008; Meng et al., 2009; Spiller et al., 2010) were tested on the parental cultivars "Red New Dawn" and " Morden Centennial". The microsatellite reaction mixtures used for Li-Cor analysis (Li-Cor Biosciences, Lincoln, NE, USA) contained 10 ng genomic DNA, 2 µl 10x Tag PCR buffer, 0.2 mM of dNTP, 10 pmol of each (labelled) forward and reverse primer, 0.5 U of Tag polymerase, in a final volume of 20 µl. PCR conditions were initial denaturation at 94°C for 180s, then 35 cycles of 94°C for 30s, ramp to 55-58°C (1°C/s), 55-58°C for 30s, ramp to 72°C (1°C/s), 72°C for 60s and final extension at 72°C for 7 min. The 20x diluted amplification products were analysed on a Li-Cor 4200 or 4300 analyser (Li-Cor Biosciences, Lincoln, NE, USA). From the tested markers, 32 showed a high level of polymorphism and these were chosen to genotype all offspring. Genotyping of the offspring was performed on an ABI 3730 DNA analyser (Applied Biosystems, Foster City, California). Amplification reactions used were performed in 10µl containing 8 ng DNA, 5µl multiplex kit (QIAGEN, Germany) and 4 pmol of each forward (labelled) and reverse primer. Amplification was under the following conditions: an initial denaturation at 95°C for 15 min. following with 30 cycles of 94°C for 30 sec, ramp 1°C/s to 50°C, 50°C for 30 sec, ramp 1°C/s to 72°C, 72°C for 120 sec and final extension at 72°C for 10min. One µl of 100x diluted PCR product was mixed with Hi-Di formamide (Applied Biosystems) containing GeneScan-500 LIZ size standard (Applied Biosystems) and run on an ABI 3730 DNA analyser. Output from the ABI platform was analysed with Genemapper 4.0 software (Applied Biosystems). The allele dosage was scored co-dominantly (Vukosavljev et al., 2012).

SNP markers

For development of a tetraploid garden rose map we used the WagRhSNP Axiom SNP Array (Koning-Boucoiran et al., in preparation), which contains 68,893 SNPs probed

from both directions. Hybridisations of all offspring plants and the parents were performed by Affymetrix (California). Dosage scoring and genotype calling were done as described by Smulders et al. (2014). In brief, we used fitTetra (Voorrips et al. 2011) to score allele dosages using a mixture model approach. The two probes for each SNP were fitted as independent markers. The software was used to determine dosages for each of the SNPs and for both probes per SNP. Missing scores were assigned if the dosage of a sample could not be assigned with sufficient confidence (assignment probability smaller than 0.95) or if the total signal intensity was too low. SNPs were also rejected if they contained more than 40% missing values over all samples, or if they were (nearly) monomorphic.

We implemented a stringent selection so that map building would start based on a relatively small set of high quality markers. For each of the two probes of each SNP we tested if the quality was acceptable based on a combination of the following aspects: (1) the number of missing data, (2) the number of conflicting scores for replicated samples, (3) match of F1 progeny segregation to one of the 20 expected disomic or tetrasomic segregation patterns, and (4) match of parental dosages with the F1 segregation. We selected those SNPs where the two probes both passed these quality checks, where less than 4% of the F1 dosages differed between the probes and where both probes matched the same segregation pattern. For those SNPs we compared for each sample the dosage fitted for the two probes; in cases where the fitted dosage differed or where only one of the two probes resulted in a dosage score the dosage with the highest probability was selected.

The results of step 3, the match for segregation, prompted us to recheck whether all plants were really offspring plants of intended parents, since there was a very large number of SNPs for which the segregation in the progeny did not agree with the expectation based on the scored parental dosages. We generated PCO plots of the population offspring, based on pairwise genetic distances among the progeny calculated for 18653 SNPs that should have been monomorphic based on putative parental scores, but which not all were monomorphic in the progeny, using NTSYS 2.10 (Rohlf, 2000), a software for assessment and visualization of genetic relationships among genotypes. We found evidence of two putative populations: RNDxHP (Red New Dawn with an unknown genotype, here called Hypothetical Parent HP) and a selfing population RNDxRND. These two populations were then used for map construction.

Map construction

For our strategy to generate a genetic map, of interest are SNPs that follow a particular dosage in the parents: SxN (simplex x nulliplex; one dose in one parent, allele absent in the other), DxN (duplex x nulliplex; two doses in one parent, absent in the other) and SxS (simplex x simplex; in both parents one copy of a SNP allele is present).

In the first step we assigned simplex x nulliplex (SxN) segregating markers to linkage groups. Since, apart from expected low frequency occurrences of double reduction, estimates of recombination frequencies and LOD scores for SxN markers in coupling phase are the

same for tetraploids as for diploids (Hackett et al., 2013), JoinMap 4.1 (Van Ooijen, 2006) was used for preliminary LG detection. A Chi square goodness-of-fit test was performed on the segregation data of all markers and the markers deviating significantly from the expected 1:1 segregation were removed from the analysis. The SNPs were grouped to linkage groups and homologs on the basis of a logarithm of odds ratio (LOD) threshold. Within the preliminary groups the markers were ordered using the Maximum likelihood algorithm. The markers were ordered using the Kosambi mapping function.

The sets of homologous linkage groups were separated into four homologous chromosomes using the assigned phase and the recombination frequency estimates, where it was possible. The recombination frequency between markers at the same position on different homologs is expected to be 1/3 (Qu & Hancock; 2001), what corresponds to about 39.5 cM, according to the Kosambi mapping functions. If markers of different homologs are mapped together (as if they were on the same homolog), gaps of around 40 cM are expected between different homologous chromosomes; therefore these observed gaps help in the separation of homologs. Each parental map is expected to have a total of 28 linkage groups, corresponding to the seven chromosomes times four coupling phase linkage groups per chromosome. The expected average number of intervening chromosomal crossovers in a single generation is 0.01 (Lodish et al, 2004) and therefore the expected average size of the chromosomes is 100 cM. In order to connect homologs within parental genomes and chromosomes between parental genomes, we subsequently added to the SxN map so-called bridge markers that were segregating as duplex x nulliplex (DxN) and simplex x simplex (SxS). Recombination frequencies and LOD scores between DxN and SxS, as well as between those with SxN markers, in coupling phase were estimated by in-house scripts written in R (R Core Team, 2012). These then were used as so-called ’pairwise data files’ in JoinMap 4.1 (Van Ooijen, 2006). A grouping tree was generated on the base of a LOD threshold of 4. For map construction we used the regression algorithm and Kosambi mapping function.

We used the same linkage group numbering as was used for the Integrated Consensus Rose Map (ICM, Spiller et al., 2011). The assignment of linkage group numbers was done by two approaches. The chromosome assignment was done indirectly, through the genetic map of cut roses (Koning-Boucoiran et al., in preparation). For that cut rose map, in addition to the WagRhSNP Axiom SNP Array, 26 SSR markers were also used that previously had been mapped on the ICM map. In addition, for the SNPs indirect anchoring was done by comparing the position of the DNA sequence surrounding the SNPs in our map with that of the most similar sequence in the sequenced *Fragaria vesca* genome (Shualev et al., 2011), using BLASTN, selecting the highest hit and a cut-off E-value of 10^{-5}. This also enabled visualization of the synteny between rose and *Fragaria* using visualisation tool Circos (Krzywinski et al., 2009).

Mode of inheritance

The mode of inheritance of the population was studied to evaluate whether disomic, tetrasomic, or intermediate inheritance models best fit the segregation of the markers and whether this was different from chromosome to chromosome, and between both parents. Five different lines of evidence were used for inferring the mode of inheritance: 1) segregation of DxN SNPs, 2) presence of non-segregating DxN SNPs, 3) estimates of recombination frequencies of SxN SNPs on different homologs of the same chromosome, 4) segregation of DxD SNPs, and, finally 5) the occurrence of double reduction.

Approach 1: DxN markers. DxN markers have a different expected segregation under tetrasomic inheritance with random pairing than under disomic inheritance with preferential bivalent pairing (Table 1). For estimation of the mode of inheritance we tested if tetrasomic or disomic inheritance better fitted the observed segregation in the population, using a χ^2 test for expected segregation ratios under tetrasomic and under disomic inheritance (1:4:1:0:0 versus 1:2:1:0:0 for nulliplex up to quadruplex dosages).

Table 1. Expected segregation ratios for DxN markers under tetrasomic and disomic inheritance, for disomic inheritance under the two strictly preferential pairing orientations that are possible.

	Tetrasomic		**Disomic**			
	Random Pairing		Pairing AB/AB		Pairing AA/BB	
Gametes	Frequency	Ratio	Frequency	Ratio	Frequency	Ratio
AA	0.17	1	0.25	1	0.0	0
AB	0.67	4	0.50	2	1.0	1
BB	0.17	1	0.25	1	0.0	0

Approach 2: DxN non-segregating markers. Non-segregating DxN markers cannot be mapped, but their existence is evidence for disomic inheritance, as under tetrasomic inheritance they would always segregate in the progeny (Table 1). To infer the position of the DxN non-segregating markers, the contigs on which they resided were blasted against the *Fragaria vesca* genome sequence (Shulaev et al., 2011) to indirectly infer to which rose LG they belong.

Approach 3: Recombination between SxN markers on different homologs. According to Wu et al. (1992) at meiosis the scored allele of a SxN marker from a locus A will be transmitted to 50% of the gametes. If another locus B also has a SxN segregation, and under the assumption that the association between the two loci is random (unlinked or very distant), four classes of gametes will occur in equal frequency (Table 2). In such a case the ratio of non-recombinant and recombinant individuals in the progeny is expected to be 1:1; if the proportion of non-recombinant individuals deviates significantly from that of the recombinants, loci A and B are expected to be linked on the same chromosome (coupling phase) or on different homologous chromosomes (here called repulsion phase, although not exactly the same interpretation as in a diploid). Systematic association between two SxN loci can be estimated with χ^2 using the equation of Mather (1951):

$\chi^2=(a-b-c+d)^2/(a+b+c+d)$

in which a, b, c, and d represent the observed numbers of plants per category in Table 2. The value of χ^2 is compared to a χ^2 distribution with 1 degree of freedom. A significant result indicates that A and B are linked in either coupling or repulsion phase; the phase is then decided upon using the following criterion: a pair of SNPs is considered to be in coupling phase when r_1 is smaller than 0.5 and in repulsion when r_1 is larger than 0.5, where r_1 is the estimated recombination frequency under the assumption of coupling phase linkage. r_1 is calculated as the proportion of recombinants over the total number of individuals assuming coupling phase.

r_1= (b+c) / (a+b+c+d)

and it has the same value under tetrasomic and disomic inheritance.

Table 2. Expected frequencies of SxN markers A and B linked in coupling or repulsion phase in tetraploids

Gamete type	Expected frequency Coupling	Expected frequency Repulsion (random pairing)	Expected frequency Repulsion (bivalent pairing)	No. Observed
--	½ (1-r1)	⅙ + ⅙ r2	½ r3	a
A-	½ r1	⅙ + ⅙ (1-r2)	½ (1-r3)	b
-B	½ r1	⅙ + ⅙ (1-r2)	½ (1-r3)	c
AB	½ (1-r1)	⅙ + ⅙ r2	½ r3	d

r_1 - recombination frequency in coupling

r_2 - recombination frequency in repulsion under tetrasomic inheritance:

$r_2=[3(a+d)/(a+b+c+d)]-1$

r_3 - recombination frequency in repulsion under disomic inheritance; $r_3=(a+d)/(a+b+c+d)$

In diploids and allopolyploids with disomic inheritance, recombination between markers on homologous chromosomes occurs only by crossing-over of paired homologs, while recombination with markers on non-homologous chromosomes is at random. In contrast to this, in autopolyploids recombination in repulsion can occur by crossing-over of paired homologs but also by independent assortment of non-paired homologs and thus the frequency of repulsion-phase recombinants (R) consists of recombination frequency caused by crossing-over (Rc) and recombination resulting from independent assortment (Ri)

R= Ri+Rc = (h-2)/[2(h-1)]+ r_2/(h-1) = 1/3 + 1/3*r_2

where h represents the number of homologues, 4 for a tetraploid (Qu & Hancock; 2001). Practically, Rc is a variable parameter which depends on the genetic distance and Ri is a

fixed value which depends on the ploidy level. The Ri value for autotetraploids is 0.33 (under random pairing of two pairs of two homologous chromosomes, two markers will be on non-paired homologs in two out of three possible pairings. Random assortment leads in only half of these cases to a recombinant individual, therefore 1/3 is the expected proportion of recombinants by random pairing with independent assortment). Furthermore, if a pair of markers in repulsion phase is completely linked, r2, and consequently Rc will have a value of 0 and all recombinant progeny will occur through independent assortment (R=Ri) and is expected to be 1/3:

R= Ri+Rc = (h-2)/[2(h-1)]+ r2/(h-1) = (4-2)/[2(4-1)]+0/(4-1) = ⅓+0 =0.33

In that situation, as Ri is dependent on the ploidy level, the calculation of h may be indicative for detecting the type of pairing. Namely, under strict multivalent pairing (tetrasomic inheritance) of tetraploids h will have a value of 4, while under strict preferential pairing (disomic inheritance) h will be close to 2.

In the case that two markers in repulsion phase are not linked (r2=0.5) the expected frequency of repulsion phase recombinants (R) is 0.5

R= Ri+Rc = (h-2)/[2(h-1)]+ r2/(h-1) = (4-2)/[2(4-1)]+0.5/(4-1) = ⅓+⅙ =0.5

On the other hand, if two markers from repulsion phase are completely linked (r2=0) in diploids and tetraploids with complete preferential pairing (allopolyloids), the expected frequency of repulsion phase recombinants (R) is 0:

R= Ri+Rc = (h-2)/[2(h-1)]+ r2/(h-1) = (2-2)/[2(2-1)]+0/(4-1) = 0

while in case if a pair of markers are unlinked (r2 = 0.5), R will be 0.5

R= Ri+Rc = (h-2)/[2(h-1)]+ r2/(h-1) = (2-2)/[2(2-1)]+0.5/(2-1) = 0.5

To determine the mode of inheritance, a Binomial test was performed for every marker pair within a chromosome to determine whether the observed frequency of repulsion phase recombinants (R) was lower than 1/3 (H0: R=1/3, H1: R<1/3). The null hypothesis was rejected if the P value was lower than 0.01. If all R values are significantly larger than 0.33 ($r2 \geq 0$) for a chromosome, this suggests that there is completely random pairing of homologs, in agreement with tetrasomic inheritance, while if R is smaller than 0.33 and $r2<0$, preferential pairing (disomic inheritance) is indicated. A special situation arises when two SNP markers are known to be from the same contig and therefore should definitely be genetically very tightly linked. It is expected that the genetic distance between markers from the same contig is 0 cM. In this case the R of the markers in repulsion linkage can be taken as the Ri. The degree of preferential pairing can then be estimated using the equation $h'=2(Ri-1)/(2Ri-1)$, where $2 \leq h' \leq 4$. The smaller the h' (closer to 2), the stronger the preferential pairing. If these two markers are on different homologs, the observed repulsion recombination frequency should either be exactly 0 for disomic inheritance (apart from an occasional scoring error), or close to 1/3 (depending on sampling of pairs of homologs among

four possible homologs) for tetrasomic inheritance. Therefore these pairs of SNPs from identical contigs are highly valuable in polyploids to determine the mode of inheritance.

The level (ratio) of disomic and tetrasomic inheritance can also be estimated by observing the level of independent assortment and linkage between markers. The amount of independent assortment (Ri) is dependent on the ploidy level and on the mode of inheritance. In case of true disomic inheritance with disomic estimates the distribution of -10logP values of markers linked in coupling and repulsion phase is equal, while this is not the case if in reality there was tetrasomic inheritance (Figure 1), where much lower levels of significance of association are expected for markers in repulsion. In order to estimate the level of disomic versus tetrasomic inheritance we plotted -10log value for a Chi-square linkage test and -10logP value for a Chi-square independence test for both coupling and repulsion phase in Genstat 16 (VSN International; 2013). Much lower significance is expected for pairs of repulsion phase linked markers than for coupling phase linked markers under tetrasomic inheritance, while under disomic inheritance the distribution of significance levels is expected to be identical.

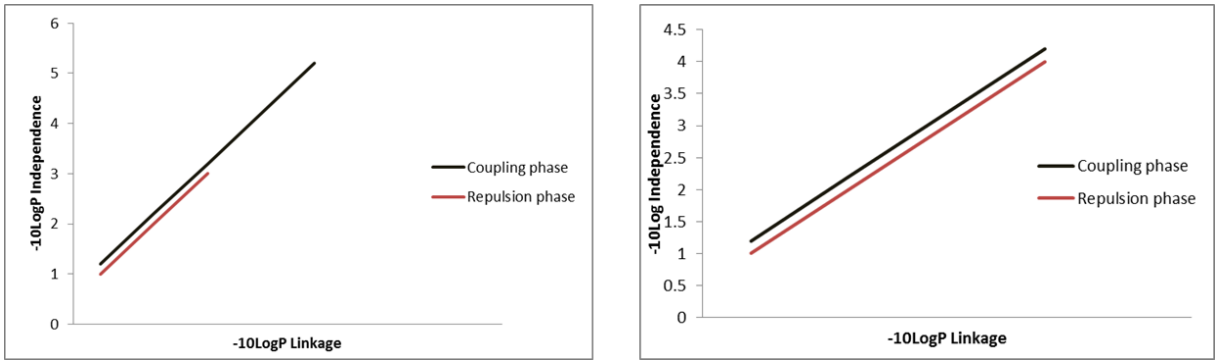

Figure 1. Expected distribution of significance values of recombination frequencies for markers in coupling and repulsion phase (estimates under a disomic model) for A) tetrasomic and B) disomic inheritance

Approach 4: DxD Markers. Like DxN markers, DxD markers segregate differently under tetrasomic inheritance with random pairing than under disomic inheritance with preferential bivalent pairing . For estimation of the mode of inheritance we tested if tetrasomic or disomic inheritance better fits the observed segregation in the population, using a $\chi 2$ test for expected segregation ratios under tetrasomic and under disomic inheritance (1:8:18:8:1 versus 1:4:6:4:1).

Approach 5: Double reduction. Double reduction is a specific phenomenon associated with the formation of quadrivalents in meiosis and thus can be used as convincing evidence of tetrasomic inheritance. For detection of double reduction and estimation of its frequency we used SxN markers. In absence of double reduction only nulliplex or simplex allele dosages are expected in the F1 progeny, so observation of duplex allele dosages, especially in

multiple markers on the same homolog fragment in an individual are indicative of double reduction. Thus all duplex dosages from SxN markers were scored over individuals and chromosomes. Duplex scores were only taken into consideration as evidence of double reduction when at least three consecutive markers with duplex scores from SxN markers were observed for a particular individual and a particular homolog. Isolated occurrences in the middle of the chromosome were not taken as evidence of double reduction but regarded as misscores. The frequency of double reduction was expressed as the fraction of occurrences per individual for a certain chromosome.

QTL mapping

A field experiment was carried out in Mali Iđoš, Serbia (lat. 45°42'30"N; long. 19°40'2"W). For the purpose of QTL analysis 85 genotypes of the RNDxRND and 61 genotypes of the RNDxHP population were grafted here on *Rosa laxa* rootstocks in June 2012. Between 8 and 42 plants per genotype were successfully grafted and grown under commercial production conditions. The experimental design was not randomized due to logistics, but the experimental plot was part of a large rose field, placed in the middle of the field (Figure 2). Two important morphological traits: recurrent blooming and prickle shape were evaluated during spring and summer 2013. Recurrent blooming was evaluated based on multiple observations of the presence (1) or absence (0) of flowers and buds during summer and fall 2013. Prickle shape was evaluated for each plant at the stage when the prickle reached complete development, as straight (1) or downward curved (0), while upward curved prickles were not detected.

Figure 2. A field experiment carried out in Mali Iđoš, Serbia (lat. 45°42'30"N; long. 19°40'2"W). For the purpose of QTL analysis 85 genotypes of the RNDxRND and 61 genotypes of the RNDxHP population were grafted here on Rosa laxa rootstocks.

QTL analysis was performed by using analysis of variance on both presence/absence and dosage per marker and by regression analysis per marker of the trait on the allele dosage. These analyses were done in R 2.12.2 (R Core Team, 2012). Significance thresholds were estimated from a permutation test with 1000 permutations of the phenotypic trait data and from running simulations with random normally distributed data. The threshold was calculated from the 95-percentile of the distributions of test statistics. Since the thresholds for both recurrent blooming and prickle shape based on phenotypic (4.1) and simulated data (3.9) did not differ much, 4.0 was used as the threshold value for significance. The proportion of the total phenotypic variance among genotypes explained by a marker was estimated by R2.

If two QTLs were found, a multiple regression approach was performed using GenStat 16 (VSN International; 2013), following the model:

$y=\mu+M1+M2+e$

in which y represents the phenotypic trait, μ represents the expected mean, M1 and M2 represent main effects of tentative QTLs at marker positions and e represents statistical error.

Results

Method to distinguish subpopulations based on SNP scores only

After running a set of 32 SSR markers (Appendix 1) we noticed that 47 offspring amplified 1-3 alleles not present in parental genotypes. These 47 offspring were assumed to be outcrosses with another parent and discarded from the population. However, the remaining 177 offspring plants amplified SSR alleles that were present in the parents, but the amplification rates for some alleles were variable and quantification of allele dosage (according to Vukosavljev et al. 2012) was difficult. Many SNP markers also did not segregate as expected based on the parental genotypes (Table 3).

Only a small fraction of the markers from each category of the mother, “Red New Dawn”, fitted the expected segregation ratios, while the ratios for fitting markers in the pollen donor, “Morden Centennial”, showed even larger irregularities. Additionally in the set of SxN markers there were many missing values over markers and individuals, mainly caused by triplex and quadruplex allele dosages that had to be removed. Comparison of the genotyping results with those of the K5 cut rose F1 population (Koning-Boucoiran et al., in preparation), generated using the same WagRhSNP Axiom array, indicated that not only the garden roses had many more missing values (19.8% compared to 4.8% in cut rose) but also that a smaller number of markers had passed the quality criteria (3893 compared to 6161 in cut rose; Koning-Boucoiran, personal communication). Reports on DNA quality indicated that the amount of DNA in the cut rose population was larger compared to the garden rose

population. Explanation for differences in efficiency between two populations may be due to variations in DNA amount or to biological reasons, such as: aneuploidy, diversity, outcrossing, etc. As most signal intensities for both populations were within acceptable ranges this suggested that the problem was not due to differences in DNA amount but possibly due to outcrosses not belonging to the intended cross RNDxMC.

Table 3. Overview of SNPs that were selected for mapping using the whole set of 177 offspring. A χ^2 test indicated that only few of the available markers fitted the expected segregation in the progeny.

SNP type	(♀)		(♂)	
	Available	Fit expected ratio	Available	Fit expected ratio
SxN	2688	1490	1205	211
DxN	2604	2	14	0
SxS	5198	304	5198	304

Considering that the garden rose population contained clearly visible offspring that were the result of outcrossing events with other parents, and on the presence of 47 plants with SSR genotypes not found in the parents, it was possible that the remaining ‘population’ contained more of such plants, but that these could not easily be spotted based on the SSRs alone. We therefore went back to the SNPs that had been filtered away. To test the parentage of “Morden Centennial” we selected markers for which the mother plant “Red New Dawn” was nulliplex or quadruplex for a specific SNP. If one parent is nulliplex, a progeny individual can inherit at most two copies of the alternative allele from the second parent. Progeny with triplex or quadruplex allele dosages were flagged. On the other hand if a parent was quadruplex for a specific SNP, offspring can be duplex, triplex or quadruplex, and the nullliplex and simplex offspring were flagged. These rules should not be taken strict and some triplex (for a nulliplex parent) and duplex scores (for a quadruplex parent) may be allowed, as double reduction and occasional errors in quantification of dosage may occur. Evidence should not come from a single marker in a single individual but from many markers indicating the same individual as putative outcross. Thus, off type scores were summed for each progeny plant.

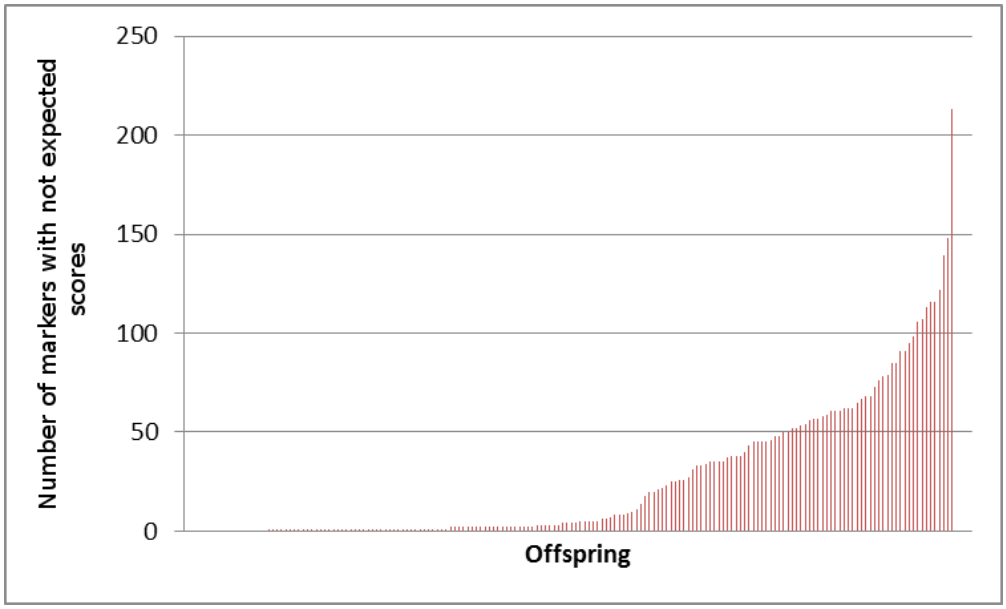

Figure 3. Population analysis using 18683 markers (9117 nulliplex and 9536 quadruplex in "Red New Dawn") that were expected to produce monomorphic markers in the offspring. Not expected dosages in the offspring based on parental genotypes (triplex and quadruplex for markers in nulliplex in one of the parents, and nulliplex and simplex for markers in quadruplex in one of the parents) were counted per offspring plant. Offspring plants have been ordered by the number of markers with not expected scores on the X axis.

In total we included 18683 monomorphic markers (9117 nulliplex and 9536 quadruplex) for "Red New Dawn" and counted the number of markers with non-allowed dosages (Figure 3): 103 offspring had between 0 and 10 non-allowed scores, 3 had between 11 and 19, while 71 offspring had between 20 and 213 non-allowed scores. Then to test the parentage on "Red New Dawn" we performed the same analysis for intended pollen donor "Morden Centennial" on a set of 17935 (8597 nulliplex and 9338 quadruplex) SNP markers (Figure 4). The corresponding bar chart for "Morden Centennial" is quite different, with few plant having not expected scores (which can be explained by double reduction). Such results indicated that our population may consist of more than one subpopulation.

Figure 4. Population analysis using 17935 markers (8597 nulliplex and 9338 quadruplex in "Morden Centennial") that were expected to produce monomorphic markers in the offspring. Not expected dosages in the offspring based on parental genotypes (triplex and quadruplex for markers in nulliplex, and nulliplex and simplex for markers in quadruplex in "Morden Centennial") were counted per offspring plant. Offspring plants have been ordered by the number of markers with not expected scores on the X axis.

To visualize the population structure we generated 2 PCO plots for all markers for which "Red New Dawn" (Figure 4a) or "Morden Centennial" (Figure 4b) were nulliplex or quadruplex. PCO plots indicated that there was no differentiation from the maternal side (plot based on markers that were nulliplex or quadruplex in "Morden Centennial"; Fig 5b), while the paternal PCO plot indicated two possible pollen donors (plot based on markers that were nulliplex or quadruplex in "Red New Dawn"; Figure 5a). Additionally, for one population the offspring is grouped together in PCO more closely, as if the plants were genetically more closely related (Figure 5a). Comparative analysis of the PCO output and the results of the analysis of non-allowed scores indicated that the same set of plants were separated in both analyses, indicating two paternal parents. Thus we divided the initial population into two populations: A (consisting of 103 offspring) and B (consisting of 74 offspring). In both populations the genotype of "Morden Centennial" could not explain the segregation in progeny and thus it was rejected as pollen parent. Population B was named RNDxHP, a cross of "Red New Dawn" and a Hypothetical Pollen Donor (HP). On the basis of genotype configurations of "Red New Dawn" and offspring the marker genotype for HP was reconstructed.

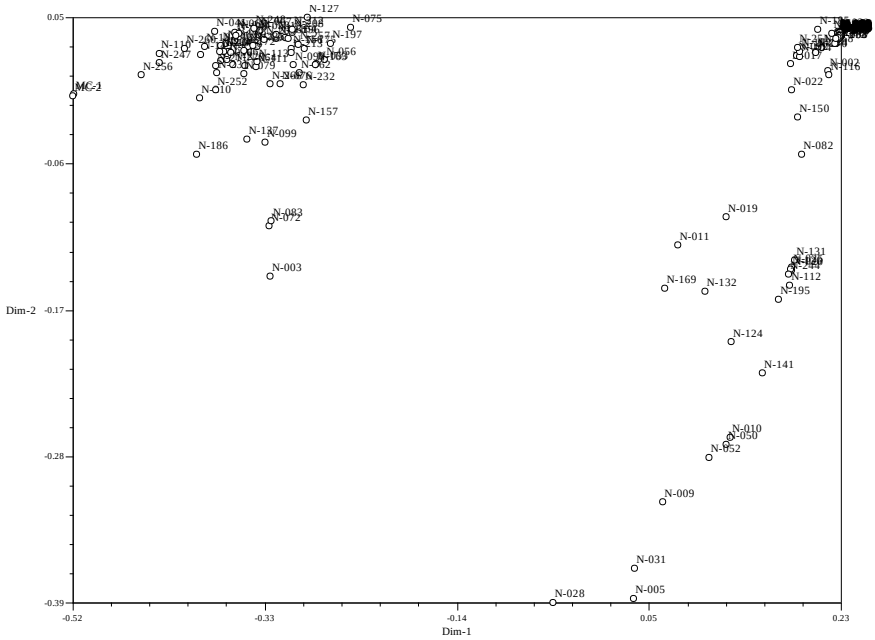

Figure 5a. PCO analysis of the mapping population using all simplex and quadruplex markers for "Red New Dawn" showing two separate clusters of offspring. The first principal coordinate explained 52%, the second 29% of the variation. Results indicate that the population consists of two populations and that 2 pollen donors have been involved in crosses: A (103 offspring) and B (74 offspring).

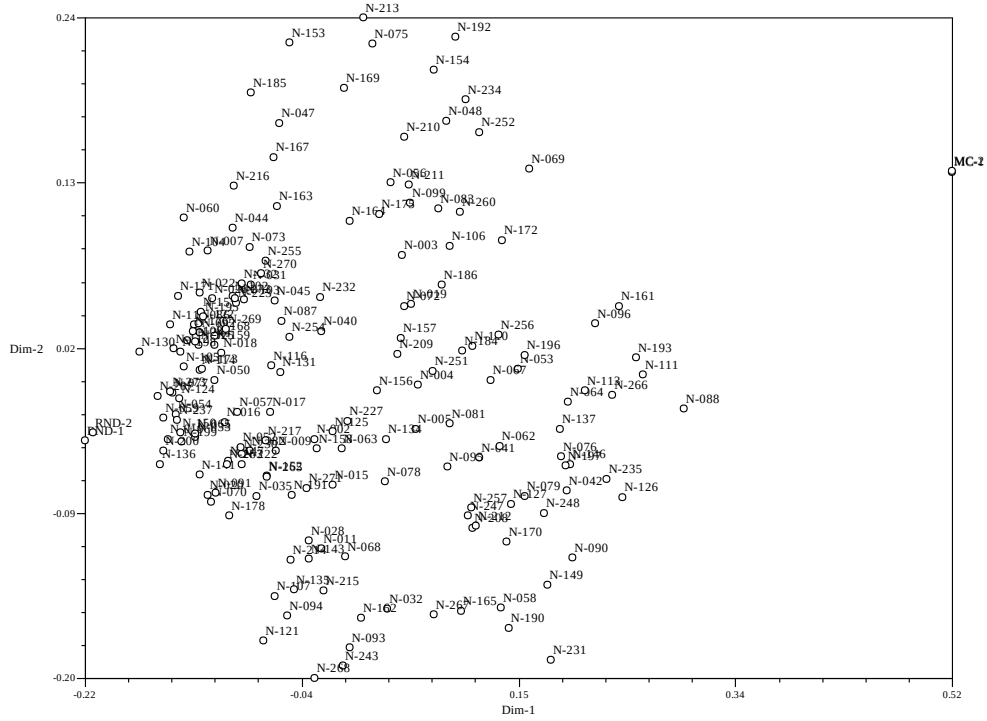

Figure 5b. PCO analysis of the mapping population using all simplex and quadruplex markers of "Morden Centennial". The first principal coordinate explained 56%, the second 27% of the variation.

For the larger of the two populations (A), the set of segregating markers that passed the quality criteria consisted of 13941 markers, of which 2455 NxN, 20 SxN, 3188 SxS, 2004 DxD, 3049 TxT, and 2686 QxQ markers (Table 4). For this population no DxN markers were detected. Since the vast majority of markers had the same inferred dosage for both parents, we hypothesized that this population must be the result of selfing. To confirm our assumption we tested whether all SNPs which are simplex for RND in the RNDxHP population are indeed SxS in this population. Of 1411 SxN markers in RNDxHP 1099 were also scored in the A population, and 1061 of them showed the SxS pattern. Of 943 SxS markers in RNDxHP population 689 were in also scored the A population and all of them showed the SxS pattern. Therefore we concluded that the A population of 103 individuals originated from selfing of RND and named it RNDxRND. To further check this, we analysed the segregation and position in the *Fragaria* genome of the 38 remaining markers which did not show a SxS pattern. The distortion of expected segregation (1:2:1:0:0), might be explained with skewed segregation and in this case it is expected that skewed markers are from the same region. Furthermore, if the population is a result of selfing, segregation of few markers which deviate from the expected segregation might result in scoring and thus they should be positioned randomly over chromosomes. From a set of 38 deviating markers 8 markers fitted 0:0:0:1:1 or 1:1:0:0:0, 26 followed 1:5:5:1:0 or 0:1:5:5:1 and 4 followed 1:4:10:0 or 0:0:1:4:1 segregation. Additionally, in this set of 38 deviating markers 14 had a large number of missing scores (between 20 and 54). Blasting against related genome positioned them on *Fragaria* chromosomes 2, 3, 4, 5, 6, and 7. Additionally, we detected a significant loss of heterozygosity in the progeny of RNDxRND compared to the heterozygosity in RND (0.55 versus 0.69; t=6.7, P=0.001), which is expected when selfing occurs.

Table 4. Frequency of different categories of SNP markers in population RNDxRND

Marker category	NxN	NxS	NxD	NxT	NxQ	SxN	SxS	SxD	SxT	SxQ	DxN	DxS	DxD	DxT	DxQ	TxN	TxS	TxD	TxT	TxQ	QxN	QxS	QxD	QxT	QxQ	Total
Number of markers	2455	0	0	0	0	20	3188	212	0	0	0	30	2004	27	0	0	0	268	3049	2	0	0	0	0	2686	13941

Therefore in the following we considered two different mapping populations with the maternal parent in common instead of a single mapping population.

Map construction

For the map construction of the RNDxHP mapping population SxN, SxS, and DxN markers were used. A total of 2513 SNPs, including 1411 SxN, 942 SxS, and 160 DxN markers were used for the construction of a genetic linkage map of RND, while for parental HP map 1760 SNPs (615 SxN, 942 SxS, and 203 DxN markers) were used. The resulting parental map for RND covers a total length of 1072.2 cM, with linkage groups varying in size from 12.5 to 94.4 cM (Appendix 2, Table 5). The RND map contains 1121 loci assigned to 23 linkage groups integrated over 4 homologs per chromosome, with median distance between markers of 0.96 cM and maximum distance between 2 markers of 17.5 cM. The

distribution of different marker types (SxN, SxS, and DxN) over chromosomes and their homologs is shown in Appendix 3. The 23 linkage groups have been assigned to the 7 chromosomes of the ICM map and for 5 chromosomes (1, 2, 4, 6, and 7) one homolog is missing. The HP map contains 522 SNPs (Appendix 4, Table 5) distributed over 18 linkage groups integrated over 4 homologs per chromosome spanning 738.3 cM (Table 5). The distribution of SxN, SxS, and DxN markers over LGs and their homologs is shown in Appendix 3. Only for chromosome 6 all four homologs have been detected; for chromosomes 2, 4, and 7 one homolog is missing, for chromosomes 3 and 5 two homologs, and for chromosome 1 three homologs are missing. The linkage group length varied from 9.1 to 107.6 cM with mean interval distance between loci of 1.4 cM and maximal distance between 2 markers of 13.2 cM.

Table 5. Map length and number of markers for the paternal HP and maternal RND map of the RNDxHP population and integrated map of the RNDxRND population.

LG	Homolog	RNDxHP -RND		RNDxHP -HP		RNDxRND	
		Length (cM)	Number of markers	Length	Number of markers	Length	Number of markers
LG1	*H1*	12.5	18	35.9	46	15.3	44
	H2	23.3	9				
	H3	28.2	7				
	H4						
LG2	*H1*	30.7	25	73.3	64	63.2	46
	H2	53.6	43	14	18	91.2	72
	H3	72.3	83	9.1	22	82.4	64
	H4					79.7	50
LG3	*H1*	58.6	68	107.2	63	115.3	102
	H2	76.3	86	62.5	66	98.2	261
	H3	53.3	102			64.8	99
	H4	36.8	30			34.4	43
LG4	*H1*	49.4	68	89.9	67	108.7	127
	H2	33.1	49	15.5	8	42.1	76
	H3	20.8	34	12.5	6	34.7	19
	H4					15.2	55
LG5	*H1*	67.4	50	71.8	39	107.6	138
	H2	52.1	54	27.1	31	91.4	65
	H3	23.2	29			87.7	53
	H4	19	16			118.2	46
LG6	*H1*	94.4	77	21.6	8	79.2	92
	H2	85.1	73	16	11	60.4	49
	H3	58.5	68	43.4	16	38.9	102
	H4			43	17	45.8	52
LG7	*H1*	68.2	46	32.3	14	84.5	141
	H2	22.3	34	42.5	19	31.9	28
	H3	33.2	52	20.6	17	71.5	66
	H4					74.1	40
Total		***1072.3***	***1121***	***738.2***	***532***	***1736.4***	1930

On the RNDxRND map (Figure 6), 1930 SNPs were mapped to 25 linkage groups (LG) integrated over 4 homologs per chromosome, spanning 1736.3 cM (Table 5). The length of the linkage groups varies from 15.2 to 118.2 cM and the average marker density is 0.9 cM and maximal distance between markers of 25.4 cM. On the RNDxRND map most of the markers are SxS, with the exception of 6 SxN markers that were mapped to LG 3 (Appendix 3).

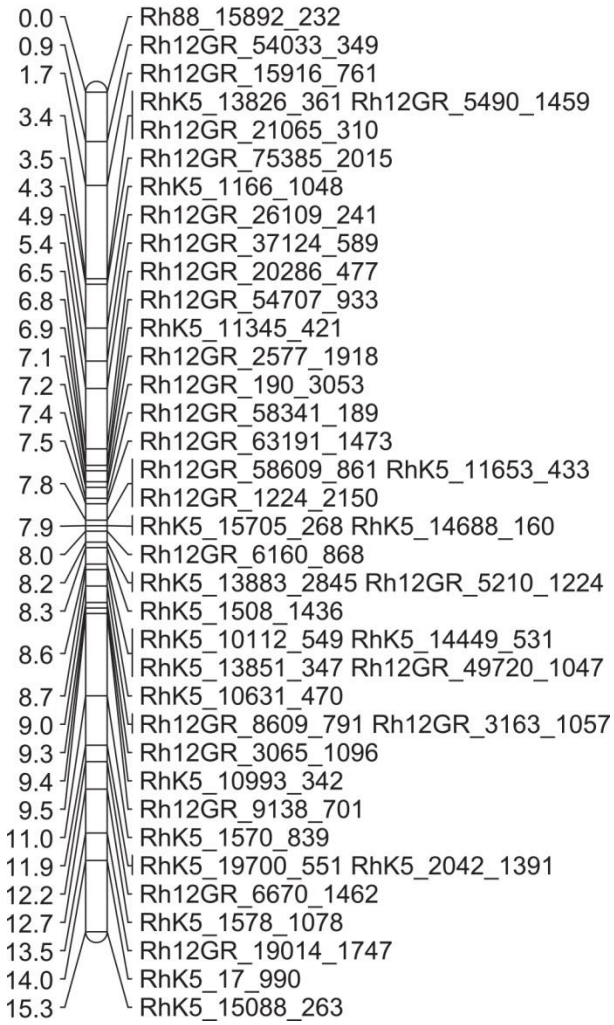

Figure 6. Linkage map of the tetraploid RNDxRND population constructed with biallelic SNP markers. Linkage groups are numbered from 1 to 7 following the ICM (Spiller et al., 2011), containing each 1, 2, 3, or 4 homologous groups (H). Markers are indicated to the right of each LG and map positions of markers (cM) are given to the left of each linkage group.

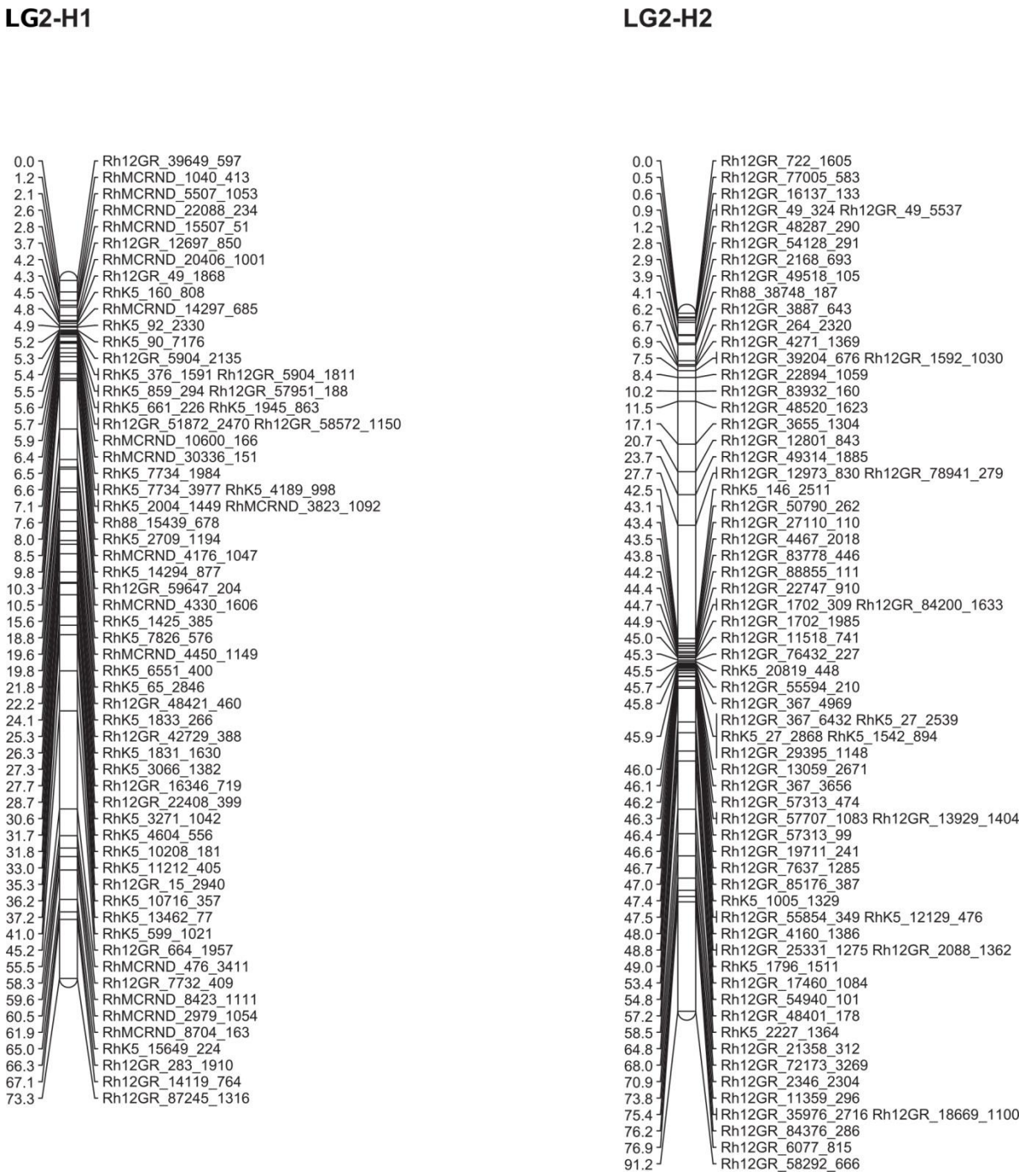

Figure 6 (*continued*). Linkage map of the tetraploid RNDxRND population constructed with biallelic SNP markers.

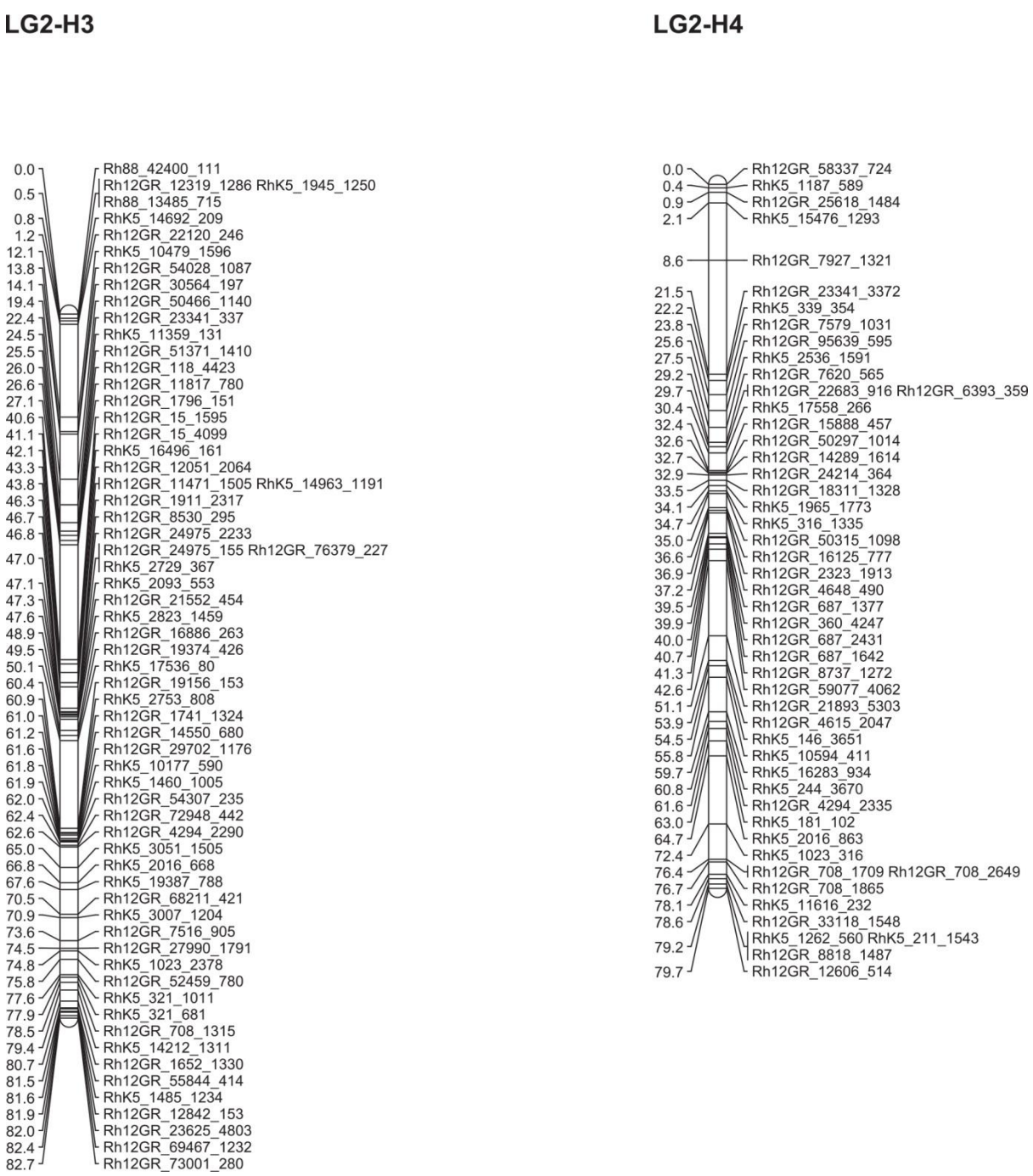

Figure 6 (*continued*). Linkage map of the tetraploid RNDxRND population constructed with biallelic SNP markers.

LG3-H1

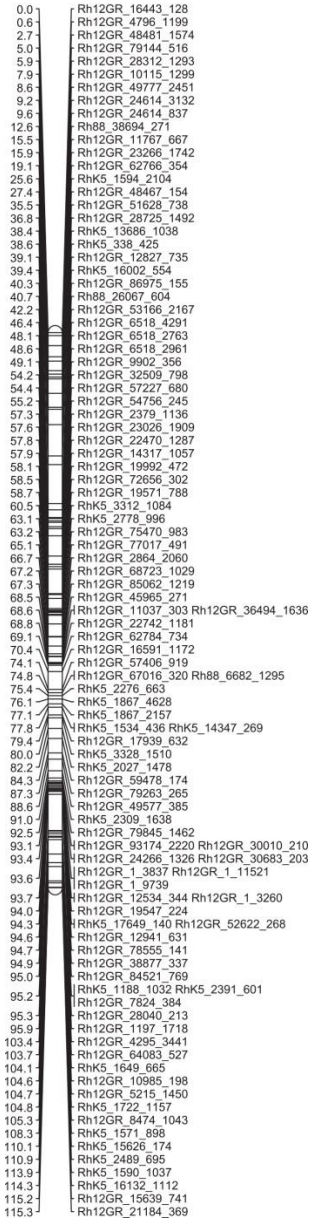

Figure 6 (*continued*). Linkage map of the tetraploid RNDxRND population constructed with biallelic SNP markers.

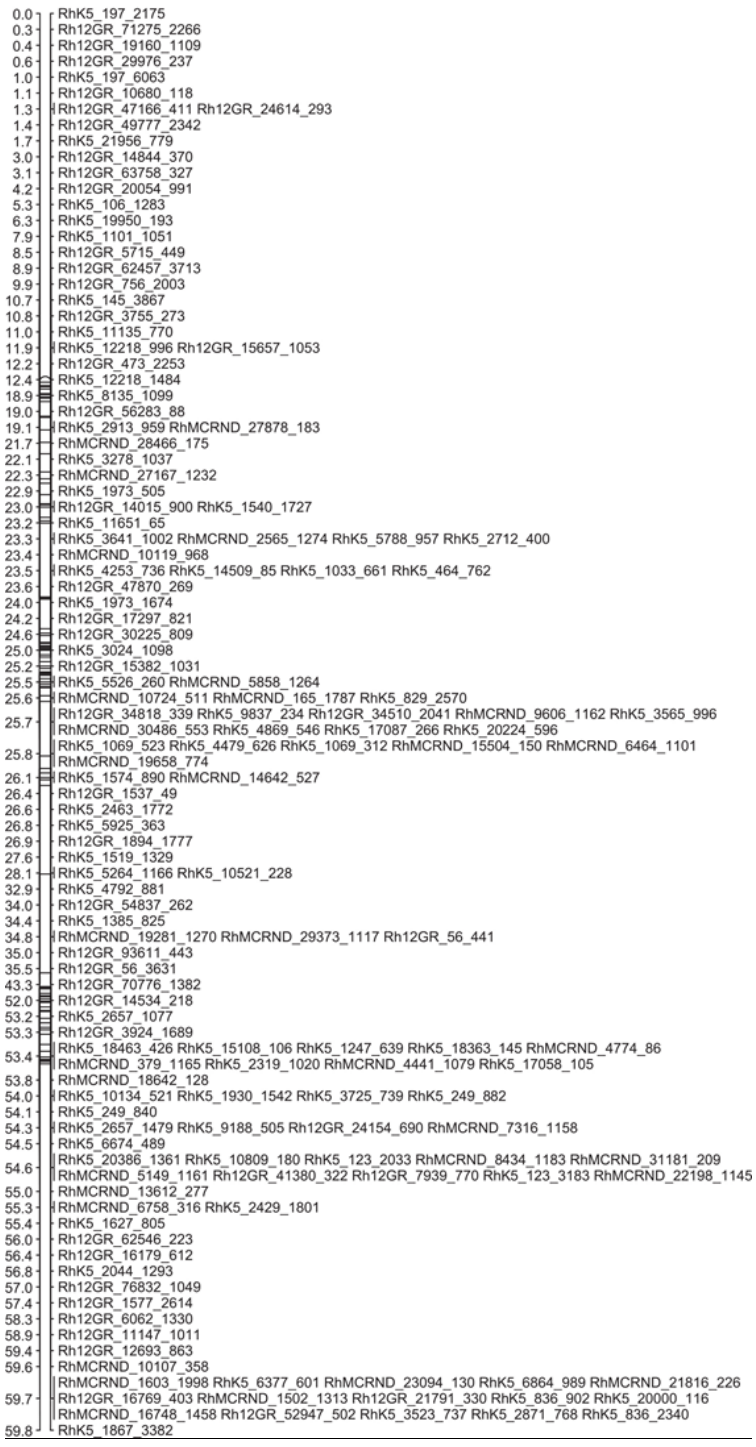

Figure 6 (*continued*). Linkage map of the tetraploid RNDxRND population constructed with biallelic SNP markers.

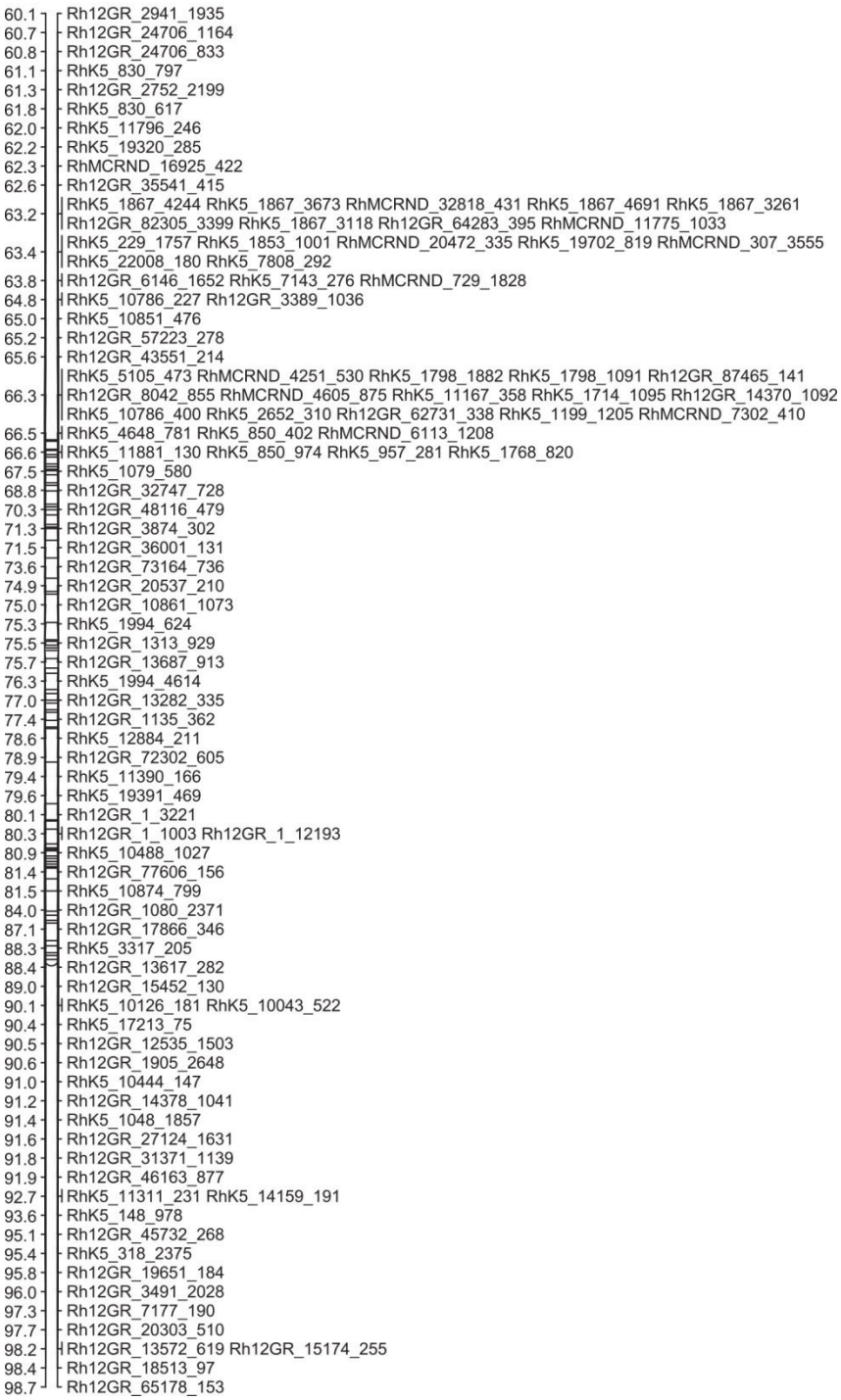

Figure 6 (*continued*). Linkage map of the tetraploid RNDxRND population constructed with biallelic SNP markers.

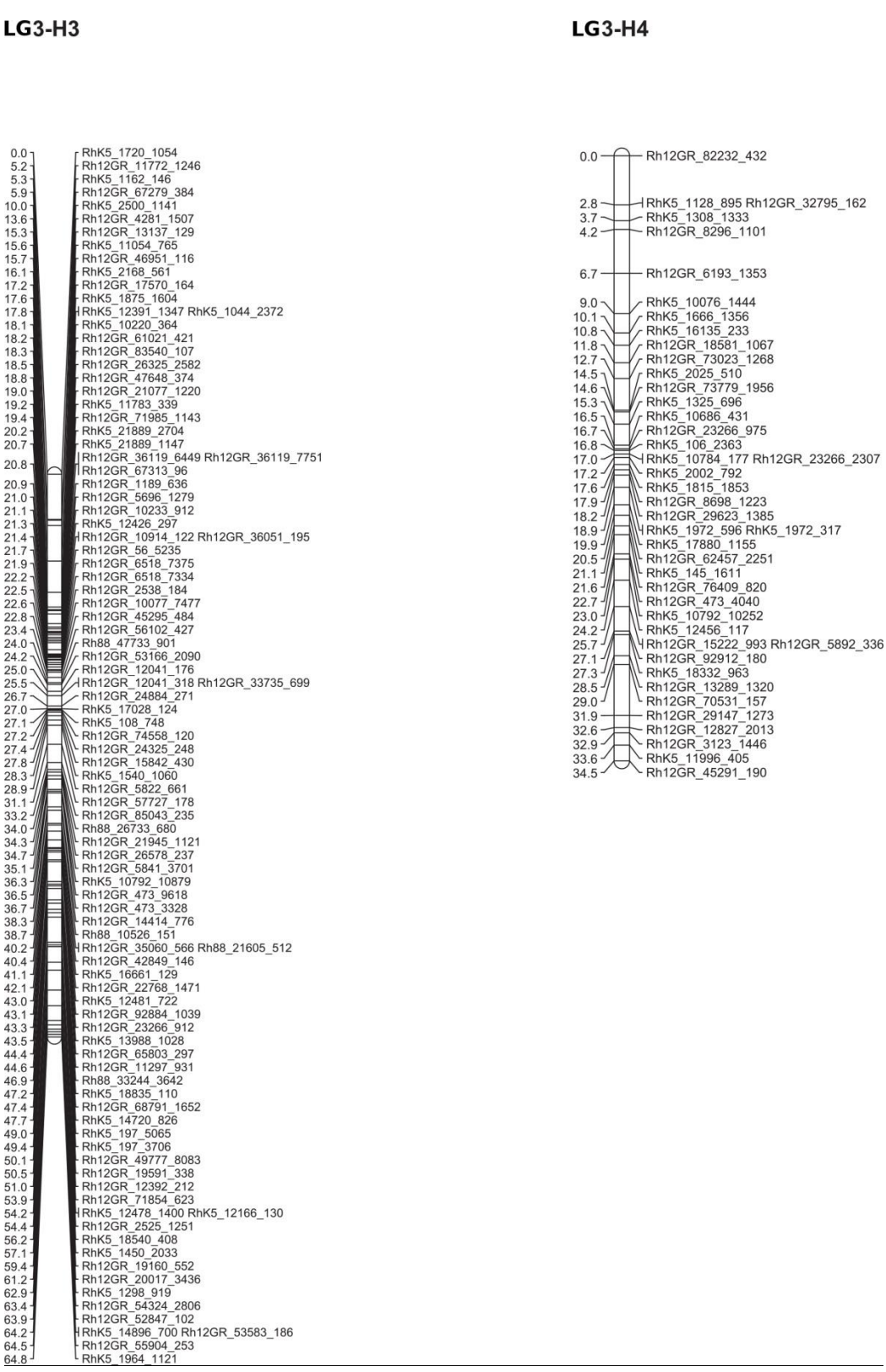

Figure 6 (*continued*). Linkage map of the tetraploid RNDxRND population constructed with biallelic SNP markers.

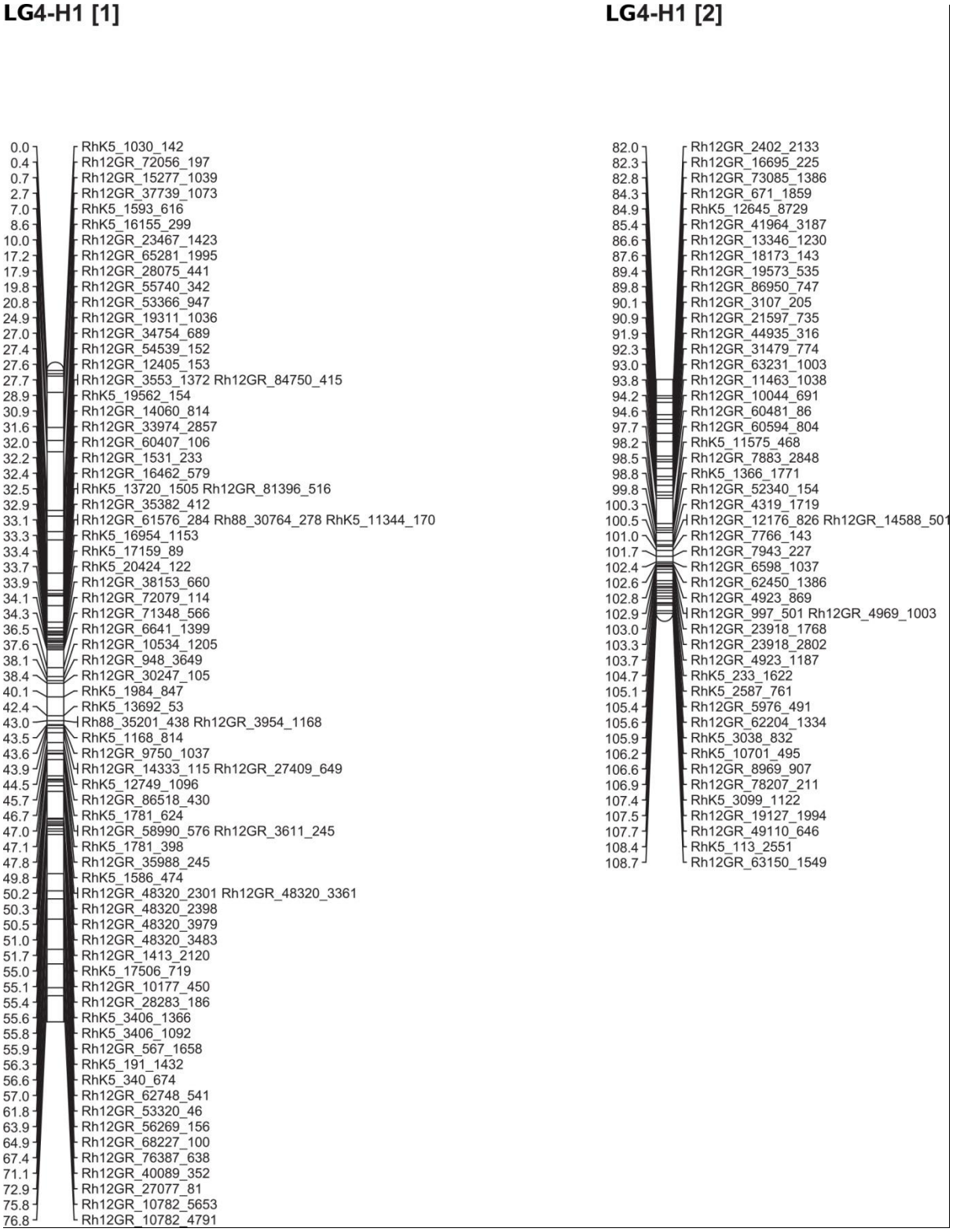

Figure 6 (*continued*). Linkage map of the tetraploid RNDxRND population constructed with biallelic SNP markers.

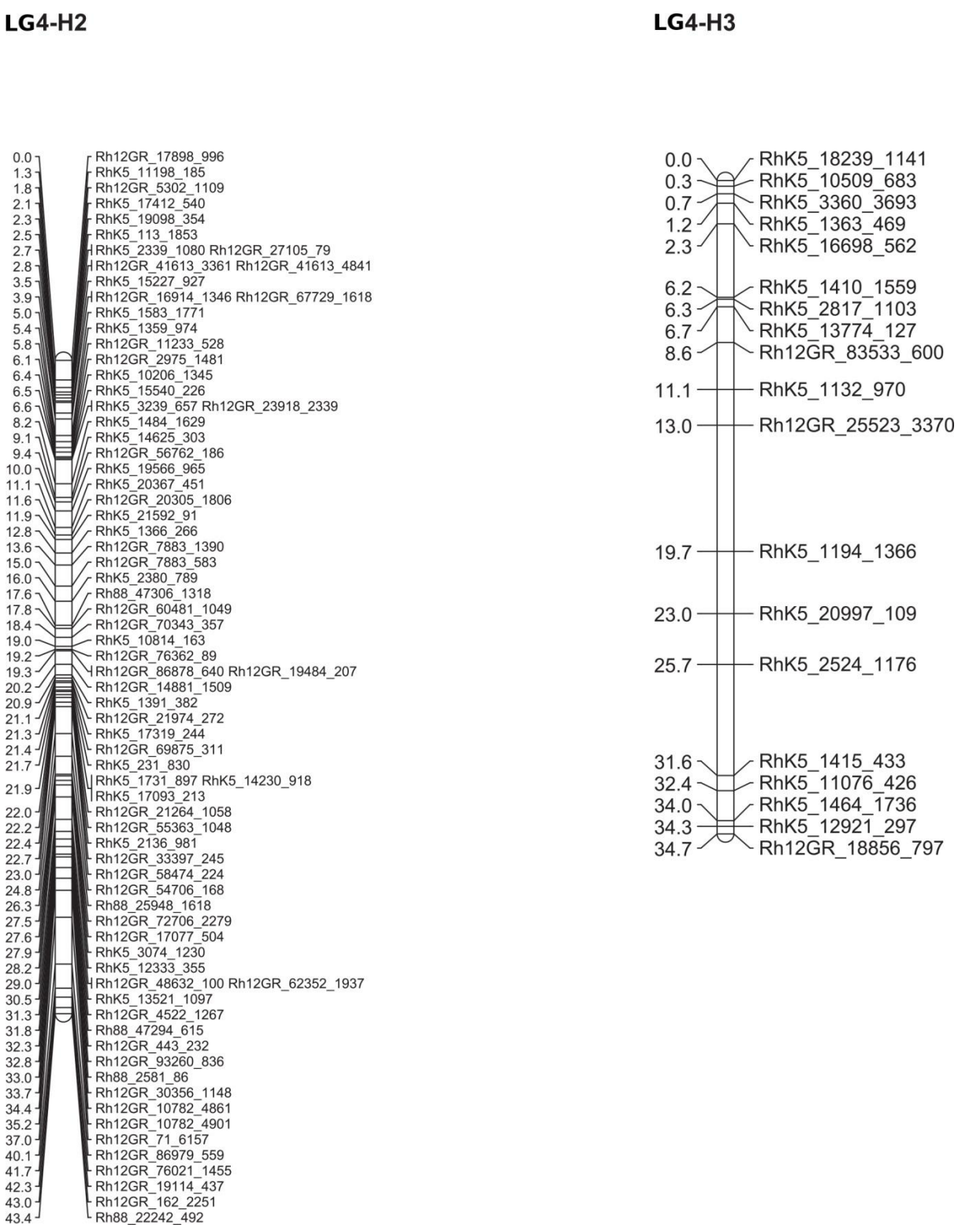

Figure 6 (*continued*). Linkage map of the tetraploid RNDxRND population constructed with biallelic SNP markers.

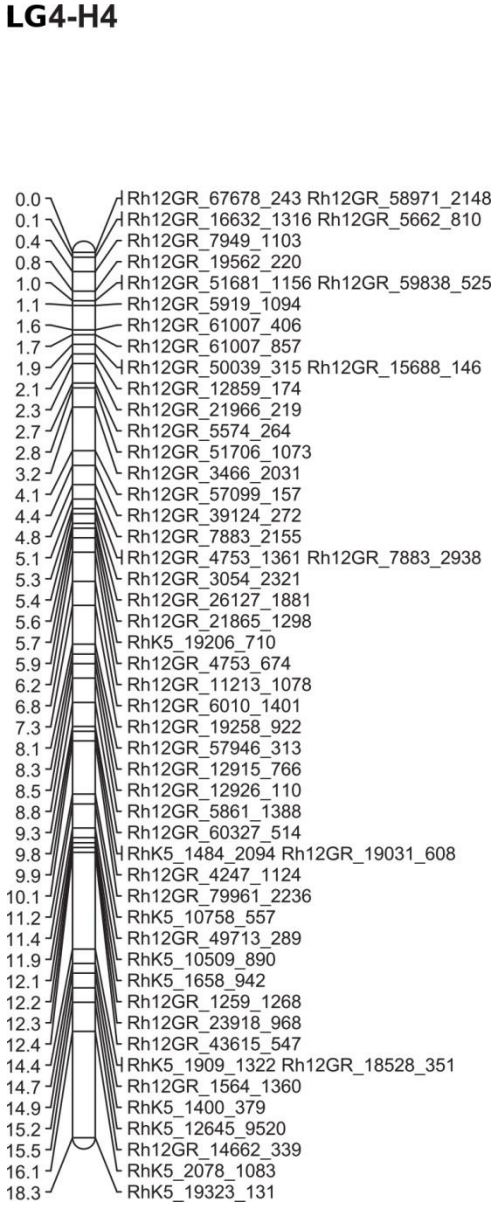

Figure 6 (*continued*). Linkage map of the tetraploid RNDxRND population constructed with biallelic SNP markers.

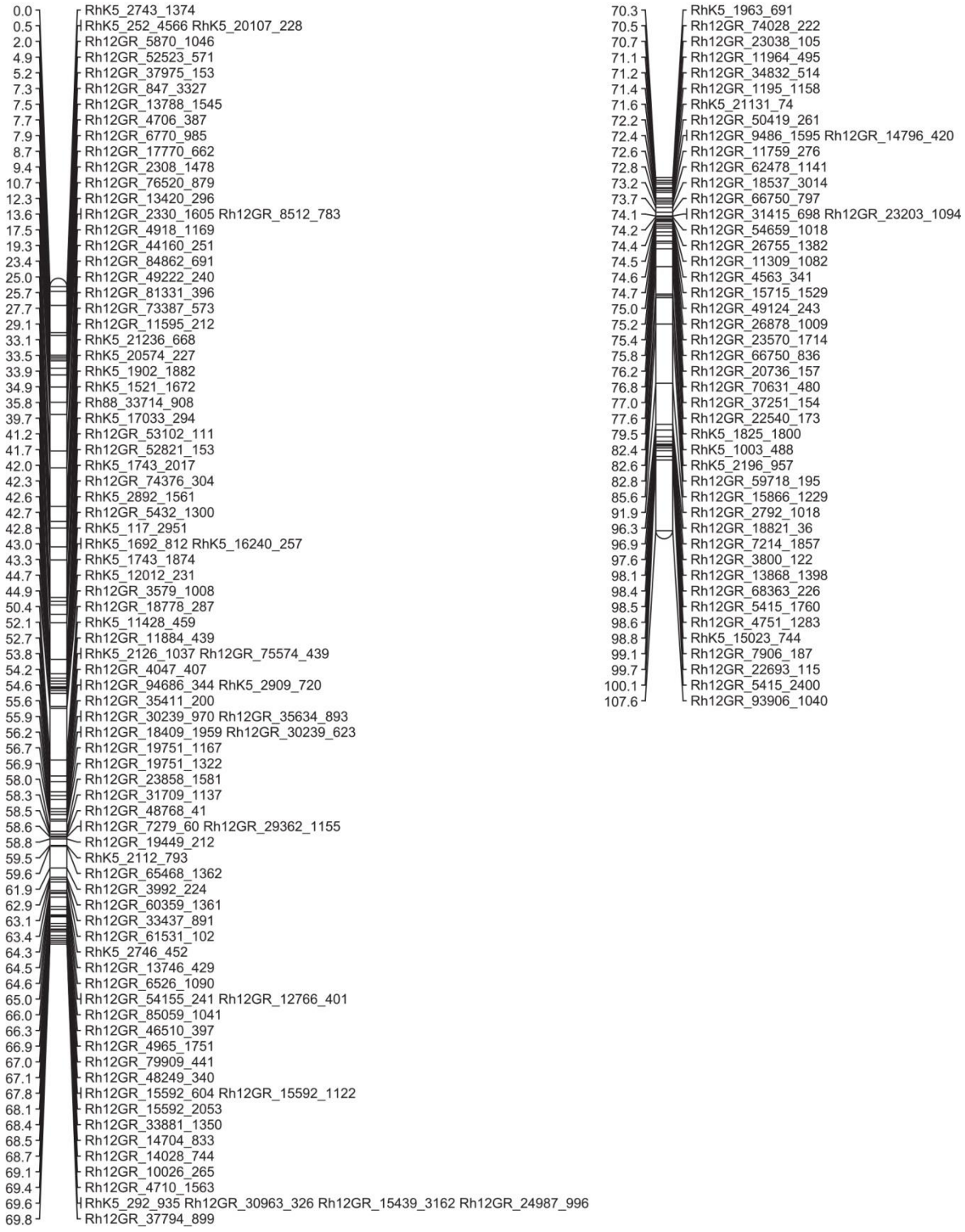

Figure 6 (*continued*). Linkage map of the tetraploid RNDxRND population constructed with biallelic SNP markers.

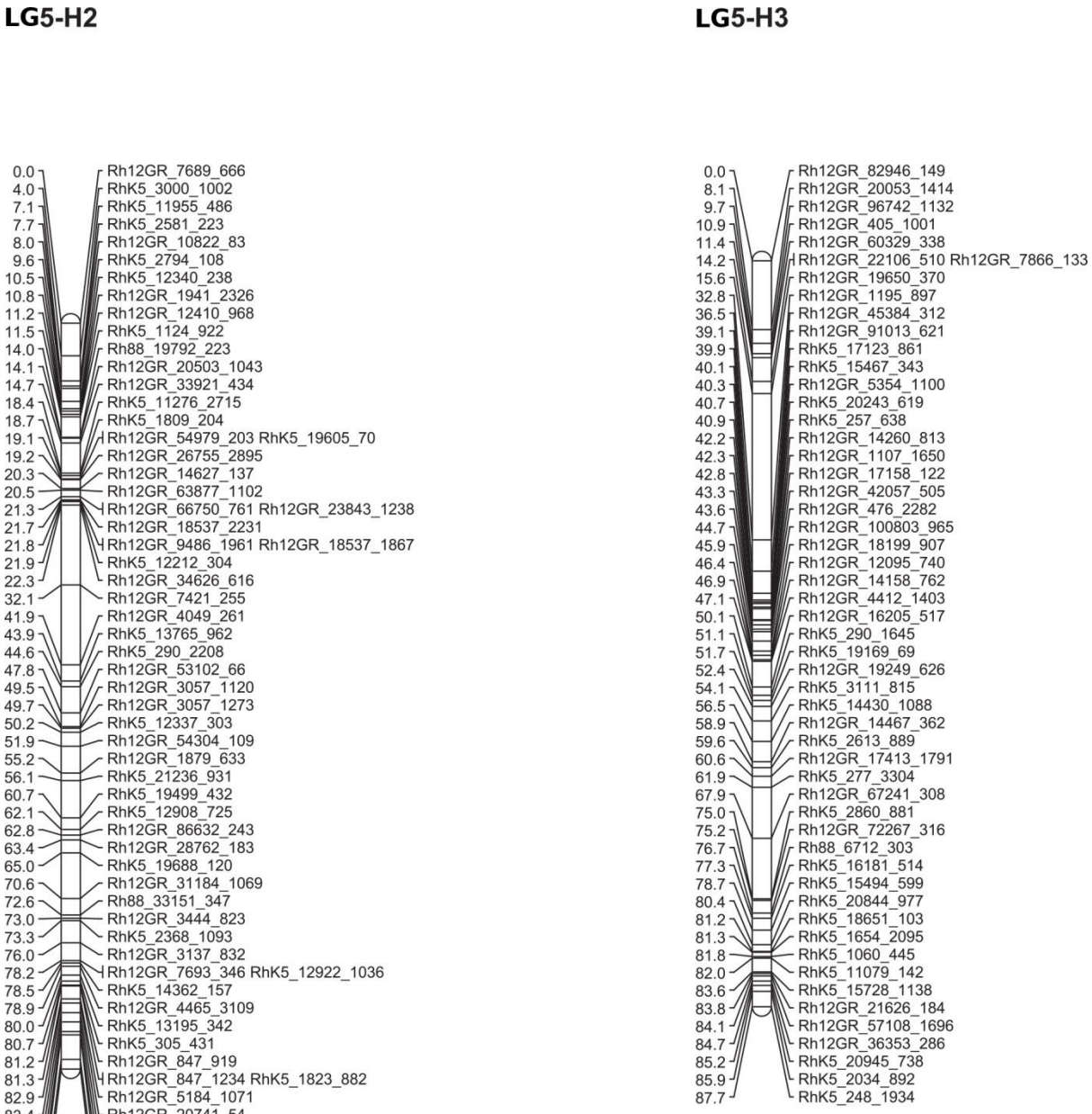

Figure 6 (*continued*). Linkage map of the tetraploid RNDxRND population constructed with biallelic SNP markers.

LG5-H4

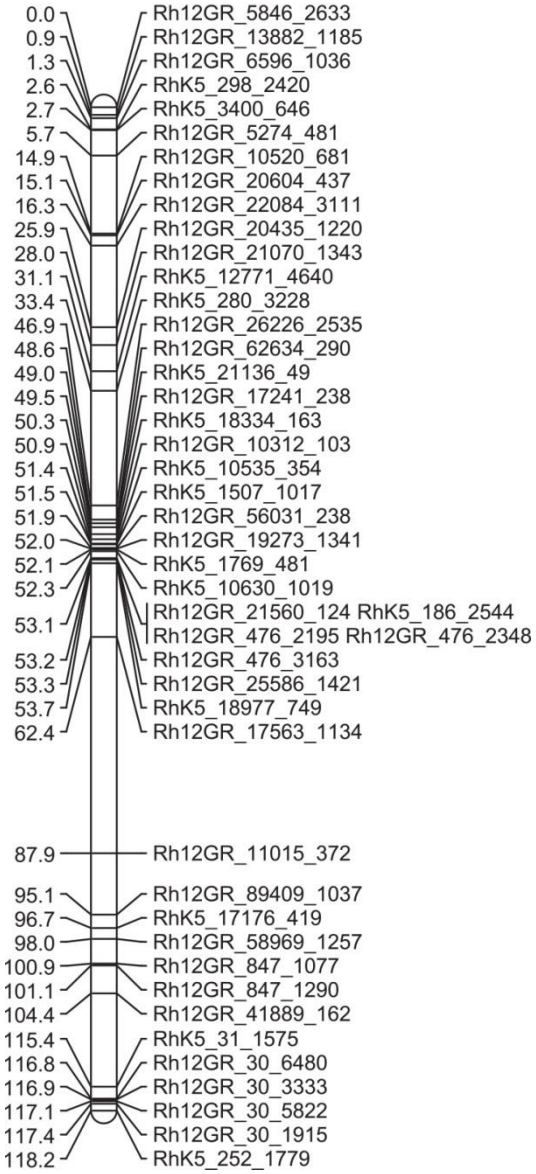

Figure 6 (*continued*). Linkage map of the tetraploid RNDxRND population constructed with biallelic SNP markers.

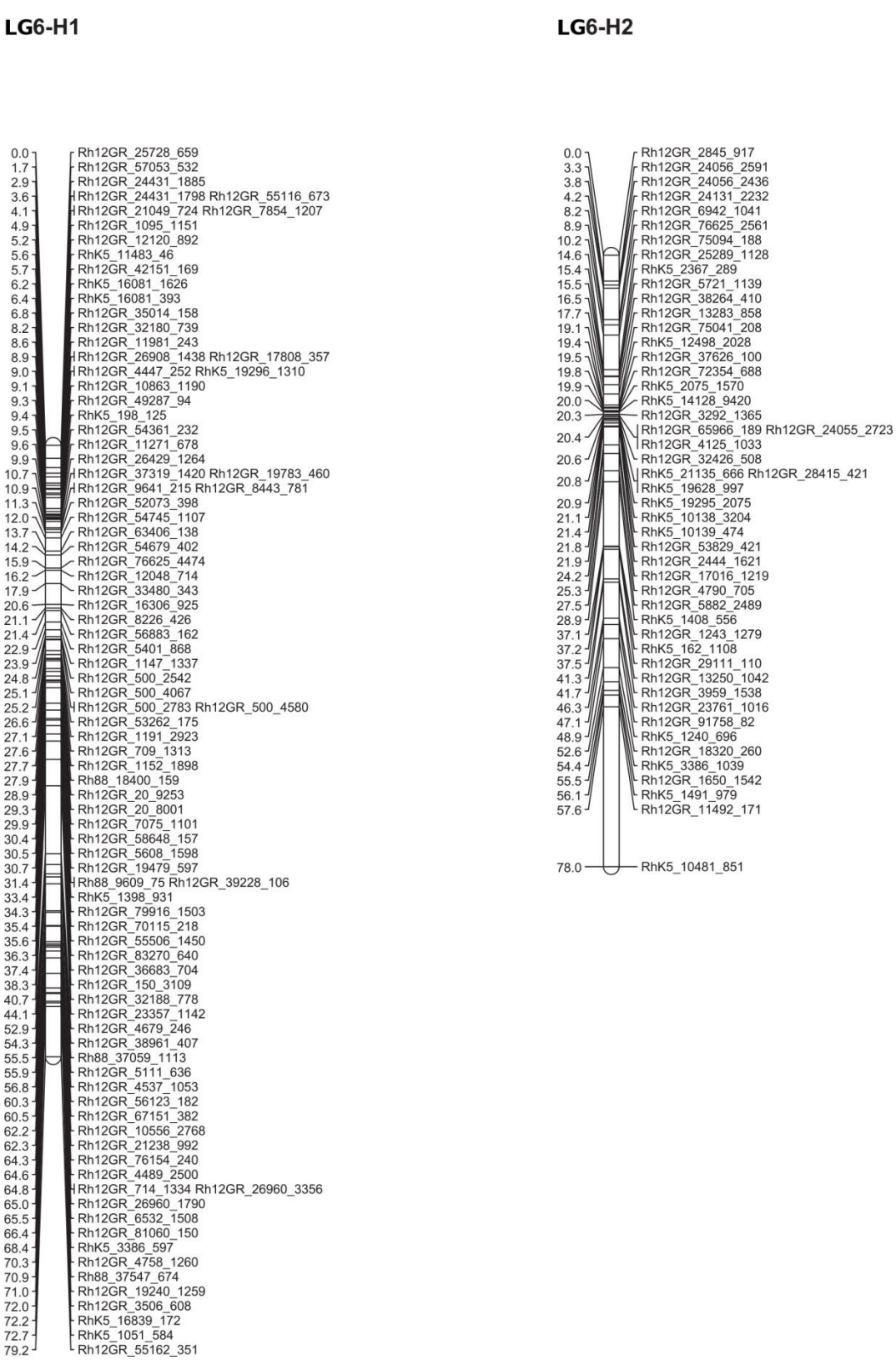

Figure 6 (continued). Linkage map of the tetraploid RNDxRND population constructed with biallelic SNP markers.

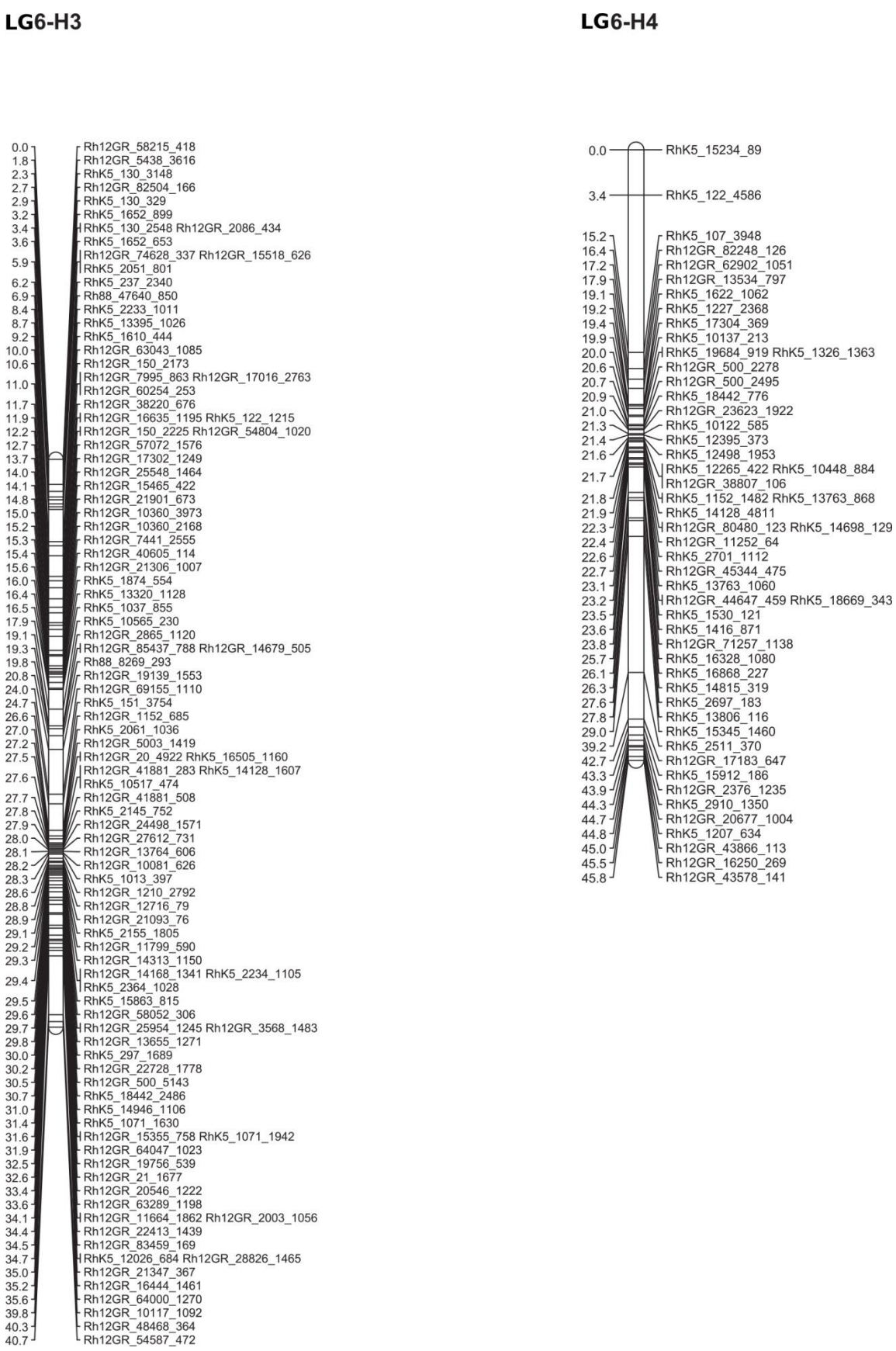

Figure 6 (*continued*). Linkage map of the tetraploid RNDxRND population constructed with biallelic SNP markers.

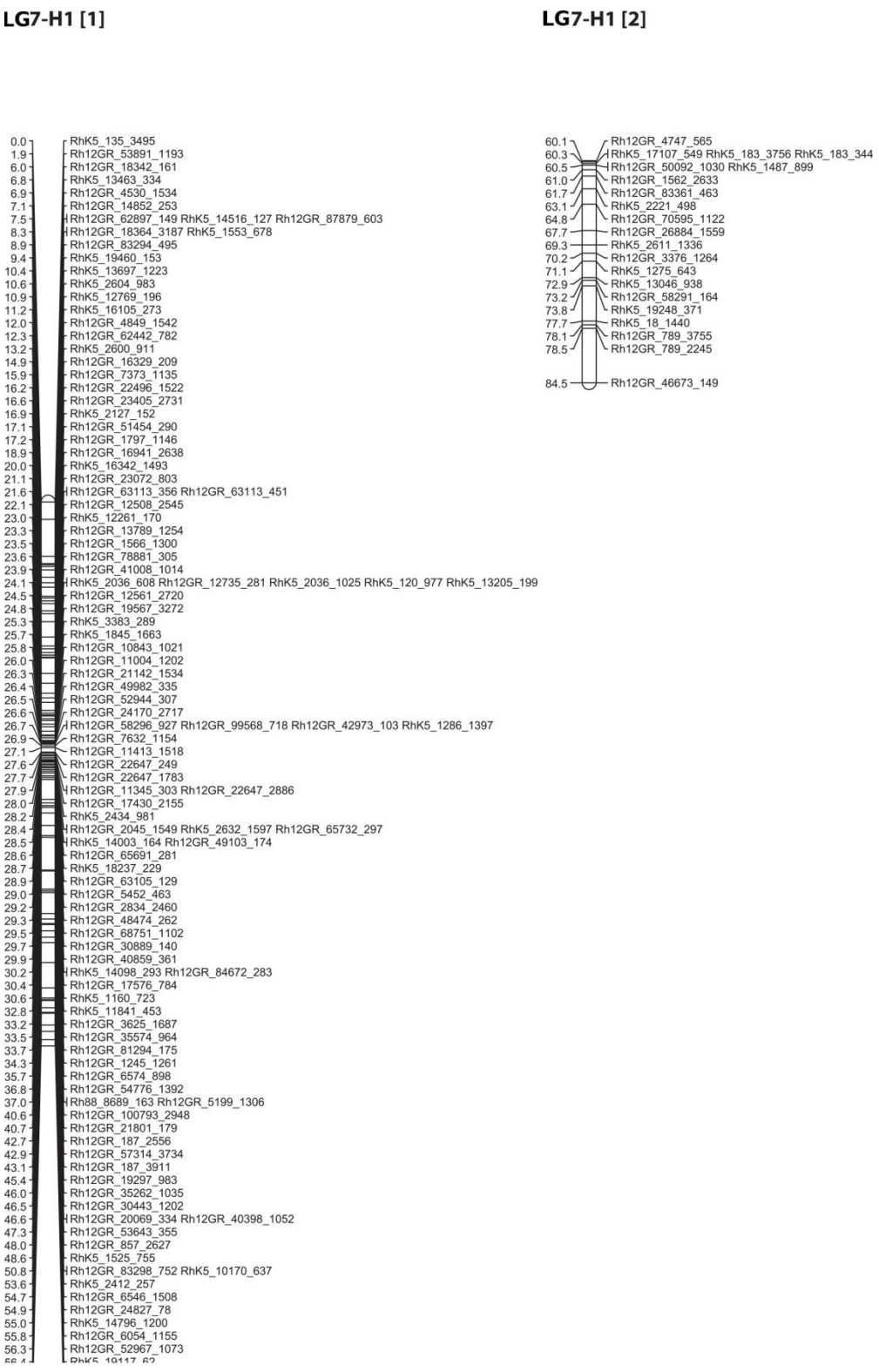

Figure 6 (*continued*). Linkage map of the tetraploid RNDxRND population constructed with biallelic SNP markers.

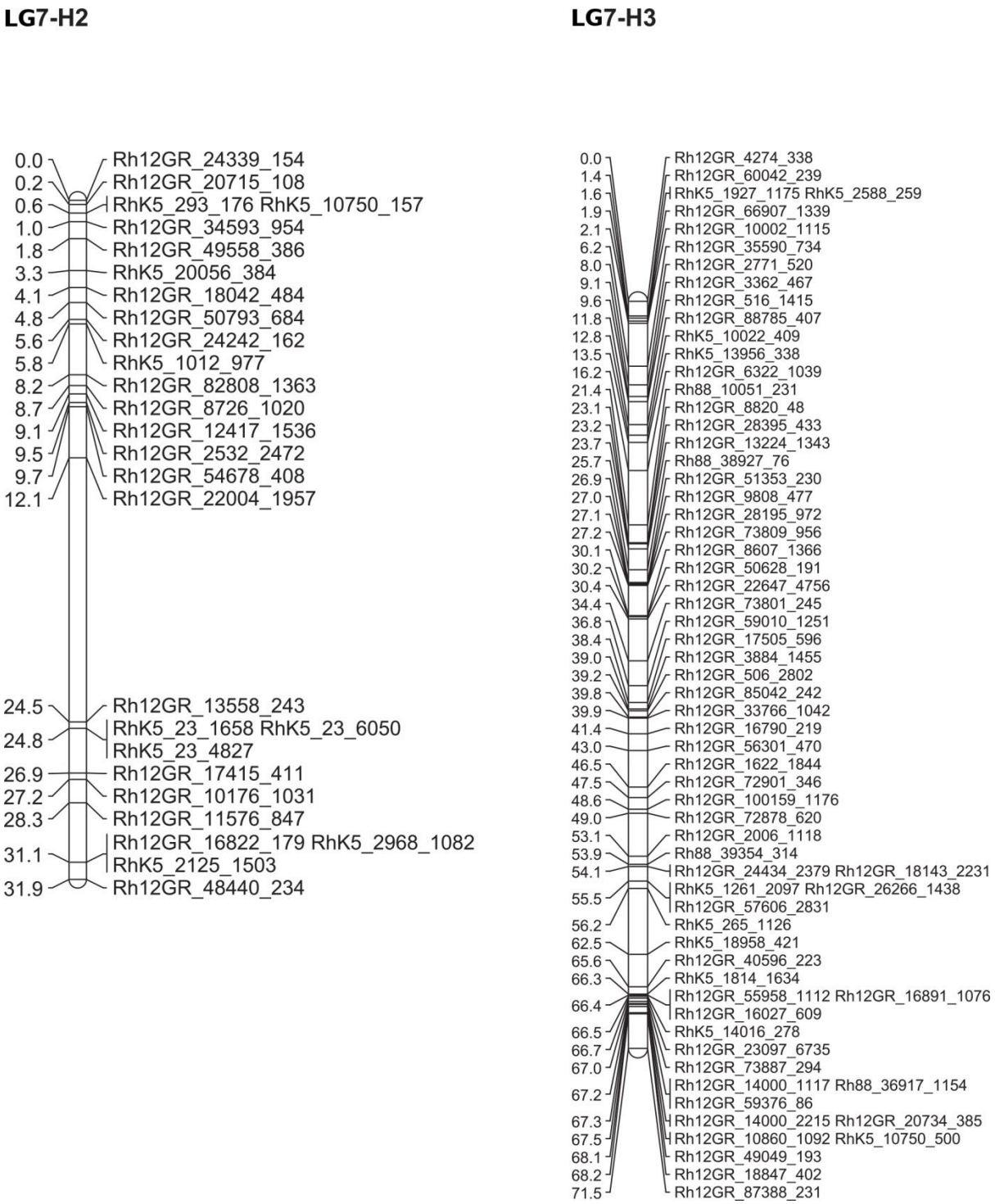

Figure 6 (*continued*). Linkage map of the tetraploid RNDxRND population constructed with biallelic SNP markers.

GL7-H4

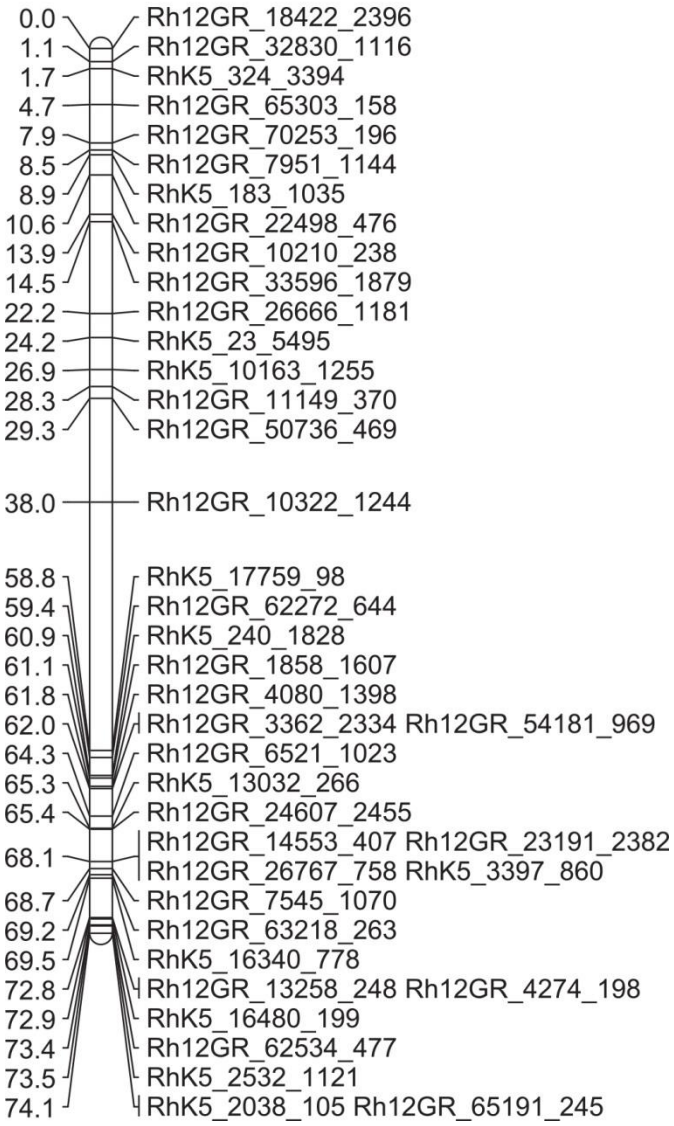

Figure 6 (*continued*). Linkage map of the tetraploid RNDxRND population constructed with biallelic SNP markers.

Mode of inheritance

To study the mode of inheritance we analysed the segregation according to five approaches, as described in the Materials and Methods section: segregating DxN SNPs; non-segregating DxN SNPs; recombination frequencies of repulsion-phase SxN SNPs, segregation of DxD markers, and occurrence and frequency of double reduction.

The mode of inheritance in RNDxHP population was studied using 4 different approaches: segregating DxN SNPs; non-segregating DxN SNPs; recombination frequencies of repulsion-phase SxN SNPs, and the occurrence and the frequency of double reduction, whereas the absence of DxD markers prevented using the fifth approach. To estimate the mode of inheritance using segregating DxN markers two markers types can be used: DxN and their "mirror" DxQ. In population RNDxHP we detected 81 DxN and 81 DxQ markers for RND. The χ^2 test indicated that for more than half of the markers tetrasomic inheritance is not rejected (49 and 48 for DxN and DxQ respectively, Table 6) while only in 2 cases disomic inheritance was not rejected. For a fairly large number of duplex markers, both hypotheses were not rejected. This can be understood since in a fairly small population 1:2:1 is not too different from 1:4:1 and skewness in segregation of some markers is expected. In the case of HP none of 90 DxN and 111 DxQ markers are indicating disomic inheritance (Table 6), which suggests also tetrasomic inheritance.

Table 6. Mode of inheritance in garden roses based on segregating DxN markers.

Conclusions of χ2 test	RNDxHP - RND		RNDxHP - HP	
	DxN	DxQ	DxN	DxQ
Tetrasomic Not Rejected	49	48	64	81
Disomic Not Rejected	2	0	0	0
Both Not Rejected	22	28	7	14
Both Rejected	8	5	19	16
Total number of markers	81	81	90	111

Mode of inheritance was estimated using DxN and DxQ markers for each parent (RND and HP) of RNDxHP population. For estimation of the mode of inheritance we tested if tetrasomic or disomic inheritance was fitted using a χ2 test for expected segregation ratios under tetrasomic and under disomic inheritance.

The second line of evidence we used for determination of the mode of inheritance was the possible presence of non-segregating DxN markers, only expected under disomic inheritance. We detected as few as five non-segregating DxN markers for RND, while for the pollen donor (HP) we did not detect any of these non-segregating markers. Non-segregating markers cannot be mapped on the rose map but their position can be indirectly inferred from the *Fragaria* genome sequence. Three did not have a hit to the *Fragaria* genome sequence, while one was located on *Fragaria* pseudochromosome FvCh2, the other remaining one on FvCh5. As the number of non-segregating DxN markers among the total number of DxN markers is very low this does not provide convincing evidence for disomic inheritance.

The recombination frequency of repulsion-phase SxN SNPs was used as a third line of evidence for the mode of inheritance. The binomial test results indicated that for RND tetrasomic inheritance was not rejected for chromosomes 1, 2, 4, 5, 6, and 7. Furthermore, the minimum observed frequency of repulsion phase recombinants (R) and minimum recombination frequencies in repulsion, assuming tetrasomic inheritance (r_2), for these chromosomes were around the expected 0.33 and 0 respectively, as expected for tetrasomic inheritance (Table 7). In contrast to this, for chromosome 3 minimal frequencies of recombination phase recombinants (0.13) and the minimal recombination frequency estimate in repulsion under the assumption of tetrasomic inheritance (-0.6) suggested disomic inheritance. For 98 out of 7885 pairs of markers on chromosome 3, recombination frequency estimates were between 0.15 and 0.17, while for the remaining markers they were around 0.33. These results indicated that on chromosome 3 both disomic and tetrasomic inheritance might occur. To indicate which homologs are involved in disomic inheritance we looked at marker positions and detected that the proximal part of homolog 2 and the distal part of homolog 3 follow disomic inheritance. The facts that markers with disomic inheritance are concentrated at a single region on a chromosome and that 'parts of chromosomes' are not expected to have a different segregation than other parts of the same chromosome may be indications for skewed marker segregation at these chromosomal regions rather than for disomic inheritance.

Table 7. Mode of inheritance in garden rose based on RND parental map.

RND	Chromosome	ICM1	ICM2	ICM3	ICM4	ICM5	ICM6	ICM7
R	Min	0.36	0.3	0.13	0.26	0.21	0.22	0.36
r2	Min	0.09	-0.1	-0.6	-0.21	-0.37	-0.33	0.074
-LOG10(P-value Coupling)	Max	16.23	16.01	16.23	16.23	16.23	16.23	16.01
-LOG10(P-value Repulsion)	Max	4.62	2.71	9.08	3.92	5.32	4.98	4.8
Binomial Test Smallest R	P(R≥1/3)	0.75	0.34	1.09E-04	0.14	0.02	0.04	0.71
Test Result 0.01	H0: R≥1/3 H1: R<1/3	NOT Reject	NOT Reject	Rejected	NOT Reject	NOT Reject	NOT Reject	NOT Reject
No Rejecting/Total No Repulsion		0/435	0/4559	98/7885	0/7875	0/7875	0/14937	0/25425

Mode of inheritance was estimated in the population RNDxHP based on frequencies of repulsion phase recombinants (R) and recombination frequencies in repulsion (r_2) under the assumption of tetrasomic inheritance. To determine the mode of inheritance, a Binomial test was performed for every marker pair to determine whether the observed recombination frequency (R) is lower than 1/3 (H_0: $R \geq 1/3$) at the significance level of 0.01. If all R values are significantly larger than 0.33 ($r_2 \geq 0$) for a chromosome, this suggest that there is completely random pairing of homologs (tetrasomic inheritance), while if R is smaller than 0.33 and $r_2 < 0$, preferential pairing (disomic inheritance) is indicated.

To confirm our assumption that disomic inheritance is present for chromosome 3 of RND we looked at recombination frequencies of markers from the same contig that mapped on different homologs. In our data set we detected only a single pair of markers (RhK_5_8_4164 mapped on homolog 2 and RhK_5_8_7997 mapped on homolog 3) and this pair fitted the tetrasomic model of inheritance (R=0.4, r2=0.21).

To estimate levels of disomic inheritance the level of independent assortment and linkage between markers have been used and their -10logP values have been plotted for

chromosome 3 (Figure 7). The figure is in agreement with what would be expected for tetrasomic inheritance.

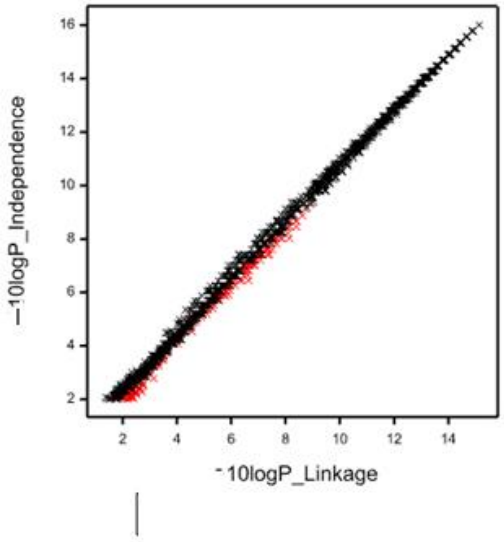

Figure 7. The level of disomic inheritance for chromosome 3 of RND. The -10logP values for Independence test and Linkage test have been plotted for coupling (black colour) and repulsion (red colour) phase. In a case of true disomic inheritance with disomic estimates the distribution of -10logP values of markers linked in coupling and repulsion phase are expected to be equal, while repulsion pairs of markers (shown in red) are expected to occupy the lower range of significance values in the case of tetrasomic inheritance.

The recombination frequencies of repulsion-phase SxN SNPs in the HP parent were also calculated. For chromosome 1 only one homolog was mapped and thus this chromosome was excluded from analysis. For chromosomes 2, 3, 4, 6, and 7 minimal frequencies of repulsion phase recombinants were between 0.30 and 0.38, as would be expected if the true situation was tetrasomic inheritance; and recombination frequencies under the assumption of repulsion, were in a range from -.01 to 0.15, also indicative of tetrasomic inheritance (Table 8). Negative estimates for a recombination frequency occurred because the estimator corrects for exactly 1/3 recombinants by independent assortment of homologs, but in reality there will be variation around this 1/3 since it is a random process. For chromosome 5 a small distortion in the recombination frequency estimate was detected (0.43 for coupling and 0.29 for repulsion phase), which can be explained by the fact that it was calculated based on only two homologs. The binomial test results confirmed that all pairs of markers follow tetrasomic inheritance.

Table 8. Mode of inheritance in garden rose based on HP parental map.

HP	Chromosome	ICM1	ICM2	ICM3	ICM4	ICM5	ICM6	ICM7
R	Min		0.36	0.3	0.38	0.43	0.35	0.38
r2	Min		0.09	-0.1	0.15	0.29	0.05	0.13
-LOG10(P-value Coupling)	Max		16.23	16.23	16.23	16.01	16.23	16.01
-LOG10(P-value Repulsion)	Max			3.08				
Binomial Test Smallest R	P(R≥1/3)		0.74	0.32	0.85	0.96	0.68	0.81
Test Result 0.01	H0: R≥1/3 H1: R<1/3		NOT Reject	NOT Reject	NOT Reject	NOT Reject	NOT Reject	NOT Reject
No Rejecting/Total No Repulsion			0/667	0/2775	0/2485	0/904	0/904	0/435

Mode of inheritance was estimated based on frequencies of repulsion phase recombinants (R) and recombination frequencies in repulsion (r2) under the assumption of tetrasomic inheritance. To determine the mode of inheritance, a Binomial test was performed for every marker pair to determine whether the observed recombination frequency (*R*) is lower than 1/3 (H_0: $R \geq 1/3$) at the significance level of 0.01. If all *R* values are significantly larger than 0.33 ($r2 \geq 0$) for a chromosome, this suggest that there is completely random pairing of homologs (tetrasomic inheritance), while if R is smaller than 0.33 and $r2 < 0$, preferential pairing (disomic inheritance) is indicated.

The fourth and last line of evidence is based on double reduction, which is a phenomenon associated with quadrivalent formation as can occur in tetrasomic inheritance but not during disomic inheritance. As evidence of double reduction for a particular individual first we used as a criterion the occurrence of at least two consecutive SxN markers with duplex scores in consistent regions on the same homolog. We also calculated the frequency of double reduction considering only as evidence of double reduction individuals with at least three SxN markers from the same homolog with duplex scores. The conclusions of the two approaches were similar. For different chromosomes double reduction was detected in different individuals. In RND double reduction was detected in 37 offspring. The average occurrence of double reduction over all chromosomes and homologs together was 7.2. We detected double reduction on every chromosome of RND (Table 9). The frequency of double reduction varied per homolog from 1.0% (chromosome 6, homolog 3) to 8.7% (homolog 3 of chromosome 7). The region of chromosomes (distal or proximal) on which double reduction was detected also varied between chromosomes and homologs (Figure 8). Interestingly, double reduction was detected for all markers on homolog 3 of chromosome 7, which might indicate that only the distal end of this homolog of this chromosome (where there is a higher expectation of double reduction occurrence) is recovered and that still part of the chromosome is missing.

Table 9. Occurrence and frequency of double reduction for RND.

Chromosome	Homolog	DR (no)	DR (%)
1	3	4	3.9
2	2	5	4.9
2	3	6	5.8
3	2	5	4.9
3	3	0	0.0
3	4	4	3.9
4	1	1	1.0
4	2	5	4.9
4	3	2	1.9
5	2	4	3.9
6	2	6	5.8
6	3	1	1.0
7	1	0	0.0
7	2	0	0.0
7	3	9	8.7
Average		**52**	**7.2**

As evidence of double reduction (DR) was taken the occurrence of duplex scores in a set of SxN markers in regions close to the end of chromosome. The frequency of double reduction was calculated per homolog of a particular chromosome and expressed in numbers (nr) and percentage (%).

In the HP parent the occurrence of double reduction was detected in only 9 offspring. Double reduction was detected on chromosomes 3 (homolog 1) and 6 (homolog 2) with frequencies of 4.1% and 8.1% respectively and average value over all chromosomes and homologs of 3.0% (Table 10). Double reduction was detected on the distal region of chromosome 6, while on chromosome 3 evidence for tetrasomic inheritance was detected at the proximal region (Figure 8).

Table 10. Occurrence and frequency of double reduction for HP.

Chromosome	Homolog	DR (no)	DR (%)
3	1	3	4.1
6	2	6	8.1
Average		**9**	**3.0**

As evidence of double reduction was occurrence of duplex scores in a set of SxN markers in consistent regions close to the end of chromosome. The frequency of double reduction was calculated per homolog of particular chromosome and expressed in numbers (no) and percentile (%).

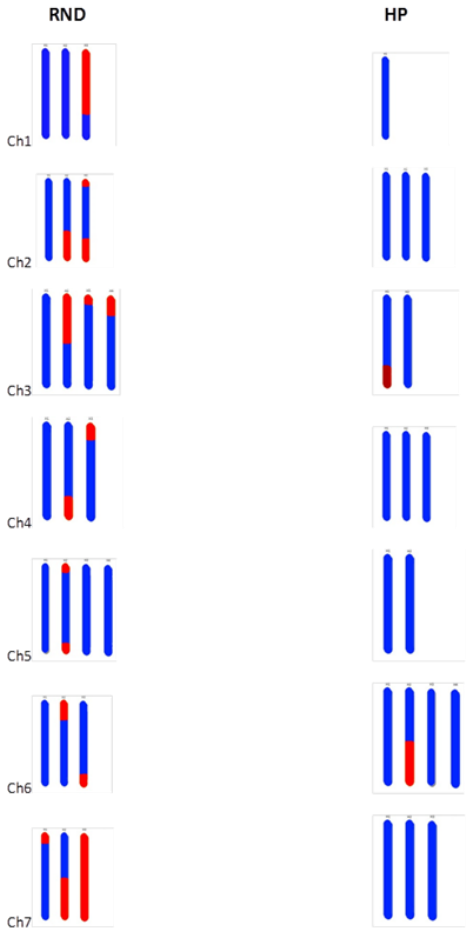

Figure 8. Overview of the areas in which markers showed evidence of double reduction across all individuals in RND and HP for each recovered homolog. The double reduction occurrence was calculated combining all occurrences. Double reduction is marked with red colour.

As the core analysis for the mode of inheritance estimation we used the segregation pattern of the DxN and SxN markers. As these markers are not present (DxN) or their number is very low (SxN) in the RNDxRND population, an additional approach based on DxD markers was performed to study the mode of inheritance in this population. From 2004 DxD markers the majority follow tetrasomic inheritance (1587), while for 177 DxD markers disomic inheritance was detected (Table 11). In case these 177 DxD markers belong to the same chromosome this would provide evidence for disomic inheritance. However, a strategy how to map DxD markers has not been developed yet, but as an alternative contig sequences of these markers can be blasted against the strawberry genome sequence (Shulaev et al., 2011) to estimate marker positions. Blasting results indicated that 100 of the markers with disomic inheritance have the highest similarity to genes mapped on strawberry pseudo-chromosome 6 (FvCh6), which corresponds to rose chromosomes 2 and 3. On the same FvCh6 chromosome also 267 markers with clear tetrasomic inheretance were mapped.

Table 11. Mode of inheritance of DxD markers in population RNDxRND.

Conclusion	Number of markers
Both rejected	17
Both not rejected	223
Disomic not rejected	177
Tetrasomic not rejected	1587
Total	2004

For estimation of the mode of inheritance we tested if tetrasomic or disomic inheritance was fitted using a $\chi 2$ test for expected segregation ratios under tetrasomic and under disomic inheritance.

The mode of inheritance might be wrongly interpreted when many markers from a particular region have a skewed segregation. In order to remove uncertainty in interpretation due to skewedness we checked how many of the 6237 found SxS markers were skewed from the expected 1:2:1:0:0 segregation ratio and 581 (9.3%) of them considered skewed and were rejected for the expected segregation ratio (Table 12). If all skewed markers are randomly distributed over chromosomes it might indicate mistakes in scoring, while concentration on a particular chromosomal region might indicate distorted segregation due to selection. Taking into consideration both mapped and unmapped markers we blasted markers against the *Fragaria* genome sequence (Shulaev et al., 2011) and detected that most markers with skewed segregation were mapped on pseudo-chromosomes 6 and 7 (Table 15).

Table 15. Number of SxS markers with skewed segregation in population RNDxRND placed on the strawberry pseudo-chromosomes (FvCh1-6).

Chromosome	Number of markers
FvCh1	63
FvCh2	8
FvCh3	15
FvCh4	24
FvCh5	55
FvCh6	153
FvCh7	155
UnM	108
Total	581

Significance of differences between expected (1:2:1:0:0) and observed segregation patterns were tested at the level of 0.05 using a $\chi 2$ test. Unmapped markers are marked as UnM.

Synteny with *Fragaria*

According to Gar et al. (2011) markers mapped to a single locus on the *Fragaria* map correspond to a single position on *Fragaria* genome sequence. For synteny comparison of the garden rose genome to the *Fragaria* genome sequence, three derived rose maps were used: the parental maps from RND and HP of the population RNDxHP and the integrated map from RND of the selfing RNDxRND population. The rose sequence contigs from which the SNPs had been derived were BLASTed to the *Fragaria* genome sequence and the best hit was used if above the threshold (Koning-Boucoiran et al. in preparation). For the parental RND map, 940 of 1121 markers contig sequences could be blasted to the strawberry genome with the minimal cut-off E-value of 10^{-5} (Appendix 5). In case of the integrated RND map, 1560 of 1930 markers had a hit (Table 13), while 458 of 533 markers mapped on HP map were located on the *Fragaria* genome sequence (Appendix 6).

Table 13. Number of markers in population RNDxRND placed on the strawberry pseudo-chromosomes (FvCh1-7).

	FvCh1	FvCh2	FvCh3	FvCh4	FvCh5	FvCh6	FvCh7	UnM
GR1	0	0	0	0	1	0	40	3
GR2	53	4	0	3	6	147	2	17
GR3	171	2	14	6	7	211	5	89
GR4	1	1	8	153	27	1	2	84
GR5	0	41	197	1	4	3	3	53
GR6	0	216	6	1	4	9	1	58
GR7	8	0	0	14	184	2	1	66

The rose sequence contigs from which the SNPs had been derived were BLASTed to the *Fragaria* genome sequence (Shulaev et al., 2011) and the best hit was used if above the threshold. Unmapped markers are marked as UnM.

The distribution of markers mapped in all three maps showed that most of the markers located on a single rose linkage group are located on a corresponding single *Fragaria* pseudo-chromosome indicating a high level of macro-synteny between rose and strawberry. Garden rose linkage group 1 (GR1) corresponded to *Fragaria* pseudo-chromosome 7 (FvCh7), GR4 to GvCh4, GR5 to FvCh3, GR6 to FvCh2, GR7 to FvCh5, while garden rose chromosomes 2 and 3 correspond to parts of *Fragaria* pseudo-chromosomes 1 and 6 (Figure 9, Appendix 7).

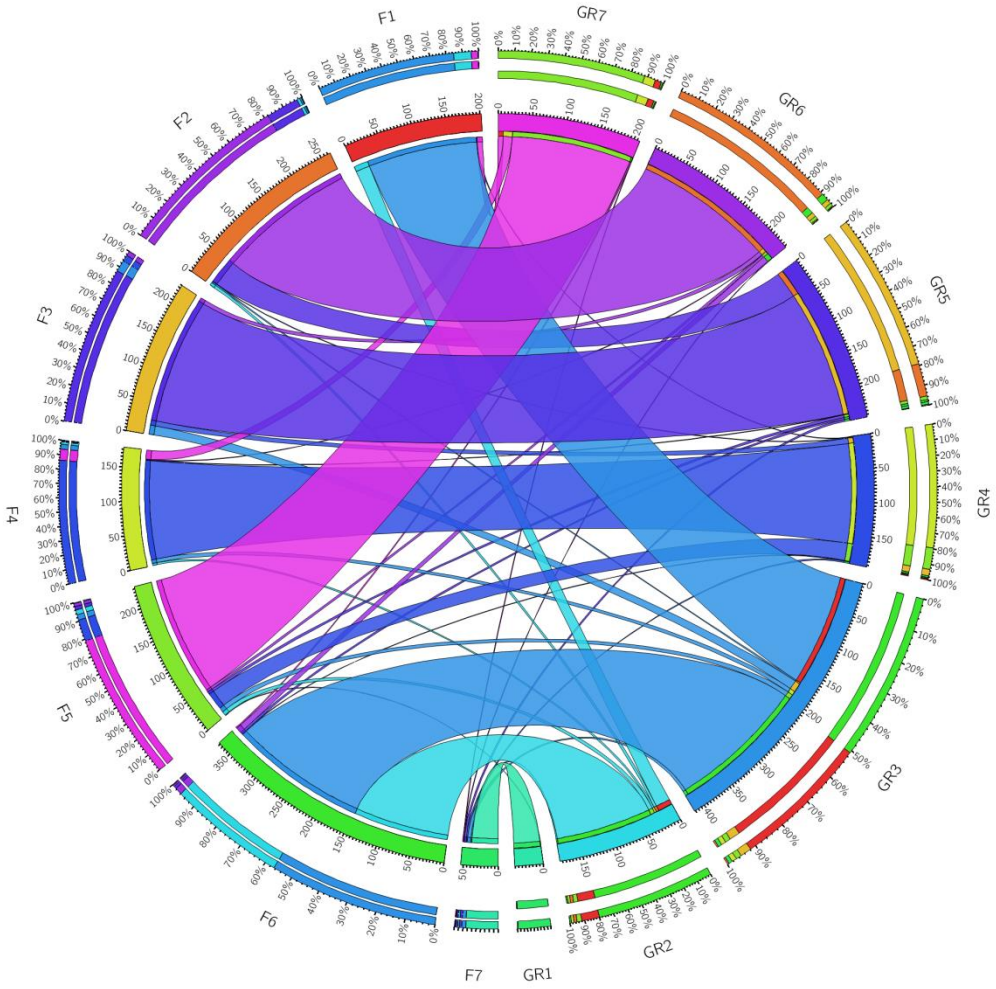

Figure 9. Synteny between *Fragaria vesca* (genome sequence) and garden rose (integrated RNDxRND map). The synteny was established by linking the contigs on which the mapped rose SNPs reside to the homologous region of the *Fragaria vesca* genome sequence by Blast.

QTL mapping

A per-marker QTL analysis was done for two morphological traits: recurrent blooming and prickle shape. In the RNDxHP population RND is the donor of recurrent blooming and recurrent versus non-recurrent blooming segregated in a 1:3 ratio (16 versus 45, χ^2 (P) = 0.8; Table 14). Taking into consideration monogenic inheritance and dominance of non-recurrent blooming, as was observed previously (Semeniuk, 1971; Debener et al., 2001; Crespel et al., 2002; Shupert & Byrne, 2007; Hibrand-Saint Oyant et al., 2008; Kawamura et al., 2010) a 1:3 segregation is expected under tetrasomic inheritance only if both parents have the non-recurrent blooming (Aaaa). Additionally, in the selfing population

RNDxRND recurrent blooming segregated in a 1:1 ratio (Table 14). The 1:1 segregation under tetrasomic inheritance may be obtained only if parental genotypes are in simplex and nulliplex, a segregation type not possible for a selfing and this suggests that more than 1 gene should be involved in recurrent blooming determination.

For prickle (straight) shape segregation patterns in RNDxHP (3:1) and RNDxRND population (3:1) can be explained by monogenic inheritance if both parents have an Aaaa genotype (Table 14).

Table 14. Segregation of studied morphological traits in progeny of RNDxHP and RNDxRND populations.

Population	Recurrent blooming				Prickle shape			
	R	NR	Ratio	χ^2 (P)	St	Cu	Ratio	χ^2 (P)
RNDxHP	16	45	3:1	0.8	38	20	3:1	0.26
			5:1	0.08			1:1	0.09
RNDxRND	35	44	1:1	0.26	57	15	3:1	0.41
							5:1	0.34

For each trait the number of individuals in each phenotypic category was counted. The significance of differences between expected and observed values were calculated using a 2-tailed $\chi 2$ test for all possible parental genotypes and their theoretical segregation ratios in progeny for tetraploid population in a case of full dominance. Conclusion was drawn for level of 0.05. The Table only shows those ratios that were not rejected for each trait.
R – recurrent blooming, NR – non- recurrent blooming
St – straight prickle shape, Cu – curved prickle shape

Per marker QTL analyses were done using regression on allele dosage, ANOVA on dosage classes and ANOVA on presence/absence. A putative QTL for recurrent blooming was found on LG 3 (Table 15) in the RNDxHP population. In the same population indications for QTLs for recurrent blooming were detected on chromosomes 5 and 6.. The prickle shape was studied as straight or curved and association with the straight phenotype was detected on chromosome 2 (-10logP=5.1). The next highest significance values were found on LG3 and LG5 but these are not confirmed in a multiple QTL analysis using multiple regression.

Table 15. Marker-trait associations in RNDxHP population.

	Maximum value											
	Regression				ANOVA dosage				ANOVA presence/absence			
	$-^{10}\log$(p-value)	LG	R2	Marker	$-^{10}\log$(p-value)	LG	R2	Marker	$-^{10}\log$(p-value)	LG	R2	Marker
Recurrent blooming	2.5	3	12.9	RhK5_14294_877	2.5	3	12.9	RhK5_14294_877	3.1	3	17.4	RhK5_14294_877
	2.5	5	12.5	Rh12GR_1107_1650	2.4	5	12.5	Rh12GR_42057_505R	2.8	5	15.0	Rh12GR_1107_1650
	2.5	6	16.6	RhK5_1152_1482	2.5	6	12.2	RhK5_1152_1482	2.5	6	12.2	RhK5_1152_1482
Prickle shape	2.0	2	10.0	RhK5_9196_81	5.1	2	33.4	RhMCRND_5507_1053	2.0	2	10.0	RhK5_9196_81
	2.9	3	16.9	RhK5_7699_516	2.8	3	18.9	RhK5_7699_516	1.8	3	8.7	RhK5_17058_105
	2.1	5	10.4	RhK5_14067_261	2.0	5	12.4	RhK5_14067_261	2.5	5	13.8	RhK5_14067_261

Traits are scored as presence or absence of specific trait and marker-trait association was run on regression on dosage, ANOVA on dosage, and ANOVA on presence/absence. For each trait thresholds are calculated running 1000 and 5000 rounds of permutations on data set and simulations on normal distributed data and threshold was set up at -10LogP of 4.

Marker/trait association in RNDxRND population confirmed indications for QTL for recurrent blooming on chromosome 3 (Table 16), while on chromosome 7 a QTL was detected with a larger effect for the same trait. The associations between straight prickle shape and markers were detected on chromosomes 4 and 6.

Table 16. Marker-trait association in RNDxRND population.

	Maximum value											
	Regression				ANOVA dosage				ANOVA presence/absence			
	$-^{10}\log$(p-value)	LG	R2	Marker	$-^{10}\log$(p-value)	LG	R2	Marker	$-^{10}\log$(p-value)	LG	R2	Marker
Recurrent blooming	3.9	7	16.4	Rh12GR_88785_407	2.8	7	13.8	Rh12GR_6322_1039	3.2	7	13.2	RhK5_13956_338
	2.1	3	7.2	Rh12GR_5415_2400	2.3	3	10.8	Rh12GR_1135_362	2.0	3	6.9	Rh12GR_23858_1581
Prickle shape	4.0	4	17.6	Rh12GR_52340_154	3.5	4	18.3	Rh12GR_14588_501	4.1	4	19.1	Rh12GR_14588_501
	3.0	6	12.2	Rh12GR_2444_1621	3.4	6	16.5	Rh12GR_2444_1621	3.4	6	14.2	Rh12GR_25289_1128

Traits are scored as presence or absence of specific trait and marker-trait association was run on regression on dosage, ANOVA on dosage, and ANOVA on presence/absence. For each trait thresholds are calculated running 1000 and 5000 rounds of permutations on data set and simulations on normal distributed data and threshold was set up at -10LogP of 4.

Discussion

In map construction we were faced with the problem of F1 offspring plants that were not in agreement with the genotypes of the putative parents. After exclusion of plants with non-parental SSR alleles the quantification of allele dosage could still not be done without assuming many markers with null alleles. Problems in population uniformity are not always reported, but off type offspring are a common side-product in breeding and a situation that many companies are faced with. Here we have developed a method which can identify groups of plants belonging to offspring of other parents, in a few steps, using markers for which no segregation was expected from one or both intended parents. With this procedure we were able to reconstruct subpopulations with different parentage in the absence of

genotype information on the putative parents. It is therefore a powerful tool to study pollination systems.

One of the two populations turned out to be the result of selfing of the mother, variety "Red New Dawn". This was concluded based on three lines of evidence: the absence of DxN segregating markers, the fact that more than 97% of the "Red New Dawn" markers that segregated SxN in the RNDxHP population behaved now as SxS, and the fact that more than 98% of the 5141 "Red New Dawn" markers that would not be expected to segregate from that parent (NxN and QxQ) indeed were nulliplex or quadruplex in the offspring. Even more, the progeny of RNDxRND population expressed significant reduction of heterozygosity by 20.3% compared to RND, an observation expected under selfing. To date a study on self-compatibility in garden roses has not been conducted and breeders' experiences are limited and kept confidential. A study on diploid *R. rugosa* Thunb. indicated that self-pollinated flowers wilt after pollination, suggesting gametophytic self-incompatibility (Ueda and Ando, 1996). In contrast to this, a microsatellite characterization of 24 offspring plants from open-pollinated seeds of tetraploid *R. damascena* indicated that they originated either from self-pollination of the mother plant or from cross-pollination with another *R. damascena* plant (Rusanov et al., 2005). Comparison of self-fertility between rose species indicates that the level of selfing can increase with the ploidy level (Nybom et al., 2005). A potential explanation is that higher ploidy weakens self-incompatibility. Self-compatibility, at least in some cultivars, could possibly allow the production of homozygous lines, which would open the way to using hybrids in rose production and also offer a possibility to fix highly valued traits by forced selfing. It may also highlight a need to improve quality control of crosses during breeding.

We constructed 3 linkage maps for garden roses employing SxN, SxS, and DxN SNP markers. On the parental map of RND (RNDxHP) 1121 markers were mapped, spanning 1072 cM, with an average marker distance of 0.96 cM. On the HP parental map fewer markers were mapped (522), affecting the total map length (738.3 cM) and the average distance between markers (1.4 cM). Finally, the integrated map of the RNDxRND population included 1930 loci, with a total map length of 1736 cM and an average marker distance of 0.9 cM. Comparing our map density to previously constructed tetraploid maps, where the average marker distance is between 2.4 cM (integrated map; Gar et al., 2011) and 5.3 cM (map per homolog; Rajapakse et al., 2005), coverage of the map and marker density are clearly improved. It is also evident that in each map we still miss some of the 28 homologs, so that this map still needs further improvement. We speculate that this should be done first by increasing the population size, as the number of high quality segregating markers from the WagRhSNP array is probably sufficient, as only few markers were now excluded because of unclear segregation patterns. Larger populations will also greatly increase the statistical power for QTL analyses in tetraploid mapping progenies. In addition, software specifically designed for polyploid maps would enable using some of the marker types other than SxN, SxS, and DxN, which have now been left unused. However, it should be realized that these other marker segregation types (DxS and DxD) are far less informative, both for map

construction and for QTL analysis, due to the large ambiguity of the origin of the marker alleles in these types of segregations.

The study of the mode of inheritance was done using five approaches observing segregation ratios of segregating DxN markers, occurrence of non-segregating DxN markers, recombination frequencies of SxN markers from different homologs, segregation pattern of DxD markers, and evidence of double reduction for SxN markers. There were hardly any non-segregating DxN markers in RND. This observation indicates evidence of tetrasomic inheritance. The segregating DxN markers lead to the same conclusion: for only 4 of 163 markers in RND tetrasomic inheritance was rejected. Also the SxN markers provided evidence for tetrasomic inheritance of most chromosomes: with the exception of chromosome 3, the recombination frequencies of pairs of SxN markers in repulsion all indicated tetrasomic inheritance. Only on linkage group 3 of RND for 98 pairs of markers (out of 7885) tetrasomic inheritance was rejected. This represents around 1.24% of all marker pairs of this chromosome; given that at a 99% test level per marker pair, we would allow for 1% of all pairs to reject the hypothesis of tetrasomic inheritance even when in reality there was tetrasomic inheritance, we consider this still to be compatible with tetrasomic inheritance. Additional analysis of -10LogP values (for both coupling and repulsion phase) distribution together with double reduction occurrence also indicated tetrasomic inheritance in garden roses. For the selfed RNDxRND population tetrasomic inheritance was detected for all chromosomes, while for chromosome 3 disomic inheritance could not be discarded. Further analysis on segregation patterns for SxS markers indicated that on garden rose chromosome 3 there is an aggregation of skewed markers. The skewness of some markers from chromosome 3 might be explained by the fact that the locus for self-incompatibility is mapped on this chromosome (Spiller et al., 2012). In the HP population, all evidence indicated tetrasomic inheritance. In tetraploid cut rose (Koning-Boucoiran et al., 2012) segregation patterns of markers and detection of double reduction also excluded disomic inheritance. For another polyploid representative of the *Rosaceae* family, blackberry, tetrasomic inheritance was also reported (Castro et al., 2013). In contrast to this, in the tetraploid black cherry disomic inheritance was detected (Pairon and Jacquemart, 2005).

Double reduction, a phenomenon related to tetrasomic inheritance was detected on all chromosomes at a frequency varying from 1.0% (homolog 3 of chromosome 6) to 8.7% (on chromosome 7, homolog 3). The occurrence of double reduction under theoretically expectations is at most 16.6% (Stift et al. 2008). Double reduction in cut rose was also estimated to have occurred in 39 out of 184 offspring (Koning-Boucoiran et al., 2012), but due to the small number of markers and restricted information due to dominant scoring, a precise estimate of double reduction frequencies has not been calculated. The highest frequency of double reduction in *Rorippa*, a tetraploid species with an intermediate mode of inheritance, was 2.5% (Stift et al., 2008). Interestingly, the frequency of double reduction was higher in the meiosis of the female parent than for the male parent, which is in agreement with our findings.

Garden roses, as members of the genus *Rosa*, are closely related to the genus *Fragaria* (Potter et al., 2007) and thus the *Fragaria vesca* FvH4 sequence could be used as a

reference for validation of markers mapped in rose mapping populations (Gar et al., 2011). Our synteny approach indicates a high level of conservation between rose and strawberry. The majority of markers that map on one linkage group in rose have their highest sequence similarity with the sequence of a single pseudo-chromosome of strawberry, with the exception for rose linkage groups 2 and 3: their markers corresponded to strawberry pseudo-chromosomes 1 and 6, indicating translocations have occurred in one of the two species. The macro-synteny observed in this study are in agreement with outcomes of a study on *Rosa hybrida* (Gar et al., 2011), in which also translocations for 2 chromosomes were detected. Furthermore, studies of synteny between members of *Rosacea* family indicated a high level of synteny among the genera *Malus*, *Fragaria*, and *Prunus* and demonstrated the existence of conserved syntenic blocks (Villanova et al, 2008; Illa et al., 2011). This high level of synteny is favourable for searches of candidate genes and for comparative mapping. In our study the high synteny level was used as an advantageous tool especially for giving marker positions of non-segregating markers and the study of inheritance using that information.

The occurrence of flowers in the first year after sowing, within 1 to 2 months after germination, is a good indicator of recurrent blooming (De Vries and Dubois, 1971). Our segregation analysis suggested that the inheritance of recurrent blooming cannot be explained by a single gene. In both populations we have indications for a QTL for recurrent blooming on LG 3. Recurrent blooming was previously mapped on the diploid rose map (Crespel et al., 2002; Hibrand-Saint Oyant et al., 2008; Kawamura et al., 2010) on a position on chromosome 3 (Spiller et al., 2011). Even though in many previous studies on recurrent blooming a monogenic inheritance of this trait was reported, distortion from expected segregation ratios was detected in a number of crosses (Debener, 1999; Rajapakse et al., 2001; Crespel et al., 2002; Shupert and Byrne, 2007; Kawamura et al., 2010). This distortion may be explained by multiple gene interaction, the (interspecific) nature of certain populations, or strong selection (unfavourable genotypes germinate poorly or die at an early stage, according to Shupert and Byrne, 2007). Additionally, recurrent blooming was studied on diploid material. The final expression of a trait in tetraploids is more complex and might depend on allele dosage. In tetraploids dominance relationships between alleles might be more complex and the influence of other genes may become apparent. Alternatively other genes may be involved that have a different allele action than simple dominance. Furthermore, tetraploid roses might have a different origin (source) of the trait than diploid roses. It indicates that QTLs from studies on diploid species cannot be simply translated to polyploids and that separate QTL analysis on polyploids is needed.

In the RNDxHP population we detected a QTL for prickle shape on LG2, while in RNDxRND populations two QTLs were found on LGs 4 and 6. Although QTLs for prickle presence/absence and prickle numbers were studied thoroughly before in other studies (Crespel at al., 2002; Koning-Boucoiran et al., 2012), prickle shape has not been studied before, which can be explained by the fact that decorative prickles in garden roses have aesthetic value, while in cut rose it is thornlessness that is valued highly. Crespel et al. (2002) detected a QTL for prickle number on the equivalent of ICM LG7. In contrast to this in cut rose QTLs for prickle number are detected on ICM LG2 and LG3 (Koning-Boucoiran et al.,

2012), suggesting that prickle-related traits are coded by multiple genes located on different chromosomes.

Conclusions

The first prerequisite for successful linkage map generation and QTL mapping in tetraploid rose is population uniformity and exclusion of outliers with other pollen donors or unintended selfed progeny. We developed a method for distinguishing subpopulations that share parents using SNP data only. Using this method we confirmed that selfing occurs in garden rose, which opens new possibilities for strategies in rose breeding. As a first step in setting up the basis for marker assisted breeding we detected tetrasomic inheritance and developed three dense genetic linkage maps for garden roses, which in comparison with previous maps, significantly improved coverage of the rose genome.

Acknowledgements

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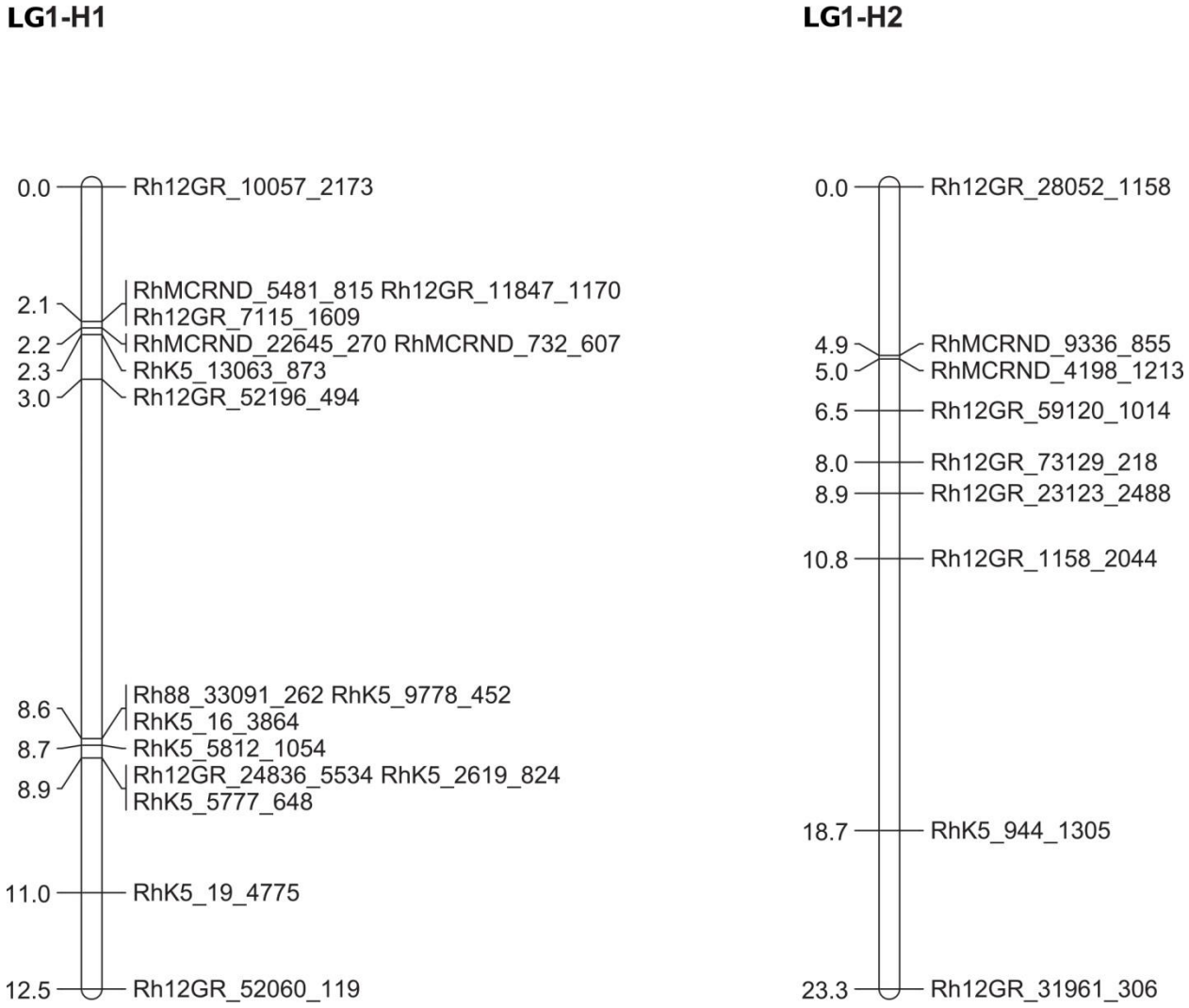
Appendix 1. List of SSR markers used in a study.

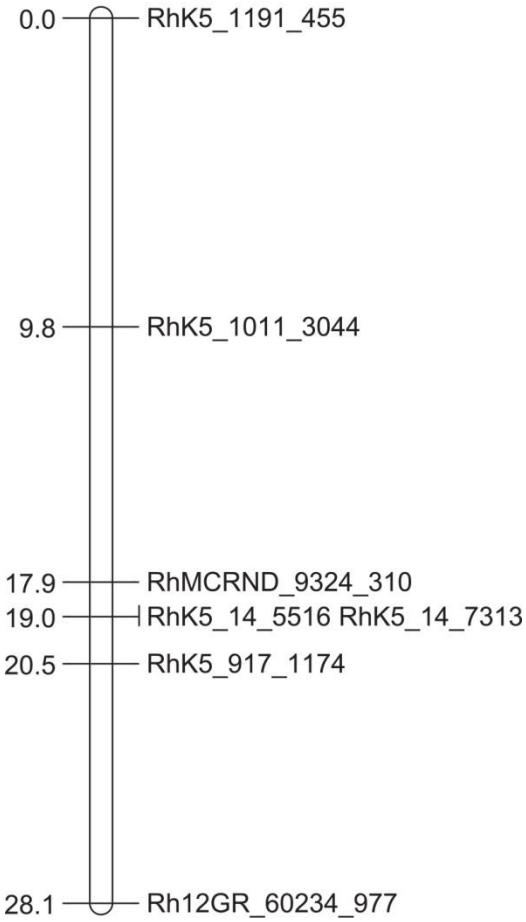
Marker	Reference
RhAB40	Esselink et al., 2003
RhO517	Esselink et al., 2003
RhP518	Esselink et al., 2003
RhD221	Esselink et al., 2003
RhO506	Esselink et al., 2003
RhB303	Esselink et al., 2003
RhD201	Esselink et al., 2003
RhP50	Yan et al., 2005
RhE2b	Esselink et al., 2003
H23O17	Hibrand Saint Oyant et al., 2008
Rw59A12	Hibrand Saint Oyant et al., 2008
Rh80	Yan et al., 2005
Ctg623	Hibrand Saint Oyant et al., 2008
Rw55E12	Hibrand Saint Oyant et al., 2008
RhABT12	Yan et al., 2005
Rh58	Yan et al., 2005
Rh48	Yan et al., 2005
Cl2980	Hibrand Saint Oyant et al., 2008
RMS082	WO 20030979869 A3*
Rw12J12	Hibrand Saint Oyant et al., 2008
Rh60	Yan et al., 2005
Rh91	Yan et al., 2005
RMS097	WO 20030979869 A3*
RMS120	WO 20030979869 A3*
RMS138	WO 20030979869 A3*
RhAB26	Esselink et al., 2003
Rh98	Yan et al., 2005
Rh59	Yan et al., 2005
RhAB28	Yan et al., 2005
ROG27	Meng et al, 2009
ROG22	Meng et al, 2009
ROG26	Meng et al, 2009

* Microsatellite markers for genetic analyses and the differentiation of roses

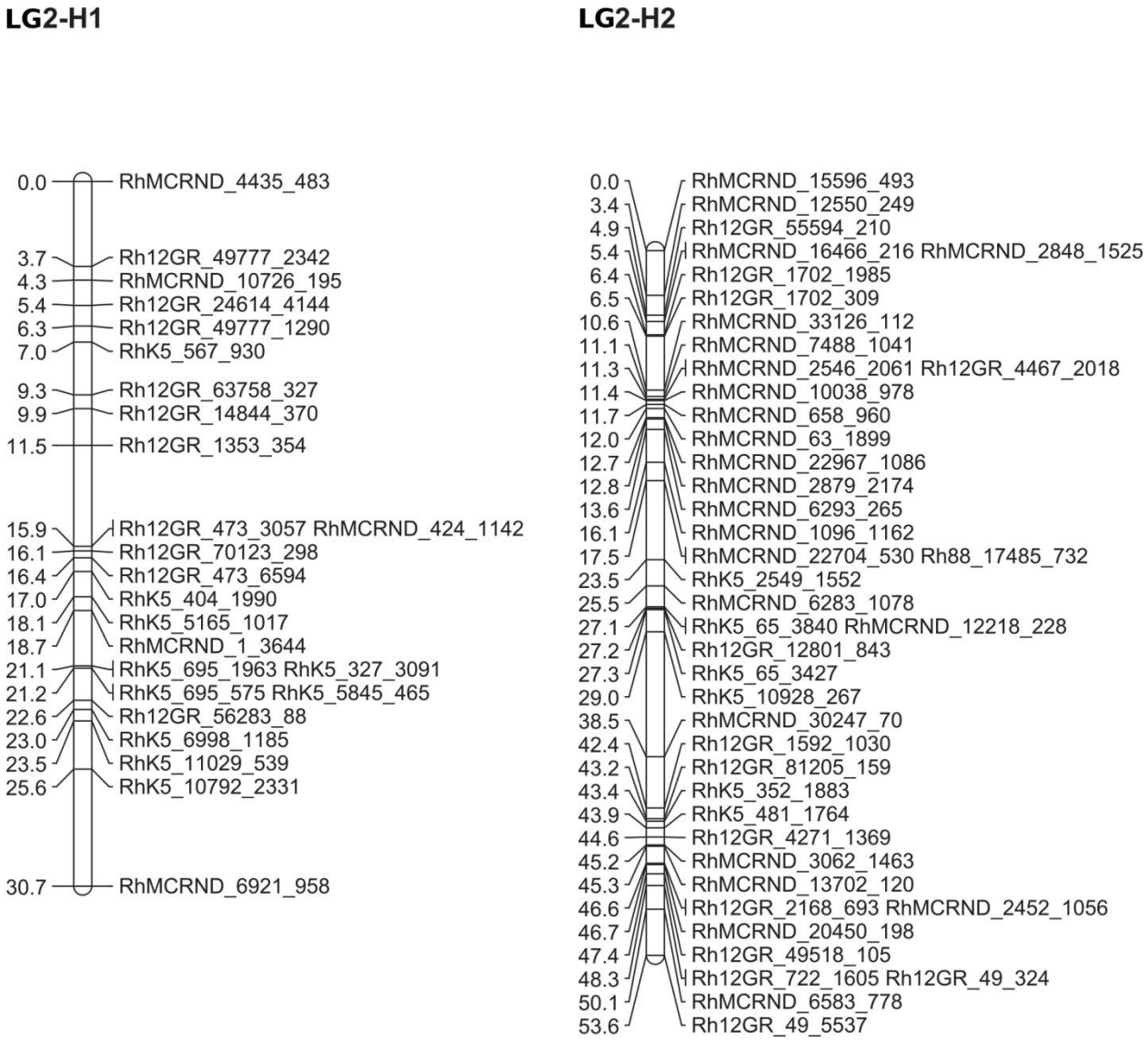
Appendix 2. Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers. Linkage groups are numbered from 1 to 7 following the ICM (Spiller et al., 2011), containing each 1, 2, 3, or 4 homologous groups (H). Markers are indicated to the right of each LG and map positions of markers (cM) are given to the left of each linkage group.



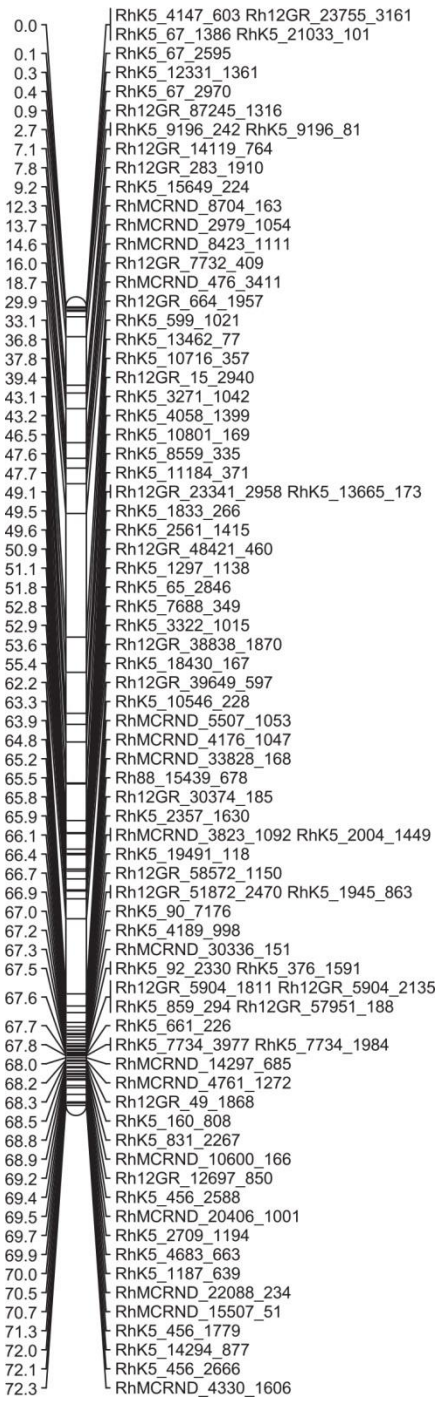
LG1-H3


0.0
RhK5_1191_455
9.8
RhK5_1011_3044
17.9
RhMCRND_9324_310
19.0
RhK5_14_5516 RhK5_14_7313
20.5
RhK5_917_1174
28.1
Rh12GR_60234_977

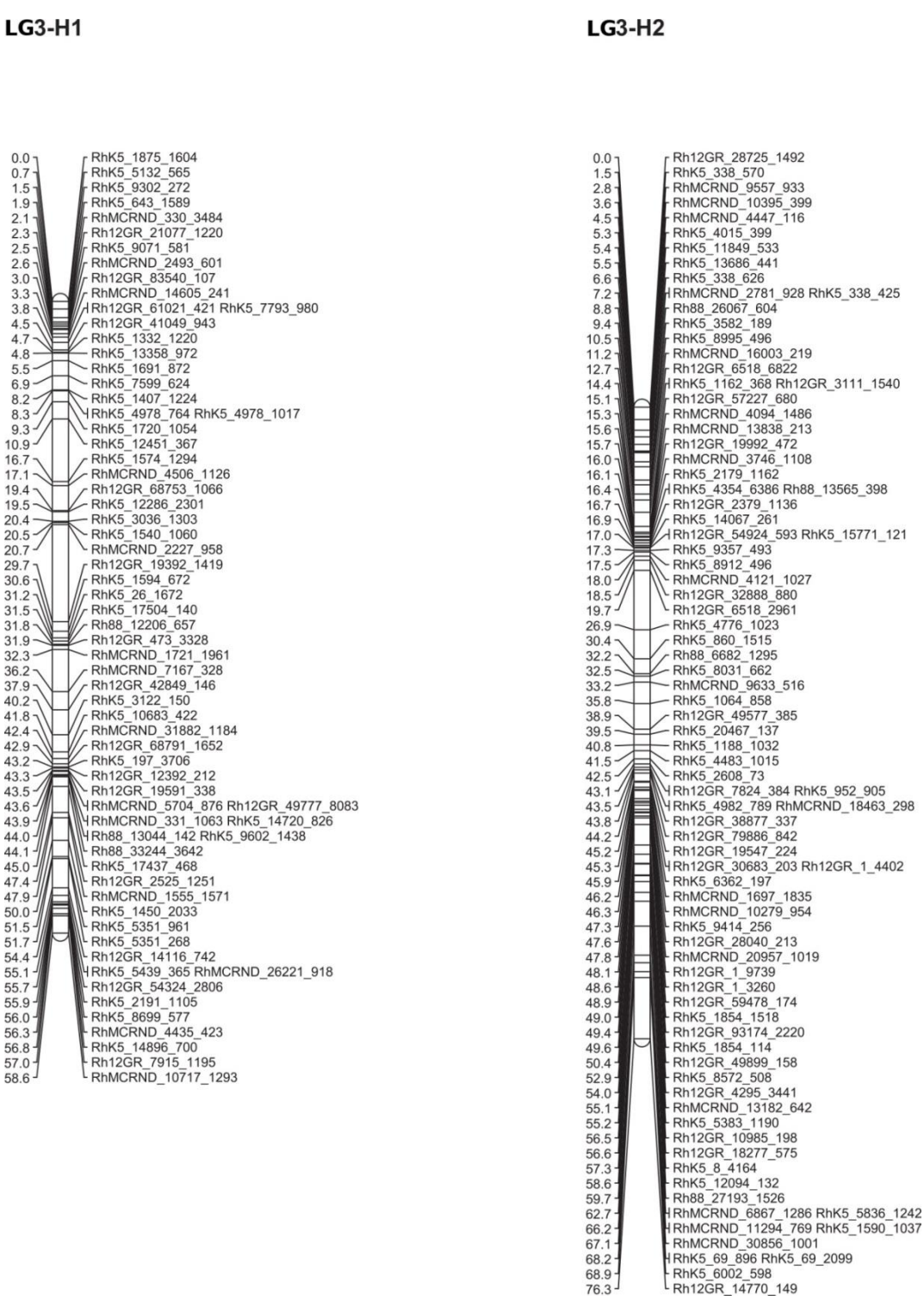
Appendix 2 (*continued*). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.



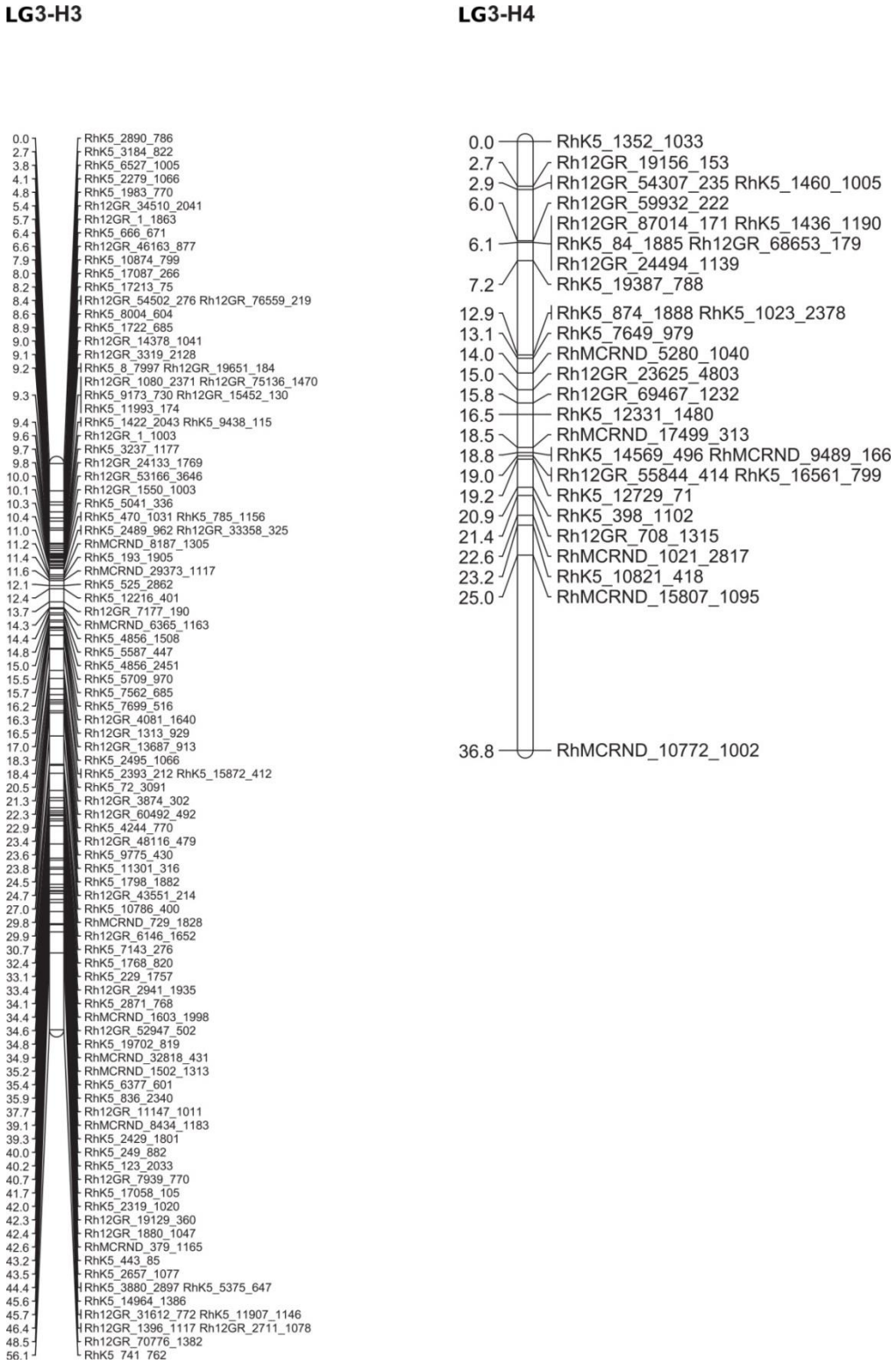
Appendix 2 (continued). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.



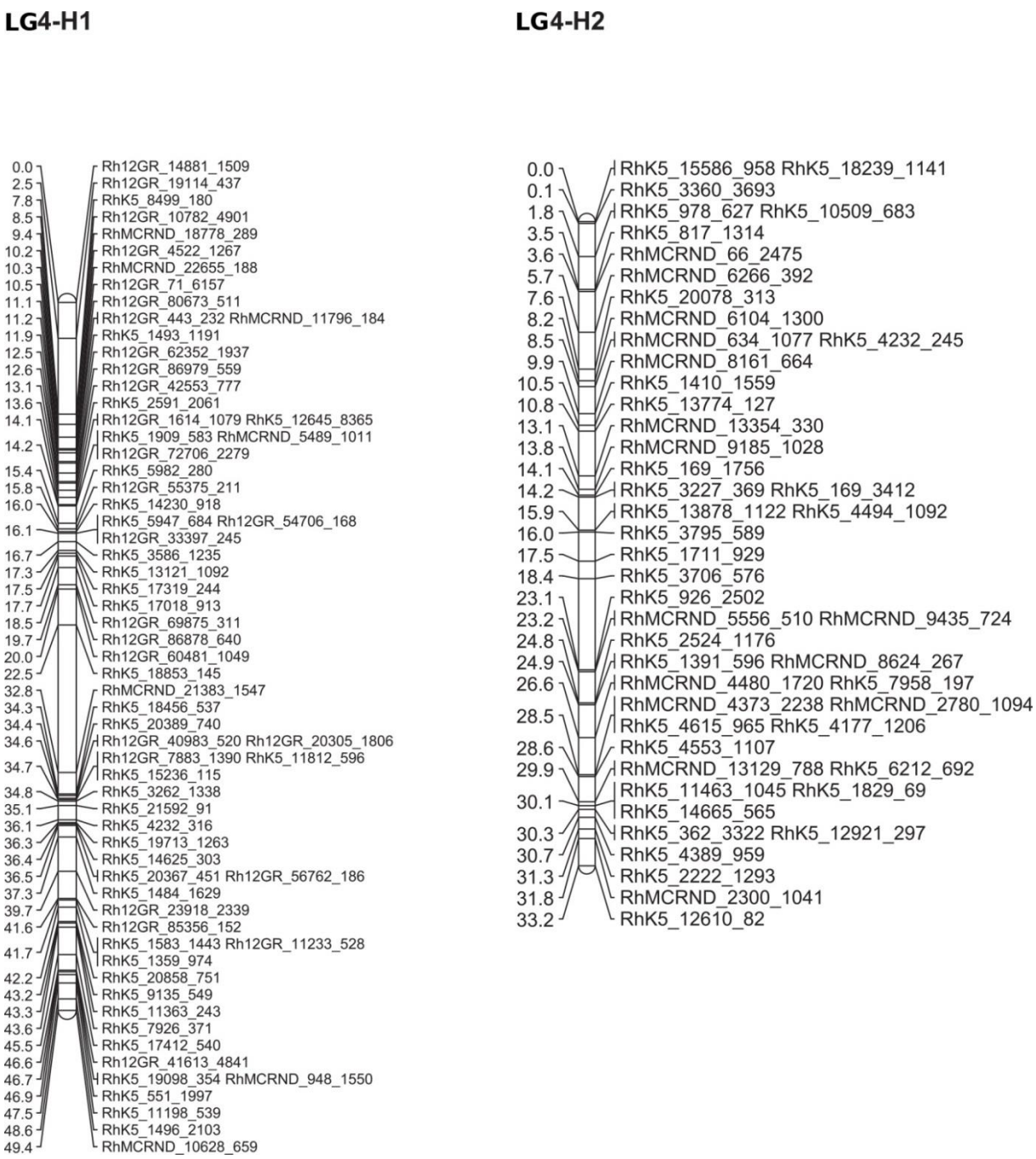
Appendix 2 (continued). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.



Appendix 2 (continued). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.

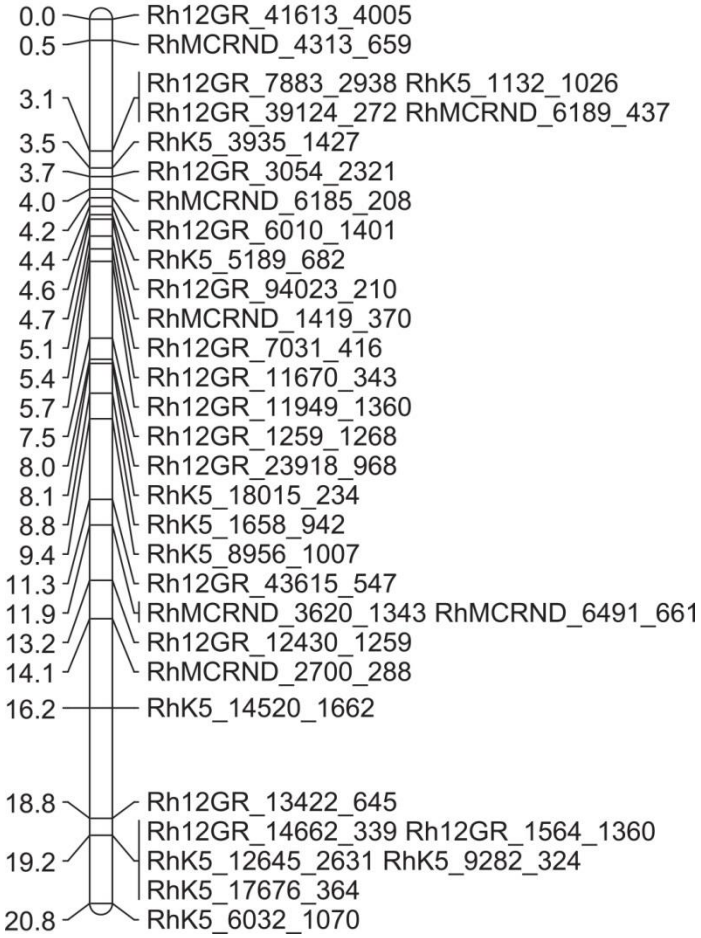


Appendix 2 (continued). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.

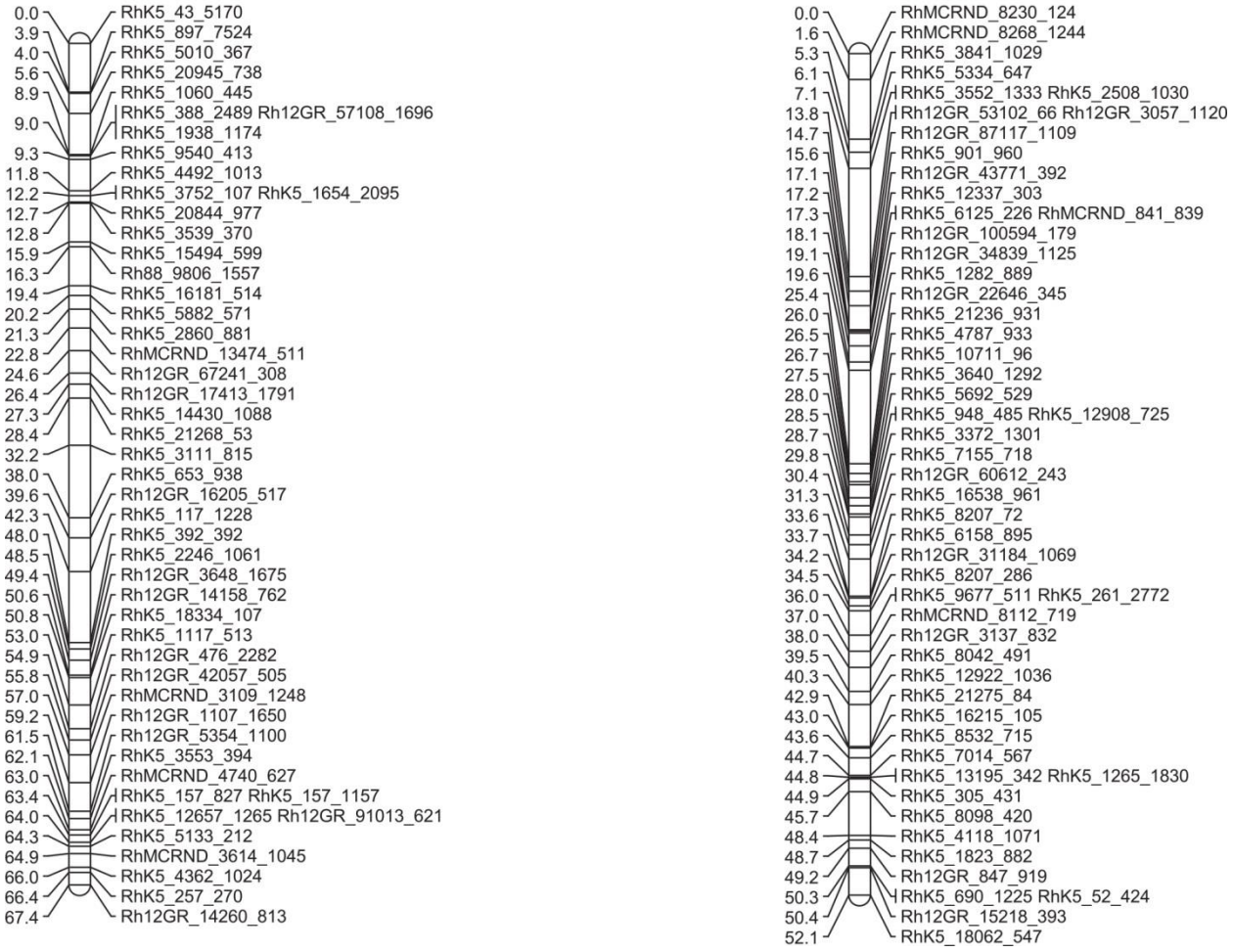


Appendix 2 (continued). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.

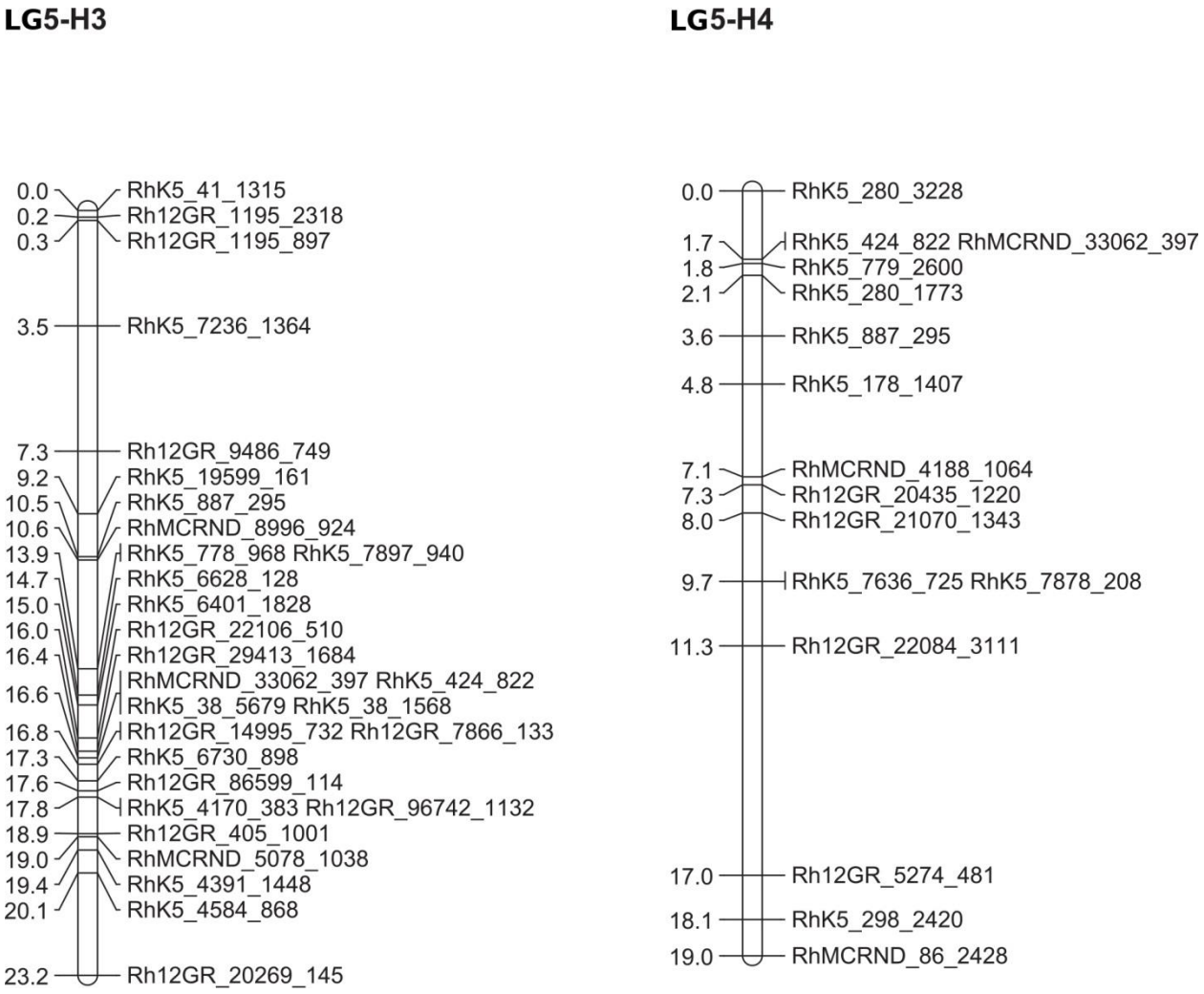
LG4-H3



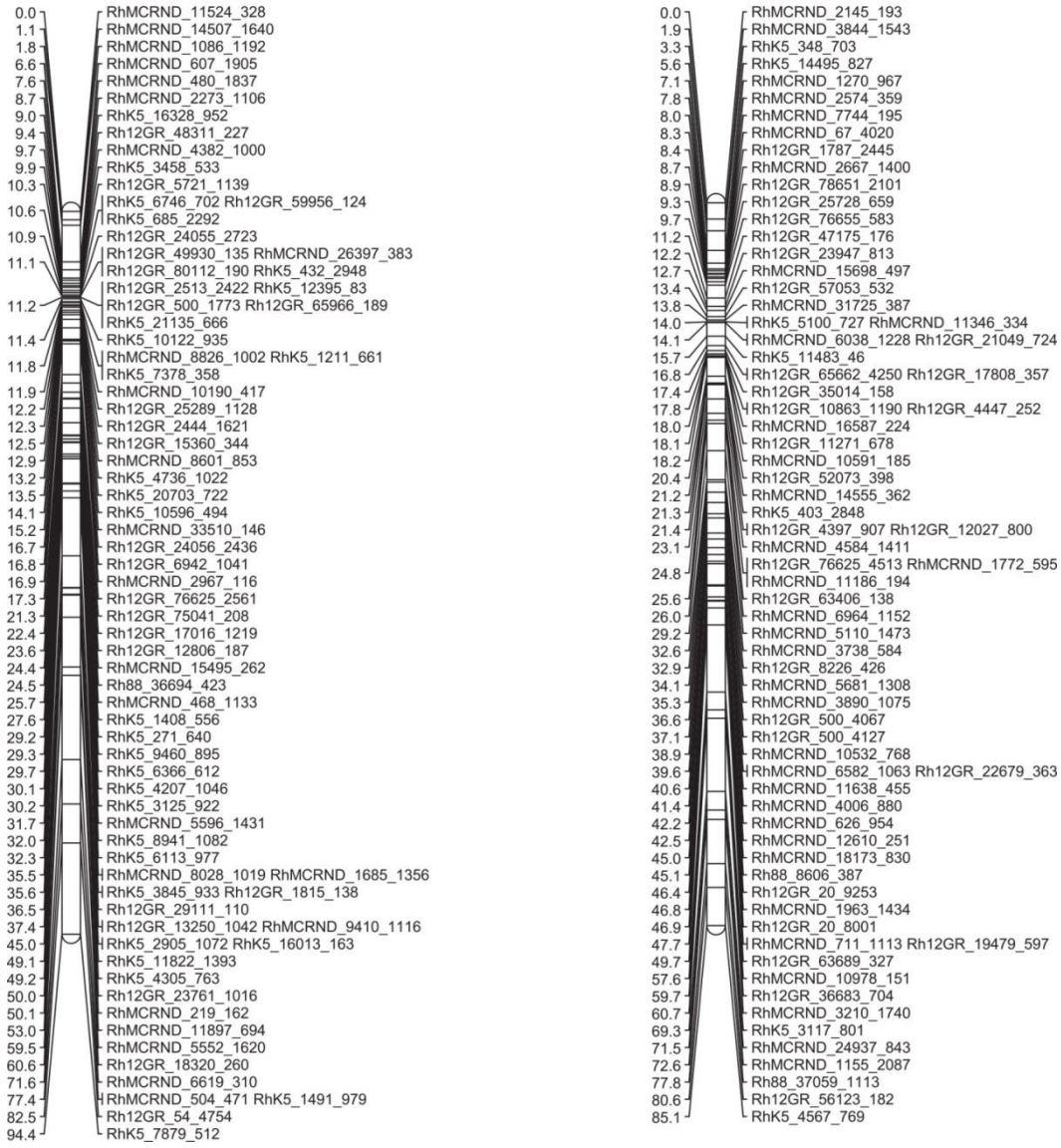
Appendix 2 (continued). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.



Appendix 2 (continued). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.

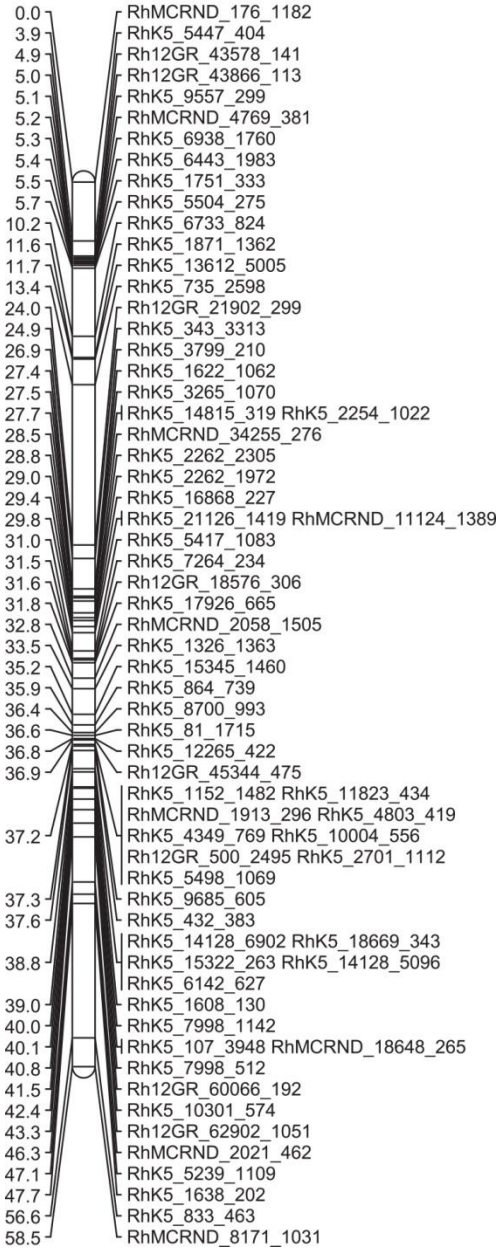


Appendix 2 (continued). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.

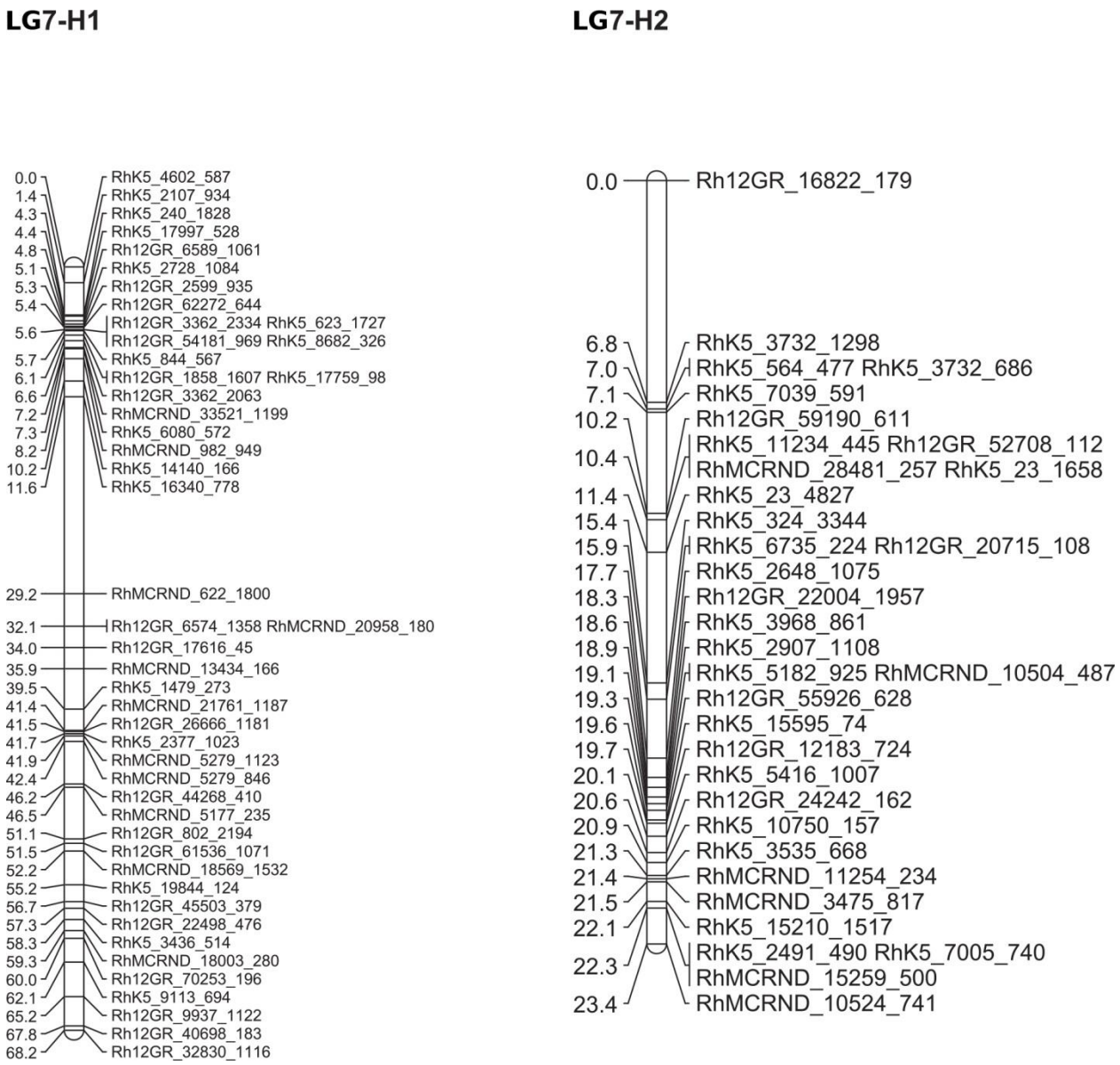


Appendix 2 (continued). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.

LG6-H3

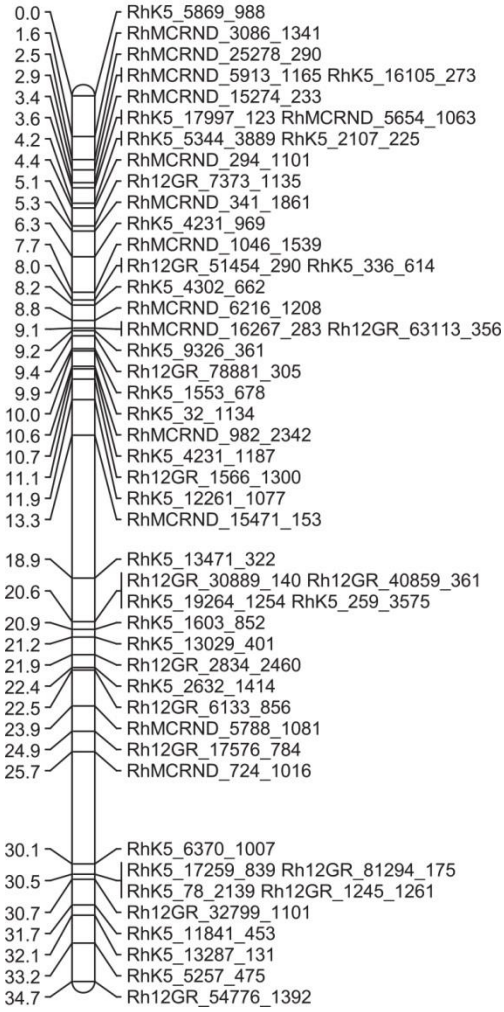


Appendix 2 (continued). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.



Appendix 2 (continued). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.

LG7-H3

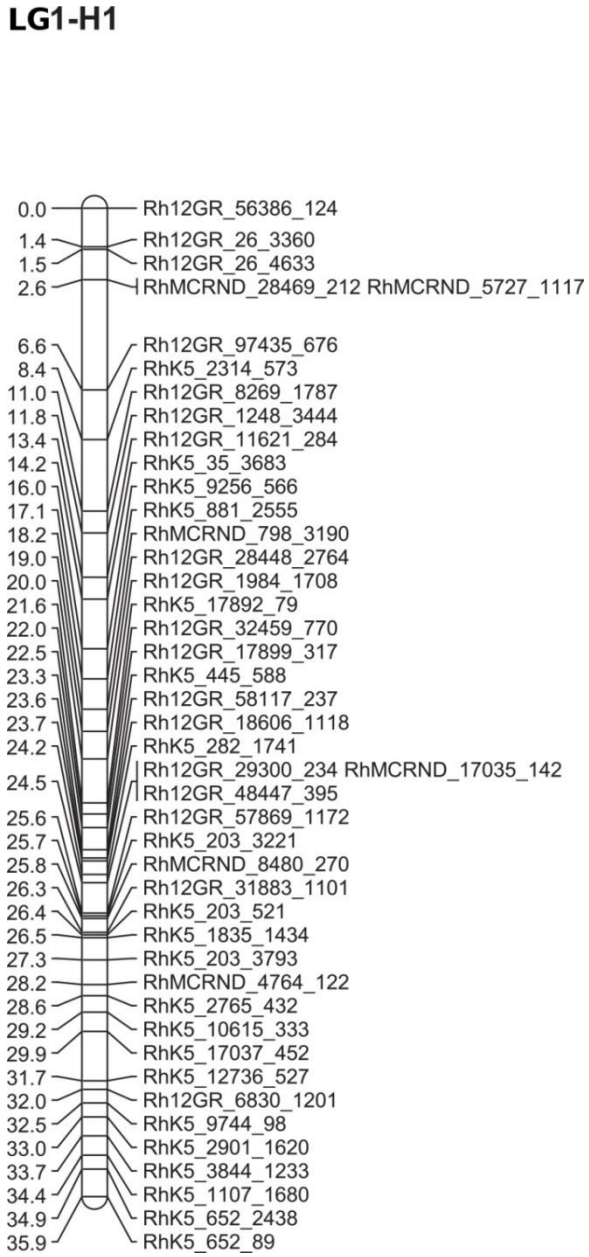


Appendix 2 (continued). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.

Appendix 3. Distribution of different marker types on parental linkage maps of RND and HP (RNDxHP population) and integrated linkage map for RND (RNDxRND) over chromosomes and homologs.

		RNDxHP-HP					RNDxHP-RND					RNDxRND			
LG	Homolog	SxN	SxS	DxN	Total		SxN	SxS	DxN	Total		SxN	SxS	DxN	Total
LG1	H1	36	0	10	46		17	1	0	18		0	44	0	44
	H2						8	1	0	9					
	H3						6	1	0	7					
	H4														
LG2	H1	14	50	0	64		22	0	3	25		0	46	0	46
	H2	17	1	0	18		42	1	0	43		0	72	0	72
	H3	20	0	2	22		32	0	51	83		0	64	0	64
	H4											0	50	0	50
LG3	H1	50	2	11	63		7	61	0	68		0	102	0	102
	H2	25	41	0	66		59	23	4	86		6	255	0	261
	H3						46	56	0	102		0	99	0	99
	H4						14	16	0	30		0	43	0	43
LG4	H1	50	13	4	67		61	7	0	68		0	127	0	127
	H2	6	0	2	8		47	0	2	49		0	76	0	76
	H3	6	0	0	6		18	13	3	34		0	19	0	19
	H4											0	55	0	55
LG5	H1	21	14	4	39		33	17	0	50		0	138	0	138
	H2	11	0	20	31		54	0	0	54		0	65	0	65
	H3						21	0	8	29		0	53	0	53
	H4						13	0	3	16		0	46	0	46
GL6	H1	8	0	0	8		77	0	0	77		0	92	0	92
	H2	11	0	0	11		29	40	4	73		0	49	0	49
	H3	16	0	0	16		68	0	0	68		0	102	0	102
	H4	16	0	1	17							0	52	1	52
LG7	H1	13	0	1	14		46	0	0	46		0	141	0	141
	H2	15	0	4	19		34	0	0	34		0	28	0	28
	H3	17	0	0	17		52	0	0	52		0	66	0	66
	H4											0	40	0	40

Appendix 4. Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers. Linkage groups are numbered from 1 to 7 following the ICM (Spiller et al., 2011), containing each 1, 2, 3, or 4 homologous groups (H). Markers are indicated to the right of each LG and map positions of markers (cM) are given to the left of each linkage group.



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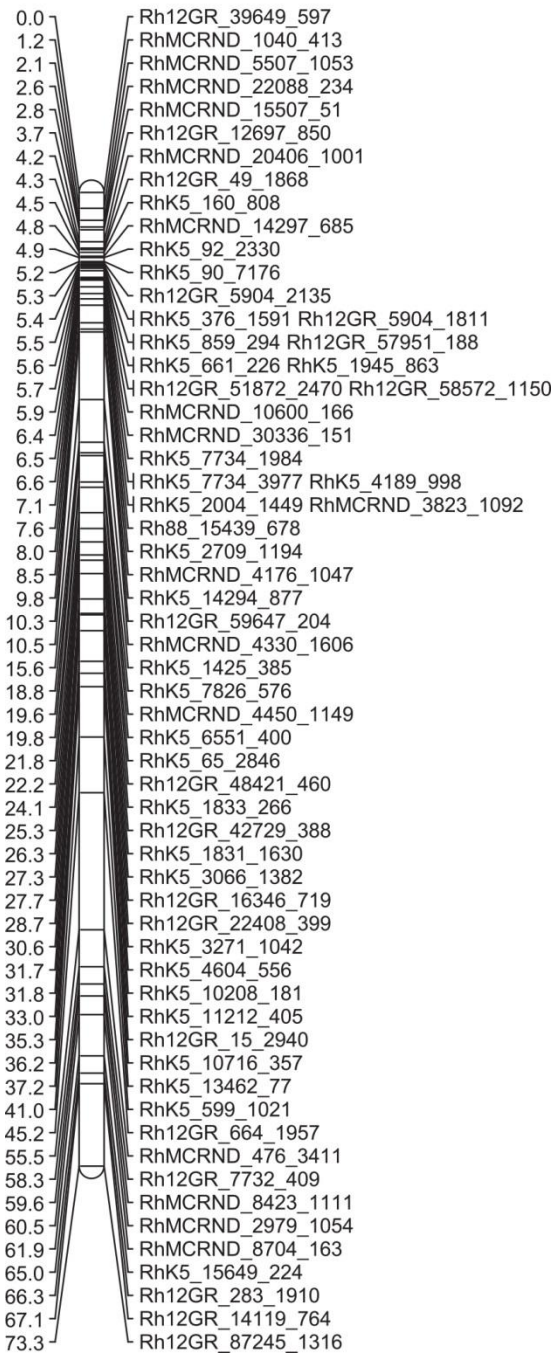
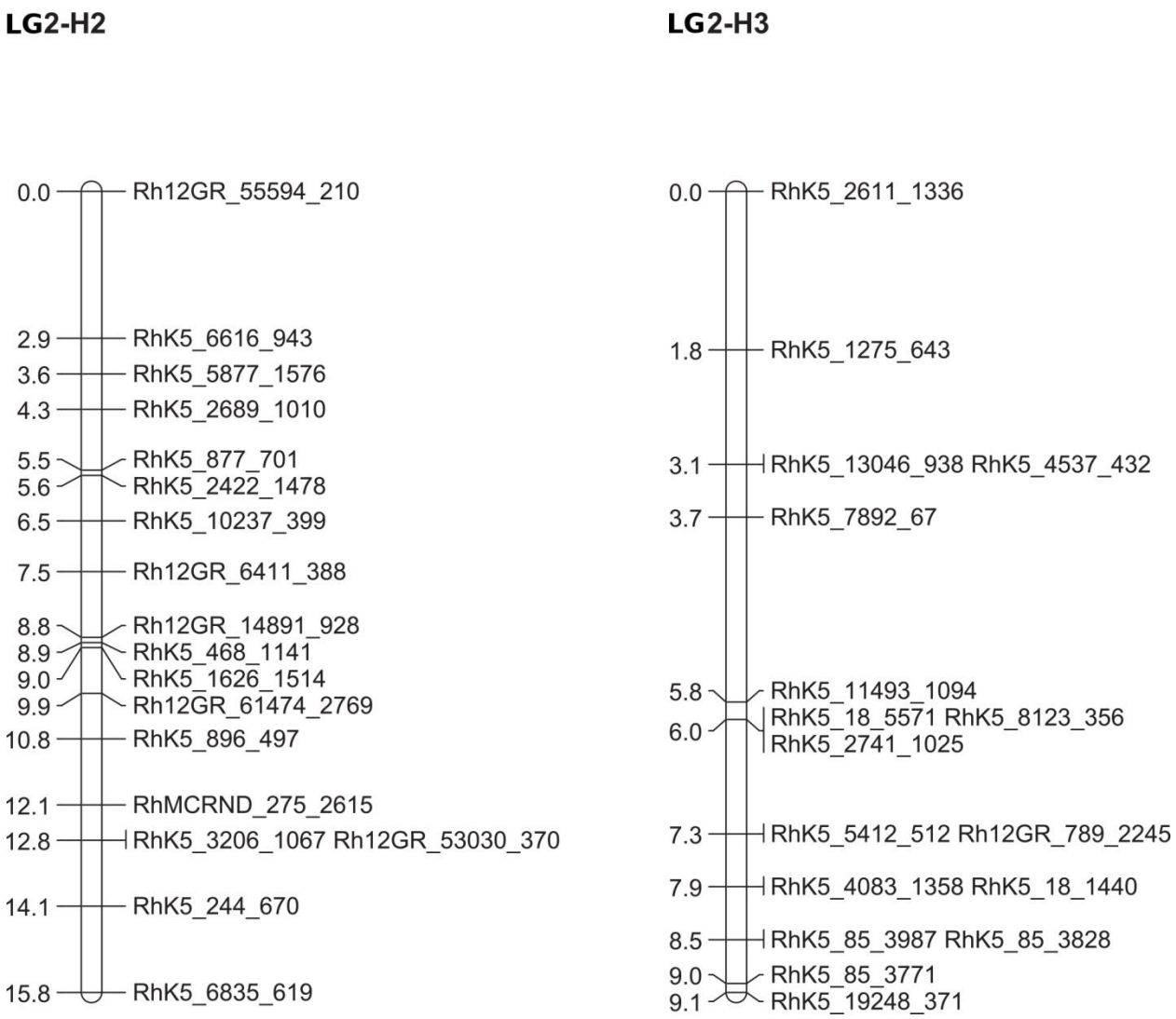
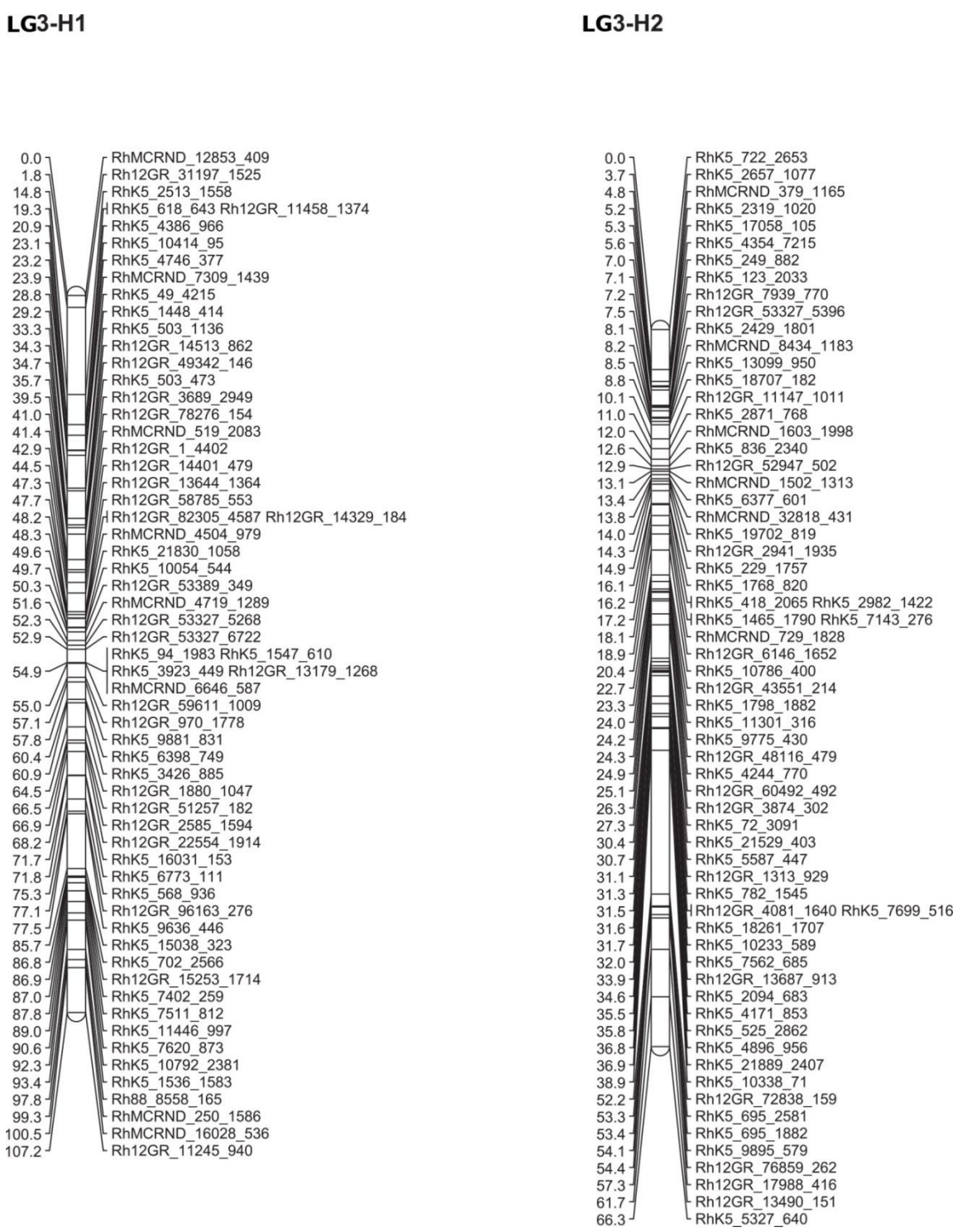


Appendix 4 (*continued*). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.

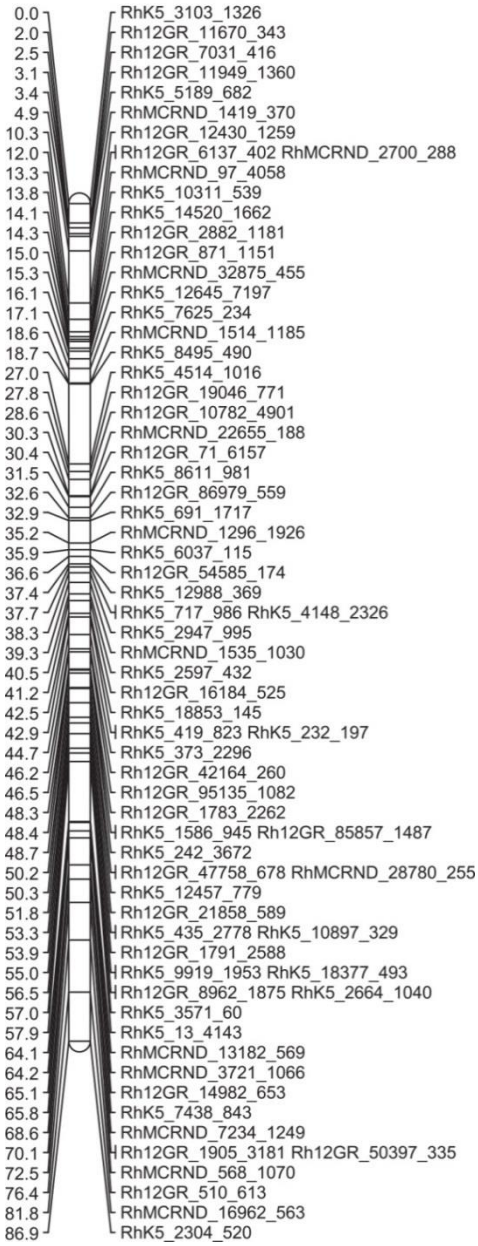


Appendix 4 (*continued*). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.

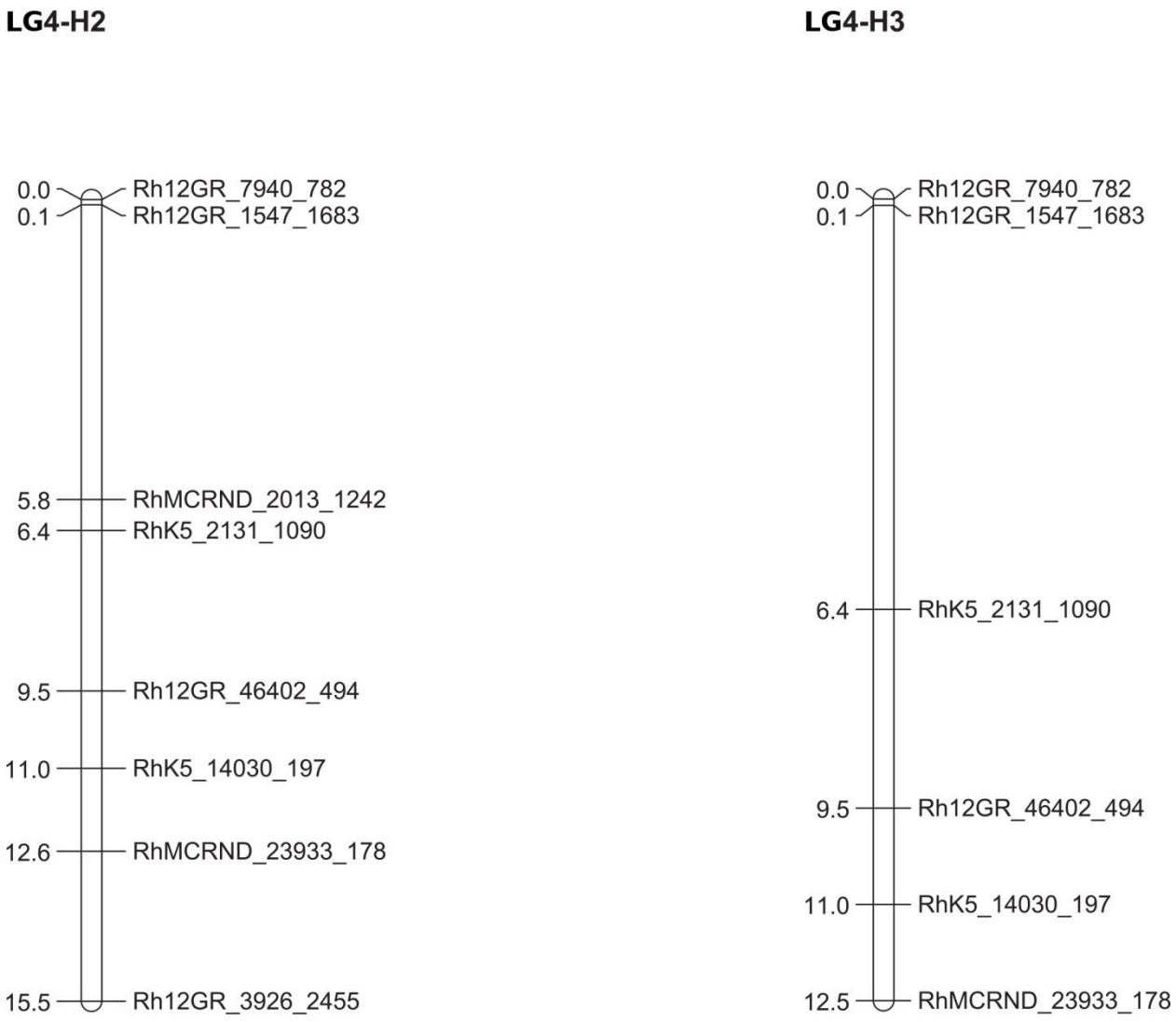


Appendix 4 (*continued*). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.

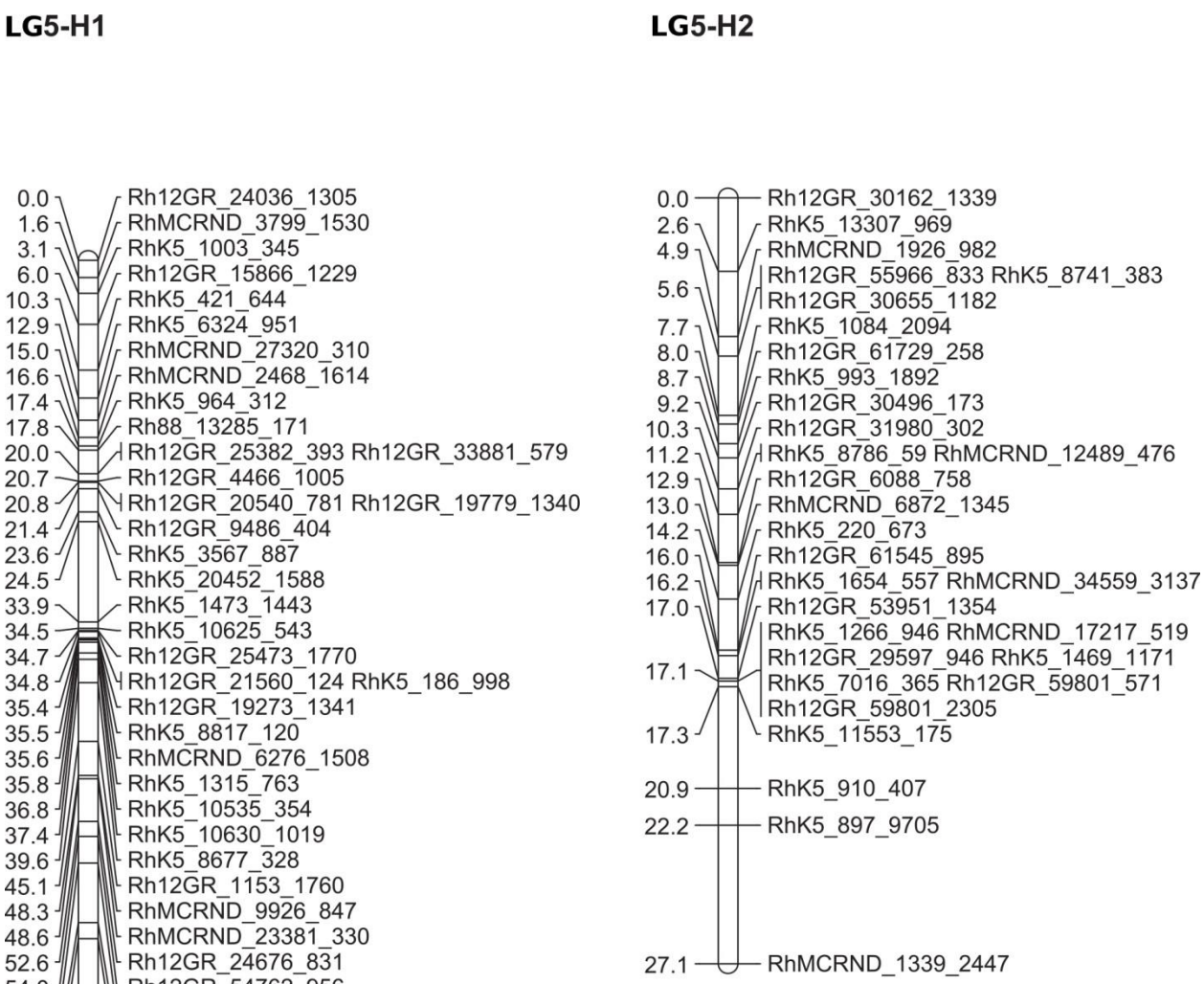
LG4-H1



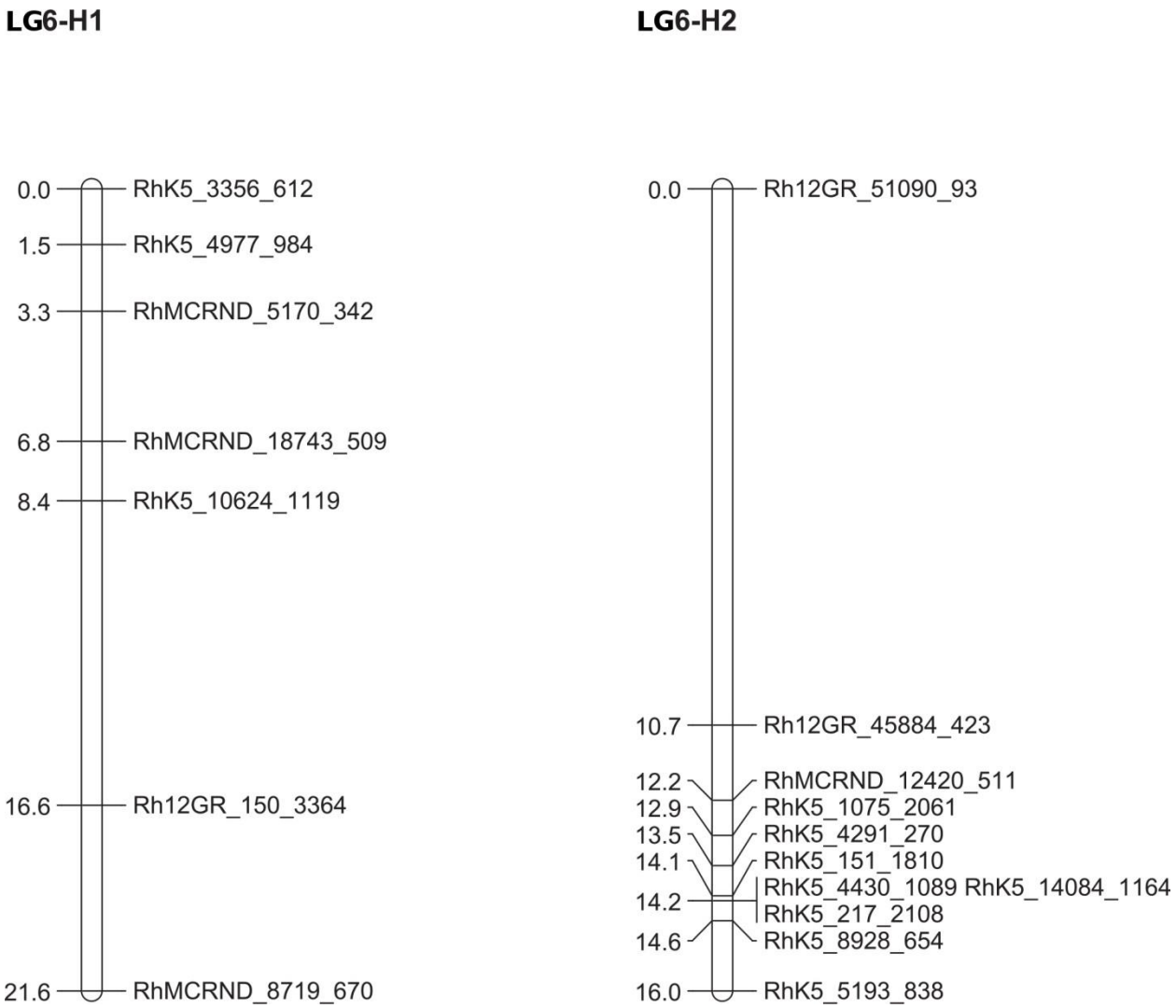
Appendix 4 (*continued*). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.



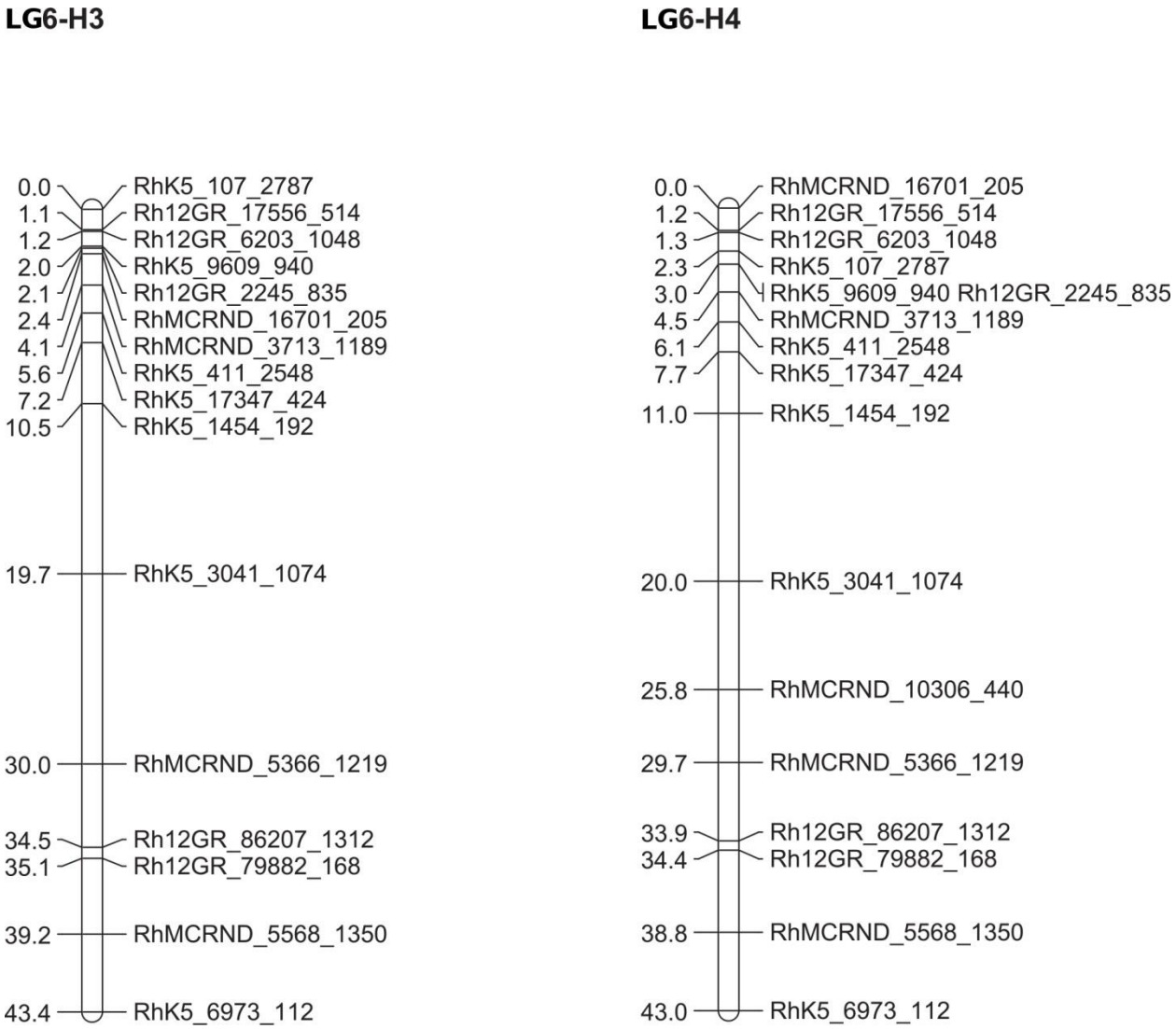
Appendix 4 (*continued*). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.



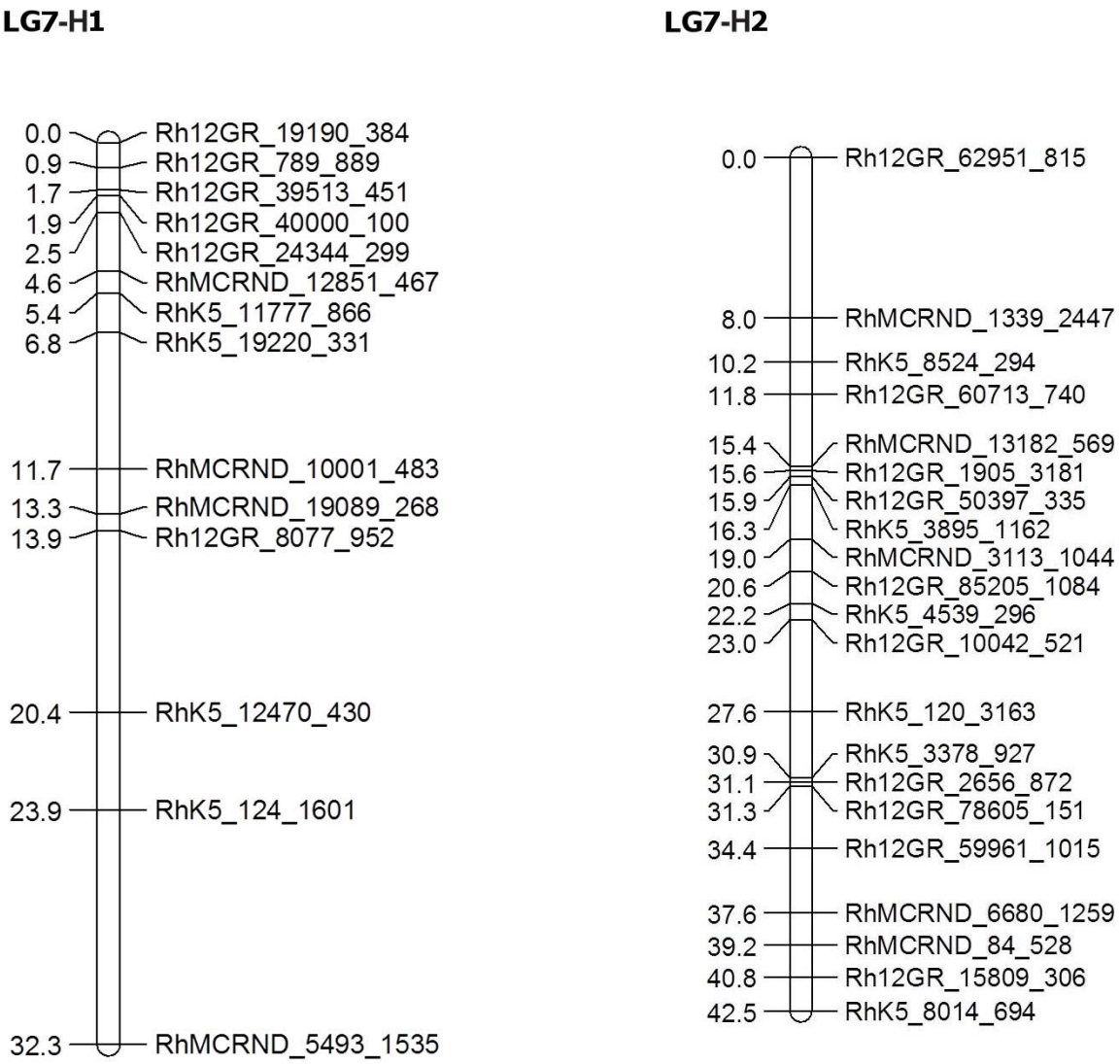
Appendix 4 (*continued*). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.



Appendix 4 (*continued*). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.

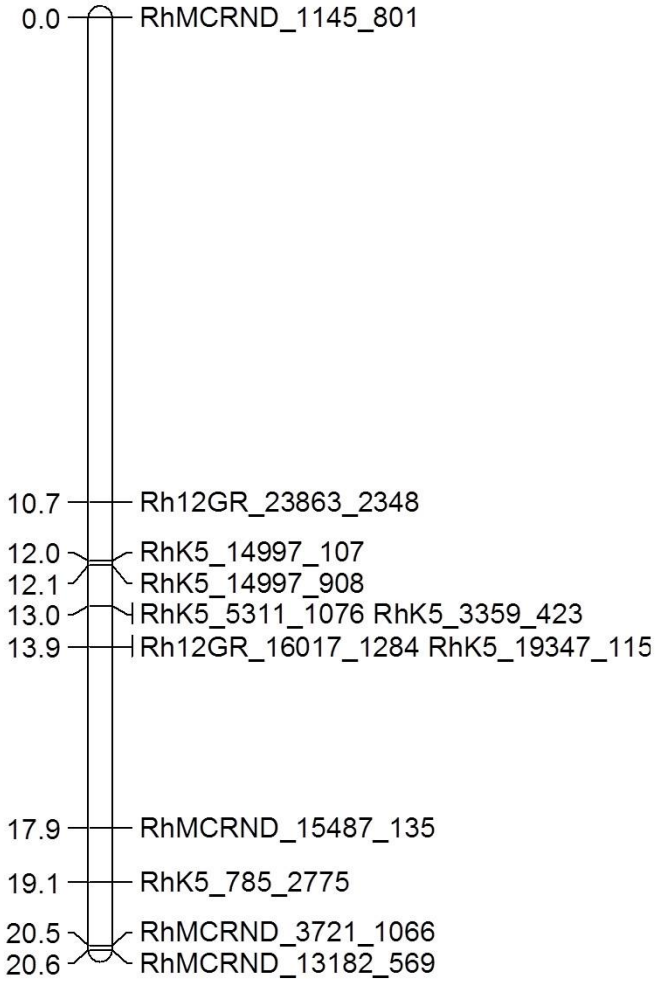


Appendix 4 (*continued*). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.



Appendix 4 (*continued*). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.

LG7-H3



Appendix 4 (*continued*). Parental linkage map for RND (RNDxHP population) constructed with biallelic SNP markers.

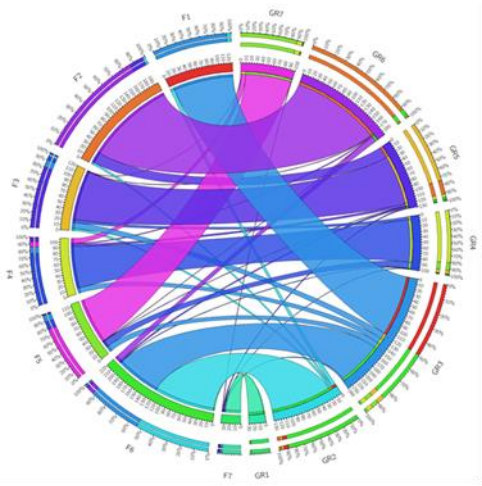
Appendix 5. Number of markers in parental RND map of population RNDxHP placed on the strawberry pseudo-chromosomes (FvCh 1-7). The rose sequence contigs from which the SNPs had been derived were BLASTed to the Fragaria genome sequence (Shulaev et al., 2011) and the best hit was used if above the threshold. Unmapped markers are marked as UnM.

	FvCh1	FvCh2	FvCh3	FvCh4	FvCh5	FvCh6	FvCh7	UnM
GR1	0	0	0	0	0	2	29	3
GR2	24	4	4	0	0	118	0	1
GR3	118	0	12	10	11	86	3	46
GR4	0	0	6	82	12	1	1	49
GR5	0	22	97	5	1	3	2	19
GR6	0	172	1	2	1	11	0	31
GR7	0	0	1	9	89	0	1	32

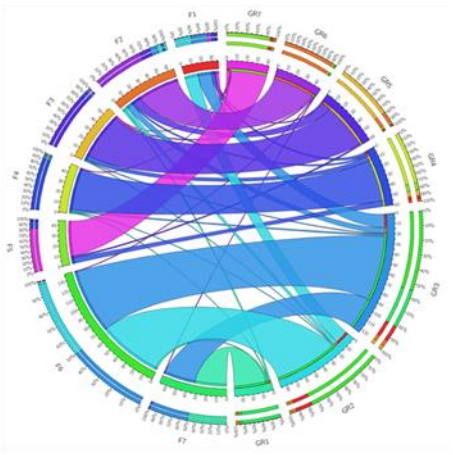
Appendix 6. Number of markers in parental HP map of population RNDxHP placed on the strawberry pseudo-chromosomes (FvCh 1-7). The rose sequence contigs from which the SNPs had been derived were BLASTed to the Fragaria genome sequence (Shulaev et al., 2011) and the best hit was used if above the threshold. Unmapped markers are marked as UnM.

	FvCh1	FvCh2	FvCh3	FvCh4	FvCh5	FvCh6	FvCh7	UnM
GR1	0	2	0	1	0	2	33	8
GR2	14	4	1	1	0	66	0	8
GR3	14	6	1	2	0	68	33	16
GR4	3	2	5	44	6	0	0	21
GR5	2	9	48	0	2	0	0	9
GR6	0	43	2	0	0	1	1	5
GR7	3	0	1	1	37	0	0	8

Appendix 7. Synteny between *Fragaria vesca* (genome sequence) and garden rose (A parental RND and B parental HP map of RNDxHP population). The synteny was established by linking the contigs on which the mapped rose SNPs reside to the homologous region of the *Fragaria vesca* genome sequence by Blast.



A



B

Chapter 6

Surviving in the cold: quantitative trait loci associated with winter hardiness in tetraploid garden roses

Vukosavljev M, ArensP, van de Weg WE, Visser RGF, Voorrips RE, MaliepaardC, van de Wouw M, Srećkov Z, Smulders MJM

Surviving in the cold: quantitative trait loci associated with winter hardiness in tetraploid garden roses

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Abstract

Winter hardiness is a complex trait and one of the most important limiting factors for garden rose growth and distribution in areas characterized by a continental climate. This research was undertaken to determine the genetic regions underlying winter hardiness of garden roses, and to identify linked markers. For this purpose we exposed two segregating populations, RNDxRND and RNDxHP, to temperatures below -15˚C in a cold chamber and in the field in Serbia. Frost damage was estimated directly at the phenotypic level (proportion of dieback) and at the non-visible physiological level indirectly (through the potential for meristem production in spring; regrowth). Two tentative QTLs for winter hardiness were detected in the RNDxRND population and also two in the RNDxHP population, one in common between the two populations. The ability of plants to regrow in spring was associated with genomic regions on two linkage groups of the RNDxRND population and two different linkage groups in the RNDxHP population. A comparison of the ability for regrowth and level of damage caused by low temperature revealed that these two traits are inherited independently and that cold tolerance depends on the ability of plants to withstand low temperature and to regrow fast in spring.

Introduction

Garden roses are among the most economically important ornamentals (Gudin, 2010). It is estimated that between 80 and 100 million grafted garden roses are sold every year. In addition to this, garden roses are multiplied and introduced to the market as cuttings and pot plants, in total representing a value of 560 million € (Peter Cox, "Roath", Eindhoven; personal communication; Irma van der Hoeven, "FloraHolland", Naaldwijk ; personal communication). Recently the markets in Eastern Europe have shown a steady growth (Peter Cox, "Roath", Eindhoven; personal communication). Unfortunately most commercial cultivars are not well adapted to the continental climate of Eastern Europe, characterized by long, cold winters and hot summers. While hot summers do not reduce the rose growth drastically, the cold winters are one of the major limiting factors for rose growth and

distribution. Thus, there is a need to understand the genetic background of winter hardiness in roses in order to more efficiently breed new cultivars tolerant to low temperatures.

Cold tolerance or winter hardiness (both terms are used more or less for the same adaptation in the scientific literature) is the plant's ability to adapt its metabolism and growth as a response to suboptimal temperature (NDong et al., 1997). Cold tolerance is often mixed with frost tolerance. Frost is the expression for several types of ice coatings and accumulations that may form in humid, cold conditions (mostly overnight). In continental climates it most commonly appears as crystals or frozen dew drops (Oliver, 2005). Thus cold tolerance is the broad term involving hardiness at the whole spectrum of suboptimal (both positive and negative) temperatures, while frost tolerance is practically the resistance to ice formation and consequently temperatures below 0ºC (freezing temperatures, Galiba et al., 2013).

During winter plant metabolism is at low profile but still some amount of water is lost in a process of respiration. Low temperature in combination with wind and poor soil moisture causes that amount of lost water exceeds water uptake by roots. These circumstances result in desiccation. Additionally, snow increases the rate of desiccation. Even though snow protects plant parts that are below the snow, at the same time it reflects sunlight warming up the parts above the snow. Even though desiccation is not always seen at the level of phenotype, it drastically reduces plant ability to withstand low temperature (Fuchinoue, 1982).

In cold tolerant plants low temperature induces physiological and biochemical changes which result in achieving hardiness through acclimation. The importance of winter hardiness for plants may be best described by the estimation that more than 70 COld Responsive (COR) genes (either by up- or down-regulation) and 300-450 metabolites are involved in the response to low temperature (Kreps et al., 2002; Cook et al., 2004; Kaplan et al., 2004; Hannah et al., 2005; Vogel et al., 2005; Kosova et al., 2007; Moellering et al.; 2010; Li et al., 2011). Cell membrane stability is a crucial factor. During acclimation membrane stability is modified by alterations in sugar and protein level, fatty acids (chain length, isomerization, cis-trans ratio, level of unsaturated fatty acids), and lipid content (Zhou et al., 2009; Heidarvand & Amiri, 2010; Preston & Sandve, 2013). Additionally, during acclimation, changes have been detected in the carbohydrate level, in protein composition (dehydrins, antifreeze proteins, heat shock proteins, cold shock proteins, domain proteins, etc.) and in fatty acid composition (especially proline), which have an effect on redirecting plant metabolism (Obrist et al., 2001; Welling et al., 2004; Yan et al., 2006; Wellin et al., 2006; Burbulis et al., 2008; Renaul et al., 2008; Woldendorp et al., 2008; Park et al., 2009; Heidarvand & Amiri 2010; Pagter & Arora 2013).

The suitability of a particular cultivar for cultivation at below-zero temperature depends on the maximum winter hardiness level and on the timing of acclimation and de-acclimation. Studies on the relation between the ability for acclimation, winter hardiness, and de-acclimation indicated that these processes are probably inherited independently and thus it is recommended to evaluate them separately during selection and breeding for cold tolerance (Arora & Rowland, 2011).

Acclimation to low temperatures in the fall is brought about through changes at biochemical and physiological levels in plants. Many compounds that have a protective role are accumulated during acclimation, while metabolism-related biochemical pathways are suppressed. Both ABA-dependent and ABA-independent genes participate in plant acclimation (Talanova et al., 2011), with cross-talk between these pathways (Heidarvand &

Amiri, 2010). Indeed, genetic studies indicate that acclimation is a multigenic trait (Arora & Roeland, 2011; Arora et al., 2000; Pan et al., 1994). Zuzek et al. (1997) reported that timing and rate of acclimation have been limiting factors for rose growth in the Minnesota Arboretum.

De-acclimation occurs in response to increased temperatures in spring and results in de-hardening. When cold acclimated plants are exposed to warm temperatures the level of carbohydrates and proteins rapidly decreases, resulting in a plant that no longer possesses high level of cold tolerance (Trischuk et al., 2014).De-acclimation is a fast process and winter hardiness can be lost in a few days. If de-acclimated plants are again exposed to cold spells damage may occur. This depends on the depth of de-acclimation and the ability of the plant to re-acclimate (Arora & Rowland, 2011; Pagter & Arora, 2013). The degree of temperature fluctuation is probably the most important factor for determining the rate of de-acclimation. The induction of de-acclimation depends on plant geographic distribution (as plants from regions characterized by temperature alternations have developed more de-acclimation resistance), climate, genotype, dormancy level, early plant growth in spring, and progression of winter (as plants de-acclimate faster in late winter/early spring than they do in mid-winter; Leinonen et al., 1997; Kalberer et al., 2007; Arora & Rowland, 2011; Pagter & Williams, 2011; Pagter & Arora, 2013).

De-acclimated plants still can survive stress induced by low temperature if they have the capacity for re-acclimation. Re-acclimation is the process in which de-acclimated plants recover part of the winter hardiness lost during de-acclimation. Re-exposure to low temperatures results in re-accumulation of carbohydrates and proteins (Trischuk et al., 2014). Resilience against cold will be higher if re-acclimation is quicker (Kalberer et al., 2006). Insight into kinetics suggests that de-acclimation is a faster process (taking days to weeks) than acclimation (which may take weeks to months). These differences may be explained by different energy requirements (Browse & Lange, 2004; Kalbere et al., 2007b). Comparison of the capacity for de-acclimation and re-acclimation of plants did not show any correlation between them (Arora & Rowland, 2011). In azalea (Kalberer et al., 2007a) re-acclimation could be achieved only if a minimal level of de-acclimation (de-hardening) had been reached. In apple the duration and level of higher temperature as well as the de-acclimation stage influenced (limited) the re-acclimation ability (Howell & Weiser, 1970). Limitation of re-acclimation can be due to irreversible developmental changes after de-acclimation (e.g., bud burst) and to the lack of energy substrates necessary for re-acclimation (Arora & Rowland, 2011).

In rose winter hardiness probably is the result of a combination of several physiological processes and escape mechanisms, including frost tolerance itself and a delay in bud break in spring (so that damage due to late spells of frost can be avoided). This would mean that cultivars that flower later in spring may more often be more winter-hardy. During the past 50 years a large set of Canadian cultivars has been produced in two programs, the Explorer and Parkland Series. Some of these cultivars can withstand up to -45° C (Ogilvie et al., 1999; http://www.helpmefind.com/rose, accessed on 04.04.2014.). Winter hardy offspring could be obtained in one to three generations of breeding, which suggested that winter hardiness in roses is controlled by a limited number of major genes or closely linked genetic factors. This was supported by the lack of variation in hardiness level among offspring of various hardy parents (all are winter hardy) at the diploid and tetraploid level (Svejda, 1974; Svejda, 1979).

The possibility to detect QTLs for winter hardiness depends on the experimental design and the observation and quantification of traits involved in winter hardiness. Experiments set up under uncontrolled conditions in the field can assess overall winter hardiness, but in most cases they do not allow to distinguish (separate) effects of various stresses which cause plant loss. For better understanding of single-stress effects experiments under controlled conditions (climate chambers) are needed. However, there is not a single component that by itself determines the genotype's ability to survive low temperature, and combined effects cannot be estimated in climate chambers (Croser et al., 2003). This suggests that the most appropriate method to estimate winter hardiness is to conduct both field and climate chamber experiments. To get a better insight into such a complex trait as winter hardiness, it is necessary to dissect the trait into components and analyze these separately. The injury caused by low temperature often is observed at the level of phenotype as a change of color (necrosis, dieback; Nejad 2005). However, some changes at cellular or biochemical level cannot be observed by eye, but influence the pace of growth (Arora et al., 2011). Even more, some genotypes developed a strategy to survive by compensating damage caused by low temperature with fast meristem growth in spring (Croser et al., 2003). Therefore, estimation of damage level in combination with evaluation of potential for meristem growth in spring gives a better and more accurate insight into plant winter hardiness.

The aim of our study was to determine the genetic regions underlying winter hardiness of tetraploid garden roses, and to identify linked markers. For this purpose we exposed two segregating populations, for which a high-density SNP-based genetic maps are available (Chapter 5), to temperatures as low as -16 °C in a cold chamber. The frost damage in the hardened plants was estimated, both at the phenotypic level (proportion of dieback) and at the non-visible physiological level indirectly through the potential for meristem production in spring (regrowth). The level of damage and regrowth were used in a QTL analysis. As older plants show a higher level of winter hardiness, we included both one- and two-year old cuttings of these plants, grown on their own roots. In addition, the population was grown in the field in Serbia to evaluate winter hardiness across the season.

Materials and Methods

Plant materials and maps

For the purpose of detecting genome regions responsible for winter hardiness in garden roses two populations were available: RNDxRND, a selfed population of European cultivar "Red New Dawn" which consists of 103 offspring plants and RNDxHP, a cross between RND and hypothetical pollen donor (HP) which consists of 74 offspring plants (Chapter 5). For both populations we developed dense genetic maps employing the WagRhSNP Axiom SNP Array (Chapter 5).

Winter hardiness evaluation

To assess winter hardiness two types of trials have been utilized: in the first trial plants were subjected to cold storage in a cold chamber; in the second, plants were subjected to field conditions over winter in a location with a continental climate.

Cold chamber experiment

In order to determine the optimal temperature for the main experiment, a pilot experiment was carried out to determine LT_{50}, a temperature at which 50% of the plants would die. For this purpose one- and two-year old cuttings of 6 commercial cultivars of *Rosa hybrida* ("Morden Centennial", " Moje Hammarberg", " The Fairy", "Henry Kelsey", "Snow Ballet", and "New Dawn") from different USDA Plant Hardiness Zones (http://planthardiness.ars.usda.gov/PHZMWeb/) were exposed to -10, -12, and -20°C for 24 hours. Temperatures were measured inside the boxes using temperature data loggers. Results indicated that -15°C (reached after 13 hours) was the temperature closest to LT_{50} for roses (Appendix 1).

For the main experiment one- and two-year old cuttings of 99 offspring of the RNDxRND and 69 offspring of the RNDxHP population (Chapter 5) were used. For most genotypes 20 one-year old and 12 two-year old cuttings were used. The two-year old cuttings were made in the summer of 2011. In May-July 2012 an additional set of cuttings (1-year plants) was made. They received additional light from October until December 2012. Both 1- and 2- year old plants were grown in pots filled with standard commercial potting mix in an unheated, frost-free greenhouse at Wageningen University, The Netherlands. Roses were kept in the greenhouse until the end of January 2013. After this period it was assumed that roses had acclimated to low temperatures and become dormant.

Plants of each genotype were randomly distributed within each age group over 5 batches. Within each batch the roses were arranged in cardboard boxes (60x40x40cm) in a completely randomized design. Finally, all boxes per batch were randomly distributed over 21 (1-year roses) and 22 (2-year roses) Euro-pallets. Each pallet contained four boxes next to each other and two or three boxes stacked on each other. At the end of January 2013 all the boxes were transported to the freezing company "Vriesoord", ('s-Hertogenbosch, The Netherlands) and stored in a cold chamber at 0°C in darkness. At least five boxes per batch contained a data logger and additionally the freezing cells were equipped with a thermometer. Each week, starting from the end of February, one batch was exposed to -15 °C for 28 hours. After this exposure all boxes of the batch were placed at 0°C for 24 hours. Subsequently, the plants were transported to a greenhouse (near 's-Hertogenbosch, The Netherlands) with controlled 18°/10°C day/night temperature and additional light. All plants were immediately watered on arrival and damage was estimated by assigning codes from 0-5 (0 meaning high damage and 5 meaning no damage; Table 1, Figure 1). Low temperature injury may cause necrosis and change of tissue color (browning) due to oxidation (Faust, 1997). Hence the change of stem color (green into brown) can be used as a morphological indication of damage.

Table 1. Description of codes for cold tolerance ascribed to the roses after moving from cold chamber.

Code	Description
0	Dead, no visible green parts
1	Dead, stem (partly) green (with closed buds)
2	Dead, stem (partly) green with small leaves forming that are dying
3	Alive, green stem with fresh green leaves forming, plant has dieback
4	Alive, green stem with fresh green leaves, no dieback, but some visual damage to the leaves
5	Alive, no visible damage, fresh green plant

Five weeks after moving plants to the greenhouse, height and dieback of the longest branch was measured in cm and frost damage was calculated as proportion of dieback compared to whole branch length. Damage caused by low temperature is not always morphologically visible, but can affect the pace of growth in spring. Thus, three weeks after the first assessment of damage, the frost damage of plants and extent of regrowth were measured.

Figure 1. Level of damage attributed to codes used for evaluation the effect of low temperature.

For regrowth the average new shoot length was expressed as percentage of the initial length of the branch.

Field trial

The experiment was carried out in Mali Iđoš (lat. 45° 42' 30" N; long. 19° 40' 2" W), Serbia during winter 2012-2013. The survival and injury caused by low temperature were evaluated on successfully budded rose plants of 146 genotypes (61 genotypes of the RNDxHP population and 85 genotypes of the RNDxRND population). To obtain a sufficient (adequate) number of replicates, between 20 and 50 budding eyes of each genotype had been grafted on *Rosa laxa* rootstocks in June 2012. This finally resulted in between 8 and 42 replicates per genotype, which were grown under standard conditions. Plants of the two populations were kept separate, so random in the field (Figure 2). The genotypes were not randomized over the experimental plots due to logistics, but the trial was part of a larger rose field. Hence, the plants were surrounded by other cultivars. The fact that plants were surrounded by other cultivars does not compensate for the lack of randomization, at most for lack of borders. It means that all replicates of a genotype were in the same position. In that case, position effects and genotype effects are confounded, and there are no replicate observations, but there will be only one (independent) observation per genotype. The distance between rows was 105 cm, the distance between plants was 10 cm.

Figure 2. Trial field set set up in Mali Iđoš (lat. 45° 42' 30" N; long. 19° 40' 2" W), Serbia during winter 2012-2013.

In March 2013 frost damage was calculated as proportion of dieback compared to whole branch length (%). Regrowth was measured in April 2013. Both were measured as in the cold chamber experiment described above.

Data analysis and QTL mapping

The percentage of damage (D) caused by low temperature was estimated per branch over multiple branches per plant using the equation:

$D=100*l_d/l_t$

in which l_d is the length of dieback and l_t the length of whole branch (total).

The percentage of regrowth (R) for each branch was estimated using the equation:

$R=100*l_n/l_{in}$

in which l_n is the length of the new shoot and l_{in} the initial length of the branch (l_t from the previous equation) and the final regrowth of each genotype was calculated as average regrowth of all branches over replicates.

Significance of differences in regrowth and damage triggered by low temperature between the two mapping populations was tested with a 2-sample Welch's t-test (GenStat 16, VSN International, 2013).

QTL analysis was performed using in-house written scripts run in R 2.12.2 (R Core Team, 2012). Scripts were written for marker/trait association based on regression on marker dosage, ANOVA on marker dosage and ANOVA on presence/absence of a marker allele. A significance threshold was calculated by a permutation test by running 1000 permutations of the real data and, alternatively, by running simulations with random normally distributed data (1000 and 5000 simulations); the threshold for the $-^{10}\log$(p-value) was computed from the 95-percentiles from the empirical distributions. Based on these results ($-^{10}\log P$ of 4.1 for permuted real data, 3.9 for simulated normally distributed data), the threshold was set to a $-^{10}\log$(p-value) of 4.0. The proportion of the total phenotypic variance among genotypes explained by a marker was estimated by R^2.

In case of two observed QTLs, a multiple regression approach was performed to quantify the effect of both QTLs, using GenStat 16 (VSN International; 2013), following the model:

$y=\mu+M_1+M_2+e$

in which y represents the phenotypic trait, μ represents the expected mean M_1 and M_2 represent tentative QTLs at marker positions and e represents statistical error. No interaction of the two QTLs was modelled.

QTL regions were plotted using MapChart 2.2 (Voorrips, 2002) along (parts of) the linkage maps for each of the two populations (see Chapter 5).

Results

Cold chamber experiment

The plants were put into the -15 °C cold chamber in five batches. The temperature loggers inside the boxes indicated that the temperature dropped slowly and that the temperature of -15°C was not reached for batch 1 and thus this set of plants was excluded from analysis. The lowest temperature for batches 2 to 5 were -15°, -12°, -16.5°, and -16° C

respectively. The data loggers output (Figure 3) indicated that after moving plants to the freezing chamber the temperature dropped gradually and once the freezer was switched off temperature increased fast (a period of one hour) until a plateau was reached (at around -1° to -4°C). The position of pallets inside the cold chamber had some influence on the actual temperature inside the box, but no clear pattern could be detected.

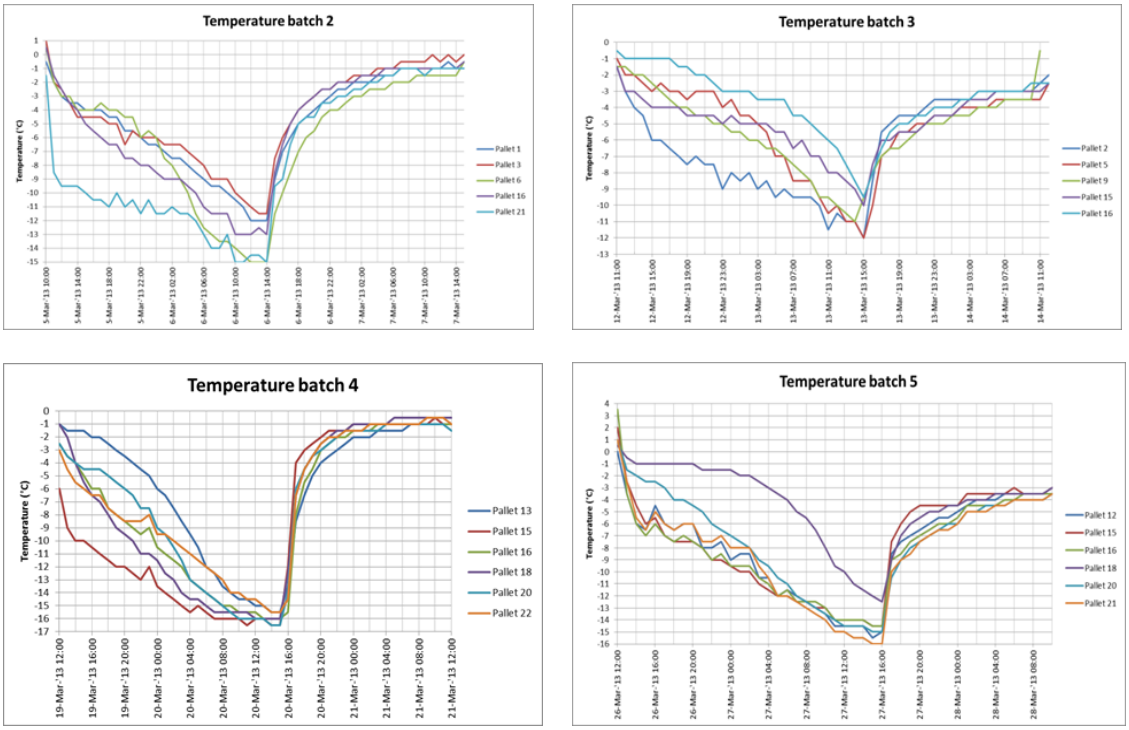


Figure 3. Measured temperatures inside the boxes during exposure to -15°C and subsequent de-freezing at 0°C for batches 2-5. Data loggers were put inside randomly chosen boxes across pallets (individual loggers are depicted by separate colors).

Genotypes of the RNDxRND population were most cold sensitive and proportions of damage caused by low temperature after 5 weeks in the greenhouse were 88.5% for 1-year and 98.3% for 2-year old cuttings, while the damage in the RNDxHP population was 73.1% and 90.7%, respectively, for one- and two-year old plants (Table 2). Differences in damage between the two populations for both one-year old ($p=0.04$) and two-year old roses were significant ($p=0.009$; Welch's t-test). Additionally, damage caused by low temperature differed between batches. For both populations and both age groups the highest proportion of damage was detected for batch 5, while plants from batch 3 showed the highest survival rate. The correlation (r) between the damage (%) of one- and two-year old roses was only 0.07 for RNDxRND and 0.23 for the RNDxHP population. Correlation coefficients varied over batches and for both populations the highest correlation coefficient was detected for batch 3 (Appendix 2).

Table 2. Damage (%) of roses from the populations RNDxRND and RNDxHP, 5 and 8 weeks after exposure to low temperature. Damage was calculated as the proportion of dieback of the longest branch compared to the length of that branch.

	After 5 weeks				After 8 weeks			
	RNDxRND		RNDxHP		RNDxRND		RNDxHP	
	1-year-old (%)	2-year-old (%)	1-year-old (%)	2-year-old (%)	1-year-old (%)	2-year-old (%)	1-year-old (%)	2-year-old (%)
Batch 2	86.2	98.1	67.6	87.7	100.0	100.0	98.8	100.0
Batch 3	71.8	95.0	48.5	79.6	100.0	100.0	99.7	100.0
Batch 4	98.1	100.0	81.8	96.1	99.6	100.0	97.6	100.0
Batch 5	98.1	100.0	94.5	99.4	98.7	100.0	95.5	100.0
Average	88.5	98.3	73.1	90.7	99.6	100.0	97.9	100.0

Eight weeks after moving plants from the cold chamber to the greenhouse a second evaluation was done. Damage after eight weeks was significantly higher than damage after five weeks for all genotypes (two-sample t-test; $p<0.0001$) and none of the 2-year cuttings of either population survived (Table 2). One-year-old genotypes from the RNDxHP population showed a significantly higher rate of survival ($p= 0.016$) than roses of the same age from the RNDxRND cross, but with severe damage (97.9% of the branches damaged or died). In both populations most damage was recorded for roses from batch 2, while roses from batch 5 were characterized with highest survival ability.

Comparison of the best survival rates of 1- and 2-year cuttings of the same population after the cold chamber treatment indicated that different genotypes within the populations showed the best survival. The top 5 plants after five weeks are all different genotypes (correlation $r=0.29$).

A few days after the cuttings were returned from the cold chamber to the greenhouse, initiation of bud growth (regrowth) could be detected, but it was not quantified. However, when the plants were assessed after five weeks, all buds were already affected by necrosis. Possible genetic differences in regrowth potential were thus not recorded.

Results of damage in the cold chamber experiment (Table 2) suggest that in batches 4 and 5, the temperature was too low to survive. Overall hardly any two-year old plants survived, so we performed QTL mapping of damage after five weeks on the one-year old plants of batches 2 and 3 only. The correlation (r) between the level of damage for batches 2 and 3 was 0.64. Comparison of damage rate caused by low temperature in both populations for batches 2 and 3 (Table 3) indicated that offspring of the RNDxHP cross had significantly less damage (63.47% damage) than those of RNDxRND (82.6%; $p < 0.001$).

Table 3. Summary statistics for cold damage (%) from cold chamber (batches 2 and 3) and trial field experiments and regrowth at field (%) for populations RNDxRND and RNDxHP.

	RNDxRND			RNDxHP		
	Cold damage - cold store	Cold damage - field	Regrowth	Cold damage - cold store	Cold damage - field	Regrowth
Mean	82.6	3.65	43.9	63.47	1.88	30.74
Standard deviation	16.88	11.93	13.48	21.7	4.43	15.71
Variance	285.4	142.3	181.8	458.1	15.71	247
Standard error of mean	1.71	1.29	1.47	2.6	0.57	2.01

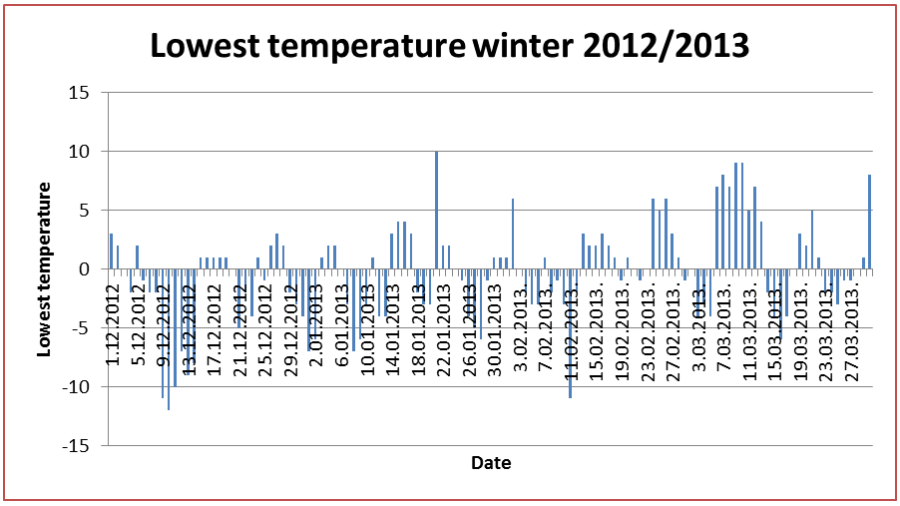
Field experiment

The field experiment was carried out in the winter of 2012-2013. Meteorological data on temperature in Serbia for the period December 1st 2012 to March 30th 2013 (http://www.wunderground.com; Figure 4) indicated a mild winter (average temperature of -0.3°C) with two cold peaks: the first in mid-December (lowest temperature -12°C, the length of cold spell 3 days) and the second in mid-February (lowest temperature -11°C for 1 day). During these two cold periods snow cover reached a height of 40 cm. Due to this relative warm winter, atypical for Serbia, the level of damage caused by low temperature (Table 3) was low (mean values 3.65% for RNDxRND and 1.88% for RNDxHP), and only few genotypes in both populations suffered from low temperature. Furthermore, significant differences between the two populations in mean values for cold temperature initiated damage were not detected ($p=0.21$; Welch's two-sample t-test). Detailed comparison of top 10 plants that performed best for level of damage caused by low temperature in cold chamber experiment did not show the lowest level of damage caused by low temperature at the field.

The field experiment was maintained for another year, but also the winter 2013-2014 was exceptionally warm. In fact, it was even warmer than the previous year (average temperature of 0.9°C for the period December 1st 2013 to March 30th 2014; http://www.wunderground.com), and there was only one cold peak at the end of January (the lowest temperature -10°C, duration of 1 day), the level of damage caused by low temperature was very low and insufficient to perform a QTL study.

In sharp contrast to the level of damage there were noticeable differences in meristem growth (regrowth) in the trial field. Roses of the RNDxRND population had a larger potential for regrowth in spring (44.57% versus 30.73% in RNDxHP). Differences in regrowth between the two populations were significant (Welch's 2-sample t test: $p<0.001$). Detailed comparison indicated that plants that performed best for regrowth in population RNDxRND did not necessarily show the lowest level of damage caused by low temperature in cold chamber (for the four plants with the largest regrowth the damage was 81.3, 26.7, 6.3, and 37.5%). Offspring of population RNDxHP showed a similar tendency: the best three genotypes in terms of regrowth capacity had varying degrees of low temperature damage (49.7, 73.3, and 100% of damage).

A



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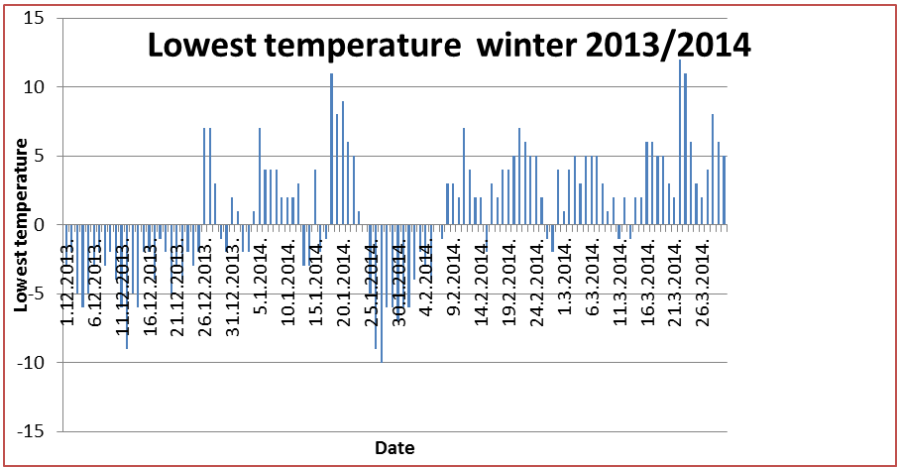

Figure 4. Fluctuation of lowest temperature in Serbia for period from 01.12.2012.-30.03.2013. (A) and 01.12.2013.-30.03.2013. (B). Data obtained from http://www.wunderground.com.

QTL analysis

For QTL analyses in the tetraploid rose populations, associations between markers and traits were studied using regression analysis on marker dosage, ANOVA on presence/absence of a marker allele and ANOVA on allele dosage. A general problem with these analyses is that the population sizes of the two populations are small, so that the power of these tests are low. In population RNDxRND (Table 5) no statistically significant associations between markers and damage caused by low temperature could be detected, only slight indications for QTLs on linkage groups 4 and 6 (Table 5). An additional test (multiple regression with markers RhGR12_33397_245 and Rh12GR_19567_3272 of each of these two linkage groups) showed a significant association with damage caused by low temperature ($p=0.03$).

A QTL for regrowth was detected on LG 6 (Table 5). Association between markers and trait indicated additional QTL for regrowth on LG 4. Multiple regression with additive effects of both QTLs (markers RhK5_16328_1080 and Rh12GR_10782_4661 were included in the multiple regression analysis) for regrowth showed a significant association ($p=0.01$).

These results indicate that LG 6 may be the location of potential QTLs for both rate of damage caused by low temperature and regrowth. Alleles of tentative QTLs are positioned on different homologs of the map of RND (damage caused by low temperature on homolog 4 and regrowth on homolog 3) and correlation (r) between damage rate and regrowth was very low: 0.013.

Table 5. Marker/trait associations for components of winter hardiness in the RNDxRND population. The associations between markers and traits were studied implementing regression, ANOVA on allele dosage and ANOVA on allele presence/absence. The strongest association was attributed to a certain linkage group (LG) and the highest scoring marker is given. The proportion of phenotypic variation explained by a marker is indicated for each putative QTL.

	Maximum value											
	Regression				ANOVA dosage				ANOVA presence/absence			
	$-^{10}\log$(p-value)	LG	R2	Marker	$-^{10}\log$(p-value)	LG	R2	Marker	$-^{10}\log$(p-value)	LG	R2	Marker
Winter hardiness (cold store)	2.3	4	6.9	RhK5_10509_683	2.7	4	11.0	Rh12GR_33397_245	2.4	4	7.6	Rh12GR_14333_115
	2.1	6	6.7	Rh12GR_2376_1235	2.2	6	8.9	Rh12GR_19567_3272	1.8	6	5.2	Rh12GR_13534_797
Regrowth	4.0	6	15.8	RhK5_2701_1112	3.4	6	15.7	RhK5_16328_1080	3.8	6	14.7	RhK5_16868_227
	2.3	4	7.9	Rh12GR_53102_111	2.6	4	9.6	Rh12GR_10782_4861	2.1	4	8.7	Rh12GR_71_6157

Similarly, for the RNDxHP population marker/trait associations were found for four regions for components of winter hardiness (Table 6) with maximum $-^{10}\log$(p-value) between 2.0 and 3.9. Multiple regression with two LGs for each trait (RhK_860_1515 and Rh12GR_11949_1366 for damage caused by low temperature and RhRMCRND_9489_166 and Rh12GR_40698_183 for regrowth) showed a significant relationship between markers and damage caused by low temperature (p= 0.01) and regrowth (p=0.01).

On the genetic map of HP 522 SNPs out of a total of 1760 were mapped, spanning a length of 738.26 cM (Chapter 5). The map is not very dense and rather fragmented, for reasons discussed in Chapter 5 (small population size in combination with individual missing values and possible misscores). Therefore QTL analysis was done per marker on the whole set of 1760 markers. In order to derive positions of unmapped markers a BLAST against the wild strawberry (*Fragaria vesca* FvH4) genome sequence (Shulaev et al., 2011) was performed. Results indicated that on strawberry pseudochromosomes 1 and 6 there are clusters of unmapped markers in rose, with $-^{10}\log$(p-value) values in the range of 3.5-4.7. According to our synteny results (Chapter 5) the translocations happened between *Fragaria* pseudo-chromosomes 1 and 6 and they correspond to rose linkage groups 2 and 3 (in the numbering according to the ICM map). The QTL analysis in the RNDxHP population thus indicated a possible QTL for regrowth on rose LG 3 (Table 6). Position on *Fragaria* genome of both mapped and unmapped markers with highest $-^{10}\log$(p-value) values indicated that they belong to the same region (Figure 5).

Table 6. Marker/trait associations for winter hardiness and regrowth in the RNDxHP population. The associations between markers and traits were studied with regression, ANOVA on allele dosage and ANOVA on allele presence/absence and the strongest association was attributed to certain linkage group (LG) and marker. The proportion of phenotypic variation explained by a marker is indicated for each putative QTL.

	Maximum value											
	Regression				ANOVA dosage				ANOVA presence/absence			
	$-^{10}\log$(p-value)	LG	R^2	Marker	$-^{10}\log$(p-value)	LG	R^2	Marker	$-^{10}\log$(p-value)	LG	R^2	Marker
Winter hardiness (cold store)	2.1	5	9.5	RhK5_860_1515	2.1	5	9.5	RhK5_860_1515	2.1	5	9.5	RhK5_860_1515
	2.2	4	9.8	Rh12GR_11949_1360	2.0	4	10.5	Rh12GR_11949_1360	2.5	4	11.7	Rh12GR_11949_1360
Regrowth	2.8	3	15.2	RhMCRND_9489_16	3.1	3	20.2	RhMCRND_9489_166	3.9	3	21.5	RhMCRND_9489_166
	2.5	7	13.2	Rh12GR_40698_183	2.5	7	13.0	Rh12GR_40698_183	2.5	7	13.0	Rh12GR_40698_183

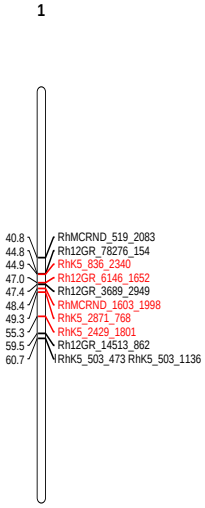

Figure 5. Positions of the markers with the highest -10log(p-value) values for regrowth for population RNDxHP on *Fragaria vesca* pseudo-chromosome 1 (Shulaev et al., 2011). The position of markers follows that on the strawberry pseudo-chromosome after an arbitrary translation of bp into cM (250000bp= 1 cM). To make the marker names on this map readable, only markers with -10log(p-value) values above 3.0, both unmapped (markers in red) and mapped (black) on rose linkage group 3, are shown.

In both populations potentially a QTLs for damage caused by low temperature was detected on LG4. To confirm that potential QTLs are located at the same regions in both populations the markers with highest $-^{10}\log$(p-value) value were compared. Inopportunely, each of these markers was only mapped in one of the populations. Therefore we also blasted these markers against the *Fragaria vesca* FvH4 sequence (Shulaev et al., 2011). The position of the markers on the strawberry pseudo-chromosomes was translated into cM by an arbitrary division of bp positions by 250000. Results indicated that these markers are in the same region in strawberry (Figure 6) and we expect that these markers are closely linked on the rose genome as well.

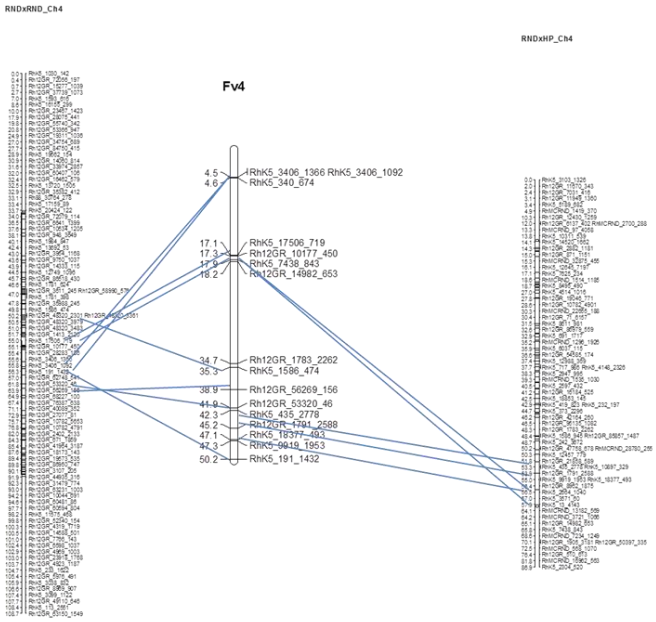

Figure 6. The positions of the mapped markers significantly linked to cold damage in both rose populations on the Fragaria vesca genome sequence FvH4 (Shulaev et al., 2011). The linkage group numbering is that of the rose ICM (Spiller et al.). The position of markers follows that on the strawberry pseudo-chromosomes after an arbitrary translation of bp into cM (250000bp= 1 cM).

Discussion

To estimate components of plant winter hardiness (damage caused by low temperature and regrowth) we used plants grafted on *R. laxa* and plants from cuttings. Garden roses are vegetatively propagated plants and with few exceptions (such as Canadian Parkland roses, which are grown on their own roots) they are grown as grafted plants. Grafting on rootstocks resistant to diseases, or nematodes and tolerant to abiotic stresses improves their performance, also in terms of winter hardiness. As most garden roses in gardens or in production fields are grown as grafted plants the best way to study their hardiness and potential performance is to use grafted roses in field studies. Under such circumstances, the overall level of winter hardiness in a period from autumn to spring is the result of a combination of winter hardiness components of the cultivar itself and of the rootstock. To estimate individual winter hardiness components of cultivars directly, the use of cuttings under controlled conditions (cold chambers) may be a valid approach. These approaches would be expected to complement each other.

Both types of experimental design have benefits and drawbacks. While field trial experiments can be inclusive as they may assess the combination of various winter hardiness components, and many plants may be assessed, their main disadvantage is the complete dependence on weather conditions. In our case, atypically high minimal temperatures for winter at the trial location in combination with a relatively high snow cover (40 cm) negatively influenced the possibility to assess winter hardiness. Snow acts as a natural insulator (Zuzek et al., 1997). In our trial it completely covered the plants and thus gave additional protection to temperatures that were not very low anyway. A breakdown of climate

components in winter 2012/2013 indicated that the average temperature (-0.3°C) was not much higher than average winter temperature for period 2002-2012 (-0.6°C), but the minimum temperature was (the coldest temperature being only -12°C compared to -24°C in a typical winter in Serbia and –30° to -38° C in a severe winter; http://www.wunderground.com). Furthermore, the length of the cold spells was much shorter than the 10-year average (1-3 days compared to 5-9 days). As the level of damage caused by low temperature mainly depends on the lowest temperature and the duration of exposure (Larcher, 2005), it is no surprise that our field experiments showed very little damage.

Experiments in cold chambers are independent of climate circumstances and thus many experiments or replications may be conducted in one year, but they are limited by size of chambers and space for plants to acclimate to the cold. Under controlled conditions it is not possible to imitate all the combinations and variability of factors present in nature, so here we focused on the effect of the lowest temperature. In the cold chamber experiments we included both one-year and two-year old plants, as according to breeders' experience (P. Cox, "Roath", Eindhoven; personal communication) and our pilot experiment, two-year-old plants may show better survival than one-year-old plants. However, we observed lower damage rates in one-year (88.53% damaged in RNDxRND and 73.08% in RNDxHP population) than in two-year old roses (98.26 in RNDxRND and 91.71% in RNDxHP). The difference in survival rates might be due to inappropriate cold acclimation, as the two-year roses were moved to an unheated greenhouse to acclimatize prior to the cold chamber exposure two months before the 1-year plants, which still were growing to become sufficiently large. Longer exposure to limiting conditions (mainly dehydration and low light intensity) might have had an effect on these plants. Alternatively, older plants might need more harsh conditions for acclimatization and an optimal level of winter hardiness may not have been reached in the two-year old plants because temperatures were too high. It is known that even a few degrees higher temperature may affect the level of winter hardiness achieved. For instance, the mean temperature at Geisenheim Research Center during winter 2011/2012 was warmer (by 2.3-2.4°C) than the mean temperature in last 30 years, which affected the winter hardiness of strawberry cultivars (Krüger and Josuttis, 2014). Similarly, in a study on peach and apricot Szalay et al. (2012) found a negative correlation between the level of winter hardiness and the temperature during acclimation.

Even though for some genotypes a low level of damage was detected immediately after cold treatment, the assessment of damage 8 weeks after removing plants from cold chambers showed that most of plants were lost (survival of 1-year roses: only 0.41% for RNDxRND and 2.09% for RNDxHP). The discrepancy between these two time points may be related to dehydration, although the plants were watered right from the moment they were removed from the cold chamber, and they showed bud opening and growth at the first measurement. A possible mechanism is root failure. Plants in the field can develop strong rooting system, which can penetrate to the deeper layers of the soil. During winter the temperature of deeper layers of soil is cooling slower than the temperature at surface (Huggett, 2003), so only part of roots can be injured. In contrast, in potted plants root development is limited by pot size, and freezing temperatures can lower the temperature of the whole pot to those below what is experienced in the field. If so, rooting system injury in cold chambers might be more severe than injury in the field under the same temperatures. This may suggest that in cold chamber experiments more attention should be paid to protection of the roots.

The level of damage after exposure to low temperature differs between the two populations. Five weeks after removing from cold chamber the offspring of RNDxHP had a

significantly higher rate of winter hardiness (73% damage compared to 88.53% in RNDxRND for one-year-old plants). Explanation for this might be inbreeding depression in a selfed RNDxRND population (Chapter 5). Alternatively, this indicates that the main donor of winter hardiness in RNDxHP population is HP, which is probably a Canadian winter hardy cultivar (Chapter 5). Indeed, in the relatively small, RNDxHP population possible QTLs for winter hardiness are located on linkage groups 4 and 5. The QTL study however also found indications for QTLs on LGs 6 and 4 of RNDxRND, even though "Red New Dawn" is relatively susceptible to low temperatures. This suggests that the winter hardiness components of the Canadian cultivars are different from those in European cultivars.

Comparative analysis of European and Canadian cultivars supports the idea that different regions of the genome may be responsible for expression of winter hardiness. Even though Canadian cultivars are extremely hardy roses, under European climate conditions Hybrid Rugosa cultivars, such as "Moje Hammarberg" and "Frau Dagmar Hastrup", are most winter hardy. Hybrid Rugosas are shorter, more compact cultivars than the Canadian ones, and characterized by many side branches. A series of German cold tolerant cultivars, the Pavement series, also represents small, compact roses with many branches suitable for borders and ground cover. Production of large number of branches might be a kind of escape mechanism: if some branches die off, there are still plenty left. Furthermore, at European trial fields characterized with continental climate only the lower part of the branches of Canadian roses, where they are usually covered by snow, can withstand low temperatures. On the basis of pedigree analysis, growth type and performance in the field, we therefore suggest that there may be two sources of winter hardiness. Bushy, vigorous (almost climbing) roses of Parkland and Explorer series survive well in severe Canadian winter conditions with thick snow cover, but the snow coverage may be an essential component. During European winters (at continental climate areas), characterized by a thinner layer of snow, European winter hardy cultivars perform better. The lowest temperatures in this region of Europe are not so low as in Canada, but without snow coverage the temperature of exposed branches may actually become lower as snow has an insulating effect; alternatively or in combination with this, dehydration may become a severe problem. In other words, winter hardiness is a multi-faceted trait that can differ under different conditions (snow cover or no cover) and that involves not just physiology but also plant morphology. Since different mechanisms are likely to be involved it may not be surprising that QTL regions found under different conditions are different. A study of the damage caused by low temperature in various strawberry cultivars from different regions also indicated that different strategies have been developed to withstand low temperature (Shokaeva, 2008), including compactness, high crown density, and late crown branching.

Our earlier study on genetic diversity of garden roses (Chapter 3; Vukosavljev et al. 2013) indicated that the highest genetic differentiation between Canadian Explorer (winter hardy) and European (non-winter hardy) roses as well as European winter hardy (Olesen's) and cold susceptible cultivars (Austin's and Harkness') was for LG5. Thus, LG5 is a potential location for a QTL for winter hardiness. The current study found an indication for a QTL on LG5 in the RNDxHP population, coming from parent HP. In both populations other potential QTLs for winter hardiness are mapped on LG 4. Many markers with an association to plant survival could not be mapped in this small mapping population, but based on synteny of the markers with the strawberry genome sequence (Shualev et al. 2011) these QTLs probably are in the same chromosome region, which corroborates the presence and map position of these QTLs.

The genetic basis of aspects of winter hardiness has been intensively studied in the last few decades in a number of species (Kalberer et al., 2006). Novillo et al. (2007) identified seven QTLs, one of which, FTQ4, mapped to the C-repeat/drought-responsive

element Binding Factor (CBF) locus and accounted for about 20% of the variation in freezing tolerance in *Arabidopsis*. Among grasses the long arm of chromosome 5 presented a hot spot for genes involved in winter hardiness (Tondelli et al., 2011). Comparative analysis indicated that the Frost Resistance-1 (FR1) gene of barley and wheat co-segregated with VRN-1, whose function is to protect floral primordia from low temperature. Basically, VRN-1 is a major gene in vernalization regulation and it is responsible for delaying of flowering until vernalization is sufficient. As the tolerance to cold stress in *Graminae* is lower after transition from vegetative to reproductive phase it is more likely that FR-1 has a pleiotropic effect on VR-1 rather than that another gene is involved (Tondelli et al., 2011). Recently, Frost Resistance-2 (FR-2), another QTL responsible for winter hardiness in barley, was mapped 30 cM proximal of FR-1 (Francia et al., 2004), while Frost resistant-H3 was detected on the short arm of chromosome 1 (Fisk et al., 2013). Application of existing QTLs in barley breeding did not bring fast improvement (Galiba et al., 2013), which may suggest that detection of small phenotypic variation is difficult, that selection already has proceeded by classical breeding, or that these QTLs cannot always be combined. Detection of loci with small effect on the phenotype in barley revealed that QTLs with a minor effect on winter hardiness are located on chromosomes 1, 2 and 3 (Tondelli et al., 2014) and are candidates for pyramiding. A QTL study in oat detected 8 QTLs related to winter hardiness on three chromosomes (Maloney et al., 2011), while a ryegrass study revealed as many as 26 QTLs that control winter survival and freezing tolerance, distributed over five chromosomes and explaining between 10.2 and 22.1% per region of phenotypic variation (Xiong et al., 2007). Similarly, Arbaoui et al. (2008) found in faba bean (*Vicia faba* L.) five putative QTLs related to low temperature tolerance. Winter hardiness of alfalfa (Medicago sativa L.) was mapped to chromosomes 1, 3, 5, and 8 as genomic regions responsible for cold tolerance (Brouwer et al., 2000). Apparently, in general, several genomic regions distributed over a few chromosomes contribute to winter hardiness, consistent with the notion that winter hardiness is derived from the combination of several processes.

One of the components of winter hardiness is regrowth, which is the plant's ability for fast shoot growth in spring. It plays an important role in plant recovery after exposure to suboptimal temperature. We considered that the rate of regrowth, as a reflection of the capacity of the plant to sustain growth, could be an indicator of effects of low temperature exposure that would not be visible morphologically. Offspring of population RNDxRND expressed a significantly higher rate of regrowth (44.57% compared to 30.73% in RNDxHP), which may indicate that the capacity for early regrowth comes from RND. Marker/trait analysis for regrowth revealed indications for QTLs on LGs 4 and 6 of RNDxRND, but there was also a significant QTL on LG 3 of parent HP in the RNDxHP population. All markers with the highest $-^{10}\log$(p-value) scores in both populations, even though the values are not statistically significant, have synteny with the same region of strawberry pseudo-chromosome Fv1 (LG 3 in rose). The parental maps for RND and HP for the RNDxHP population cover a lower proportion of the genome and are less dense compared to the integrated map for RNDxRND (Chapter 5). Hence in the case of RNDxHP additional QTLs may have been missed and/or unfavourable linkage between markers and QTLs had a negative effect on QTL detection (in a case when parent have marker "A", but lacking trait of interest (QTL), for instance AQ/Aq/aQ/aq, where "a" is a marker and "q" is a QTL: Bradshaw et al., 1998). Corresponding to our results in the RNDxRND population, a QTL for growth rate was detected on rose LG 6 by Yan et al. (2007).

Interestingly, in a recent study on alfalfa, Robins et al. (2007) highlighted the quantitative nature of regrowth and showed that the winter hardiness gene *MsaciB* was associated with autumn regrowth. QTL analysis of *Lotus japonicus* (Gondo et al., 2007)

confirmed the polygenic nature of regrowth by mapping two QTLs responsible for this trait on chromosomes 4 and 6.

A comparison of rose genotypes in this study indicated that plants with the ability to grow fast in spring do not necessarily have high levels of winter hardiness. It indicates that these two traits are inherited independently. This is supported by low positive correlation between damage rate and regrowth (r=0.013). Although the QTL mapping confirmed their independence by mapping them on different linkage groups or different homologs of the same LG, the low $-^{10}\log$(p-value) values raise doubts that these may not be true QTLs. For false positives it would not be unexpected to be mapped at different locations. Confirmation of these QTLs (using larger population sizes and further optimization of the phenotyping) is necessary to confirm credibility of this statement.

With the exception of the QTLs for regrowth in both populations RNDxRND and RNDxHP, the detected $-^{10}\log$(p-value) values for other traits were below the significance thresholds and the detected marker/trait associations can only be considered as preliminary indications. To confirm these putative QTLs, multiple QTL analysis might be implemented (Jansen et al., 1995). If pre-selected markers of putative QTLs (markers with largest $-^{10}\log$(p-value)s) are used as cofactors, the major part of variation induced by these putative QTLs would be accounted for in the statistical model. Such a cofactor analysis reduces residual variance, leading to an increase in the power of QTL detection. Such multiple QTL models can be fitted with multiple regression or mixed model approaches. In a multiple regression model we found that models with additive effects of markers on two linkage groups were statistically significant. In theory, for detecting major QTLs in tetraploids the optimal population size is around 250 individuals (Bradshaw et al., 199; Hackett at el., 1998). We started with more than 300/400 seedlings, but the final sizes of the two populations (85 for RNDxRND, 61 for RNDxHP) was a limiting factor for the detection of marker/trait associations. It is still possible that a strong winter may provide clear segregation for winter hardiness in the plants of the two populations, which are still in the trial field in Serbia, but that will be beyond the time frame of this thesis.

Conclusion

The most important prerequisite to estimate the effects of low temperature on plant survival is to set up an adequate experiment. We carried out a multi-year experiment on lowest temperature tolerance in Serbia, but the low temperatures necessary to induce winter hardiness differentiation in the trial field experiment were not reached in the past two winters, which were both exceptionally mild. As an alternative, experiments under controlled conditions in a cold chamber were used. Our study suggests that acclimation is crucial for winter hardiness development and that plants of different age need a different acclimation regime. A comparative study on two components of plant survival after winter, low temperature damage and regrowth, indicated that they significantly varied between the two populations, indicating that RND is donor of regrowth and HP of winter hardiness. For winter hardiness we detected tentative QTLs on LGs 5 and 4 in one population and 2 tentative QTLs for winter hardiness (on LGs 4 (the same region as in first population) and 6) in the other population. The ability of a plant to produce meristems in spring was associated with genomic regions on LGs 4 and 6 of the RNDxRND population, and LGs 3 and 7 of the RNDxHP population. Additional comparison of the ability for regrowth and the level of damage caused by low temperature revealed that these two traits are likely to be inherited independently and that the final cold tolerance depends both on the ability to withstand low temperature and to produce meristems fast in spring.

Acknowledgements

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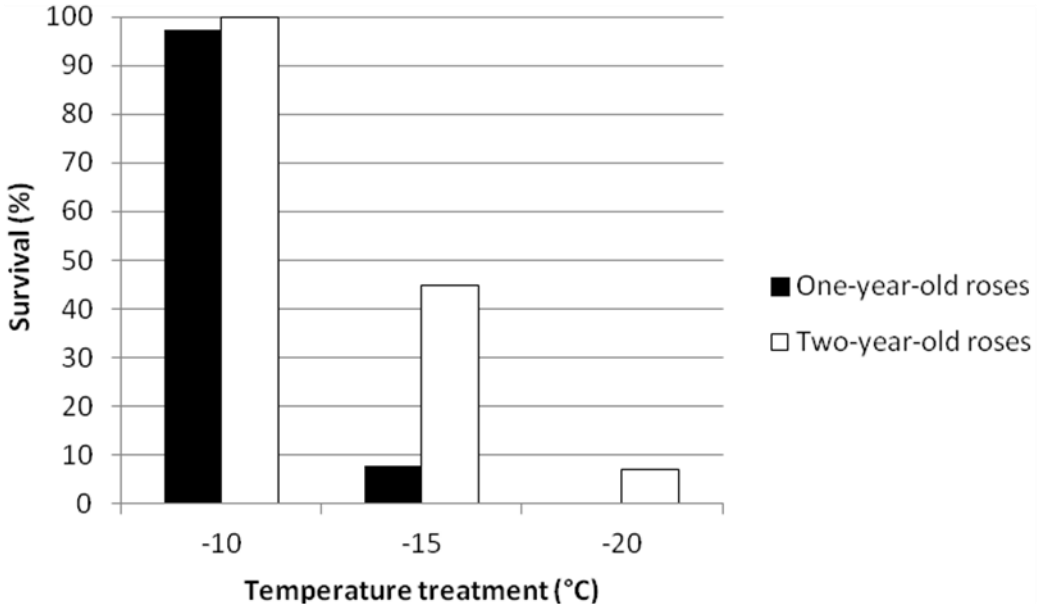
Appendix 1. Survival (%) of the roses after the treatments of -10°C, -15°C and -20°C. The used cultivars were: Henry Kelsey, Morden Centennial, Moje Hammarberg, New Dawn, Snow Ballet and The Fairy. The survival of the roses was assessed five weeks after the temperature treatments.



Appendix 2. Coeffecient of correlation (r) between survival of 1- and 2- year old roses of populations RNDxMC and RNDxHP.

	RNDxRND	RNDxHP
Batch 2	7.00E-05	0.039
Batch 3	8.00E-04	0.246
Batch 4	NA	0.02
Batch 5	NA	0.003
Overall	0.072	0.228

Chapter 7

General discussion

Historically, rose breeding may be divided into three phases depending on the techniques used (de Vries and Dubois, 1996). In the first stage (the period from pre-history until 1875) favourable genotypes were selected from wild species and planted in gardens, while new cultivars arose from seeds from open pollination. Under such conditions only data on female parents were available, which has as a result that the pedigrees of modern roses are only partially known. In this stage of rose breeding the genetic variation was broadened by introducing new species carried by travellers especially from Asia. The second phase of rose breeding (the period between 1875 and 1967) started with the discovery of directed crossing, and breeders started to build knowledge on the inheritance of traits. The selection of parents in combination with improving methods of selection and cross-breeding led to improvement in the gain of breeding. The third phase (from 1967 onwards) was initiated with the development of cell and tissue culture techniques and later biotechnology. In this phase many developments (knowledge about traits, new techniques in breeding and selection, etc.) led to shortening of the breeding cycle and improving the final gain. Below I will discuss how the results described in this thesis and recent results of others may contribute to further speeding up breeding in roses, and to laying the foundation for a better predictability of the traits that are targets for breeding.

Widening genetic diversity

Breeding in rose, as for many ornamentals, is based on selection from the progeny of crosses between unrelated and partly heterozygous parents. By hybridization and sexual reproduction, genetic diversity is increased. In the next step superior genotypes are selected, while asexual, vegetative propagation enables fixation of the heterozygous genotype (McKey et al., 2010). In rose breeding, selection for genotypes with preferable phenotypes is strict and only the best performing genotypes are cloned. This strict selection leads to high uniformity for the traits under selection and linked characteristics and indirectly to high yield and genotype by environment interaction (Bisognin et al., 2011). Breeders may unconsciously select those genotypes that have on average a higher level of heterozygosity, as is known in other crops (e.g. apple, Kumar et al., 2010), but this has not been studied in rose. It is also not known whether the relationship between heterozygosity and fitness is the same in tetraploids as it is in diploids. Theoretically, hybrid fitness increases with the increase of number of different alleles in a locus (Gallais, 2003). A fact that in many outbreeding crops higher ploidy levels give superior plants do suggest a correlation, but to date the level of resolution and lack of tools did not enable us to test this hypothesis in reality (Dr. Herman van Eck, Wageningen UR Plant Breeding, personal communication). However, this would depend on the availability of many different alleles in the germplasm.

In **Chapter 2**, I determined the allelic diversity and heterozygosity at microsatellite loci in a range of cut and garden roses. Linkage group (LG) 2 and LG5 had a higher F_{st}-value among breeders compared to the F_{st}-value in LG1 and LG3 which was lower than average. Clear differences in average heterozygosity were visible among linkage groups as well,

showing effects of selection (either converging or diversifying) and indicating that there is room for increasing allelic diversity in the germplasm on particular LGs.

Explorer roses had more private alleles and the largest genetic distance from all others. This is probably the result of recent introgression from wild species (*R. kordesii*, *R. acicularis*, *R. amblyotis*, *R. laxa*, *R. spinossisima*, and *R. rugosa*). It shows the potential of introgression from wild relatives, even if it concerns new sources from species that already have been used in rose breeding in the past.

Does the fact that 'only' 10-20 wild rose species were used in breeding indicate that genetic variation of modern cultivars is limited and that in unused species additional genetic variation is present? An additional reduction of diversity happened when garden rose breeding was separated from cut rose breeding due to focusing on different traits (de Vries and Dubois, 1996). Thus, although overall there is quite a large genetic diversity (increased through hybridisation and introgression), relative to the gene pool of the wild relatives only a limited amount has been introduced into the cultivated germplasm. The fact that for each wild rose species only a few genotypes have been used represents a bottleneck for each of the species when going from wild to cultivated germplasm. Therefore, there is still a large reservoir of diversity in these wild species, and that may include various useful characteristics for breeding.

Analysis of pedigrees of modern cultivars indicates that many cultivars have common ancestors. Thus, it is possible that they are the outcome of inbreeding and that some inbreeding depression may occur. Using common ancestors in a breeding program may be done without such problems, as for example is known in apple. Apple pedigrees include many common ancestors, and sometimes the same cultivar, e.g." Golden Delicious", is used more than once in a pedigree (Evans et al., 2011). Homozygous regions do occur in the genome of elite apple cultivars, but these are limited in size and may be specifically selected for, as the self-incompatibility system of apples generally prevents inbreeding. Rose is also an outbreeding crop, so it can be hypothesised that productivity and vigour also depends on heterozygosity, which is endangered by inbreeding. However, the incompatibility system of tetraploid roses is not well known. Studies on diploid material indicated that in roses gametophytic self-incompatibility is present (Debener et al., 2010) and a self-incompatible (SI) locus was mapped on chromosome 3 (Spiller et al., 2011). Nevertheless, observations from breeding practise indicate that progeny of some cultivars is phenotypically uniform and similar to the mother plant (Peter Cox, "Roath", Eindhoven; personal communication), which suggests that self-pollination in some cases does occur in tetraploid roses. In support of the contention that some degree of inbreeding may be occurring in commercial breeding programs is the fact that only a small percentage of the seedlings germinate, and that many germinated seedlings die while still juvenile. To determine the extent to which selfing affects inbreeding depression it would be interesting to compare the genotype of offspring lost in the juvenile period with mother plants; for instance, to check if the seedlings that died are predominantly selfed.

Diversity in garden roses might be enlarged by including exotic material of the cultivated species, interspecific crosses with wild underutilized or new species, and natural as well as induced mutations (Moose and Mumm, 2008). It was shown in other crops that the introgression from exotic and wild germplasm can tremendously improve traits (Gur and Zamir, 2004). The exotic germplasm in many cases has given good results, introducing new sources of traits or phenotypes. A survey on usage of wild species highlighted that in many crops (potato, rice, wheat, maize, barley, sunflower, tomato, lettuce, millet, sorghum, cassava, chickpea, cowpea, lentil, soybean, groundnut, and banana) a whole range of traits, including resistance for abiotic and biotic stresses (fungi, pest and disease resistance; drought, low temperature, and salinity resistance) have been introgressed from wild species (Hajjar and Hodgkin, 2007). Despite this, exotic material often has undesirable traits. Wild relatives of domesticated plants in many cases represent the best source of rare characteristics, as they have gone through a long period of natural selection. For instance, when breeding for winter hardiness in rose, wild rose species may play a crucial role. In the Canadian breeding program for winter hardy roses wild species have indeed been widely used. The genetic diversity study in **Chapter 2** indicated that winter hardy Explorer roses and Rugosa types are closest to the Rootstocks. This probably reflects the introgression of *R. arkansana* and *R. rugosa*, commonly used as rootstocks, into the Explorer roses. *R. laxa* is commonly used in Europe as a rootstock because it is very cold tolerant (winter hardiness zone 2a, i.e., it can withstand temperatures as low as -35ºC; http://planthardiness.ars.usda.gov accessed 13 April 2014). Based on literature and pedigree analysis *R. laxa* would be a potential source of resistance to low temperatures, but so far breeders did not have a lot of success in crossing *R. laxa* with garden rose cultivars. Other sources of winter hardiness may be obtained by including cold tolerant wild species in breeding that have not been used or that have been rarely used. For instance, tetraploid species *R. ferruginea, R. glauca, R. majalis rubrifolia* from winter hardiness zone 2b, *R. foetida, R. hispida, R. lutea bicolor, R. lutea punicea, R. mollis, R. bicolor, R. eglanterica punicae* from zone 3a and *R. gallica* and *R. moyesii* "Nevada" from zone 3b could be donors of winter hardiness. Molecular markers already have been approved as an efficient tool for introgression of black spot resistance from wild species into tetraploid rose cultivars (Debener et al., 2003). A problem of hybridization between wild and cultivated relatives is that in the F1 generation not only desired traits are transmitted, which may have an enormous impact on the phenotype. Clearly, in ornamentals, which are mainly evaluated based on phenotype, it can be a main reason for excluding wild species from breeding programs. Nowadays, markers enable reduction of wild donor germplasm and its undesired traits within two generations of backcrossing (Debener et al., 2003), which enables wider implementation of wild relatives into breeding. Also, molecular markers can be successfully employed for overcoming incompatibility barriers and for detecting close relatives of potential wild donors for specific traits. Namely, some wild species are donor for important traits, but no viable offspring can be obtained if we cross them with cultivars. For instance, *Rosa roxburghii* is resistant to all known powdery mildew and blackspot isolates, but does not give viable F1 generation when it is crossed with rose cultivars. In this case related species to *Rosa roxburghii* can be identified by markers, which may serve as a specific "bridge" for introgression (Debener et al., 2004).

Even though wild species are an appreciated source of new characteristics, in many cases strong barriers exist that may prevent crosses between related species (van Huylenbroeck, 2012). Furthermore, the polyploid nature of roses complicates cross-pollination (de Vries and Dubois, 1996). While most modern cultivars are tetraploid, the ploidy level of wild rose species varies from diploid to hexaploid. Choosing parents with the same ploidy level ensures a better rate of successful pollination, but this would severely limit the range of species that may be used. A cross of diploid and tetraploid parents produces triploid progeny, most of which are sterile. However, according to van Huijlenbroeck (2012), F1 triploid hybrids can be back-crossed with tetraploid cultivars and as a result fertile tetraploid F2 generations may routinely be obtained. He speculated that this actually was practiced widely in the 18th and 19th century (the first phase of rose breeding) but has been forgotten today. Increasing the level of ploidy in general has a positive effect on plant vigour and in this way superior characters of diploids may be introduced into new cultivars (de Vries and Dubois, 1996).

F1 rose hybrids

According to the segregation of SNP markers in **Chapter 5** it was concluded that part of the offspring analysed originated from a selfing of "Red New Dawn". In polyploids the prediction of possible consequences of selfing is difficult due to the allele dosage effect. Interesting possibilities may arise from the implementation of selfing in practical breeding: fixation of traits through round(s) of selfing, creation of inbred lines and F1 hybrid seed production. RND has been widely used as a parent in the breeding programme of "Pheno-Geno Roses" in crosses with Canadian winter hardy cultivars (Peter Cox, "Pheno-Geno Rose", Novi Sad; personal communication). A comparison of the performance in the field of 1-year-old grafted plants of RND x RND (the selfed progeny) with that of a bulk population of RND x various Canadian parents indicated that the average plant height in the selfing population was lower (21.9 cm (SD=8.4; SE=0.9) versus 24.25 cm (SD=12.3; SE=1.2)). Taking into account that RND is a climber with a final height of 305-365 cm and that all Canadian roses are bushes with a height only in the range of 90 to 150 cm (http://www.helpmefind.com/rose, accessed June 18, 2014) it was expected that the selfed progeny would have the larger height. Additionally, between mother plant (RND) and the progeny heterozygosity was reduced by 20.3% (**Chapter 5**) which may have led to reduced growth due to inbreeding depression. Basically, selfing might help in fixing valuable rare alleles, but at the same time the effects of harmful recessive alleles might be further enhanced.

If selfing is possible, the time needed to reach homozygosity at a locus is longer in polyploids than in diploids. Compared to diploids, in one generation of selfing heterozygosity in autotetraploid drops slower (17-21% compared to 50% in diploids; Bever and Felber, 1992). Indeed, the RND selfed progeny on average had a reduction of 21% in heterozygosity

as measured by the SNPs. For estimating the number of generations in autotetraploids to reach a certain level of homozygosity, the occurrence of double reduction plays an important role. Due to the occurrence of double reduction the chromosomal regions that are situated further away from the centromere will move towards homozygosity more rapidly. The level of double reduction observed in the two crosses here was 7.2 and 3.0% respectively, which indicates that this will contribute considerably to the reduction of heterozygosity.

Finally, the level of viability of homozygotes and heterozygotes plays a role in reaching homozygosity; if homozygotes are less viable than heterozygotes the progress towards homozygosity will be slower. According to Parsons (1959) to achieve inbred lines 27 to 28 generations of selfing may be needed in autotetraploids, compared to 7 to 8 generations in diploids. As an alternative, for F1 hybrid seed production in autotetraploids instead of pure inbred lines genotypes homozygous for specific traits (i.e., only particular genomic regions) of interest might be used. This strategy in combination with molecular markers linked to the trait/region of interest, would ensure that regions of interest are really homozygous, what finally would lead to speeding up the whole process.

Practical experience confirmed that inbreeding depression may have an especially bad impact in autopolyploids. Research on potato (Bradshaw, 1994) highlighted that inbreeding depression exceeds twice that predicted by the inbreeding coefficients. This was explained by their polyploid nature; namely autopolyploids may accumulate more harmful recessive alleles than diploid species. Due to the negative effect of inbreeding depression it was not recommended to expose them to self-pollination.

Propagation

Roses can be vegetatively propagated in two ways: by grafting or by cuttings. Grafting is a widely used technique for multiplication of well-performing genotypes, generally by using budding eyes on a rootstock. In Europe *Rosa laxa* is the most commonly used rootstock. Next to the use for multiplication the rootstock may also increase the grafted plant's resistance to soil-borne pathogens like nematodes, and to abiotic stresses like cold. A disadvantage of grafting lays in the fact that grafting success depends on the genotype/rootstock interaction, rootstock health, and environment (temperature, humidity). In rose multiplication by cuttings, hormones are used to stimulate rooting and cuttings grow on their own roots. The success of cutting production depends on genotype capacity and shoot position. Cuttings originated from the middle part are characterized by greater flower stem diameter, higher fresh weight, and specific fresh weight compared to plants originated from apical or basal position (Bredmose and Hansen, 1996). As grafting might improve a cultivar's stress tolerance, in estimating the effect of stress factors it is necessary to both the roses on their own roots (showing the cultivars' potential for tolerance) and grafted roses (for cultivar's tolerance under field conditions), such as was used in the design of the winter hardiness experiment in **Chapter 6**. As an alternative to these two traditional ways of vegetative propagation, tissue culture techniques offer the potential of fast propagation under

controlled conditions. Tissue culture procedures not only improve multiplication, but also enable obtaining disease-free plants. Basically, by meristem culture (aimed to get disease-free plants) and micropropagation (to generate large number of identical clones) under controlled, aseptic conditions, tissue culture techniques have been introduced into rose breeding (Bisognin, 1994).

Making rose breeding more efficient

Garden rose breeding involves many steps in developing a strategy of parents to combine, making the segregating population(s), and strict selection of the desired phenotypes (Figure 1). In the best case a new cultivar is introduced to the market after 5 years of intensive breeding. In the first stage, selected parents with complementary traits are cross-pollinated. Only part of the crosses is successful (depending on the parental combination; on average around 55%) and produce hips. Additionally, the low germination percentage (again depending on the parental combination; on average around 40%) considerably reduces the number of achieved seedlings. During the first and second year, seedlings are kept in a greenhouse and negative selection, which reduces the number of seedlings by 95-97%, is implemented. From third year onwards positive selection for traits of interest is employed at trial fields (Figure 1) and selected genotypes are vegetatively multiplied. Depending on trait and environmental conditions, phenotypic evaluation on the trial field may take an additional few years. As a result, only a small portion of offspring (around 0.1%) are introduced to the market in the form of cultivars.

Figure 1. Rose test field in Mali Iđoš, Serbia.

This process can and should be improved. It is clear that only a small portion of the cross-pollinations result in hip production, and many of these seeds fail to germinate. Before parents are pollinated a few steps may be taken in order to improve the final outcome. Firstly, the ploidy level of parents should be tested. In many cases pollination is not successful due to differences in ploidy level, most often because several cultivars are triploid (www.helpmefind.com/roses) and many of those triploids are sterile (de Vries and Dubois, 1996). Secondly, the success of cross-pollination may be improved by testing general and specific combining ability of parents. As the success of pollination largely depends on climate conditions, climate should be better controlled or testing should be repeated few times and conclusions should be drawn only on data corrected for environmental effect. This testing may be time consuming, but it will ensure a good basis for the crosses of the years ahead. In well-established companies data on pollination success and germination are already available for several years and additional analysis may be cost-effective. Thirdly, poor pollen germination might be a reason of low pollination rate. The pollen ability to germinate can be tested simply on medium in Petri dishes. Additionally, based on own observations during the making of crosses, some pollen showed better germination ability a few days after collecting, while pollen of other genotypes showed the highest germination ability immediately after collection. Knowledge on pollen germination may increase the success of pollination and improve the efficiency of the breeding program. Lastly, seed germination in roses is low. Seed ripening conditions are very important for successful germination (Koornneef et al., 2002). Many treatments on different species have been implemented and results indicated that seed germination may be improved by: microorganisms which enhance nutrient uptake (Taylor and Harman, 1990), UV radiation (Noble, 2002), scarification of achenes, and exposure to a combination of sulphuric acid and low temperature (Zhou et al., 2009).

Breeding in horticultural crops in general is based on empirics and, compared to other cultivated crops, little research on the genetic basis of traits has been conducted. Rose breeding is mainly done by private companies and gained knowledge and technologies are kept confidentially as a business secret. Thus, it is needed to improve the genetic knowledge on traits and create a common knowledge base. For improvement of breeding it is essential to share expertise. Education of breeders can be combined with training in the use of molecular techniques. Molecular markers might improve breeding success in a few stages: in selecting complementary parents for crosses by genotyping potential donors, and in selecting superior offspring for traits for which markers can be used (see below) at seedling stage, which would shorten the breeding process (in the case of introgression from wild species) or the number of plants to be evaluated (Figure 1; Bisognin, 2011).

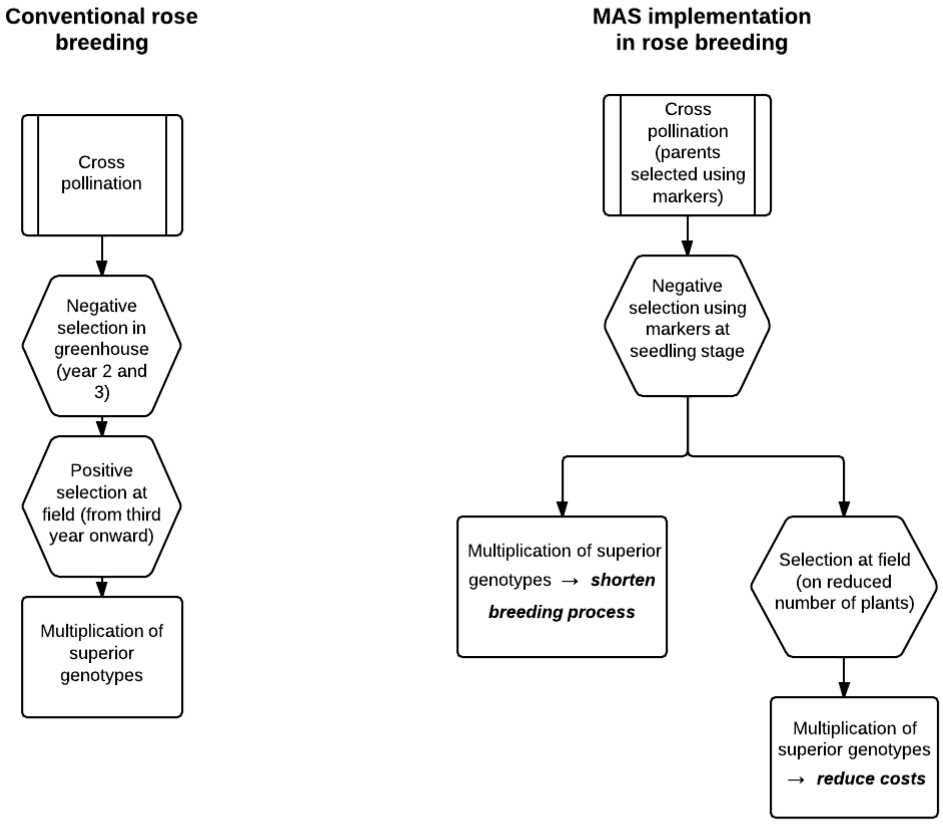


Figure 1. Main steps in conventional rose breeding and benefits of MAS implementation in rose breeding.

Another way to improve rose breeding is pedigree-based analysis. Freely available software (Pedimap; Voorrips et al., 2012) for visualization of pedigrees and allele flow from generation to generation is user-friendly and gives better insight into inheritance of traits. Furthermore, based on knowledge of pedigrees superior parents might be selected. Similarly to this concept, family-based QTL mapping provides more knowledge on the effect of a particular gene and its epistasis on phenotype expression. Family-based QTL mapping is based on a number of families, but it can deal with populations with fewer offspring. This fact is important for roses, as the seed germination rate is low. A family-based QTL approach already gave results in tetraploid wheat and in apple (Aliey al., 2013; MSc. Di Guardo, Wageningen UR Plant Breeding, personal communication).

Speeding up breeding: MAS

One of the possibilities to improve conventional breeding is to use marker-assisted selection (MAS). MAS represents indirect selection of traits using molecular markers that are linked to the genes (Xu and Crouch, 2008). MAS comprises a range of molecular methods

and approaches that can improve selection methods and might increase the efficiency of breeding by permitting earlier selection and smaller population size during selection. MAS may be incorporated in different stages of plant breeding: gene introgression from wild species, germplasm characterization, and selection of parents and progeny with superior traits (Ibitoye and Akin-Idowu, 2011). Understanding the genetic relations among germplasm may improve selection of parents (Sosinkski et al., 2000; Ibitoye and Akin-Idowu, 2011). Marker assisted introgression demands specific intellectual infrastructure/logistics, such as genetic maps, knowledge on the basic genetics of specific traits, and molecular markers linked to traits. Under such circumstances, molecular markers may be used to test potential donors, which would speed up the selection process for parents and broaden genetic diversity (Hermsen, 1994).

MAS is useful in breeding of traits that are difficult to evaluate (as monitoring is expensive, time-consuming, and/or unreliable), for traits whose selection depends on developmental stage and/or environmental conditions, for polygenic and pyramiding multiple monogenic traits (quality traits, disease, stress, and pest resistance), and in back-cross breeding for speeding up and maintaining recessive alleles (Xu and Crouch, 2008). Furthermore, selection for genotypes resistant to abiotic (cold, drought tolerance, etc.) or biotic (disease resistance) stresses is complicated by the fact that phenotype response often depend on a combination of climatic factors. It is often essential to repeat an experiment for a few years, while correcting for the effects of additional factors. In these cases selection based on allele configuration would shorten the selection process with a few to several years. Basically, offspring can be tested at the stage of seedlings, but that is relatively expensive. Alternatively, parents can be selected, e.g. by including parents that are homozygous for particular resistance genes when the goal is to stack such genes in the progeny. This may limit breeders to a lower number of parents in which they assessed diversity of focal genes and found closely linked markers.

For successful implementation of MAS it is necessary to build a logistics basis and provide genomics tools. The first step is to obtain a large set of molecular markers. With larger numbers of markers (marker density) involved in mapping the probability that some marker is in or nearby the target gene(s) is higher (Xu and Crouch, 2008). Expenses for setting up conditions for implication and application of MAS by itself used to be high, but this is quickly changing with regard to the marker development and marker detection. In rose we have now generated 60K SNP markers, on the WagRhSNP array. Using this array it was possible to develop dense genetic parental (for RND and HP) as well as integrated maps (RNDxRND) (**Chapter 5**), and use it for QTL mapping. This resource will be useful for all groups working on genetics and breeding in roses. The pedigree-genotyping based further analyses, which may quickly assess important traits like has been done in apple, now will be also executed in other *Rosaceae*.

Genetic maps are made for a specific cross and, due to allelic diversity, genetic variation and recombination patterns, might differ from population to population. For instance, the markers that were mapped in one of the RND parental maps (either in selfed RNDxRNR or RNDxHP) and on the HP map were only 16.4% the same. The SNPs were

chosen based on parents of cut and garden rose populations but also on a wide set of garden rose genotypes. A big advantage of an array with many markers is that, within any part of the germplasm, the same array can be used, and markers for the different populations can be selected based on whether and how they segregate.

In polyploids of commercial importance, such as potato, wheat, sugarcane, MAS breeding already gave results. In ornamental breeding MAS selection is hardly implemented and if so, then only in the initial stage. A survey on molecular marker implementation in fruit and ornamental breeding programs (Byrne, 2007) indicated that in 39% of them markers have been used, while in 10% of the companies it was considered to involve markers within the next couple of years. In ornamentals in most cases markers were involved in diversity and cultivar identification studies (45%), mapping, gene tagging and isolation (15%), while in 8% of the cases markers were used in MAS. It is worthy to highlight that especially in ornamentals MAS was rather more under development than being applied. Interestingly, in ornamental and fruit breeding SSR markers were predominantly used, which indicates that for breeders SSRs are an user-friendly molecular tool and thus there is a need for their fast development. As a contribution to this field, in **Chapter 4** I described a novel model for fast development of highly polymorphic SSRs using transcriptome reads. A further contribution of this thesis to SSR-based MAS is an improved method for determination of allele dosage or quantitative scoring of SSRs (**Chapter 3**). As it was shown in the genetic diversity section of this thesis, in most cases less than 4 alleles are detected on a single locus. It means that some alleles are present in 2 or 3 doses (duplex or triplex). With dominant scoring only presence or absence of an allele can be notified. Implementation of additional information collected based on dosage would improve the final outcome of MAS.

The benefit of MAS depends on precision of (QTL-) mapping. Markers closely linked to the trait may be selected according to two scenarios. In the first scenario the whole segregation population is genotyped with markers that cover the entire genome. After genotyping, association between markers and phenotypic data is tested. In the second scenario (so-called pooled or bulked DNA analysis) only extreme phenotypes are genotyped and differences in allelic frequency between extremes are used for estimation of association. Bulked DNA analysis saves money and time, however some pitfalls of this method are reflected into low marker density and power of QTL detection, not always accurate estimation of allele frequency, and the possibility that no marker-trait associations are found even though linkage between them is statistically significant. In a case when individuals with extreme phenotypes can be simply screened the most effective approach would be to combine selective genotyping with selective phenotyping. In this case only a subset of plants with extreme phenotypes will be selected for genotyping, which would ensure that enough information is involved (Xu and Crouch, 2008).

The implementation of MAS in polyploid crops is also hindered by the lack of adequate software for mapping and QTL analysis. With recent developments and achievements at the field of rose molecular genetic studies, a solid base for MAS is ensured. The first condition for MAS implementation: availability of molecular markers, is satisfied with a set of reasonably good SSR markers (**Chapter 2**), while an additional step was made

with the recent development of an SNP array (WagRhSNP, Ir. Koning-Boucoiran et al., in preparation; see also Smulders et al., 2013). As was shown (**Chapter 5**) with the developed markers it was possible to generate dense genetic maps and perform QTL analysis (**Chapter 5 and 6**). In case that there is still a need to develop more markers a new strategy for development of highly polymorphic markers is established (**Chapter 4**).

QTL mapping in tetraploids is more challenging than in diploids. First of all, suitable software for mapping and QTL detection does not exist. The only software for mapping specifically designed for autotetraploid populations, TetraploidMap (Hackett and Luo, 2003), has serious limitations: restriction on marker number, absence of possibilities to deal with double reduction, and the need for manual interaction and visual inspection (Voorrips and Maliepaard, 2012). As an alternative software the program JoinMap (van Ooijen, 2006) that was designed for diploids, was used in this thesis to do mapping using three marker segregating types (SxN, DxN, SxS) and a two-step mapping approach. In this approach, software for diploid mapping (JoinMap) producing a map with SxN type markers is combined with scripts written in R to estimate recombination frequencies of these SxN type markers together with DxN and SxS segregation types enabled generation of dense genetic maps (**Chapter 5**) in the second step. Further development of suitable software for mapping and QTL analysis in polyploids is ongoing at Wageningen UR Plant Breeding. Luckily, as the WagRhSNP array contains several tens of thousands of SNPs, sufficient numbers of SNPs were available to make dense maps for the two populations (**Chapter 5**). However, when breeders and researchers want to make maps of other rose populations using this array, the number of common markers between the maps may increase if, with the new software, also other segregation types can be used optimally (notably the types: DxD, SxD and its "mirror" form TxD).

Sometimes breeders are faced with the fact that markers do not predict reliably the phenotype. In many cases this is associated with low precision of QTL analysis and/or insufficient validation (Young, 1999). The cost-effectiveness of MAS depends on many factors, such as: trait and its inheritance, phenotyping method and its effectiveness, and costs of resources, field/chamber/greenhouse and labour. In principle, cost efficiency should be considered for each specific case (Dreher et al., 2003). In cases when phenotyping is straightforward and cheap (prickles, shininess of leaves, number of petals), use of MAS is not preferable. In case the inheritance is complicated and linking markers to components is difficult, such as in flower color, it is also better to select by eye. But in cases where the phenotyping is expensive and unreliable (disease resistances) it is advisable to use markers, once they have been developed. Markers are also essential to be able to combine multiple resistances into one plant (Ortega and Lopez-Vizcon, 2012). Developing markers associated to sources of disease resistance is a good topic of a joint effort of all rose breeders together, as it is particularly good example of precompetitive research for the benefit of all breeders and the production of the crop compared to alternative crops.

In the last two decades the genomic resources for representatives of Rosoideade (Fragaria, Rubus, Rose, and Potentilla) have developed rapidly. Many marker types (SSRs, RAPDs, RFLPs, AFLPs) have been used in different phases of MAS: cultivar identification

and parentage analysis, fingerprinting, genetic diversity approach, map construction and QTL detection (Longhi et al., 2014). Additionally, high-throughput sequencing has been used to develop SNP markers and SNP arrays for apple, peach, cherry, and rose (Antanaviciute et al., 2012; Verde et al., 2012; Peace et al., 2012; Koning-Boucoiran et al., 2012b). Finally, the genome sequence of one representative of *Rosoideae*, *Fragaria vesca*, is available (Shulaev et al., 2011) and because of its synteny to roses (**Chapter 5**) there is now a good basis for further development of MAS in rose (markers, maps, transcriptome sequences) .

QTL mapping

Clearly, tetrasomic inheritance of garden roses (**Chapter 5**) in combination with heterozygosity (**Chapter 2**), complicates the accumulation of desired alleles (Conner et al., 1997), which has a tremendous effect on rose breeding. In **Chapter 6** we detected QTLs for regrowth and winter hardiness in a cross of Canadian (HP) and European (RND) cultivars. The QTLs for winter hardiness are still tentative as the population size was too small. Before those QTLs can be implemented in a breeding program it is necessary to validate them in another, larger, population, to use multiple populations, or to expand the population. In our case the current population RNDxHP cannot be expanded because the Canadian male parent is unknown. Validation might be done using sets or bulks of only the most extreme plants ("plus" and "minus" plants). The QTLs detected in a single population might be used in another if population founders have the same source of the trait. In principle comparison of allele configuration for a specific trait and phenotypic evaluation of the level of winter hardiness might validate QTLs. Assuming that winter hardiness is inherited from the Canadian parent (depends on a few major loci; Svejda, 1974) and as only few wild species have been used as donor of this trait in the Canadian breeding program, candidate markers may predict the expression of the trait even if it comes from different Canadian cultivars, and the QTL may be validated in other crosses with Canadian roses. We confirmed this strategy on garden roses by conducting a diversity study employing SSR markers. Comparison of genetic distances for each chromosome separately on a panel of winter hardy/winter susceptible cultivars indicated that QTL for winter hardiness may be located on chromosomes 5, as here the roses from the Canadian program had much more genetic diversity. A marker-trait association approach employing SNPs confirmed that indication for QTL for winter hardiness is placed on chromosome 5. One complication remaining is that in other Canadian roses recombination between SNP and trait might have happened. Under such circumstances another allele should be followed, but as we scored SxN dominantly, another marker may be on any of the homologs. The problem of bi-allelic markers can be solved by implementing a haplotypes/identity-by-descent (IBD) approach.

It may be illustrative to compare the situation in garden rose with that in potato. Potato is also an autotetraploid, vegetatively propagated and highly heterozygous crop. Its genetic base is narrow due to limited foundation stock, while inbreeding depression highly influences its breeding (Barrell et al., 2013). Implementation of MAS in potato breeding program and breeders' experience might give guidelines for MAS putting into practice in

roses. Another aspect of not negligible allelic variation is decrease of possibility to transfer markers directly from mapping populations to breeding material and the need to validate markers (Milczarek et al., 2011). Validation of potato tuber quality, when 11 candidate markers were examined on a set of multi-parental material (Li et al., 2013) is a nice example how QTLs detected in single population might be used in another if population founders have the same source of the trait (if the trait is conserved). Even more, if few QTLs for a single trait are detected the combination of different alleles over markers might be used for evaluation of single marker and its epistasis on phenotypic expression. Even though the genetics of tetraploid potato is complex, MAS found implementation in nematode and multiple pathogen resistance (Barrelli et al., 2013) breeding . Unfortunately, similarly as in rose, in potato single SNP marker are in general not indicative for specific desired phenotypes. As a solution selection of few SNPs in one gene might be a better indication of phenotype. Thus selection for desired phenotype should be based on haplotype (Barrell et al., 2013).

Conclusions for rose breeding

Even though MAS in rose is in the initial stage, few QTLs for different traits have been detected. In many crops wild germplasm has been used for introgression of disease resistance. Some QTLs for disease resistance (from wild sources including also diploid material) in rose exist, but only few groups in the world work on this. Clearly more efforts are necessary to identify sources of (strong) resistance against diseases. Additionally, single dominant traits in rose are detected and some of them are scientifically interesting, but do not represent useful traits for breeders (for instance prickle presence/absence). Namely, in evaluation of the utility of marker assisted selection an important fact is the relation between invested money at the beginning and saved costs at the end of the breeding and selection process. A positive cost-benefit analysis at the end of the breeding program may be achieved through reduction of costs for trial field and greenhouse testing, in a shortening of the time-to-market for new cultivars, and in the possibility to effectively combine traits into one cultivar that otherwise would be difficult or impossible to combine. With respect to this, for traits that can be evaluated easily in an early juvenile stage (such as presence of thorns, leaf glossiness, flower colour etc.) it would not be beneficial to use MAS. In contrast to this, implementation of MAS in breeding traits controlled by single loci whose expression is in late juvenile stage (recurrent blooming) or multiple loci whose phenotyping is difficult and/or depends on climate condition (winter hardiness; drought, heat tolerance, etc) might lead to early selection and thus reduce the breeding costs. Within rose companies markers might be applied for selection of parents for crosses and to screen progeny in order to shorten the breeding period.

Genomic tools for MAS application in rose breeding are rapidly increasing. Molecular markers, an initial prerequisite for MAS implementation, exist in the form of a large set of

SSR markers and a 63K SNP array. Additionally a quick method to develop new SSR markers was developed (**Chapter 3**), so marker development is not a big issue any more. Even though the perfect software for mapping in polyploids does not yet exist, procedures and scripts have been developed to generate genetic maps for tetraploid rose cultivars based on a subset of the markers with suitable segregation types (**Chapter 4**). Currently, QTL analysis might be performed either per marker (marker/trait association) or along linkage groups as long as it is assumed that all homologous linkage groups are independent (in software for QTL mapping in diploids, e.g. MapQTL). The latter is currently the biggest shortcoming as one may expect that different alleles at the same locus on different homologs may contribute to the same trait, therefore new procedures for QTL mapping in tetraploids are being developed (Dr. Chris Maliepaard, Wageningen UR Plant Breeding, personal communication). With regard to population size, the ideal size of 250 progeny for mapping in tetraploids (Bradshow et al., 1998) is rarely being achieved in rose breeding. As a trick to overcome this pitfall multiple smaller populations may be used, as was done in apple and strawberry (Dr. Eric van de Weg, Wageningen UR Plant Breeding, personal communication). Additionally, in rose breeding negative selection is implemented in an early stage, while for QTL mapping both plants with desired and undesired characteristics ("plus" and "minus" genotypes) are needed. As an alternative, detection of QTLs could be considered as precompetitive research and several companies together should initiate it, as it is the case in many other crops, such as potato and vegetables. The research partner or partners involved in such a mutual project can introduce theoretical and practical knowledge by organizing teaching sessions or workshops, thus combining the research with training of the personnel in the breeding companies. Such collaboration might be a key factor for the establishment of MAS in rose. However, such collaboration between companies has been difficult to achieve in rose in the past and more efforts should be invested in it in the future if rose breeding should remain an economically viable activity.

References

Ali MB, Ibrahim AMH, Malla S, Rudd J, Hays DB (2013) Family-based QTL mapping of heat stress tolerance in primitive tetraplouid wheat (*Triticum turgidum* L.). Euphytica 192: 189-203.

Ansari MJ , Al-Ghamdi A, Kumar R, Usmani S, Al-Attal Y, Nuru A, Mohamed AA, Singh K, Dhaliwal HS (2013) Characterization and gene mapping of a chlorophyll-deficient mutant clm1 of Triticum monococcum L . Biologia Plantarum 57(3): 442-448.

Antanaviciute L, Fernandez-Fernandez F, Jansen J, Banchi E, Evans-KM, Viola-R, Velasco-R, Dunwell JM, Troggio M, Sargent DJ (2012) Development of a dense SNP-based map of an apple rootstock progeny using Malus Infinium whole genotyping array. BMC Genomics 13: 203.

Anstey TH, Moore JF (1954) Inheritance of glossy foliage and cream petals in green sprouting broccoli. J. Hered. 45: 39-41.

Aranzana MJ, Abbassi EK, Howad W, Arús P, (2010) Genetic variation, population structure and linkage disequilibrium in peach commercial varieties. BMC Genet. 11, 69.

Arbaoui M, Link W, Satovic Z, Torres AM (2008) Quantitative trait loci of frost tolerance and physiologically related trait in faba bean (Vicia faba L.). Euphytica 164: 93-104.

Arora R, Rowland LJ, Lehman LS, Lim C-C, Panta GR, Vorsa N (2000) Genetic analysis of freezing tolerance in blueberry. Theor Appl Genet 100: 690-696.

Arora R, Rowland LJ (2011) Physiological research on winter-hardiness: deacclimation resistance, reacclimation ability, photoprotection strategies, and a cold acclimation protocol design. HortScience 46: 1070-1078.

Ashkenazi V, Chani E, Lavi U, Levy D, Hillel J, Veilleux RE (2001) Development of microsatellite markers in potato and their use in phylogenetic and fingerprinting analyses. Genome, 44, 50-62.

Austin D (2012) Handbook of roses 2012/2013, European Edition, London, pp. 6-40.

Badawi M, Reddy YV, Agharbaoui Z, Tominaga, Y, Danyluk J, Sahran F, Houde M (2008) Structural and functional analysis of wheat ICE (inducer of CBF expression) genes. Plant Cell Physiol 49: 1237-1249.

Barrell PJ, Meiyalaghan S, Jacobs JME, Conner AJ (2013) Applications of biotechnology and genomics in potato improvement. Plant Biotechnol J 11: 907-920.

Baruah A., Naik V, Hendre S, Rajkumar R, Rajendrakumar P, Aggarwal RK (2003) Isolation and characterization of nine microsatellite markers from Coffea arabica L., showing wide cross-species amplifications. Molecular Ecology Notes, 3, 647-650.

Barzegar R, Peyvast G, Ahadi A.M, Rabiei B, Ebadi AA, Babagolzadeh A (2013) Biochemical systematic, population structure and genetic variability studies among Iranian Cucurbita (*Cucurbita pepo* L.) accessions, using genomic SSRs and implications for their breeding potential. Biochemical Systematics and Ecology 50: 187-198.

Bassil N, Davis T, Amaya I, Bellon LR, Brew F, Denoyes B, van Dijk T, Ficklin S, Iezzoni A, Jung S, Mahoney LL, Main D, Mittmann M, Monfort A, Peace C, Pirani A, Rosyara U, Sargent DJ, Webster TA, Wood DJ, Yang Y, Zhang H, Van de Weg WE (2014) Development and Preliminary Evaluation of the IStraw90 Axiom® Array in the Cultivated Strawberry (Fragaria ×ananassa). Abstract W318, Plant and Animal Genome XXII, San Diego, CA, USA. https://pag.confex.com/pag/xxii/webprogram/Paper9546.html

Becher SA, Steinmetz K, Weising K, Boury S, Peltier D, Renou JP, Kahl G, Wolff K (2000) Microsatellites for cultivar identification in *Pelargonium*. Theor. Appl. Genet. 101, 643-651.

Ben-Meir H, Vainstein A (1994) Assesssment of genetic relatedness in roses by DNA fingerprinting analysis. Sci. Hortic. 58, 158-164.

Bever JD, Felber (1992) The theoretical population genetics of autopolyploidy. In: Antonovics J, Futuyma D (Eds.) Oxford surveys in evolutionary biology, 8: 185-217. Oxford University press, New York. NY, USA.

Bingham ET, Groose RW, Woodfield DR, Kidwell KK (1994) Complementary gene interactions in alfalfa are greater in tetraploids than diploids. Crop Sci 34: 823-829.

Bisognin DA (2011) Breeding vegetatively propagated horticultural crops. Crop Bree Appl Biotechnol, S1: 35-43.

Blair MW, Hurtado N (2013) EST-SSR markers from five sequenced cDNA libraries of common bean (Phaseolos vulgaris L.) comparing three bioinformatic algorithms. Molecular Ecology Resources 13: 688-695.

Bradshaw JE (1994) Quantitative genetics theory for tetrasomic inheritance. In: Bradshaw JE, Mackay GR (Eds.) Potato genetics. CABI, Cambridge, 467-497.

Bradshaw JE, Mackay GR (1994) Breeding strategies for clonally propagated potatoes. In Bradshaw JE and Mackay GR (Eds.) Potato genetics. CABI, Cambridge, 467-497.

Bradshaw JE, Hackett CA, Meyer RC, Milbourne D, McNicol JW, Phillips MS, Waugh R (1998) Identification of AFLP and SSR markers associated with quantitative resistance to *Golbodera pallida* (Stone) in tetraploid potato (*Solanum tuberosum subsp. tuberosum*) with a view to marker-assisted selection. Theor Appl Genet 97: 202-210.

Bredmose N, Hansen J (1996) Topophysis affects the potential of axillary bud growth, fresh biomass accumulation and specific fresh weight in single-stem roses (*Rosa hybrida* L.). Ann Bot 78: 215-222.

Browse J, Lange BM (2004) Counting the cost of cold-blooded life: metabolomics of cold acclimation. Proc Natl Acad Sci USA 101: 14996-14997.

Brouwer DJ, Duke SH, Osborn TC (2000) Mapping genetic factors associated with winter hardiness, fall growth, and freezing injury in autotetraploid alfalfa. Crop Sci 40: 1387-1396.

Byrne DH (2007) Molecular marker use in perennial plant breeding. Acta Hort 751: 163-168.

Burbulis N, Kuprienė R, Blinstrubiene A (2008) Investigation of cold resistanse of winter pareseed in vitro. Sodinikystė ir daržininkystė 27: 223-232.

Burbulis N, Jonytiene V, Kupriene R, Blinstrubiene A, Liakas V (2011) Biochemical and physiological factors related to cold de-acclimation and re-acclimation in rapeseed shoots in vitro. Journal of food, agriculture & environment 9(1): 483-487.

Butruille DV, Boiteux LS (2000) Selection–mutation balance in polysomic tetraploids: impact of double reduction and gametophytic selection on the frequency and subchromosomal localization of deleterious mutations. Proceedings of the National Academy of Sciences of the USA, 97, 6608-6613.

Cao MD, Tasker E, Willadsen K, Imelfort M, Vishwanathan S, Sureshkumar S, Balasubramanian, S Bodén M (2014) Inferring short tandem repeat variation from paired-end short reads. Nucleic Acids Research, 42, e16. doi:10.1093/nar/gkt1313

Cardoso SD, Gonçalves D, Robalo JI, Almada VC, Canário AVM, Oliveira RF (2013) Efficient isolation of polymorphic microsatellites from high-throughput sequence data based on number of repeats. Marine Genomics, 11, 11-16.

Castro P, Stafne ET, Clark JR, Lewers KS (2013) Genetic map of the primocane-fruiting and thornless trait of tetraploid blackberry. Theor Appl Genet 126: 2521-2532.

Castoe TA, Poole AW, de Koning APJ, Jones KL, Tomback DF et al. (2012) Rapid microsatellite identification from Illumina paired-end genomic sequencing in two birds and a snake. PLOS ONE, 7, e30953. doi:10.1371/journal.pone.0030953.

Celton JM, Tustin DS, Chagné D, Gardiner SE (2009) Construction of a dense genetic linkage map for apple rootstocks using microsatellites developed from Malus ESTs and Pyrus genomic sequences. Tree Genetics and Genomics, 5, 93-107.

Cheng S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Reporter, 11, 113-116.

Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, Agarwal M, Zhu JK (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis. Genes Dev 17: 1043-1054.

Chinnusamy V, Zhu V, Zhu JK (2007) Cold stress regulation of gene expression in plants. Trends Plant Sci 12: 444-451.

Collard BCY, Jahufer MZZ, Brouwer JB, Pang ECK (2005) An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. Euphytica 142: 169-196.

Conner AJ, Jacobs JME Ganet RA (1997) Transgenic potato versus "traditional" potatoes: what's difference? In: McLean GD, Waterhouse PM, Gibbs MJ (Ed.) Commercialisation of transgenic crops: Risk, benefit, and trade consideration, 23-26.

Cox SE, Stushnoff C (2001) Temperature-related shifts in soluble carbohydrate content during dormancy and cold acclimation in Populus tremuloides. Can J For Res 31: 730-737.

Cook D, Fowler S, Fiehn O, Thomashow MF (2004) A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of Arabidopsis. Proc Natl Acad Sci USA 101(42): 818-822.

Crespel L, Chirollet M, Durel CE, Zhang D, Meynet J, Gudin S (2002) Mapping of qualitative and quantitative phenotypic traits in Rosa using AFLP markers. Theor. Appl. Genet. 105:1207–1214.

Croser JS, Clarke H, Siddique KHM, Khan TN (2003) Low-Temperature stress: Implications for Ccickpea (Cicer arietinum L.) Improvement. Critical Reviews in Plant Sciences, 22(2):185–219.

Daly C, Widrelechner MP, Halbleib HJ, Smith JI, Gibson WP (2012) Development of a new USDA plant hardiness zone map for the United States. Journal of Applied Meteorology and Climatology 51: 242-264.

Dami IE, Ennahli S, Zhang Y (2012) Assessment of winter injury in grape cultivars and pruning strategies following a freezing stress event. Am J Enol Vitic 63(1): 106-111.

Davarynejad GH, Aryanpooya Z, Attar S (2012) Frost injury in buds and wood of several peach and nectarine cultivars. In: Gitona J, Marsal J (Eds) Proceedings of the VIIth international peach symposium, Lleida, Spain, 217-224.

De Riek J, De Cock K, Smulders MJM, Nybom H (2013) AFLP-based population structure analysis as a means to validate the complex taxonomy of dogroses (*Rosa* section Caninae). Mol. Phylogenet. Evol. 67, 547-559.

De Riek J, Everaert I, Esselink GD, Calsyn E, Smulders MJM, Vosman B (2007) Assignment tests for variety identification compared to genetic similarity-based methodsusing experimental datasets from different marker systems in sugar beat. Crop Sci. 47, 1964-1974.

De Vries DP, Dubois AM (1978) On the transmission of the yellow flower colour from *Rosa foetida* to recurrent flowering hybrid tea-roses. Euphytica 27: 205-210

De Vries DP, Dubois LAM (1996) Rose breeding: Past, present, and prospects. Acta Hort 424: 241-248.

Debener T, Bartels C, Mattiesch L (1996) RAPD analysis of genetic variation between a group of rose cultivars and selected wild rose species. Mol. Breed. 2, 321-327.

Debener T, Mattiesch L (1999) Construction of a genetic linkage map for roses using RAPD and AFLP markers. Theor Appl Genet 99:891–899.

Debener T, von Malek B, Mattiesch L, Kaufmann H (2001) Genetic and molecular analysis of important characters in roses. Acta Hort 547: 45-49.

Debener T, B. van Malek B, Schreiber B, Drewes-Alvarez R (2003) Marker assisted background selection for the introgression of black spot resistance into cultivated roses. Europ J Hort Sci 68(6): 245-252.

Debener T, Linde M, Dohm A (2004) The utilization of molecular tools for rose breeding and genetics. Acta hort 630: 29-42.

Debener T, Linde M (2009) Exploring Complex Ornamental Genomes: The Rose as a Model Plant. Crit Rev Plant Sci 28, 267-280.

Debener T, Bretzke M, Dreier K, Spiller M, Limde M, Kaufmann H, Berger RG, Krings RG (2010) Genetic and molecular analysis of key loci involved in self incompatibility and floral scent in roses. Acta Hort 870: 183-187.

Doulati-Baneh H, Mohammadi SA, Labra M (2013) Genetic structure and diversity analysis in Vitis vinifera L. cultivars from Iran using SSR markers. Scientia Horticulturae 160: 29-36.

Du FK, Xu F, Qu H, Feng S, Tang J, Wu R (2013) Exploiting the transcriptome of Euphrates Poplar, Populus euphratica (Salicaceae) to develop and characterize new EST-microsatellite markers and construction an EST-microsatellite database. PLoS ONE, 8, e61337.

Dreher K, Khairallah M, Ribaut J, Morris M (2003) Monet matters (I): Costs of field and laboratory procedures associated with conventional and marker-assisted maize breeding at CIMMTY. Mol Breed 11: 221-234.

Dudley JW (1963) Basic problems in quantitative genetics of autotetraploids. In: Hanson WD and Robinson HF (ed.) Statistical genetics and plant breeding: a symposium and workshop sponsored by the committee of plant breeding and genetics of the agricultural board at the North Carolina state college, Raleigh, NC: 519-520. National academy of science, Washington DC.

Dufresnes C, A Brelsford, P Béziers, N Perrin (2014) Stronger transferability but lower variability in transcriptomic- than in anonymous microsatellites: evidence from Hylid frogs. Molecular Ecology Resources (in press). doi:10.1111/1755-0998.12215.

Dugo ML, Satovic Z, Millán T, Cubero JI, Rubiales D, Cabrera A, Torres AM (2005) Genetic mapping of QTLs controlling horticultural traits in diploid roses. Theor Appl Genet 111: 511-520.

Duran C, Singhania R, Raman H, Batley J, Edwards D (2013) Predicting polymorphic EST-SSRs in silico. Molecular Ecology Rssources, Doi: 10.1111/1755-0998.12078.

Durand J, Bodénès C, Chancerel E, Frigerio NM, Vandramin G, Sebastiani F, Buonamici A, Gailing O, Koelewijn HP, Villani F, Mattioni C, Cherubini M, Goicoechea PG, Herrán A, Ikaran Z, Cabané C, Ueno S, Alberto F, Dumoulin PY, Guichoux E, se Daruvar A, Kremer A, Plomion C (2010) A fast and cost-effective approach to develop and map EST-SSR markers: oak as a case study. BMC Genomics 11:570.

Edwards MD, Page NJ (1994) Evaluation of marker-assisted selection through computer simulation. Theor Appl Genet 88: 376-382.

Ellis JR, Burke JM (2007) EST-microsatellites as a resource for population genetic analysis. Heredity, 99, 125-132.

Encyclopedia Britannica, 2012. *Encyclopedia Britannica Online Academic Edition*. Encyclopedia Britannica Inc. Web. 07 Dec. 2012.

Eschbach E, S Schöning (2013) Identification of high-resolution microsatellites without a priori knowledge of genotypes using a simple scoring approach. Methods in Ecology and Evolution, 4, 1076–1082

Esselink GD, Smulders MJM, Vosman B (2003) Identification of cut rose (*Rosa hybrida*) and rootstock varieties using robust sequence tagged microsatellite site markers. Theor. Appl. Genet. 106, 277-286.

Esselink GD, Nybom H, Vosman B (2004) Assignment of allelic configuration in polyploids using the MAC-PR (microsatellite DNA allele counting-peak ratios) method. Theor. Appl. Genet. 109, 402-408.

Evans KM, Patocchi A, Rezzonico F, Mathis F, Durel CE, Fernández-Fernández F, Boudichevskaia A, Dunemann F, Stankiewicz-Kosyl M, Gianfranceschi L, Komjanc M, Lateur M, Madduri M, Noordijk Y, van de Weg WE (2011) Genotyping of pedigreed apple breeding material ith a genome-covering set of SSRs: trueness-to-type of cultivars and their parentages. Mol Breed 28: 535-547.

Faust M (1997) Physiology of temperate zone fruit trees. Academic Publishers, 338.

Fernández-Escobar R, Navarro S, Melger JC (2011) Effect of nitrogen on frost tolerance of olive trees. In: Tous J et al. (Eds) Proceedings of the olive trends symposium "From olive tree to olive oil: new trends and future challenges", Lisbon, Portugal, 41-46.

Fisk SP, Cuesta-Marcos A, Cistue L, Russell J, Smith KP, Baenziger S, Bedo Z, Correy A, Filichkin T, Karsai I, Waugh R, Hayes PM (2013) GR-H3: a new QTL to assist in the development of fall-sown barley with superior low temperature tolerance. Theor Appl Genet 126: 335-347.

Foncéka D, Hodo-Abalo T, Rivallen R, Faye I, Sall MN, Ndoye O, Fávero AP, Bertioli DJ, Glaszmann JC, Courtois B, Rami JF (2009) Genetic mapping of wild introgressions into cultivated peanut: a way toward enlargening genetic basis of a recent allotetraploid. BMC Plant Biol 9: 103.

Foucher F, Chevalier M, Corre C, Souffley-Freslon V, Legeai F, Hibrand-Saint Oyant L (2008) New resources for studying the rose flowering process. Genome: 51(10):827-837. doi: 10.1139/G08-067

Francia E, Rizza F, Vattivelli L, Stanca AM, Galiba G, Tóth B, Hayes PM, Skinner JS, Pecchioni N (2004) Two loci on chromosome 5H determine low-temperature tolerance in Nure (winter) x Tremois (spring) barley map. Theor Appl Genet 108: 670-680.

Fuchinoue H (1982) Winter desiccation of tea plant in Japan. In: Li PH, Sakai A (Eds.) Plant cold hardiness and freezing stress mechanisms and cop implications: 499-511. Academis Press INC, New York.

Gallais A (2003) Quantitative genetics and breeding methods in autopolyploid plants. INRA, Paris, 30-38.

Galiba G, Stockinger EJ, Francia E, Milc J, Kocsy G, Pecchioni N (2013) Freezing tolerance in the Triticeae. In Varshney RK, Tuberosa R (Eds.) Translational genomics for crop breeding: 99-124. Wiley, USA.

Gallowat LF, Etterson JR (2007) Inbreeding depression in an autotetraploid herb: A three cohort field study. New Phytol 173(2): 383-392.

Glémin S, Bazin E, Charlesworth D (2006) Impact of mating systems on patterns of sequence polymorphism in flowering plants. Proceedings of the Royal Society Series B- Biological Sciences 273: 3011-3019.

Gao Z, J Wu, Z Liu, L Wang, H Ren, Q Shu (2013) Rapid microsatellite development for tree peony and its implications. BMC Genomics, 14, 886.

Gar O, Sargent DJ, Tsai C-J, Pleban T, Shalev G, Byrne DH, Zamir D (2011) An autotetraploid linkage map of rose (Rosa hybrid) validated using the strawberry (Fragaria vesca) genome sequence. PLoS ONE 6(5): e20463.

Gondo T, Sato S, Okumura K, Tabata S, Akashi R, Isobe S (2007) Quantitative trait locus analysis of multiple agronomic traits in the model legume Lotus japonicus. Genome 50: 627-637.

Grabherr MG, Haas BJ, Yassour M, et al. Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q, Chen Z, Mauceli E, Hacohen N, Gnirke A, Rhind N, di Palma F, Birren BW, Nusbaum C, Lindblad-Toh K, Friedman N, Regev A (2011) Full-length transcriptome assembly from RNA-seq data without a reference genome. Nature Biotechnology, 29, 644-652. doi:10.1038/nbt.1883.

Gudin S (2010) Rose: Genetics and breeding. In: Janick J (ed): Plant Breeding Review, 17: 159-189. John Wiley & sons, New York.

Guo E, Cui Z, Wu D, Hui, M., Liu Y, Wang H (2013) Genetic structure and diversity of Portunus trituberculatus in Chinese population revealed by microsatellite markers. Biochemical Systematics and Ecology 50: 313-321.

Gur A, Zamir D (2004) Unused natural variation can lift yield barriers in plant breeding. PLOS Biol 2(10): e245.

Guy C (1999) Molecular responces of plants to cold shock and cold acclimation. J Mol Microbiol Biotechnol 1: 231-242.

Hackett CA, Bradshaw JE, Meyer RC, McNicol JW, Milbourne D, Waugh R (1998) Linkage analysis in tetraploid species: a simulation study. Genet Res 71: 143-154.

Hackett CA, Luo ZW (2003) TetraploidMap: Construction of a linkage map in autotetraploid species. J Hered 94: 358-359.

Hackett CA, McLean K, Bryan GJ (2013) Linkage Analysis and QTL Mapping Using SNP Dosage Data in a Tetraploid Potato Mapping Population. PLoS ONE 8(5): e63939.

Hackett CA, Bradshaw JE, Bryan GJ (2014) QTL mapping in autotetraploids using SNP dosage information. Theor Appl Genet, DOI 10.1007/s00122-014-2347-2.

Hajjar R, Hodgkin T (2007) The use of wild relatives in crop improvement: A survey on developments over the last 20 years. Euphytica 156: 1-13.

Hannah MA, Heyer AG, Hincha DK (2005): A global survey on gene regulation during cold acclimation in Arabidopsis thaliana. Plos Genet 1(2): 179-196.

Hardy OJ, Vekemans X (2002) SPAGeDI: A versatile computer program to analyse spatial genetic structure at the individual or population levels. Mol. Ecol. Notes 2, 618-620.

Harlan JR (1992) Crops and man. American Society of Agronomy and Crop Science Society of America, Madison.

Heidarvand L, Amiri RM (2010) What happens in plant molecular responses to cold stress? Acta Physiol. Plant 32: 419-431.

Hemming MN, Trevaskis B (2011) Make hay when the sun shine: the role of MADS- box genes in temperature-dependant seasonal flowering responses. Plant Sci 150: 447-453.

Hermsen JGT (1994) Introgression of genes from wild species, including molecular and cellular approaches. In Bradshaw JE and Mackay GR (Ed.) Potato genetics. CABI, Cambridge, 71-99.

Hessayon DG (2004) The Rose Expert. Expert Books, New York.

Heidarvand L, Amiri RM (2010) What happens in plant molecular responses to cold stress? Acta Physiol. Plant 32: 419-431.

Heinrichs F (2008) International statistics flowers and plants. AIPH/Union Fleurs 56:16–90.

Hibrand-Saint Oyant L, Crespel L, Rajapakse S, Zhang L, Foucher F (2008) Genetic linkage maps of rose constructed with new microsatellite markers and locating QTL controlling flowering traits. Tree Genetics & Genomes 4:11–23.

Hoffman JI, Nichols HJ (2011) A novel approach for mining polymorphic microsatellite markers In Silico. PLOS ONE, 6, e23283.

Hokanson SC, McNamara S (2013) Can't always get what we want! Finding and creating cold for hardiness screening at the University of Minesota. In: Van Huylenbroeck J et al. (Eds) Proceedings of the Second international symposium on woody ornamentals of the temperate zone, Ghent, Belgium, 193-202.

Holsinger KE, Weir BS (2009) Genetics in geographically structured populations: defining, estimating and interpreting Fst. Nat Rev Genet 10, 639-650.

Howe GT, Aitken SN, Neale DB, Jermstad KD, Wheeler NC, Chen THH (2003) From genotype to phenotype: unraveling the complexities of cold adaptations in forest trees. Can J Bot 81: 1247-1266.

Huggett RJ (2003) Fundamentals of geomorphology. Routledge, London, p.237.

Ibitoye DO, Akin-Idowu PE (2011) Marker-assisted-selection (MAS): a fast track to increase genetic gain in horticultural crop breeding. Afr J Biotechnol 10(55): 11333-11339.

Illa E, Sargent DJ, Lopez Girona E, Bushakra J, Ceestaro A, Crowhurst R, Pindo m, Cabrera A, van der Knaap E, Iezzoni A, Gardiner S, Velasco R, Arús P, Changé, Troggio M (2011) Comparative analysis of rosaceous genomes and the reconstruction of a putative ancestral genome for the family. BMC Evol Biol 11:9.

Iwaizumi MG, Tsuda Y, Ohtani M, Tsumura Y, Takahashi M (2013) Recent distribution changes affect geographic clines in genetic diversity and structure of *Pinus densiflora* natural populations in Japan. Forest Ecology and Management 304: 407-416.

Jablonka E, Raz G (2009) Trangenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. Q Rev Biol 84: 131-176.

Jansen RC, Van Ooijen JW, Stam P, Lister C, Dean C (1995) Genotype-by-environment interactions in genetic mapping of multiple quantitative trait loci. Theor Appl Genet 91: 33-37.

Jackson RC, Jackson JW (1996) Gene segregation in autotetraploids: prediction from meiotic configurations. Am J Bot 83: 673–678.

Jenks MA, Rashotte AM, Tuttle HA, Feldmann KA (1996) Mutants in Arabidopsis thaliana altered in epicuticular wax and leaf morphology. Plant Physiol. 110: 377-385.

Jennings TN, Knaus BJ, Mullins TD et al. (2011) Multiplexed microsatellite recovery using massively parallel sequencing. Molecular Ecology Resources, 11, 1060-1067.

Jørgensen M, Østrem L, Höglind M (2010) De-hardening in contrasting cultivars of timothy and perennial ryegrass during winter and spring. Grass Forage Sci 65: 38-48.

Jung C, Muller AE (2009) Flowering time control and applications in plant breeding. Trends in Plant Science 14(10): 563-573.

Larcher W (2005) Climatic constraints drive the evolution of low temperature resistance in woody plants. J Agric Meteor 61: 189-202.

Li Y, Böck A, Haseneyer G, Korzun V, Wilde P, Schön CC, Ankerst DP (2011) Association analysis of frost tolerance in rye using candidate genes and phenotypic data from controlled, and field phenotyping platforms. BMC Plant Biology 11: 146.

Kacperska A (1999) Plant response to low temperature: signaling pathways involved in plant acclimation. In: Margesin R, Schinner F (Eds), Cold-adapted organisms – ecology, physiology, enzymology and molecular biology. Springer-Verlag, Berlin, Germany: 79–103.

Kalberer SR, Wisniewski M, Arora A (2006) Deacclimation and reacclimation of cold-hardy plants: Current understanding and emerging concepts. Plant Sci 171: 3-16.

Kalberer SR, Arora R, Leyva-Estrada N, Krebs SL (2007a) Cold hardiness of floral buds of deciduous azaleas: dehardening, rehardening, and endodrmancy in late winter. J Am Soc Hortic Sci 123: 73-79.

Kalberer SR, Leyva-Estrada N, Krebs SL, Arora R (2007b) Frost dehardening and rehardening of floral buds of deciduous azaleas are influenced by genotypic biogeography. Environ Exp Bot 59: 264-274.

Kaplan F, Guy CL (2004) β-Amylase induction in and the protective role of maltose during temperature shock. Plant Physiol 135: 1674-1684.

Kawamura K, Hibrand-Saint Oyant L, Crespel L, Thouroude T, Lalanne D, Foucher F (2011) Quantitative trait loci for flowering time and inflorescence architecture in rose. Theor Appl Genet 122: 661-675.

Khodakovskaya M, McAvoy R, Peters J, Yi Li H (2005) Enhanced cold tolerance in transgenic tobacco expressing a chloroplast omega-3 fatty acid desaturase gene under the control of a cold inducible promoter. Planta 223: 1090-1100.

Kimura T, Nishitani C, Iketani H, Ban Y, Yamamoto T, (2006) Development of microsatellite markers in rose. Mol. Ecol. Notes 63, 810-812.

Kimura T, Nishitani C, Iketani H, Ban Y, Yamamoto T (2010) Development of microsatellite markers in rose. Molecular Ecology Notes, 6, 810-812.

Kirchhiff M, Svirshchevskaya A, Hoffmann C, Schechert A, Jung C, Kopisch Obuch FJ (2012): High degree of genetic vatiation of winter hardiness in a panel of Beta vulgaris L. Crop Science 52: 179-188.

Koehler G, Randall SK (2012) Molecular cold responses for two strawberry cultivars: comparison of proteomic and microarray analysis. In: Folta KM et al. (Eds) Proceedings of the international symposium on genomics and genetic transformation of horticultural crops, Lisbon, Portugal, 73-80.

Kellogg AA, Branaman TJ, Jones NM, Little CZ, Swanson JD (2011) Morphological studies of developing *Rubus* prickles suggest that they are modified glandular trichomes. Botany 89: 217-226.

Koning-Boucoiran CFS, Gitonga VW, Yan Z, Dolstra O, van der Linden CG, van der Schoot J, Uenk GE, Verlinden K, Smulders MJM, Krens FA, Maliepaard C (2012a) The mode of inheritance in tetraploid cut roses. Theor Appl Genet 125:591-607.

Koning-Boucoiran CF, Smulders MJM, Krens FA, Esselink GD, Maliepaard C (2012b) SNP genotyping in tetraploid cut roses. Acta Hortic 953: 351-356.

Koopman WJM, Wissemann V, De Cock, K, Van Huylenbroeck J, De Riek J, Sabatino GJH, Visser D, Vosman B, Ritz CM, Maes B, Werlemark G, Nybom H, Debener T, Linde M, Smulders MJM (2008) AFLP markers as a tool to reconstruct complex relationships: A case study in *Rosa* (Rosaceae). Am. J. Bot. 95, 353-366.

Koornneef M, Bentsink L, Hilhorst H (2002) Seed dormancy and germination. Curr Opin Plant Biol 5(1): 33-36.

Kosova K, Vitamvas P, Prasil IT (2007) The role of dehydrins in plant response to cold. Biol Plant 51(4): 601-617.

Kreps JA, Wu YJ, Chang HS, Zhu T, Wang X, Harper JF (2002) Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress. Plant Physiol 130(4): 2129-2141.

Kruissmann G (1981) The complete book of roses. Timber Press, Portland.

Krüger E, Josuttis M (2014) Effects of growing and climate conditions on berry yield and nutritional quality. Acta Hortic 1017: 351-362.

Krzywinski M, Schein J, Birol I, Connors J, Gascoyne R, Horsman D, Jones SJ, Marra MA (2009) Circos: An information aesthetic for comparative genomics. Genome Res 19: 1639-1645.

Kumar S, Volz RK, Alspach PA, Bus VGM (2010) Development of a recurrent apple breeding programme in New Zealand: a synthesis of results, and a proposed revised breeding strategy. Euphytica 173: 207-222.

Lance SL, Love CN, Nunziata SO, O'Bryhim JR, Scott DE, Flynn WR, Jones KL (2013) 32 species validation of a ne Illumina paired-end approach for the development of microcatellites. PLOS ONE, 8, e81853.

Lande R, Thompson R (1990) Efficiency of marker-assisted selection in the improvement of quantitative traits. Genetics 124: 743-756.

Lark KG, Chase K, Adler F, Mansur LM, Orf JH (1995) Interactions between quantitave trait loci in soybean in which trait variation at one locus is conditional upon a specific allele. Proc Natl Acad Sci USA 92: 4656-4660.

Larcher W (2005) Climatic constraints drive the evolution of low temperature resistanse in woody plants. J Agric Meteor 61: 189-202.

Legendre M, Pochet N, Pak T, Verstrepen KJ (2007) Sequence-based estimation of minisatellite and microsatellite repeat variability. Genome Research, 17, 1787-1796.

Leinonen L, Repo T, Hänninen H (1997) Changing environmental effects on frost hardiness of scots pine during dehardening. Ann Bot 79: 133-138.

Li W, Wang R, Li M, Li L, Wang C, Welti R, Wang X (2008) Differential degradation of extraplastidic and plastidic lipids during freezing and post-freezing recovery in Arabidopsis thaliana, J. Biol. Chem. 283: 461–468.

Li J, Kiranmoy D, Fu G, Tong C, Li Y, Tobias C, Wu R (2010) EM algorithm for mapping quantitative trait loci in multivalent tetraploids. International Journal of Plant Genomics, Vol 2010.

Li ZH, Lu X, Yun G, Liu SJ, Tao M, Xiao H, Qiao YQ, Zhang YP, Luo J (2011) Polyploidization and epigenetics. Genetics 56: 245-252.

Li Y, Böck A, Haseneyer G, Korzun V, Wilde P, Schön CC, Ankerst DP (2011): Association analysis of frost tolerance in rye using candidate genes and phenotypic data from controlled, and field phenotyping platforms. BMC Plant Biol 11: 146.

Li L, Tacke E, Hofferbert HR, Lübeck J, Strahwald J, Deaffehn AM, Walkmeier B, Gebhardt C (2013) Validation of candidate gene markers for marker-assisted selection of potato cultivars with improved tuber quality. Theor Appl Genet 126: 1039.1052.

Lian C, Hogetsu T (2002) Development of microsatellite markers in black locust (Robinia pseudoacacia) using a dual-supression-PCR technique. Molecular Ecology Notes, 2, 211-213.

Liang X, Chen X, Hong Y, Liu H, Zhou G, Li S, Gio B (2009) Utility of EST-derived microsatellite in cultivated peanut (Arachis hypogaea L.) and Arachis wild species. BMC Plant Biology, 9, 35.

Liebhard R, Gianfranceschi L, Koller B, Ryder CD, Tarchini R, Van de Weg E, Gessler C (2002) Development and characterization of 140 new microsatellites in apple (*Malus x domestica* Borkh.). Mol Breed 10: 217-241.

Liu H, Li, S, Hu, P et al. (2013) Isolation and characterization of EST-based microsatellite markers for Scatophagus argus based on transcriptome analyses. Conservation Genetics Resources, 5: 483-485. doi: 10.1007/s12686-012-9833-0.

Linde M, Mattiesch L, Debener T (2004) Rpp1, a dominant gene providing race-specific resistance to rose powdery mildew (*Podosphaera pannosa*): molecular mapping, SCAR development and confirmation of disease resistance data. Theor Appl Genet 109(6): 1261-1266.

Linde M, Hattendorf A, Kaufmann H, Debener T (2006) Powdery mildew resistance in roses: QTL mapping in different environments using selective genotyping. Theor Appl Gene 113(6): 1081-1092.

Livingston III DP, Henson CA, Toung TD, Wise ML, Tallury SP, Duke SH (2013) Histological analysis and 3D reconstruction of winter cereal crowns recovering from freezing: A unique response in oat (Avena sativa L.). LOS ONE 8(1): e53468.

Luby JL, Shaw DV (2001) Does Marker-assisted selection make dollars and sense in fruit breeding program? HortScience 36(5): 872-879.

Luo ZW, Hackett CA, Bradshaw JE, McNicoL JW, Milbourne D (2001) Construction of a genetic linkage map in tetraploid species using molecular markers. Genetics, 157: 1369-1385.

Maloy PV, Lyerly JH, Wooten DR, Anderson JM (2011) Marker development and quantitative trait loci in a fall-sown oat recombinant inbred population. Crop Sci 51: 490-502.

Maiti RK, Rao KEP, Raju PS, House L (1984) The glossy trait in sorghum: Its characteristics and significance in crop improvement. Field Crops Research 9: 279-89.

Masterson J (1994) Stomatal size in fossil plants-evidence for polyploidy in majority of angiosperms. Science 264: 421-424.

Mather K (1951) The measurement of linkage in heredity. Methuen, London, 149.

Marti AF, Athanson B, Koepke T, Forcada CF, Dhingra A, Oraguzie N (2012) Genetic diversity and relatedness of sweet cherry (*Prunus avium* L.) cultivars based on single nucleotide polymorphic markers. Front. Plant Sci. 3, 116.

McKey D, Elias M, Pujol B, Duputié A (2010) The evolutionary ecology of clonally propagated domesticated plants. New Phytol 186: 318-332.

Meng J, Li D, Yi T, Yang J, Zhao X (2009) Development and characterization of microsatellite loci for *Rosa odorata* var. gigantea Rehder & EH Wilson (Rosaceae). Conserv. Genet. 10, 1973-1976.

Milczarek D, Flis B, Przetakiewicz A (2011) Suitability of molecular markers for selection of potatoes resistant to Glodobera spp. Am J Potato Res 88: 245-255.

Miura K, Jin JB, Lee J, Yoo CY, Stirm V, Miura T, Ashworth EN, Bressan RA, Yun DJ, Hasegawa PM (2007) SIZ1-Mediated Sumoylation of ICE1 Controls CBF3/DREB1A Expression and Freezing Tolerance in Arabidopsis. The Plant Cell 19: 1403-1414.

Moellering ER, Muthan B, Benning C (2010) Freezing tolerance in plants requires lipid remodeling at the outer chloroplast membrane. Science 330(6001): 226-228.

Moghaddam HH, Leus L, De Riek J, Van Huylenbroeck J, Van Bockstaele E (2012) Construction of a genetic linkage map with SSR, AFLP and morphological markers to locate QTLs controlling pathotype-specific powdery mildew resistance in diploid roses. Euphytica 184 (3): 413-427.

Moran RE, Sun Y, Geng F, Zhang D (2011) Cold temperature tolarance of trunk and root tissue in one- or two- year-old apple rootstocks. HortScience 46(11): 1460-1464.

Morin X, Ameglio T, Ahas R, Kurz-Besson C, Lanta V, Lebourgeois F, Miglietta F, Chuine I (2007) Variation in cold hardiness and carbohydrate concentration from dormancy induction to bud burst among provenances of three European oak species. Tree physiology 27: 817-825.

Moose SP, Mumm RH (2008) Molecular plant breeding as the foundation for 21st century crop improvement. Plant Physiol 147: 969-977.

Noble RE (2002) Effects of UV-irradiation on seed germination. Sci Total Environ 299: 173-176.

Noak R (2003) Selection strategies for disease and pest resistance. In: Roberts AV, Debener T, Gudin S (eds.), Encyclopedia of Rose Science, Elsevier Ltd, Oxford, UK: 49-55.

Ma K-H, Jang D-H, Dixit A, Chung J-W, Lee S-Y, Lee J-R, Kang H-K, Kim S-M, Parks Y-J (2007) Characterization of 30 new microsatellite markers, developed from enriched genomic DNA library of zoysiagrass Zoysia japonica Steud. Molecular Ecology Notes, 7, 1323-1325.

NDong C, Quellet F, Houde M, Sarhan F (1997) Gene expression during cold acclimation in strawberry. Plant Cell Physiol 38(7): 863-870.

Nejad P (2005) Pathogenic and Ice-Nucleation Active (INA) bacteria causing dieback of willows in short rotation forestry. Doctoral thesis, Swedish University of Agricultural Sciences, Uppsala.

Nijveen H, van Kaauwen M, Esselink DG, Hoegen B, Vosman B 2013. QualitySNPng: a user-friendly SNP detection and visualization tool. Nucleic Acids Research 41, W587-W590. doi: 10.1093/nar/gkt333.

Nordström S, Hedrén M (2007) Development of polymorphic nuclear microsatellite markers for polyploid and diploid members of the orchid genus Dactylorhiza. Molecular Ecology Notes, 7, 644-647.

Novillo F, Medina J, Salinas S (2007): Arabidopsis CBF1 and CBF3 have a different function than CBF2 in cold acclimation and define different gene classes in the CBF regulon, Proc. Nat. Acad. Sci. USA 104: 21002–21007.

Nybom H (2004) Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. Mol. Ecol. 13, 1143–1155.

Nybom H, Werlemark G, Esselink GD, Vosman B (2005) Sexual preferences linked to rose taxonomy and cytology. Acta Hort. 690: 21-27.

Nybom H, Weising K, Rotter B (2014) DNA fingerprinting in botany: past, present, future. Investigative Genetics, 5, 1. doi:10.1186/2041-2223-5-1.

Obrist D, Arnone JA, Körner C (2001) In situ effects of elevated CO2 on leaf freezing resistance and carbohydrates in a native temperature grassland. Ann Bot-London 87: 839-844.

Oliver JE (2005). The Encyclopedia of World Climatology. Springer Science & Business Media. pp. 382.

Ogilvie IS, Arnold NP, Richer C (1999) Three new winter-hardy Explorer rose cultivars. HortScience 34(2): 358-360.

Ortega F, Lopez-Vizcon C (2012) Application of Molecular Marker-AssistedSelection (MAS) for disease resistancein a practical potato breeding programme. Potato Res 55: 1-13.

Otto SP, Whitton J (2000) Polyploid incidence and evolution. Annu Rev Genet 34: 401-437.

Parsons PA (1959) Some problems in inbreeding and random mating in tetrasomics. Agron J: 465-467.

Pagter M, Williams M (2011) Frost dehardening and rehardening of Hydrangea macrophylla stems and buds. HortScience 46: 1121-1126.

Pagter M, Arora R (2013) Winter survival and deacclimation of perennials under warming climate: physiological perspectives. Physiologia Plantarum 147: 75-87.

Pairon MC, Jacquemart A-L (2005) Disomic segregation of microsatellites in the tetraploid *Prunus serotina* Ehrh. (*Rosaceae*). J Amer Soc Hort Sci 130 (5): 729-734.

Pan A, Hayes PM, Chen F, Chen THH, Blake T, Wright S, Karsai I, Bedo Z (1994) Genetic analysis of the components of winter-hardiness in barley. Theor Appl Genet 89: 900-910.

Park SJ, Kwak KJ, Oh TR, Kim YO, Kang H (2009) Cold shock domain proteins effects seeds germination and growth of Arabidopsis thaliana under abiotic stress conditions. Plant Cell Physiol 50: 869-878.

Park YH, Ahn SG, Choi YM, Oh HJ, Ahn DC, Kim JG, Kang JS, Choi YW, Jeong BR (2010) Rose (Rosa hybrida L.) EST-derived microsatellite markers and their transferability to strawberry (Fragaria spp.). Scientia Horticulturae 125: 733-739.

Peace C, Bassil N, Main D, Ficklin S, Rosyara UR, Stegmeir T, Sebolt A, Gilmore B, Lawley C, Mockler TC, Bryant DW, Wilhelm L, Iezzoni A (2012) Development and evaluation of a genome-wide 6K SNP array for diploid sweet cherry and tetraploid sour cherry. Plos ONE 7: e48305.

Pérez-de-Castro AM, Vilanova S, Cañizares J, Pascual L, Blanca JM, Díez MJ, Prohens J, Picó (2012) Application of genomics tools in plant breeding. Curr Genomics 13: 179-195.

Pfeiffer T, Roschanski AM, Pannell JR, Korbecka G, Schnittler M (2011) Characterization of microsatellite loci and reliable genotyping in a polyploidy plant, Mrcurialis perennis. Journal of heredity, 102(4): 479-488.

Phillips R, Rix M (2004) The ultimate guide to roses, Macmillan, London.

Piepho HP, Koch G (2000) Codominant analysis of banding data from a dominant marker system by normal mixture. Genetics, 155: 1459-1468.

Potter D, Ericksson T, Evans RC, Oh S, Smedmark JEE, Morgan DR, Kerr M, Robertson KR, Arsenault M, Dickinson TA, Campbell CS (2007) Phylogeny and classification of *Rosaceae*. Plant Syst Evol 266: 5-43.

Preston JC, Sandve SR (2013) Adaptation to seasonality and the winter freeze. Frontiers in Plant Science 4: doi: 10.3389/fpls.2013.00167.

Qu L, Hancock JF (2001) Detecting and mapping repulsion-phase linkage in polyploids with polysomic inheritance. Theor Appl Genet 103: 136-143

Rafalski JA (2002) Novel genetic mapping tools in plants: SNPs and LD-based approaches. Plant Sci 162: 329-333.

Rajapakse S, Byrne DH, Zhang L, Anderson N, Arumuganathan K, Ballard RE (2001) Two genetic linkage maps of tetraploid roses. Theor Appl Genet 103: 575-583.

R Core Team (2012). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

Remay A, Lalanne D, Thouroude T, Le CF, Hibrand-Saint Oyant L, Foucher F (2009) A survey of Flowering genes reveals the role of gibberellins in Floral control in rose. Theor Appl Genet 119:767–781.

Richer C, Arnold NP, Davidson CG (2000) Winter-hardy Roses: Explorer and Parkland Series. Ottawa: Agriculture Canada.

Rigoldi MP, Rapposelli E, Satta D, Rau D, Resta P, De Giorgio D, Porceddu A (2011) Genetic diversity of almond cultivars and characterization of self-incompatibility alleles. *Proceedings of the Joint Meeting AGI-SIBV-SIGA Assisi, Italy.* ISBN 978-88-904570-2-9 Poster Communication Abstract – 9.40.

Roberts AV, Blake PS, Lewis R, Taylor JM, Dunstan DI (1999) The effect of gibberellins on flowering in roses. J Plant Growth Regul 18: 113-119.

Roberts AV, Gudin S, Debener T (2003) Encyclopedia of rose science, vol 3. Elsevier.

Robinson JG, Bauchan GR, Brummer EC (2007) Genetic mapping forage yield, plant height, and regrowth at multiple harvest in tetraploid alfalfa (Medicago sativa L.). Crop Sci 47: 11-18.

Rohlf FJ (2000) NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System. Version 2.1. Setauket, New York, USA: Exeter Software.

Ronfort J, Jenczewski E, Bataillon T, Rousset F (1998) Analysis of population structure in autotetraploid species. Genetics 150: 921-930.

Ronford J (1999) The mutation load under tetrasomic inheritance and its consequences for the evolution of the selfing rate in autotetraploid species. Genet Res 74: 31-42.

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds.) Bioinformatics methods and protocols: Methods in molecular biology. Humana Press, Totowa NJ: 365-386.

Rusanov K, Kovacheva N, Atanassov A, Atanassov I (2005) Lessons from the microsatellite characterization of a segregating population derived from seeds of open pollinated *Rosa damascena* Mill. F. Trigintipetala plants. Biotechnol&Biotechnol. Eq. 19(2): 72-79.

Saha MC, Cooper JD, Rouf Mian MA, Chekhovskiy K, May GD (2006) Tall fescue genomic microsatellite markers: development and transferability across multiple grass species. Theoretical and Applied Genetics, 113, 1449-1458. doi:10.1007/s00122-006-0391-2.

Scariot V, Akkak A, Botta R (2006) Characterization and genetic relationships of wild species and old garden roses based on microsatellite analysis. J. Am. Soc. Hortic. Sci. 131, 66-73.

Semeniuk P (1971) Inheritance of recurrent blooming in *R. wichuraiana*. J. Hered. 62: 203-204.

Shahin A, van Kaauwen M, Esselink D, Bargsten JW, van Tuyl JM, Visser RGF, Arens P (2012) Generation and analysis of expressed sequence tags in the extreme large genomes Lilium and Tulipa. BMC Genomics, 13, 640.

Schmieder R, Edwards R (2011) Quality control and preprocessing of metagenomic datasets. Bioinformatics, 27, 863-864.

Shokaeva DB (2008) Injuries induced in different strawberry genotypes by winter freeze and their effect on subsequent yield. Plant Breed 127: 197-202.

Schreiber SG, Ding C, Hamann A, Hacke UG, Thomas BR, Brouard JS (2013a) Frost hardiness vs. growth performance in trembling aspen: an experimental test of assisted migration. Journal of Applied Ecology 50: 939-949.

Schreiber SG, Hamann A, Hacke UG, Thomas BR (2013b) Sixteen years of winter stress: an assessment of cold hardiness, growth performance and survival of hybrid poplar clones at a boreal planting site. Plant, Cell and Environment 36: 419-428.

Schröter D, Cramer W, Leemans R, Prentice IC, Araújo NW, Arnell NW, Bondeau A, Bugmann H, Cartet TR, Gracia CA, de la Vega-Leinert AC, Erhard M, Ewert F, Glendining M, House JI, Kankaanpää S, Klein RJT, Lavorel S, Lindner M, Metzeger MJ, Meyer J, Mitchell TD, Reginster I, Rounsevell M, Sabaté S, Sitch S, Smith B, Smith J, Sykes MT, Thonicke K, Thuiller W, Tuck G, Zaehle S, Zierl B (2005) Ecosystem service supply and vulnerability to global change in Europe. Science 310: 1333-1337.

Shulaev V, Sargent DJ, Crowhurst RN, Mockler TC, Folkerts O, Delcher AL, Jaiswal P, Mockaitis K, Liston A, Mane SP, Burns P, Davis TM, Slovin JP, Bassil N, Hellens RP, Evans C, Harkins T, Kodira C, Desany B, Crasta OR, Jensen RV, Allan AC, Michael TP, Setubal JC, Celton JM, Rees DJG, Williams KP, Holt SH, Rojas JJR, Chatterjee M, Liu B, Silva H, Meisel L, Adato A, Filichkin SA, Troggio M, Viola R, Ashman TL, Wang H, Dharmawardhana P, Elser J, Raja R, Priest HD, Bryant DW, Fox SE, Givan SA, Wilhelm LJ, Naithani S, Christoffels A, Salama DY, Carter J, Girona EL, Zdepski A, Wang W, Kerstetter RA, Schwab W, Korban SS, Davik J, Monfort A, Denoyes-Rothan B, Arus P, Mittler R, Flinn B, Aharoni A, Bennetzen JL, Salzberg SL, Dickerman AW, Velasco R, Borodovsky M, Veilleux RE, Folta KM (2011) The genome of woodland strawberry (*Fragaria vesca*). Nature Genetics 43:109-116.

Schunter C, Garza JC, Macpherson E, Pascual M (2014) SNP development from RNA-seq data in a nonmodel fish: how many individuals are needed for accurate allele frequency prediction? Molecular Ecology Resources, 14, 157-165. doi:10.1111/1755-0998.12155

Shupert DA, Byrne DH (2007) Inheritance of flower traits, leaflet number and prickles in roses. Acta Hort. 751: 331-335.

Smee MR, Pauchet Y, Wilkinson P et al. (2013) Microsatellites for the marsh fritillary butterfly: de novo transcriptome sequencing, and a comparison with amplified fragment length polymorphism (AFLP) markers. PLOS ONE, 8, e54721.

Smulders MJM, Bredemeijer G, Rus-Kortekaas W, Arens P, Vosman B (1997) Use of short microsatellites from database sequences to generate polymorphism among Lycopersicon esculentum cultivars and accessions of other Lycopersicon species. Theoretical and Applied Genetics, 94, 264-272.

Smulders MJM, Esselink GD, Voorrips RE, Vosman B (2009) Analysis of a database of DNA profiles of 734 hybrid tea rose varieties. Acta Hortic. 836, 169-174.

Smulders MJM, Arens P, Koning-Boucoiran CFS, Gitonga VW, Krens FA, Atanassov A, Atanassov I, Rusanov KE, Bendahmane M, Dubois A, Raymond O, Caissard JC, Baudino S, Crespel L, Gudin S, Ricci SC, Kovatcheva N, Van Huylenbroeck J, Leus L, Wissemann V, Zimmermann H, Hensen I, Werlemark G, Nybom H (2011) Rosa, in: Kole C (Ed.), Wild crop relatives: genomics and breeding resources plantation and ornamental crops. Springer Verlag, Berlin Heidelberg, pp. 243-275.

Smulders MJM, Vukosavljev M, Shahin A, van de Weg WE, Arens P (2012) High throughput marker development and application in horticultural crops. Acta Horticulturae (ISHS), 961, 547-551. http://www.actahort.org/books/961/961_72.htm

Smulders MJM, Voorrips RE, Esselink GD, Santos Leonardo TM, van ‘t Westende WPC, Vukosavljev M, Koning-Boucoiran CFS, van de Weg WE, Arens P, Schulz D, Debener T, Bellon L, Mittmann M, Pirani A, Webster T, Brew F, Cox P, Maliepaard C (2014) Development of the WagRhSNP Axiom SNP Array based on sequences from tetraploid cut roses and garden roses. Acta Horticulturae (in press).

Swarts ND, Sinclair EA, Dixon KW (2007) Characterization of microsatellite loci in the endangered grand spider orchid Caladenia huegelii (Orchidaceae). Molecular Ecology Notes, 7, 1141-1143.

Shepherd RE (1954) History of the Rose. Macmillan, New York.

Soltis DE, Soltis PS (1995) The dynamic nature of polyploid genomes. Proc. Nat. Acad. Sci. USA 92: 8089-8091.

Song K, Lu P, Tang K, Osborn TC (1995) Rapid genome change in synthetic polyploids of Brassica and its implications for polyploidy evolution. Proceedings of National Academy of Sciemce of the USA 92: 7719-7723.

Song Y, Liu F, Zhu Z, Tan L, Fu Y, Sun C, Cai H (2011) Construction of a simple sequence repeat marker-based genetic linkage map in the autotetraploid forage grass Dactylus glomerata L. Grassland Science, 57: 158-167.

Spiller M, Linde M, Hibrand-Saint Oyant L, Tsai C-J, Byrne DH, Smulders MJM, Foucher F, Debener T (2011) Towards a unified genetic map for diploid roses. Theor Appl Genet 122: 489-500.

Staub JE, Serquen FC (1996) Genetic markers, map construction, and their application in plant breeding. HortScience 31(15): 729-738.

Stebbins GI (1956) Artificial polyploidy as a tool in plant breeding. Brookhaven Symposia in Biology 9: 37-52.

Stift M, Berenos C, KuperuS P, van TiendereN H (2008): Segregation models for disomic, tetrasomic and intermediate inheritance in tetraploids: a general procedure applied at Rorippa (Yellow Cress) microsatellite data. Genetics 179: 2113-2123.

Strimbeck GR, Schaberg PG, DeHayes DH, Shane JB, Hawley GJ (1995) Midwinter dehardening of montane red spruce during a natural thaw. Can J For Res 25: 2040-2044.

Svejda F (1974) Reproductive capacity of F1, hybrids from Rosa rugosa and Chinensis cultivars, Euphytica 23: 665-669.

Svejda F (1979) Inheritance of winterhardiness in roses, Euphytica 28: 309- 314.

Sybenga J (1994) Preferential pairing estomates from multivalent frequencies in tetraploids. Genome 37(6): 1045-1055.

Sybenga J (1996) Chromosome pairing affinity and quadrivalent formation in polyploids: do segmental allopolyploids exist? Genome 39: 1176-1184.

Szalay L, Németh Sz, Tomin B, Végvári Gy (2012) Frost hardiness of peach and apricot flower buds. Acta Hortic 962: 291-296.

Szymajda M, Pruski K, Żurawicz E, Sitarek M (2013) Freezing injuries to flower buds and their influence on yield of apricot (Prunus armeniaca L.) and peach (Prunus persica L.). Can J Plant Sci. 93: 191-198.

Talanova VV, Titov AF, Topchieva LV, Repkina NC (2011) Expression patterns of ABA-dependent and ABA-independent genes during wheat cold adaptation. Russ J Plant Physiol 58(6): 859-865.

Talanova VV, Titov AF, Topchieva LV, Frolova SA (2012) Effects of abscisic acid treatment on the expression of cysteine proteinase gene and enzyme inhibitor during wheat cold adaptation. Russian Journal of plant physiology 59(4): 581-585..

Tang J, Vosman B, Voorrips RE, van der Linden GC, Leunissen JAM (2006) QualitySNP: a pipeline for detecting single nucleotide polymorphisms and insertions/deletions in EST data from diploid and polyploid species. BMC Bioinformatics, 7, 438. doi: 10.1186/1471-2105-7-438.

Tang J, Baldwin SJ, Jacobs JM, van der Linden GC, Voorrips RE, Leunissen JAM, van Eck H, Vosman B (2008) Large-scale identification of polymorphic microsatellites using an in silico approach. BMC Bioinformatics, 9, 374. doi: 10.1186/1471-2105-9-374.

Tanksley SD, Young ND, Peterson AH, Bonierbale MW (1989) RFLP mapping in plant breeding: New tools for an old science. Biotechnology 7: 257-263.

Tantau H, Balko C, Brettschneider B, Melz G, Dörffling K (2004) Improved frost tolerance and winter survival in winter barley (Hordeum vulgare L.) by in vitro selection of proline overaccumulating lines. Euphytica 139: 19–32.

Tarumoto I (2006) Genetic studies on glossy leaves in sorghum (*Sorghum bicolor* L. Moench). JARQ 40(1): 13-20.

Taulavuori K, Bauer E, Taulavuori E (2001) Overwintering stress of Vaccinium vitis-idaea in the absence of snow cover. Environmental and experimental botany 72: 397-403.

Taylor AG, Harman GR (1990) Concepts and technologies of selected seed treatments. Ann Rev Phytopathol 28: 321-339.

Thomas GS (2004) The Graham Stuart Thomas Rose Book. Frances Lincoln Limited, London.

Trischuka RG, Schillinga BS, Lowb NH, Graya GR, Gustaa LV (2014) Cold acclimation, de-acclimation and re-acclimation of spring canola, winter canola and winter wheat: The role of carbohydrates, cold-induced stress proteins and vernalization. Environ Exp Bot 106: 156–163.

Thomashow MF (1999) Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Phys 50: 571-599.

Tong Z, Yang Z, Chen X, Jiao F, Li X, Wu X, Gao Y, Xiao B, Wu W (2012) Large-scale development of microsatellite markers in Nicotiana tabacum and construction of a genetic map of flue-cured tobacco. Plant Breeding, 131, 674-680. doi:10.1111/j.1439-0523.2012.01984.x

Tondelli A, Francia E, Barabaschi D, Pasquariello M, Pecchioni N (2011) Inside the CBF locus in Poaceae. Plant Science 180: 39-45.

Tondelli A, Pagani D, Ghafoori JN, Rahimi M, Ataei R, Rizza F, Flavell AJ, Cattivelli L (2014) Allelic variation at Fr-H1/Vrn-H1 and Fr-H2 loci is the main determinant of frost tolerance in spring barley. Env Exp Bot. http://dx.doi.org/10.1016/j.envexpbot.2014.02.014

Trick M, Long Y, Meng J, Bancroft I (2009) Single nucleotide polymorphism (SNP) discovery in the polyploidy *Brassica napus* using Solexa transcriptome sequencing. Plant Biotechnology Journal 7: 334-346.

Triwitayakorn K, Chatkulkawin P, Kanjanawattanawong S et al. (2011) Transcriptome sequencing of Hevea brasiliensis for development of microsatellite markers and construction of a genetic linkage map. DNA Research, 18, 471-482.

Turhan E, Aydogan C, Baykul A, Akoglu A, Evrenosoglu Y, Ergin S (2012) Apoplastic antioxidant enzymes in the leaves of two strawberry cultivars and their relationship to cold-hardiness. Not Bot Horti Agrobo 40(2): 114-122.

Ueda Y, Ando TT (1996) Pollination in *Rosa rugosa* Thunb. ex Murray. Acta Hort. 424: 309-310.

UPOV (2010) Rose guidelines for the conduct of tests for distinctness, uniformity and stability. International union for the protection of new varieties of plants, Geneva.

Vainstein A, Ben-Meir H, Zucker A (1993) DNA fingerprinting as a reliable tool for the identificaton and genetic analysis of ornamentals. Proceedings of the XVIIth Eucarpia Symposium "Creating genetic variation in ornamentals", San Remo, 63-68.

van Dijk T, Noordijk Y, Dubos T, Bink MCAM, Visser RGF, van de Weg WE (2012) Microsatellite allele dose and configuration establishment (MADCE): an integrated approach for genetic studies in allopolyploids. BMC Plant Biol 12: 25.

van Huylenbroeck J (2012) Species crosses in roses. Roses, what's in it for us?: 15-16 September 2013, Symposium on Floriade.

Van Ooijen JW (2006) JoinMap® 4, Software for the calculation of genetic linkage maps in experimental populations. Kyazma B.V., Wageningen, Netherlands.

van Ooijen JW (2009) MapQTL, software for the mapping of quantitative trait loci in experimental populations of diploid species. In: BV K (ed), 6edn, Wageningen, The Netherlands.

Verde I, Bassil N, Scalabrin S, Gilmore B, Lawley CT, Gasic K, Micheletti D, Rosyara UR, Cattonaro F, Vendramin E, Main D, Aramini V, Blas AL, Mockler TC, Bryant DW, Wilhelm L, Troggio M, Sosinski B, Aranzana MJ, Arús P, Iezzoni A, Morgante M, Peace C (2012) Development and evaluation of 9K SNP array for peach by internationally coordinated SNP detection and validation in breeding germplasm. Plos ONE 7: e35668.

Victoria FC, da Maia LC, de Oliveira AC (2011) In silico comparative analysis of microsatellite markers in plants. BMC Plant Biology, 11, 15.

Vilanova S, Sergent D, Arus P, Monfort A (2008) Synteny conservation between two distantly-related *Rosaceae* genomes: *Prunus* (the stone fruits) and *Fragaria* (the strawberry). BMC Plant Biol 6: 67.

Visioni A, Tondelli A, Francia E, Pswarayi A, Malosetti M, Russell J, Thomas W, Waugh R, Pecchioni N, Romagosa I, Comadran J (2013) Genome-wide association mapping of frost tolerance in barley (*Hordeum vulgare* L.). BMC Genomics 14: 424.

Vogel JT, Zarka DG, Van Buskirk HA, Fowler SG, Thomashow MF (2005) Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of Arabidopsis. Plant J 41: 195-211.

von Korff M, Wang H, Léon J, Pillen K (2005) AB-QTL analysis in spring barley. I. Detection of resistance genes against powdery mildew, leaf rust and scald introgressed from wild barley. Theor Appl Genet 111: 583-590.

von Korff M, Wang H, Léon J, Pillen K (2006) AB-QTL analysis in spring barley. II. Detection of favourable exotic alleles for agronomic traits introgressed from wild barley (*H. vulgare* ssp. *spontaneum*). Theor Appl Genet 112: 1221-1231.

Voorrips RE (2002). MapChart: Software for the graphical presentation of linkage maps and QTLs. J Hered 93 (1): 77-78

Voorrips RE, Gort G, Vosman B (2011) Genotype calling in tetraploid species from bi-allelic marker data using mixture models. BMC Bioinformatics 12: 172-182.

Voorrips RE, Maliepaard CS (2012) The simulation of meiosis in diploid and tetraploid organism using various genetic models. BMC Bioinformatics 13: 248.

Voorrips RE, Bink MC, van de Weg WE (2012) Pedimap: software for the visualization of genetic and phenotypic data in pedigrees. J Hered 103(6): 903-907.

Vukosavljev M, M. Di Guardo, W.E. van de Weg, P. Arens, M.J.M. Smulders (2012) Quantification of Allele Dosage in Tetraploid Roses. Book of abstracts of International conference on Molecular mapping & marker assisted selection, pp.27. ScienceMED (Bologna) 3(3): 277-282. http://www.medimond.com/sciencemed/v3_3_2012.pdf

Vukosavljev M, Zhang J, Esselink GD, van 't Westende WPC, Cox P, Visser RGF, Arens P, Smulders MJM (2013) Genetic diversity and differentiation in roses: A garden rose perspective. Scientia Horticulturae, 162, 320-332. http://dx.doi.org/10.1016/j.scienta.2013.08.015

VSN International (2013). GenStat for Windows 16th Edition. VSN International, Hemel Hempstead, UK. Web page: GenStat.co.uk

Vukosavljev M, GD Esselink, WPC van 't Westende, P Cox, RGF Visser, P Arens, MJM Smulders (2014) Efficient development of highly polymorphic microsatellite markers based on polymorphic repeats in transcriptome sequences of multiple individuals. Molecular Ecology Resources. http://dx.doi.org/10.1111/1755-0998.12289

Wang B, Ekblom R, Castoe TA et al. (2012) Transcriptome sequencing of black grouse (Tetrao tetrix) for immune gene discovery and microsatellite development. Open Biology, 2, 120054.

Wang JY, Song XM, Li Y, Hou XL (2013) In-silico detection of EST-microsatellite markers in three Brassica species and transferability in B. rapa. Journal of Horticultural Science & Biotechnology, 88(2), 135-140.

Welling A, Rinne P, Viherä-Aarnio A, Kontunen-Soppela S, Heino P, Palva ET (2004) Photoperiod and temperature differentially regulate the expression of two dehydrin genes during overwintering of birch (*Betula pubescens* Ehrh.). J Exp Bot 55: 507-516.

Welling A, Palva ET (2006) Molecular control of cold acclimation in trees. Physiol Plant 127: 167-181.

Wiessemann V, Hellwig FH (1997) Reproduction and hybridization in the genus *Rosa*, section *Caninae* (Ser.). Rhed Bot Acta 110: 251-256.

Wissemann V (2003) Conventional taxonomy (wild roses). In: Roberts AV, Debener T, Gudin S (eds) Encyclopedia of rose science. Elsevier, Academic, Oxford, UK, pp 111–117.

Widrlechner MP, Daly C, Keller M, Kaplan K (2012) Horticultural applications of a newly revised USDA plant hardiness Zone map. Hort Technology 22(1): 6-19.

Whitaker VM, Bradeen JM, Debener T, Biber A, Hokanson SC (2010) Rdr3, a novel locus conferring black spot disease resistance in tetraploid rose: genetic analysis, LRR profiling, and SCAR marker development. Theor Appl Genet 120(3): 573-585. Xie C, Schizhong X (1999): Mapping quantitative trait loci in tetraploid populations. Genet Res., 76: 105-115.

Woldendorp G, Hill MJ, Doran R, Ball MC (2008) Frost in a future climate: modelling interactive effects of warmer temperatures and rising atmospheric [CO2] on the incidence and severity of frost damage in a temperature evergreen (Eucalyptus pauciflora). Global Change Biol 14: 294-308.

Wu KK, Burnquist W, Sorrells ME, Tew TL, Moore PH, Tanksley SD (1992) The detection and estimation of linkage in polyploids using single-dose restriction fragments. Theor Appl Genet 83: 294-300.

Xiao J, Wu K, Fang DD, Stelly DM, Yu J, Cantrell RG (2009) New microsatellite markers for use in cotton (Gossypium spp.) improvement. Journal of Cotton Science, 13(2), 75-157.

Xing C, Schumacher FR, Xing G, Lu Q, Wang T, Elston RC (2005) Comparison of microsatellites, single-nucleotide polymorphisms (SNPs) and composite markers derived from SNPs in linkage analysis. BMC Genetics, 6(Suppl 1), S29.

Xiong Y, Fei SZ, Arora R, Brummer EC, Barker RE, Jung G, Warnke SE (2007) Identification of quamtitative trait loci controlling winter hardiness in n annualxperennial ryegrass interspecific hybrid population. Mol Breed 16: 125-136.

Xu Y, Crouch JH (2008) Marker-assisted selection in plant breeding: from publication to practice. Crop Sci 48: 391-407.

Xue W, Xing Y, Weng X, Zhao Y, Tang W, Wang L, Zhou H, Yu S, Xu C, Li X, Zhang Q (2008) Natural variation in Ghd7 is an important regulator of heading date and yield potential in rice. Nat. Genet. 40:761–767.

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Plant Biol 57: 781-803.

Yan Z, Denneboom C, Hattendorf A, Dolstra O, Debener T, Stam P, Visser PB (2005) Construction of an integrated map of rose with AFLP, SSR, PK, RGA, RFLP, SCAR and morphological markers. Theor Appl Genet 110: 766-777.

Yan SP, Zhang QY, Tang ZC, Su WA, Sun WN (2006) Comparative proteomic analysis provides new insight into chilling stress response in rice. Mol Cell Proteom 5: 484-496.

Yan Z, Visser PB, Hendriks T, Prins TW, Stam P, Dolstra O (2007) QTL analysis of variation for vigour in rose. Euphytica 154: 53-62.

Yang X, Lv Y, Pang X, Tong C, Wang Z, Li X, Feng S, Tobias CM, Wu R (2012) A unifying framework for bivalent linkage analysis of allotetraploids. Briefing in bioinformatics 14(1): 96-108.

Yokoya K, Roberts AV, Mottley J, Lewis R, Brandham PE (2000) Nuclear DNA amounts in roses. Ann Bot 85:557–561.

Young ND (1999) A catiously optimistc visio of marker-assisted breeding. Mol Breed 5:505-510.

Yuan S, Ge L, Liu C et al. (2013) The development of EST-microsatellite markers in Lilium regale and their cross-amplification in related species. Euphytica, 189, 393-419.

Zitouna N, Marghali S, Gharbi M, Chennaoui-Kourda H, Haddioui A, Trifi-Farah N (2013) Mediterranean Hedysarum phylogeny by transferable microsatellites from Medicago. Biochemical Systematics and Ecology 50: 129-135.

Zhang LH, Byrne DH, Ballard RE, Rajapakse (2006) Microsatellite marker development in rose and its application in tetraploid mapping. J Am Soc Hort Sci 131(3): 380-387.

Zhang Y, Dami IE (2012) Foliar application of abscisic acid increases freezing tolerance of field-grown Vitis vinifera Cabernet franc grapevines. Am J Enol Vitic 63(3): 377-384.

Zhang J, Esselink GD, Che D, Fougère-Danezan M, Arens P, Smulders MJM (2013) The diploid origins of allopolyploid rose species studied using single nucleotide polymorphism haplotypes flanking a microsatellite repeat. J. Hortic. Sci. Biotechnol. 88, 85-92.

Zhao Y, Williams R, Prakash CS, He G (2013) Identification and characterization of gene-based microsatellite markers in date palm (Phoenix dactylifera L.). BMC Plant Biology, 12, 237.

Zhou Z, Wang MJ, Zhao ST, Hu JJ, Lu MZ (2009) Changes in freezing tolerance in hybrid poplar caused by up- and dawn-regulation of PtFAD2 gene expression. Transgenic Res 19: 647-654.

Zhou ZQ, Bao WK, Wu N (2009): Dormancy and germination in *Rosa multibracteata*. Sci Hort 119: 434-441.

Zlesak D (2007) Rose: *Rosa x hybrida*. In: Anderson N.O. (Ed) Flower breeding and Genetics. Springer, Heidelberg, pp 695-740.

Zuzek K, Richards M, McNamara S, Pellett H (1997) Cold hardiness. In: Roses for the North. Regents of the University of Minnesota.

Summary

Over the last few decades the rose market in Eastern Europe showed a steady growth, which indicates that there is increasing demand for new cultivars that are adapted to the climate as well as to the customs and beauty criterion of that region. One of the possibilities to speed up breeding is to implement marker assisted selection (MAS). Implementation of MAS requires a specific infrastructure (molecular markers, knowledge on genetics of important traits, genetic maps) which is not yet available for tetraploid roses. In this thesis I developed some of the prerequisites for MAS in roses and discuss when and how MAS could have a positive effect on accelerating breeding and/or reducing the costs of the breeding process.

The first step in understanding the structure of the genepool of garden roses was to evaluate the relatedness among available cultivars. For the first time genetic diversity among modern garden rose cultivars was evaluated (**Chapter 2)** using a set of 24 microsatellite markers covering most chromosomes. A total of 518 different alleles were obtained in a set of 138 rose cultivars. Genetic differentiation among types of garden roses (Fst=0.022) was four times that found among cut roses, and similar in magnitude to the differentiation among breeders, due to the fact that horticultural groups and breeders overlap largely in classification. In terms of genetic diversity cut roses can be considered as a subgroup of the garden roses. Winter hardy Canadian garden rose cultivars (Explorer roses) showed the least similarities to European roses, and introgression from wild species for winter hardiness was clearly visible. Roses of two breeding programmes (Harkness and Olesen) shared a similar genepool. Comparison of the differentiation among linkage groups indicated that linkage group 5 is potentially a region containing important QTLs for winter hardiness. Linkage group 6 contains the largest amount of genetic diversity, while linkage group 2 is the most differentiated among types of garden roses.

Garden roses, as well as many other important crops (wheat, potato, strawberry, etc.) are polyploid. Genetic analyses of polyploids is complex as the same locus is present on multiple homologous chromosomes. SSR markers are suitable for mapping in segregating populations of polyploids as they are multi-allelic, making it possible to detect different alleles of the same locus on all homologous chromosomes. If a SSR marker gives fewer alleles than the ploidy level, quantification of allele dosages increases the information content. In **Chapter 3** I showed the power of this approach. Alleles were scored quantitatively using the area under the peaks in ABI electropherograms, and allele dosages were inferred based on the ratios between the peak areas for two alleles in reference cases in which these two alleles occurred together. We resolved the full progeny genotypes, generated more data and mapped markers more accurately, including markers with “null” alleles.

Even though SSR markers are one of the most appropriate marker systems for genetic studies in polyploids still few hurdles complicate (reduce) their implementation. The first major hurdle in developing microsatellite markers, the cloning step, has been overcome by

next generation sequencing techniques. The second hurdle is the testing step to differentiate polymorphic from non-polymorphic loci. The third hurdle, somewhat hidden, is that only those polymorphic markers that detect a large effective number of alleles in the germplasm to be studied, are sufficiently informative to be deployed in multiple studies. Both selection steps are laborious and still done manually. In **Chapter 4** I present a strategy in which we first screen sequence reads from multiple genotypes for repeats that show the most variation in length, and only these are subsequently developed into markers. We validated our strategy in tetraploid garden rose using Illumina paired-end transcriptome sequences of 11 roses. Out of 48 tested two markers did not amplify but all others were polymorphic. Ten loci amplified more than one locus, indicating duplicated genes or gene families. Completely avoiding this will be difficult, as the range of numbers of predicted alleles of highly polymorphic single- and multi-locus markers largely overlapped. Of the remainder, half were duplicates, indicating the difficulty of correctly filtering short sequence reads containing repeat sequences. The remaining 18 markers were all highly polymorphic, amplifying between 6 and 20 alleles in the 11 tetraploid garden roses. This strategy therefore represents a major step forward in the development of highly polymorphic microsatellite markers.

Despite that garden roses are economically very important ornamentals, breeding is still mostly conventional, mainly due to tetraploidy and the lack of genetic maps and knowledge about the genetic base of important traits. Furthermore, crosses with unintended parents occur regularly and detection of these is not always straightforward, especially when genetically related varieties are used. Moreover, in polyploids detection of off-type offspring often relies on detecting differences in allele dosage rather than the presence of new alleles. In **Chapter 5** I applied the WagRhSNP Axiom rose SNP array to generate 10,000s of SNPs for parentage analysis and to generate a dense genetic map in tetraploid rose. I described a method to separate progeny into putative populations which share parents, even if one of the parents is unknown, using PCO analysis and sets of markers for which allele dosages are incompatible. Subsequently, dense SNP maps were generated for a biparental and a self-pollinated mapping population with one parent in common. I confirmed a tetrasomic mode of inheritance for these crosses and created a starting point for implementation of marker-assisted breeding in garden roses by QTL analysis for important morphological traits (recurrent blooming and prickle shape).

Winter hardiness is a complex trait and one of the most important limiting factors for garden rose growth and distribution in areas characterized by a continental climate. In **Chapter 6** research was undertaken to determine the genetic regions underlying winter hardiness of garden roses, and to generate markers linked to them. For this purpose we exposed two segregating populations, RNDxRND and RNDxHP, to temperatures below -15C in a cold chamber and in the field in Serbia. The frost damage in the hardened plants was estimated directly at the phenotypic level (proportion of dieback) and at the non-visible physiological level indirectly (through the potential for meristem production in spring; regrowth). For winter hardiness we detected two tentative QTLs in the RNDxRND population and two tentative QTLs in the RNDxHP population, of which one was the same in both populations. The ability of plants to regrow in spring was associated to genomic regions

on three linkage groups of the RNDxRND population, and on two different linkage groups in the RNDxHP population. A comparison of the ability for regrowth and level of damage caused by low temperature revealed that these two traits are inherited independently and that the final cold tolerance depends on the plant's ability to withstand low temperature and to regrow fast in spring.

In résumé, this thesis resulted in the development of basic tools (a fast strategy for polymorphic SSR marker development), basic methods/concepts for genetic analyses in polyploids (quantification of SSR allele dosage, distinguishing outliers from population in polyploid crops, dense SNP map generation and QTL study in tetraploids), and knowledge on genetics of important traits in rose (relatedness among modern garden roses (genetic diversity approach), mode of inheritance, occurrence of selfing, QTLs for morphological traits (recurrent blooming and prickle shape) and dissection of winter hardiness (level of damage caused by low temperature and regrowth)). Additionally, potential use of markers in every phase of rose breeding was discussed (**Chapter 7**). All these aspects contribute to a solid basis for marker assisted breeding in (garden) rose.

Samenvatting

In de afgelopen decennia heeft de markt voor tuinrozen in Oost-Europa een gestage groei laten zien, wat aangeeft dat er een toenemende vraag is naar nieuwe cultivars die aangepast zijn aan het klimaat, de manier van cultiveren, en de esthetische voorkeuren van die regio. Een van de mogelijkheden om de snelheid in het veredelingsproces te vergroten is om 'marker assisted selection' (MAS) te implementeren. Implementatie van MAS vereist een specifieke infrastructuur (moleculaire merkers, kennis over de genetica van belangrijke eigenschappen, genetische kaarten) die nog niet beschikbaar is voor tetraploïde rozen. In dit proefschrift ontwikkelde ik een aantal van de benodigdheden voor het toepassen van MAS in rozen en bediscussieer wanneer en hoe MAS de veredeling kan versnellen en/of de kosten ervan kan verlagen.

De eerste stap in het begrijpen van de structuur van de genenpool van tuinrozen was om de verwantschap tussen beschikbare cultivars evalueren. Voor het eerst is de genetische diversiteit onder moderne tuinroos cultivars geëvalueerd (**Hoofdstuk 2**) daarbij gebruik makend van een set van 24 microsatelliet markers die dekkend is voor bijna alle koppelingsgroepen. Een totaal van 518 verschillende allelen werd verkregen in een set van 138 cultivars. Genetische differentiatie tussen verschillende type tuinrozen (Fst = 0.022) was vier keer zo groot als gevonden onder snijrozen en bergelijkbaar met de differentiatie tussen veredelaars, vanwege het feit dat de verschillende tuinroos types grotendeels overlappen met veredelaars. In termen van genetische diversiteit kunnen snijrozen worden beschouwd als een subgroep van de tuinrozen. Winterharde Canadese tuinroos cultivars ('Explorer' rozen) toonde de minste overeenkomsten met Europese rozen, en de introgressie vanuit wilde soorten ten behoeve van winterhardheid was duidelijk zichtbaar. Rozen van twee veredelingsprogramma's (Harkness en Olesen) deelden een overeenkomstige genenpool. Vergelijking van de differentiatie tussen koppelingsgroepen gaf aan dat koppelingsgroep 5 in potentie een gebied met een belangrijke QTL voor winterhardheid bevat. Koppelingsgroep 6 bevat de grootste genetische diversiteit, terwijl koppelingsgroep 2 het meeste onderscheid tussen de tuinroos groepen laat zien.

Tuinrozen, evenals vele andere belangrijke gewassen (tarwe, aardappel, aardbei, etc.) zijn polyploïd, wat wil zeggen dat ze meer dan één set van koppelingsgroepen hebben. Genetische analyse van polyploïden is complex omdat hetzelfde locus aanwezig is op meerdere homologe koppelingsgroepen. SSR merkers zijn geschikt voor kartering in segregerende populaties van polyploïden omdat ze multi-allelisch zijn, waardoor het mogelijk is om verschillende allelen van hetzelfde locus tegelijk te detecteren op deze koppelingsgroepen. Als een SSR merker minder allelen heeft dan het ploïdie-niveau, kun je dat zien aan de allel dosering, en daarom verhoogt kwantificering van de allel dosering het informatiegehalte. In **Hoofdstuk 3** liet ik de kracht van deze aanpak zien. Allelen werden kwantitatief gescoord op basis van het oppervlakte onder de piek in ABI elektroferogrammen en allel doseringen werden afgeleid op basis van de verhouding tussen de piekoppervlakten van de twee allelen in referentie gevallen waarin beide allelen samen voorkomen. Hiermee is

het genotype van de nakomelingen volledig terug te voeren op de ouders, worden meer gegevens gegenereerd en kunnen markers met grotere precisie worden gekarteerd, met inbegrip van merkers met "null" allelen (allelen die zelf onzichtbaar zijn maar wel mee overerven).

Ook al zijn SSR merkers een van de meest geschikte marker systemen voor genetische studies in polyploïden, er zijn nog hobbels die het gebruik compliceren. De eerste belangrijke hindernis in de ontwikkeling van microsatelliet markers, het kloneren van de DNA sequenties, is opgelost door next generation sequencing technieken. De tweede belemmering is het testen op polymorfisme. De derde, enigszins verborgen, hindernis is dat alleen die merkers die een groot effectief aantal allelen in de te onderzoeken genenpool detecteren, voldoende informatief zijn om in meerdere studies te worden ingezet. Beide laatste selectie stappen zijn bewerkelijk en worden nog steeds handmatig gedaan. In **Hoofdstuk 4** presenteer ik een strategie waarbij we eerst sequenties van meerdere genotypen screenen op de aanwezigheid van SSRs die veel variatie in lengte vertonen, en alleen deze worden vervolgens ontwikkeld tot markers. We hebben onze strategie gevalideerd in tetraploïde tuinrozen gebruik makend van Illumina ‘paired-end’ transcriptoom sequenties van 11 cultivars. Van de 48 geteste markers konden twee markers niet worden geamplificeerd, maar alle anderen waren polymorf. Tien loci amplificeerden meer dan één locus, wat aangeeft dat het gedupliceerde genen of gen families betrof. Volledig vermijden hiervan zal moeilijk zijn, aangezien het bereik van het aantal voorspelde allelen van zeer polymorfe merkers van één locus overlapt met dat van merkers die op meer loci berusten. Van de overige waren de helft duplicaten van elkaar, wat aantoont dat het lastig is om de juiste filtering van de korte sequenties vol met stukjes repeterend DNA uit te voeren. De resterende 18 markers waren allemaal zeer polymorf, met tussen de 6 en 20 allelen in de 11 tetraploïde tuinrozen cultivars. Deze strategie is dan ook een belangrijke stap voorwaarts in de ontwikkeling van zeer polymorfe microsatelliet markers.

Ondanks dat tuinrozen economisch zeer belangrijk sierplanten zijn, wordt de veredeling nog steeds grotendeels op conventionele wijze uitgevoerd, voornamelijk als gevolg van tetraploïdie, het ontbreken van genetische kaarten en van kennis over de genetische basis van belangrijke eigenschappen. Bovendien komen kruisingen met onbedoelde ouders regelmatig voor, en het opsporen van deze is niet altijd eenvoudig, vooral als genetisch verwante variëteiten worden gebruikt. Daarbij berust in polyploïden de detectie van off-type nakomelingen vaker op het detecteren van verschillen in allel dosering dan op de aanwezigheid van nieuwe allelen. In **Hoofdstuk 5** heb ik de WagRhSNP Axiom roos SNP array gebruikt om tienduizenden SNP's te genereren voor ouderschapsanalyse en om een dichte genetische kaart te genereren in tetraploïde roos. Ik beschreef een werkwijze voor het onderscheiden van afzonderlijke nakomelingen in populaties die vermoedelijk dezelfde ouders delen, zelfs indien één van de ouders onbekend is, met gebruikmaking van PCO (principale component) analyse en sets van merkers waarvoor allel doseringen onverenigbaar zijn. Vervolgens werden dichte SNP marker kaarten gegenereerd voor een biparental en een zelf-bestoven kruisingspopulatie die één ouder gemeenschappelijk hebben. Ik kon tetrasome overerving in deze kruisingen bevestigen. Hiermee is een start gecreëerd voor de

implementatie van merker-gestuurde veredeling in tuinrozen. Als voorbeeld is een QTL analyse gedaan voor enkele belangrijke morfologische kenmerken (herhaald bloeien en doorn vorm).

Winterhardheid is een complexe eigenschap en een van de beperkende factoren voor tuinrozen in gebieden die worden gekenmerkt door een continentaal klimaat. In **Hoofdstuk 6** werd onderzoek gedaan naar de genetische factoren die ten grondslag liggen aan winterhardheid van tuinrozen, met de bedoeling om gekoppelde merkers te genereren. Hiervoor hebben we twee segregerende populaties, RNDxRND en RNDxHP, blootgesteld aan temperaturen onder -15C in een vriescel en in het veld in Servië. De vorstschade in de planten werd direct geschat op fenotypisch niveau (welk deel is afgestorven?) en indirect op het niet-zichtbare fysiologisch niveau (hoe is de hergroei in het voorjaar?). Voor winterhardheid hebben we twee mogelijke QTLs in de RNDxRND populatie gedetecteerd en twee mogelijke QTLs in de RNDxHP populatie, waarvan één dezelfde was in beide populaties. Het vermogen van planten voor hergroei in het voorjaar werd in verband gebracht met drie genomische regio's op verschillende koppelingsgroepen van de RNDxRND populatie, en op twee verschillende koppelingsgroepen in de RNDxHP populatie. Bij vergelijking van het vermogen tot hergroei en de omvang van de schade veroorzaakt door lage temperaturen bleken deze twee eigenschappen onafhankelijk over te erven, zodat de uiteindelijke koude-tolerantie afhankelijk is van het vermogen van de plant om lage temperaturen weerstaan en het vermogen om snel terug te groeien in het voorjaar.

Samenvattend heeft dit proefschrift geleid tot de ontwikkeling van fundamentele instrumenten (een snelle strategie voor polymorfe SSR marker ontwikkeling), basale methoden voor genetische analyses in polyploïden (kwantificering van SSR-allel dosering, herkenning van uitschieters in een populatie van een polyploïd gewas, een dichte SNP kaart), en kennis over de genetica van belangrijke eigenschappen in de roos (verwantschap tussen moderne tuinrozen, wijze van overerving, vóórkomen van zelfbestuiving, QTLs voor morfologische kenmerken (terugkerende bloei en de vorm van dorens) en winterhardheid (niveau van schade veroorzaakt door lage temperaturen en hergroei)). Daarnaast is het potentiële gebruik van markers in elke fase van de rozenveredeling bediscussieerd (**Hoofdstuk 7**). Al deze aspecten dragen bij aan een solide basis voor merker-gestuurde veredeling in (tuin) roos.

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About the author

Mirjana Vukosavljev was born on March 1st, 1982 in Pančevo, Serbia. From 2000-2005 she studied Biology at Faculty for Mathematics and Natural Science in Novi Sad, Serbia. In 2005 Mirjana defended her BSc thesis at the field of Human Genetics and enrolled for Master at Agricultural Faculty in Novi Sad, filed Genetics and Plant Breeding. As a master student she received a scholarship from the Ministry of Science and Technological Development and was hired as a teaching assistant at the field of Genetics. For the practical experiment for master thesis Mirjana joined Institute for Field and Vegetable Crops in Novi Sad and conducted research on tissue culture in wheat. In 2009 she started PhD in the Plant Breeding Department, Biodiversity group at Wageningen University. She was focused on winter hardiness (Hyperrose Project) and development of new methods for statistical analysis in roses (Polyploid Project). This thesis presents the outcome of her PhD research work. Since July 2014 Mirjana has been working as Manager of Research and Development department of Pheno Geno Rose DOO, Novi Sad.

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)
- Winter hardiness (2013)

Writing of project proposal (4.5 ECTS)
- Hyperrose (2009)

Post-graduate courses (4.3 ECTS)
- Statistical learning methods for DNA-based prediction of complex traits; PE&RC, WUR (2011)
- Mix model based QTL mapping in GenStat; Biometris, WUR (2012)
- SPICY Symposium/workshops; Biometris, WUR (2012)

Laboratory training and working visits (1.5 ECTS)
- Visiting rose breeders in Serbia; Pheno-Geno Roses, Serbia (2009)

Deficiency, refresh, brush-up courses (1.5 ECTS)
- Basic statistics (2011)

Competence strengthening / skills courses (3.3 ECTS)
- Competence assessment; WGS, WUR (2010)
- Scientific writing; WGS, WUR (2012)
- Techniques for writing and presenting a scientific paper; WGS, WUR (2013)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.8 ECTS)
- PE&RC Weekend (2010)
- PE&RC Symposium: traits a s a link between systematics and ecology (2012)
- PE&RC Days (2012, 2013)

Discussion groups / local seminars / other scientific meetings (6 ECTS)
- Plant science seminars (2009-2014)
- Literature discussion (2009-2014)

International symposia, workshops and conferences (8.1 ECTS)
- 23th EUCARPIA Symposium: colourful breeding and genetics (2009)
- TTI Symposium (2011)
- International conference: molecular mapping and marker assisted selection; Vienna (2012)
- Floriade (2012)
- Symposium: roses, what's in it for us; Floriade, Venlo (2012)
- Fascination day, 110 years of plant breeding (2012)
- Conference: next generation plant breeding; Wageningen (2013)

Supervision of a MSc student
- Quantitative scoring of SSRs in tetraploid roses

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