Chromosome Segregation Analysis for Disomic and Tetrasomic Inheritance In K5 Tetraploid Rose Population

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Chapter 1 General introduction

1.1 Rose

Roses have been cultivated as ornamental plant, admired for their petals and fragrance, and used as medical plants or even edible hips for 5000 years by the ancient civilization of China, in west Asia and northern Africa (Shepherd 1954; Gudin 2000). During thousands of years selection took place, after 1867, first Hybrid Tea rose, more than 33000 modern cultivars have been described. Rose is in the top five of ornamental plants, partly because of the vast diversity in plant habit and flower characteristics (De Vries, 2003; Cairns, 2001; Esselink, et al, 2003; Leus et al 2004; Young and Schor, 2007).

1.1.1 Taxonomy and classification

The Rosa genus belongs to the family Rosaceae and it is closely related to important economic fruit crops like apple, pear, cherry, strawberry, etc (Debener, 2009). Rose is a perennial flowering shrub, and climbing or trailing plants, with stems prickles. It has various colors, commonly white, yellow, orange, pink or red color flowers with four to five petals, open pollination, and mostly scented (Crane and Byrne 2003; Debener, 2009).

Taxonomy in this genus has been quite complicated. According to Rehder's classification system, the genus *Rosa* is divided into four subgenera: *Hulthemia* (1 species), *Platyrhodon* (1 species), *Hesperhodos* (2 species) and *Rosa* (Rehder, 1940; Koopman et al, 2008). Roses belong to the genus *Rosa (L)*, which compromise around 180 woody perennials species, and these species are classified into 10 sections: *Banksiae*, *Bracteatae*, *Chinensis* (Indicae), *Laevigatae* (2n=2x), *Synstylae* (2n=2x), *Carolinae* (2n=2x or 4x), *Pimpenellifoliae* (2n=2x or 4x), *Caninae* (2x=4x, 5x or 6x), *Cinnamomeae* (2n=2x, 4x, 6x or 8x), and *Rosa* (2n=4x) (Darlington and Wylie, 1955; Wssemann, 2003).

Actually section Rosa only contains one wild species *R. gallica* (Nybom, 2009), most of cultivated rose today are not true species, which is derived by multiple inter-specific hybridization. About ten species from genus *Rosa* contributed to the development of cultivated rose, which are *R. chinensis*, *R. foetida*, *R. gallica*, *R. gigantean*, *R. moschata*, *R. multiflora*, *R. phoenicea, R. rugosa, R. wichurana* and *R. rubra* (Crespel and Mouchotte 2003).

Due to the continuous variation of characteristics, especially extensive inter-specific and intra-specific hybridization of modern roses, classification based on morphological characters is difficult. Even Linnaeus stated that 'the species of *Rosa* are very difficult to determine and those who have seen few species can distinguish them more easily than those who have examined many' (Shepherd, 1954).

In last two decades, various molecular and biotechnology techniques developed have been used for unraveling taxa differentiation of *Rosa*. It has been reported that by comparing the taxonomic relationships of 119 accessions from subgenera *Platyrhodon*, *Hesperhodos* and *Rosa* by conducting RAPD-based phenetic analysis, subgenera *Platyrhodon* and *Hesperhodos* should be grouped into subgenera *Rosa* (Jan et al., 1999).

In a more recent study Koopman et al. analyzed 93 accessions from 47 species, comprising 3 subgenera, 6 sect. *Rosa* and 6 subsections of sect. *Caninae* by using AFLP data. The Result supported that species of sect. *Carolinae* and sect. *Cinnamomeae* should be merged. Meanwhile, the results also suggested that subsection *Rubigineae* is a monophyletic group within sect. *Caninae* (Koopman, et al., 2008).

So far, few molecular markers studies have been conducted to investigate the intra-sectional differentiation of taxa in sect. *Caninae*, due to its peculiar *canina* meiosis described 90 years ago, which involves heterogamy and matroclinal offspring caused by an asymmetrical meiosis. These studies indicated that mainly European section *Caninae* (dogroses plays an essential role in the production of root stocks for ornamental rose breeding) are complex allopolyploids by analyzing nuclear ribosomal DNA data, and suggested that the pentaploid *Caninae* genome originated from different non-dogroses species (Penland 1923; Nybom, Esselink, et al., 2005; Ritz, Schmuths, et al., 2005).

The changes in Rosa phylogenetic relationships demonstrate the complexity of modern cultivars genetic background caused by various factors (Koopman, 2008). Therefore, distinguish morphological traits and identify the origin source of the cultivars, these molecular assisted breeding and selection are very important for rose breeders to breed new varieties, and which also help UPOV (International Union for the Protection of New Varieties) to protect breeder's rights.

1.1.2 Cultivation and major economic uses

Rosa originates from temperature regions of northern hemisphere, between 20 and 70 degrees latitude, including North America, Europe, Northwest Africa, Asia and the Middle East (Phillips and Rix 1988). Fossil records of Rosa can be traced back to 35 million years ago (Oligocene epoch) (Cox, 1999). Roses are frequently mentioned in records from Middle East around 3000 years ago. *R. moschata* and *R. foetida* were cultivated extensively for their reputed medicinal value and ornamental during Roman era. After 1200 AD, the rose has been regarded as both ornamental and medicinal plants in Europe (Joyeaus, 2003). Commercial modern roses used to be bred and grew mainly in Northern hemisphere, but now they are being produced around the world.

Because of their high economic and ornamental value, a large number of rose cultivars have been developed for different purposes, i.e. garden plant, cut rose, indoors pot plants, rose hips, and even food additives (vitamin C). In 2003, world

rose growing area under protected cultivation is 8500 ha (Blom and Tsujita, 2003). In 2007, the turnover of roses exportation from EU was estimated 723 million Euros, which accounted for 20% total ornamentals exportation (Heinrichs, 2008).

Depending on the different breeding strategies and the divergent with overlapping gene pools, there are mainly 3 horticultural groups, which are garden roses, cut roses, pot roses (Debener, et al., 2010).

1.1.3 Garden rose

Roses are woody shrub plants, which are mostly propagated asexually by bud grafting or semi-hardwood cutting (Hartmann, 2002). It takes 1 to 3 years to produce salable plants. Presently, garden rose are produced economically in subtropical and all temperate region (Nybom, 2009).

Garden roses derived from China and Tea rose, Floribunda groups, and some Miniature and Shrub types, which have European, Mediterranean, and East Asian origin, commonly known as Hybrid Tea rose. The dwarf stature of garden roses is determined by a dominant allele at one major locus (Dubois and de Vries, 1987). The major type of modern garden roses belongs to *Rosa hybrida L.*, which is mainly used for commercial cut rose production (Kimura, et al., 2006). Old garden rose (ORG, 'The rose obtained before the breeding of La France in 1867, the first Hybrid Tea rose, are generally called old garden roses') is commonly more hardy and resistant than modern roses, and are used extensively for Garden and Landscape plants. Although it has been reported that some black spot (*Diplocarpon rosae* Wolf) resistance has been found in wild species and in some Old Garden Roses (Valentina, et al., 2006, Carlson-Nilsson, 2002), none of modern roses cultivars are fully resistant to this serious fungi disease (Marchant et al., 1998). Presently, black spot is often regarded as the most destructive disease in garden roses (Carlson-Nilsson, 2006).

1.1.4 Pot rose

Pot roses have been grown for hundreds of years, but large-scale commercially production started in the 1970s. Pot roses are commonly produced from softwood cut under protection environment. In 2003, world pot rose production reach to 60-80 million pots (Pemberton et al. 2003). Pot roses used to be mainly grown outdoors, but it is also sold as indoor pot plants now. They are overall smaller than garden and cut roses, commonly with smaller flowers and leaves (Nybom, 2009).

Pot roses have been derived from *R. chinensis var. minima* (Miniature groups), Koster roses, Dwarf Plyanthas and compactas (De Vries, 2003). They have been crossed with Hybrid Tea and Floribunda cultivars. The breeding goals in this rose group are increasing flower size without raising plant size, resistance against blackspot, powdery mildew, gray mold and prolonged shelf life. It has been reported

that influence of seasonal growing environment on pot rose growth and post harvest performance, most cultivars exhibited better flower performances (quick flowering, compact growth, smaller leaf area) on summer grown plants than winter grown plants at flower stage 2 (Grossi and Pemberton, 2004). In addition, due to pesticide resistance and environment friendly concern, by studying biological control of gray mold on pot roses, it has been found that pot rose gray mold protection can be obtained by combining a foliar bio-control agent and an arbuscular mycorrhizal fungus (Møller, et al., 2009).

1.1.5 Cut rose

Commercial cut rose production started 100 years ago. Roses are cultivated in greenhouses or in the regions where the plants can be harvested throughout the year depending on light, temperature, water, and fertilization. World cut rose annually production is estimated to be 15-18 billion stems, which accounts for approximately 31% of all cut flowers traded at European auctions (Blom and Tsujita, 2003, Heinrichs, 2008).

The major commercial cut roses are among Hybrid Tea varieties (*Rosa hybrida L.*), since the first Hybrid tea rose was introduced, thousands of varieties have been released (Esselink, et al., 2003). Flower morphology, such as color, size and multi-petal (double flower 'more than five petals'), etc. is among the most important characters in cut roses breeding (Debener, 2009). Genetic analysis in several populations indicated that the number of flowers within double flowers is determined by 1 major gene, 1 QTL for the number of petals, another QTL for blooming date (Hibrand Saint Oyant et al., 2007). Commonly it takes 6-8 years to breed new cultivars by intercrossing between superior genotypes (Noak, 2003). Cut roses are mainly cultivated under climate protection condition, which is exposed to potential disease threatened environment, i.e. high humidity, suitable temperature and light for pathogen, and their narrow genetic origination in comparison with wild spectrum of genetic resources in the wild (Wylie, 1954), they can be easily infected by pathogenic fungi (Linden, et al., 2009).

In addition, flower color and scent, vase life, water efficiency, flower yield, and low energy cost for cut roses production, etc., all these factors need to be concerned during cut rose breeding and production process. In 2002, a first overview of genes expressed in rose petals was published. It described the EST show similarity with the genes involved in metabolism are related to flower scent production (Channeliere et al., 2002). Metabolic profiling has identified more 500 scent volatiles from rose flowers so far, studies of differences in gene expression between scented and non-scented roses have led to the identification of several genes coding for the enzymes involved in the biosynthesis of these scent volatiles (Knudsen, et al. 2006). In addition, the pattern of inheritance of these volatiles was also studied recently (Spiller, et al., 2010).

1.1.6 Rootstock

Presently, commercial garden roses and cut roses are usually grafted on rootstocks, which is the key to successful rose growing, as it affects the scion performance and flower quality (Karnataka, 2007). The primary traits required for rootstock are 'compatibility with a wide array of cultivars, ease of bud grafting over an extend season, tolerance to biotic and abiotic stress' (Zlesak, 2007).

Although universally adaptable rootstocks would be preferred by growers, they may not be feasible. In addition, the performance of commercial rose cultivars is not determined by the rootstocks, several studies suggest that the performances is influenced by a myriad of factors, including the combination of scion and rootstock, rootstock clonal effects, environmental effects, growing media, etc. (Cabrera, 2002). So different rose rootstock cultivars will be favored in different producing region. Commonly used rootstocks cultivars are *R. canina*, *R. indica major*, *R. x odorata*, *R. multiflora*, *R. manetti*, 'Natal Briar', and 'Dr. Huey'. In USA, R. manetti and 'Dr. Huey' are the common choices for greenhouse and outdoor garden roses. Cultivar Natal Brial is widely acceptable to hydropoic production system in the Netherlands, USA and Colombia. *R. manetti* is favored by greenhouse growers, because of its reduced dormancy requirements over years(Hanan, et al., 1987; Zlesak, 2007).

1.1.7 Rose oil and hips

Among the natural perfume oils, rose oil is the most essential oil, which has been used as body and environment fragrance, and pharmaceutical components for a long time. Rose oil contains more than 500 volatile substances, which is usually extracted from rose petals, with annual wholesale value of 1 billion dollars (Guterman et al., 2002; Knudsen, et al., 2006). *R. damascena*, *R. gallica* and *R. centifolia* are the major high-value aromatic oil producing species (Younis, et al., 2006).

The chemical composition of rose scent compounds, scent accumulated floral tissue etc. have been extensively studied (Flament et al., 1993). Rose petals contain high levels of antioxidants, anti-hypochlorite and anti-peroxynitrite, and the similar levels of antioxidants with those in green tea (Friedman, et al., 2010). Genomic approaches have been used to analyze the genes involved in rose scent biosynthesis with establishing EST databases that represent the transcriptomes of petals from different rose varieties (Guterman et al., 2002; Channeliere et al., 2002). The phenolic methyl ether 3,5-dimethoxytoluene (DMT) is a major scent compound of many modern rose varieties. DMT biosynthesis was operated by two highly similar orcinol O-methyltransferases (OOMT) 1 and 2, it has been report that both OOMT1 and 2 derived from Chinese roses (e.g., *Rosa chinensis* cv Old Blushit). After analyzing OOMT gene family in 18 rose species, it has been found that the emergence of the OOMT1 gene may be a key step in the evolution of scent production in Chinese roses (Scalliet et al., 2008).

Rose hips have been used in food, like jam, tea, ornamental fruits, etc. for a long time. Most of the commercial rose hips are harvested from wild dog roses. Because of their potential health benefits, such as remarkably high content of carotenoid, vitamin C and phenolic compounds, which have been used as powerful antioxidants with preventing lipid peroxidation associated with arterosclerosis, cancer and chronic inflammation (Olsson et al., 2005).

1.2 Genetics and breeding

1.2.1 Microsatellites SSR and phylogenetic relationship

Microsatellites, or simple sequences repeats, are stretches of DNA containing of tandem repeated short units of 1-6 units in length, which are abundantly distributed across the whole genome and which demonstrate high levels of allele polymorphism (Chistiakov, et al., 2006). Microsatellite markers are codominant, highly polymorphic and reproducible. They can be easily amplified with polymerase chain reaction (KIMURA, et al., 2006).

Microsatellites can be found both in coding region and non-coding DNA. But they are rare in the coding DNA, account for 7-10% in higher plants. In cereals, 1.5-7.5% of the ESTs contain SSRs (Thiel, et al., 2003). These ESTs are involved in a series of cell functions, such as metabolic enzymes, disease signaling, structural and storage proteins. It has been speculated that the repast have effects on plants metabolism and gene evolution (Li et al., 2004). It could make creatures adapt fast to new environments during evolution (Trifonov, 2003). In humans, expansion of trinucleotide repeats beyond a certain length in certain genes leads to particular diseases. Because of their multi-allelic property, extensive genome coverage and codominant inheritance, microsatellites have been used extensively for genetic mapping, phylogeny identification, and epidemiology analysis (Chistiakov, et al., 2006).

1.2.2 Cytogenetic –The evolutionary consequences of polyploidy

Polyploidy, is the heritable condition of possessing more than two complete sets of chromosome; it is a major force in the evolution of many eukaryotes. As a result of either the doubling of chromosomes arise within a same species (autopolyploidy) or upon an interspecific hybridization (allopolyploidy) between closely related species containing homologous (homoeologous) genomes, polyploidy is well tolerated in many eukaryotes groups (Comber et al., 2010; Sarah, 2007). The majority of flowering plants have descended from polyploid ancestors, including ancient events

as well as many recent allopolyploidy events in various plant groups, including wheat, cotton, soybean, etc. (Blanc and Wolfe 2004; Adams, 2007; Lim et al., 2008). In Rosa the ploidy levels vary from diploid (2n=2x) to hexaploid (2n=6x) (Wissemann and Hellwig, 1997). Most of the wild species are diploid and the basic chromosome number is 7 (Nybom, 2009), whereas the majority of cultivated roses tend to be triploid and tetraploid (Rout et al, 1999). Polyploidy does not always lead to stable genomes. In wheat the polyploidy state is maintained by suppressing recombination between the genomes. The gene (Ph1 locus in polyploid wheat) responsible for the suppression of bivalent paring of the chromosomes has begun to be unraveled (Griffiths, et al., 2006).

1.3 Disease resistance

1.3.1 Plant disease resistance protein signaling: NBS-LRR

In contrast to animals, plants lack the adaptive immunity system to respond to pathogens. To successfully detect and guard off the pathogens, plants evolved a specific effecter-triggered immunity mechanism. So-called 'disease resistance genes' (R-genes) are the specificity determinants of the plant immune response, which involves a specific interaction between host R genes and pathogen avirulence genes (avr) (Youssef, 2004; Brody and Roger, 2006). Their actions, in most cases, lead to localized programmed cell death, which is termed Hypersensitive Response (HR) (Nimchuk, et al., 2003). The majority of R-genes encode plant resistance proteins containing a nucleotide-binding site (NBS), a series of leucine-rich repeats (LRR), and a putative amino-terminal signaling domain, and they are termed NBS-LRR proteins (Meyers, et al., 2003). In public database, over 1600 NBS-LRR gene-encoding sequences are amplified based on the conserved sequences within the NBS domain. These sequences spread in non-vascular plants, angiosperms, and gymnosperms. (Leah, et al., 2006).

Each NBS-LRR protein encodes a highly conserved NBS domain for ATP hydrolysis and release of signal (Tameling et al., 2002). LRR domains are located at the carboxy termini of plant NBS-LRR protein, containing tandem LRRs. In contrast to no frequent gene-conversion events in NBS domain, the LRR region tends to be highly variable, due to unequal crossing-over and gene conversion, which generated the variation in the number and position of LRRs (Leah et al., 2006). The LRR domain is a common motif, which has been found in over 2000 proteins. The amino-terminal and carboxy-terminal domains in LRR are responsible for modulating activation and bacterial recognition (Youssef et al., 2006). Several lines of evidence indicate the regulatory function of LRR domains, for example, truncation of potato R gene Rx (resistance to Potato Virus X) LRR domain lead to an increase in the hypersensitive response (Bendahmane et al., 2002).

Introgression of R genes from wild species is very important for the disease resistant cultivars creation. To date, 3 single dominant R loci have been characterized in roses, 2 black spot R loci, *Rdr1*, *Rdr3*, and one powder mildew resistant locus *Rpp1* (von Malek and Debener 1998; Linde et al., 2004; Whitaker et al., 2010).

1.3.2 Black spot resistance

Black spot disease caused by *Diplocarpon rosae Wolf* (a phytopathogenic ascomycete fungus) is one of the most widespread diseases among others (powdery mildew, rust and crown gall) of roses. It is very difficult to control and causes significant losses every year (Horst, 1983; Gachomo and Simeon, 2010). It can be found on both wild species and most cultivars, the major solution of this serious disease is regular systemic fungicides spray (Drewes-Alvarez, 2003; Gachomo, 2005). However, with the increasing of public concern and fungicides legal restriction, rose breeders are more interested in developing attractive roses cultivars with high disease resistance. A prerequisite of this object is to find a resistant line as the resource of resistant genes. Due to limited genetic diversity among rose cultivars, wild species become to be the principle resistance genes resources (Debener, 2000).

The mechanisms of physiological and biochemical evidence of the roses infected by *Diplocarpon rosae* has been unraveled (Gachomo and Simeon, 2010). The genetics of partial resistance to black spot has yet to be elucidated, multiple pathogenic races have been differentiated in some countries (Debener, 1998; Uggla Carlson-Nilsson; Whitaker and Stan, 2009). The first dominant resistance gene against black spot (Rdr1) and its location within a contig of four overlapping BAC clones has been characterized recently (Terefe and Debener, 2010).

Objective

Of all possible subjects relevant to rose breeding and to the study of genetic diversity in roses, this thesis will deal with the following subjects:

- The mode of inheritance in garden roses
- Compare genetic diversity of LRR region of Rdr1 paralogues among rose cultivars and wild rose species.
- Compare genetic differentiation among European garden roses, Canadian garden roses, and cut rose cultivars by using neutral microsatellite markers.

Chapter 2 The mode of inheritance in garden roses

2.1 Introduction

Meiosis is the basic process for all sexual species. It contributes to maintaining the genome stability and promoting the genetic diversity (Opperman, et al., 2004; Cifuentes, et al., 2010). Meiosis relies on the recognition of interconnected homologous chromosomes, recombination, intimated association and synapsis (de Muyt et al., 2009). Correct segregation of chromosomes requires stable bivalent formation from metaphase I. This is especially demanding in polyploid species, which contain more than 2 sets of chromosomes. Without stable bivalents, unbalanced segregation of chromosomes (multiple or illegitimate chiasmatic association) will lead to aneuploidy and partial infertility (Armel et al., 2010; Cifuentes, et al., 2010).

Cytological diploidization, the process that in polyploid species' meiosis leads to balanced gametes, has been recognized as a critical step for autopolyploid species and allopolyploid species. If more than 2 copies of duplicated chromosomes in autopolyploid species have the same chance of chromosome recombination during meiosis, then each chromosome will pair randomly with any of its homologous. Since the pairing can start at different chromosome sites, homologous chromosome may switch the partners, leading to multivalent during meiosis (Wu et al., 2001), and crossovers can be formed between more than 2 homologous chromosomes, for the tetraploid species. This is called tetrasomic inheritance (Comber, et al., 2010). It is generally considered as indicative of autotetraploidy. If the homologous chromosomes in an autopolyploids pair following the multivalent model, two chromatids of a single chromosome could pass to the same gamete, which cause the phenomenon, called double reduction (Mather, 1936). It is a very important cytological characteristic of polyploids undergoing multivalent chromosome paring (Wu and Ma, 2005). Double reduction is caused by a combination of three major events during meiosis: crossing over between non-sister chromatids, an appropriated pattern of disjunction, and the subsequent migration of the chromosomal segments carrying a pair of sister alleles to the same gamete (Wu et al., 2001).

In contrast, allopolyploid species combine different sets of related but distinct chromosomes. Pairing and crossovers will be formed only between homologous chromosomes (Comber, et al., 2010). This lead to diploid-like meiotic behavior, generally considered as indicative of allopolyploidy; namely of disomic inheritance.

However, the distinction between disomic and tetrasomic inheritance is not clear-cut, and it could be influenced by taxonomy and genome similarity of parents. For example, disomic, tetrasomic and intermediate inheritance has been found in a tetraploid Rorippa (Yellow Cress) population (Stift, et al., 2008). In addition, with

the evolution of the species, the inheritance may shift from disomic to tetrasomic or tetrasomic to disomic (or vice versa), which may take several generations to million years. In autotraploids, four homologous chromosomes can differentiate into two sets of preferentially paring chromosome, leading to cytogenetic diploidization. For allotetraploid, the meiotic paring may not always follow strict preferential paring, so that crossover between non-homologous chromosomes can homogenize the genome (Stift, et al., 2008; Sybenga, 1975; Udall, et al., 2005). It has been reported that fewer multivalents are formed in the newly established autotetraploid lines of *Arabidopsis thaliana* ecotype Columbia after more than 13 generations (Santos et al., 2003). Theoretical models suggest that evolution of pairing genes is not essential for the establishment of disomic inheritance, but it does require high rates of mutation, recombination and inversions. According to their evolutionary model, run over 10 000 generations, the population exhibits tetrasomic inheritance at low frequency. Once the disomic inheritance is established, the population tends to act like an amphidiploid and it becomes gradually more and more unlikely that chromosomal mutations move between groups (Comber, et al., 2010; Sybenga, 1996). It is compatible with the description of the evolutionary contribution of a shift in ploidy to the adaptation, i.e. masking deleterious mutations (Wendel, 2000; Storchova and Pellman, 2004; Fuijiwara, et al., 2005).

The relationship between superior traits and polyploidy has also been investigated in Rosaceae. It was found that polyploidization is associated with increased species richness, as polyploid clades are more evolutionary successful than diploid clades, etc. (Jana and Timothy, 2006).

The exact mode of inheritance in tetraploid roses is not yet known. Investigation of this population follow disomic or tetrasomic inheritance is not only useful for the genetic linkage mapping, but also may help to unravel the origin of garden rose or cut rose cultivars. This study will focus on distinguishing the inheritance model of a tetraploid hybrid rose population and try to find out the appropriate way to build a haplotype of rose chromosomes.

To be able to study the inheritance of a locus in tetraploid species, it will be perfect that marker can distinguish all four alleles from one parent and another four alleles from the other parent will be perfect. A remarkable feature of NBS-LRR gene is high variability in the LRR domain, which can be used for recognition specificity of resistance genes. It has been reported that its application for the study of genetic diversity among wild rose species and cultivars. (Terefe and Debener, 2010). Therefore, I also used the multilocus Rd1LRR microsatellite marker that is associated with black spot resistant gene Rdr1. It is embedded in a highly conserved part of the LRR region of 9 completely sequenced Rdr1 paralogues. According to Terefe and Debener (2010), different length of $(CT)_n$ repeat are present in exon 4 of eight of out of the 9 Rdr1 paralogues within a contig, about 300kb, of four overlapping BAC clones (Biber et al., 2010). Compared with the conventional SSRs (Hibrand-Saint Oyant et al. 2008), Rd1LRR marker maybe an ideal marker for detecting genetic diversity in roses. Because among the wild roses species and even the cultivars within the same species, the variability was detected, most of the

cultivars showed diverse banding patterns, ranging from 5 bands to 26 bands (Terefe and Debener, 2010). Hence, chances are that the patterns that this marker produces can be interpreted as being indicative for multiple haplotypes, and that there are five or more different haplotypes, whose inheritance can be followed in all progeny plants.

2.2 Material and methods

2.2.1 Plant material

The tetraploid (K5) population was made by crossing between two tetraploid genotypes, P540 x P867. The F1 population, consisted of 146 progenies, together with the parents was used for analyzing the mode of inheritance. Both Hybrid Tea rose parents, are partially resistant to powdery mildew (Yan, 2005) and black spot disease. According to Yan (2005), P867 appeared to be more resistance to powdery mildew than P540. The F1 population shows segregation for many morphological traits, for example, flower color (Koning-Boucoiran, et al., 2009).

DNA Extraction

Young leaves harvested from single plant and frozen at -80°C immediately. Genomic DNA was extracted by using a modified QIAGEN DNeasy Plant Mini Kit (Westburg, Netherlands) protocol (Esselink et al., 2003). All DNA samples were diluted into 2 ng/µl.

2.2.2 Microsatellites marker

Rd1LRR

Genomic DNA samples isolated from F1 and parental line were used as template for PCR amplification. Rd1LRR primer (MicLRR_R: GGAATTTCACTTCCAGGA and MicLRR_F: AGGCTTCAACAATTGCC) was designed from the conserved region flanking the $(CT)_n$ repeat region by T. Debener (Terefe and Debener, 2010), labeled fluorescently with IRDye 700 was applied for the PCR amplification, which performed with the following protocol (Table 1).

Table 1. PCR reaction protocol for QIAGEN Multiplex kit.

Component	Concentration for 1 reaction
Multiplex Kit	5 µl
Primers (R+F) (10pmol/ µl)	0.4 µl
DNA samples (2ug/ µl)	4 µl
Milli-Q	0.6 µl

Total	10 µl

The PCR program was performed on the MJ Research PTC-200 (PTC200) DNA Engine Thermal Cycler and consisted of 2 min hot start at 95°C followed by 40 cycles, see Table 2.

Table 2. PCR conditions for Rd1LRR primer

Steps	Temperature	Time	Cycles
Hot Start	95°C	120 s	
Denature	94°C	30 s	
RAMP	1°C/s to 60°C		
Annealing	60°C	30 s	40
RAMP	1°C/s to 72°C		
Elongation	72°C	45 s	

The PCR amplified products were diluted in 1:10 in the loading buffer and electrophoresed on a 6.5% polyacrylamide gel by using LI-COR 4200 analyzer and LI-COR 4300 analyzer (LI-COR® Biosciences, Lincoln, NE)(http://www.LI-COR.com/). The bands were scored visually from gel pictures. Joinmap version 4.0 (Plant International B.V. and Kyazma B.V.) was used for linkage analysis between the established map and the Rd1LRR primer markers.

2.3 Results

Only bands that could be clearly resolved on the polyacrylamide gels were scored. Amplification of the 146 progeny samples resulted in 19 distinct bands, the fragments size ranging from 257bp to 366bp, Figure 1.

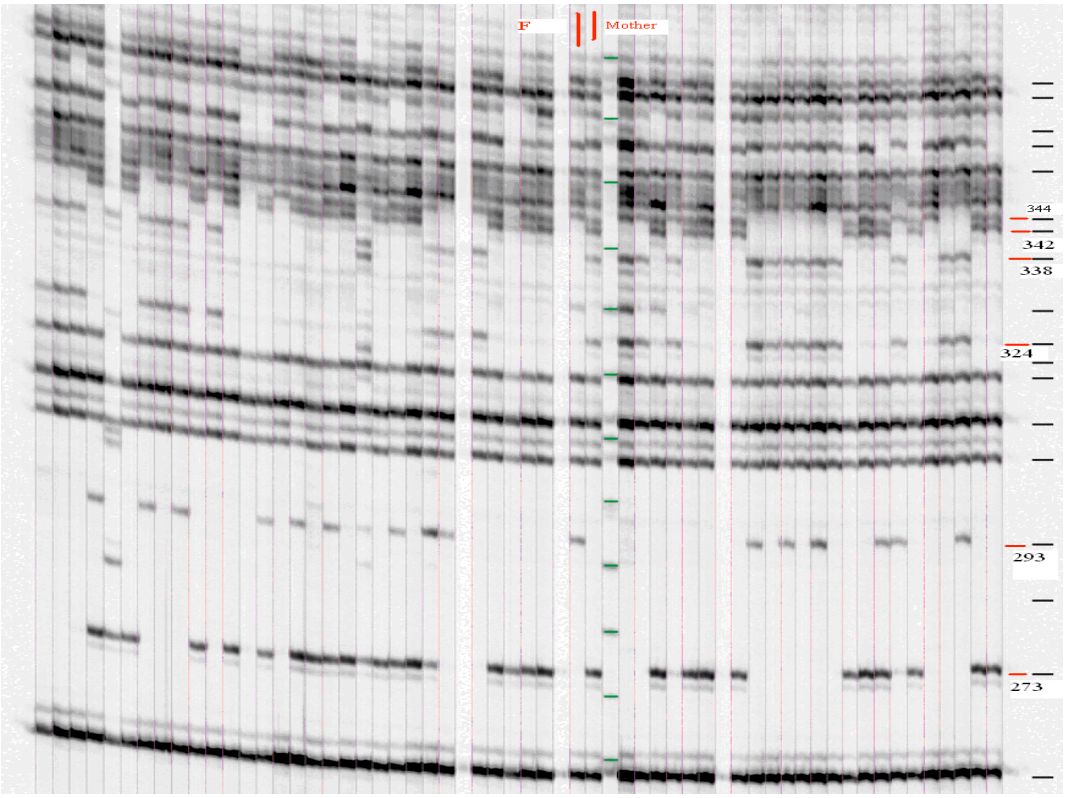


Figure 1. Sample polyacrylamide gels of Rd1LRR fragment patterns for K5 population. The two vertical red lines on the gel indicate the parents' bands pattern. Six informative bands are the right side.

Six informative (polymorphic) fragments were observed on the polyacrylamide gel picture, of estimated size 344bp, 342bp, 338bp, 324bp, 293bp, and 273bp. Of these informative fragments, the band 344bp is present in both parents, 293bp comes from the male parent, and the rest of bands belong to the female plant. These 6 bands would be expected to come from the same multi-band locus, but depending on their location in various haplotypes. If they are from different haplotypes, then they expected to be inherited independently. To test this, a Yates' chi-square test (Yates, 1934), corrected version of Pearson's chi-square statistic, was performed for the various expected segregation ratios (Table 3). All the combinations of the 6 bands, which are associated with different expected segregation ratios, were calculated. The chi-square values for the expected segregation ratio were calculated as follows:

$$\chi^2_{Yates} = \sum_{i=1}^{N} \frac{(|O_i - E_i| - 0.5)^2}{E_i}$$

Where O_i is an observed number, E_i the expected number, and N is the number of distinct events. If the χ^2 value of the expected segregation is smaller than $\chi^2_{0.05,1}=3.84$, then the progeny follow the expected segregation. The smaller the chi-

square value is, the more similar the observed number and the expected number are.

The contingency table is used to analyze the relation between two or more categorical variables. In the contingency table (table 3), of all six informative bands combinations were tested for identifying whether these bands segregate independently. The degree of freedom of all combinations are all (2-1) x (2-1)=1. Hence if the combination of two bands' $\chi 2$ is larger than 3.84, suggesting these two bands didn't segregate independently. It indicated that these two bands shouldn't locate on different chromosomes. However, all bands from female plants showed $\chi 2$ is larger than 3.84 and the p value is smaller than 0.001, whereas band 297bp from father plant showed various results. For instances, combinations of 293bp and 273bp, 338bp and 293bp, their $\chi 2$ are smaller than 3.84 (df=1). Additionally, all the combinations consisting of band 293bp, P-value is larger than other bands combinations. The results suggest that all the bands from female plants didn't segregate independently, whereas band 293bp is on the same chromosome or not still need to be approved.

Table 3 Contingency table used in testing the independence of different combinations of six informative bands observed on the gels

Combination of bands 344bp and 342bp										
Independent assortment		344bp			Independent assortment		344bp			χ2=39.06172059
The expected seg ratio		Present	Absent	Sum	The observed seg ratio		Present	Absent	Sum	
342bp	Present	48.2	20.8	69	342bp	Present	66	3	69	P-value(df=1) <0.001
	Absent	53.8	23.2	77		Absent	36	41	77	
	Sum	102	44	146		Sum	102	44	146	
Combination of bands 344bp and 338bp										
Independent assortment		344bp			Independent assortment		344bp			χ2=45.09373297
The expected seg ratio		Present	Absent	Sum	The observed seg ratio		Present	Absent	Sum	
338bp	Present	53.1	22.9	76	338bp	Present	34	42	76	P-value(df=1) <0.001
	Absent	48.9	21.1	70		Absent	68	2	70	
	Sum	102	44	146		Sum	102	44	146	
Combination of bands 344bp and 324bp										
Independent assortment		344bp			Independent assortment		344bp			χ2=39.06172059
The expected seg ratio		Present	Absent	Sum	The observed seg ratio		Present	Absent	Sum	
324bp	Present	53.8	23.2	77	324bp	Present	36	41	77	P-value(df=1) <0.001
	Absent	48.2	20.8	69		Absent	66	3	69	
	Sum	102	44	146		Sum	102	44	146	

Combination of bands 344bp and 293bp										
Independent assortment		344bp			Independent assortment		344bp			χ2=6.74080107
The expected seg ratio		Present	Absent	Sum	The observed seg ratio		Present	Absent	Sum	
293bp	Present	42.6	18.4	61	293bp	Present	35	26	61	0.001<P-value(df=1)<0.01
	Absent	59.4	25.6	85		Absent	67	18	85	
	Sum	102	44	146		Sum	102	44	146	
Combination of bands 344bp and 273bp										
Independent assortment		344bp			Independent assortment		344bp			χ2=38.07822068
The expected seg ratio		Present	Absent	Sum	The observed seg ratio		Present	Absent	Sum	
273bp	Present	52.4	22.6	75	273bp	Present	70	5	75	P-value(df=1) <0.001
	Absent	49.6	21.4	71		Absent	32	39	71	
	Sum	102	44	146		Sum	102	44	146	
Combination of bands 342bp and 338bp										
Independent assortment		342bp			Independent assortment		342bp			χ2=55.24606424
The expected seg ratio		Present	Absent	Sum	The observed seg ratio		Present	Absent	Sum	
338bp	Present	35.9	40.1	76	338bp	Present	13	63	76	P-value(df=1) <0.001
	Absent	33.1	36.9	70		Absent	56	14	70	
	Sum	69	77	146		Sum	69	77	146	
Combination of bands 342bp and 324bp										

Independent assortment		342bp			Independent assortment		342bp			χ2=58.84931507
The expected seg ratio		Present	Absent	Sum	The observed seg ratio		Present	Absent	Sum	
324bp	Present	36.5	36.5	73	324bp	Present	13	64	77	P-value(df=1) <0.001
	Absent	36.5	36.5	73		Absent	56	13	69	
	Sum	73	73	146		Sum	69	77	146	
Combination of bands 342bp and 293bp										
Independent assortment		342bp			Independent assortment		342bp			χ2=4.483984628
The expected seg ratio		Present	Absent	Sum	The observed seg ratio		Present	Absent	Sum	
293bp	Present	28.8	32.2	61	293bp	Present	22	39	61	0.01<P-value(df=1)<0.05
	Absent	40.2	44.8	85		Absent	47	38	85	
	Sum	69	77	146		Sum	69	77	146	
Combination of bands 344bp and 273bp										
Independent assortment		342bp			Independent assortment		342bp			χ2=58.69762587
The expected seg ratio		Present	Absent	Sum	The observed seg ratio		Present	Absent	Sum	
273bp	Present	35.4	39.6	75	273bp	Present	59	16	75	P-value(df=1) <0.001
	Absent	33.6	37.4	71		Absent	10	61	71	
	Sum	69	77	146		Sum	69	77	146	
Combination of bands 344bp and 324bp										
Independent assortment		338bp			Independent assortment		338bp			χ2=130.2933326

The expected seg ratio		Present	Absent	Sum	The observed seg ratio		Present	Absent	Sum	
324bp	Present	40.1	36.9	77	324bp	Present	75	2	77	P-value(df=1) <0.001
	Absent	35.9	33.1	69		Absent	1	68	69	
	Sum	76	70	146		Sum	76	70	146	
Combination of bands 338bp and 293bp										
Independent assortment		338bp			Independent assortment		338bp			χ2=3.665760749
The expected seg ratio		Present	Absent	Sum	The observed seg ratio		Present	Absent	Sum	
293bp	Present	31.8	29.2	61	293bp	Present	38	23	61	0.05<P-value(df=1)<0.1
	Absent	44.2	40.8	85		Absent	38	47	85	
	Sum	76	70	146		Sum	76	70	146	
Combination of bands 338bp and 273bp										
Independent assortment		338bp			Independent assortment		338bp			χ2=89.23183166
The expected seg ratio		Present	Absent	Sum	The observed seg ratio		Present	Absent	Sum	
273bp	Present	39	36	75	273bp	Present	10	65	75	P-value(df=1) <0.001
	Absent	37	34	71		Absent	66	5	71	
	Sum	76	70	146		Sum	76	70	146	
Combination of bands 324bp and 293bp										
Independent assortment		324bp			Independent assortment		324bp			χ2=4.483984628
The expected seg ratio		Present	Absent	Sum	The observed seg ratio		Present	Absent	Sum	

293bp	Present	32.2	28.8	61	293bp	Present	39	22	61	0.01<P-value(df=1)<0.05
	Absent	44.8	40.2	85		Absent	38	47	85	
	Sum	77	69	146		Sum	77	69	146	
Combination of bands 324bp and 273bp										
Independent assortment		324bp			Independent assortment		324bp			χ2=86.85787816
The expected seg ratio		Present	Absent	Sum	The observed seg ratio		Present	Absent	Sum	
273bp	Present	39.6	35.4	75	273bp	Present	11	64	75	P-value(df=1) <0.001
	Absent	37.4	33.6	71		Absent	66	5	71	
	Sum	77	69	146		Sum	77	69	146	
Combination of bands 293bp and 273bp										
Independent assortment		293bp			Independent assortment		293bp			χ2=0.018440127
The expected seg ratio		Present	Absent	Sum	The observed seg ratio		Present	Absent	Sum	
273bp	Present	25.9	36.1	62	273bp	Present	26	36	62	0.95<P-value(df=1)<0.90
	Absent	35.1	48.9	84		Absent	35	49	84	
	Sum	61	85	146		Sum	61	85	146	

Haplotype construction - Disomic and tetrasomic

Gamete formation model

The two types of inheritance will produce different gametes. Gamete reconstruction, and analysis of occurrence and frequency of the gametes from one of the parents or from both parents will give important information about the mode of inheritance. To analyze all possible and relevant cases, I will first elaborate all possibilities theoretically.

All chromosomes of tetraploid roses crossing parents are represented by different alleles; here the genotype of one parent is denoted as ABCD (female plant) and the other as EFGH (male plant) (Table 4). Under complete tetrasomic inheritance without double reduction, six different gametes carrying the allelic combinations AB, AC, AD, BC, BD, CD, each will occur in relative frequency of 1/6, and the same with the other parent (EF, EG, EH, FG, FH, and GH).

Table 4. Chromosomes pattern in tetraploid roses population

	Mother					Father			
Allele	A	B	C	D		E	F	G	H
344bp									
342bp									
338bp									
324bp									
293bp									
273bp									

In the case of disomic inheritance, bivalents will be formed, but a priori it is not known which alleles are on a set of chromosomes that forms a pair. Hence, if A and B are on homologous chromosomes, allele A and B will exclude each other and never end up in gamete AB, the same with CD. This AB/CD pattern would produce gametes carrying allele combinations AC, AD, BC, BD in equal proportion ¼. The other possible pair patterns, namely of AC/BD and AD/BC, would produce gametes AB, AD, BC, CD and AB, CD, BD, CD, respectively. The '/' is used here to separate the two pairs of chromosomes that do pair and recombine, and from which only one allele is transmitted to the gamete and to the progeny. The only exception is the case of double reduction. It should be noted that double reduction could only happen in multivalent polyploids. Simultaneous paring among homologous chromosomes at meiosis results in this unique cytological phenomenon. Because of the frequency of double reduction depends on the crossover between centromere and the locus. Therefore, the distance between the centromere and the locus could influence the frequency of double reduction (Ronfort et al. 1998; Wu et al. 2001). According to Ronfort (1998), quadrivalent can lead to 3 types of segregation pattern depend on the crossover between the centromere and the locus, and double reduction is one of

these three types. If the double reduction is present, the genotype ABCD produces gamete AA, BB, CC, DD, same with other parent (EE, FF, GG, HH).

No attempt to make the general polyploidy model in this research. Until now, no general polyploidy models, suitable for different species, have been created, especially for the species with complex genetic background, like rose. In addition, lacking of the genetic background information of the parents of this tetraploid roses population, and the marker is not co-dominant; all these factors preclude us to construct a model for this population. Hence, we will only analyze the chromosome inheritance in this rose population based on the observed bands information on the polyacrylamide gel pictures, which is just the opposite way to construct the model. Now we are ready to interpret the data from the six bands in the K5 population, 146 offspring plants and two parents will be tested.

Chi-square test is used to identify the association between the observed value and expected value. But if the fact that chi-square value is larger than some threshold, i.e. 3.84(df=1), can always say the observed value doesn't fit in the expected value, is doubted. For example, if we found 30% of recombination in the progeny, the expected segregation is probably not consistent with the observed segregation based on the chi-square value. Hence, in this research, we would not only study the segregation based on the chi-square value, but also consider other factors (linkage or recombination) to build the haplotype and define the mode of inheritance in this tetraploid K5 population.

Based on the results of the Yates' chi-square test (table 5) and the contingency table chi-square test (table 4), we can conclude that the majority of the bands inherit from each other and follow the expected segregation ratio (table 5). However, based on the chi-square value, for combination of bands 344 and 293, bands 342 and 293, bands 342 and 273, bands 342 and 324, bands 342 and 338, the P-value is smaller than 0.05. It means that not all the progenies are consistent with the expected segregation, which would be discussed in the following part. Of these combinations, combination of bands 344 and 293 exhibited the smallest P-value (<0.001) under one possible expected segregation ratio, and the other 3 combinations' chi-square value is close to 3.84, and P-value is larger than 0.05. Now we are going to construct the haplotype based on these six informative fragments.

Table 5. Yates' chi-square test of Rd1LRR fragments expected segregation ratio

Bands (size) Combination	P1 (Mother)	P2 (Father)	Rd1LRR fragment Pattern (Progeny)				Total number	χ^2
344	X	X	X	X				1.02
342	X		X		X			0.5<P-value(df=2)<0.7
Observed number			66	36	3	41	146	
Expected number			73	36.5	0	36.5	146	
Expected segregation ratio			2	1	0	1		
344	X	X	X	X				1.07
338	X		X		X			0.5<P-value(df=2)<0.7
Observed number			34	68	42	2	146	
Expected number			36.5	73	36.5	0	146	
Expected segregation ratio			1	2	1	0		
344	X	X	X	X				1.02
324	X		X		X			0.5<P-value(df=2)<0.7
Observed number			36	66	41	3	146	
Expected number			36.5	73	36.5	0	146	
Expected segregation ratio			1	2	1	0		
344	X	X	X	X				21.6
293		X	X		X			P-value(df=3)<0.001
Observed number			35	67	26	18	146	
Expected number			58.4	43.8	29.2	14.6	146	
Expected segregation ratio			4	3	2	1		
Observed number			35	67	26	18	146	25.1
Expected number			36.5	73	36.5	0	146	P-value(df=2)<0.001
Expected segregation ratio			1	2	1	0		
344	X	X	X	X				0.63

273	X	X		X			0.7<P-value(df=2)<0.8
Observed number		70	32	5	39	146	
Expected number		73	36.5	0	36.5	146	
Expected segregation ratio		2	1	0	1		
342	X	X	X				4.97
338	X	X		X			0.01<P-value(df=1)<0.05
Observed number		13	56	63	14	146	
Expected number		0	73	73	0	146	
Expected segregation ratio		0	1	1	0		
342	X	X	X				13.68
338	X	X		X			0.001<P-value(df=3)<0.01
Observed number		13	56	63	14	146	
Expected number		24.33	48.67	48.67	24.33	146	
Expected segregation ratio		1	2	2	1		
342	X	X	X				4.72
324	X	X		X			0.01<P-value(df=1)<0.05
Observed number		13	56	64	13	146	
Expected number		0	73	73	0	146	
Expected segregation ratio		0	1	1	0		
342	X	X	X				15.12
324	X	X		X			0.001<P-value(df=3)<0.01
Observed number		13	56	64	13	146	
Expected number		24.33	48.67	48.67	24.33	146	
Expected segregation ratio		1	2	2	1		
342	X	X	X				4.308219
273	X	X		X			0.01<P-value(df=1)<0.05

Observed number		59	10	16	61	146	
Expected number		73	0	0	73	146	
Expected segregation ratio		1	0	0	1		
338	X	X	X				0.308219
324	X	X		X			0.5<P-value(df=1)<0.7
Observed number		75	1	2	68	146	
Expected number		73	0	0	73	146	
Expected segregation ratio		1	0	0	1		
338	X	X	X				1.349315
273	X	X		X			0.2<P-value(df=1)<0.3
Observed number		10	66	65	5	146	
Expected number		0	73	73	0	146	
Expected segregation ratio		0	1	1	0		
324	X	X	X				1.568493
273	X	X		X			0.2<P-value(df=1)<0.3
Observed number		11	66	64	5	146	
Expected number		0	73	73	0	146	
Expected segregation ratio		0	1	1	0		

X stands for the fragments is present.

Band 344bp

According to the band pattern observed on the K5 population polyacrylamide gel picture, only band 344bp is shared by both parents and informative. Firstly, we decide to denote the chromosome with this band as A (mother) and E (father).

If only 1 copy of allele 344bp is present in each parent, then the gamete ratio would be Present (P): Absent (A) =1:1 for each of the two parents. The expected progeny segregation ratio should then be Present: Absent=3:1. The data observed on the gels are consistent with this, as (66+36):(41+3) ≈3: 1, (see table 3). Neither disomic nor tetrasomic can be rejected based on the first step of calculation (see table 6).

Following the same calculation, if 2 copies of allele 344bp are present in both parents, the expected segregation ratio would be Present: Absent=15: 1 in case of tetrasomic. Another two expected ratios would be possible depending on whether these two copies are located on two homologous chromosomes or not. One expected value would be the same as tetrasomic inheritance, the other expected value would be of P: A = 1:0. Neither expectation is supported by the data.

The third possibility is 1 copy of 344bp in male plant, 2 copies in the female plant, and alleles are following disomic inheritance, the expected value will be Present: Absent=7:1 if these two copies are on non-homologous allele. Otherwise, the expected value will be Present: Absent= 1:0. The result is identical if 2 copies in father plant, and 1 copy in mother. This possibility can also be rejected.

The fourth possibility is 3 copies or 4 copies of 344bp from either of the parents, in which case the expected segregation ratio would be Present: Absent=1:0. So this possibility can be rejected.

Table 6 The possible expected segregation comparison between disomic and tetrasomic inheritance based on band 344bp

Band	Copy number	Mode of inheritance	Gamete pattern	Gamete segregation ratio (Present:Absent)	Expected Progeny segregation ratio (Present:Absent)
	1	Disomic	Aaaa(♀)xAaaa(♂)	1:1	3:1
	1	Tetrasomic	Aaaa(♀)xAaaa(♂)	1:1	3:1
	2	Disomic (homologous)	AAaa(♀)xAAaa(♂)	1:0	1:0
	2	Disomic (non-homologous)	AaAa(♀)xAaAa(♂)	3:1	15:1
	2	Tetrasomic	AAaa(♀)xAAaa(♂)	3:1	15:1
	2+1	Disomic (homologous)	AAaa(♀)xAaaa(♂)	1:0(♀)x1:1(♂)	1:0
	2+1	Disomic (homologous)	Aaaa(♀)xAAaa(♂)	1:1(♀)x1:0(♂)	1:0
344bp	1+2	Disomic (non-homologous)	AaAa(♀)xAaaa(♂)	3:1(♀)x1:1(♂)	7:1
	1+2	Disomic (non-homologous)	Aaaa(♀)xAaAa(♂)	1:1(♀)x3:1(♂)	7:1
	1+2	Tetrasomic	AAaa(♀)xAaaa(♂)	3:1(♀)x1:1(♂)	7:1
	2+1	Tetrasomic	AAaa(♀)xAaaa(♂)	1:1(♀)x3:1(♂)	7:1
	3	Disomic	AAAa(♀)xA_ _ _(♂)	1:0(♀)xany ratio (♂)	1:0
	3	Tetrasomic	AAAa(♀)xA_ _ _(♂)	1:0(♀)xany ratio (♂)	1:0
	4	Disomic	AAAA(♀)xA_ _ _(♂)	1:0(♀)xany ratio (♂)	1:0
	4	Tetrasomic	AAAA(♀)xA_ _ _(♂)	1:0(♀)xany ratio (♂)	1:0

In conclusion of the first step, based on the calculation of band 344bp, the copy number of this band is 1 in each parent, and we assign them to chromosomes A and E (see table 7). Tetrasomic or disomic inheritance cannot be distinguished.

Table 7. The position of band 344bp on the 'haplotype' of the chromosomes

	Mother					Father			
Allele	A	B	C	D		E	F	G	H
344bp	X					X			
342bp									
338bp									
324bp									
293bp									
273bp									

X stands for the fragments is present.

Bands 344bp+342bp combination

Following the same kind of calculation in the first step, we looked at band 342 from mother plant. Three possible allele combinations would exist, one is that both bands are located on the same chromosome A, the second is that it is on B, a homologous chromosome in disomic inheritance (so that either 344 or 342 are being inherited), the third is that it is on a non-homologous chromosome, say C, in disomic inheritance (342 and 344 do not pair but are inherited independently). Finally, it can also be on different chromosomes in tetrasomic inheritance (Table8).

If 344bp and 342bp co-exist on chromosome A, and 342bp is only 1 copy, then the expected segregation ratio of the progeny will be present (344bp+342bp): Present (344bp): Absent=2:1:1 (see table 5.), which is close to the segregation ratio determined in the progeny (344bp+342bp): Present (344bp): Absent= 66:36:41≈2:1:1, yates' chi-square is 1.017123288, 0.5<P-value(df=2)<0.7. It means that the real segregation ratio is consistent with the expected one. This indicates these two bands co-segregate. However, we also found that 3 progenies (2%) of the plants have Present (342) only, which does not fit in this expectation.

If 1 copy of band 342bp is located on C, the non-homologous chromosome of A, then the expected segregation ratio between disomic and tetrasomic will be the same. Because the ratio of gamete pair in mother plant is Present (344bp+342bp, 342bp): Absent=5:1. In the male plant, the ratio of the gamete will be Present (344bp): Absent=1:1. And the expected Absent ratio in the progeny should be 1/12, which clearly is not (the real value is close to ¼). If band 342bp is on the homologous allele of allele A, then these two bands should never end up together in the same gamete, which is clearly not the case. So this possibility can be rejected.

If 1 copy of band 342bp is on the non-homologous chromosome C and the other one is not, then the expected gamete ratio of mother should be Present (344bp+342bp): Present (344bp): Present (342bp)=2:1:3, and father plant will be Present (344bp):

Table 8 The possible expected segregation comparison between disomic and tetrasomic inheritance based on combination of bands 344bp and 342bp

Band	Copy number	Mode of inheritance	Gamete pattern	Gamete segregation ratio (Present:Absent)	Expected Progeny segregation ratio (Present:Absent)
344bp+342bp	1+1	Disomic (same chromosome)	Aaaa(♀)xAaaa(♂)	1:1(♀)x1:1(♂)	2 (both) :1 (344bp): 0 (342bp) :1 (absent)
			Bbbb(♀)xbbbb(♂)	1:1(♀)x0:1(♂)	
		Disomic (non-homologous)	Aaaa(♀)xAaaa(♂)	1:1(♀)x1:1(♂)	3 (both) :3 (344bp):1 (342bp) :1 (absent)
			bbBb(♀)xbbbb(♂)	1:1(♀)x0:1(♂)	
		Disomic (homologous)	Aaaa(♀)xAaaa(♂)	1:1(♀)x1:1(♂)	1 (both) :2 (344bp):1 (342bp) :0 (absent)
			bBbb(♀)xbbbb(♂)	1:1(♀)x0:1(♂)	
		Tetrasomic (same chromosome)	Aaaa(♀)xAaaa(♂)	1:1(♀)x1:1(♂)	2 (both) :1 (344bp): 0 (342bp) :1 (absent)
			Bbbb(♀)xbbbb(♂)	1:1(♀)x0:1(♂)	
		Tetrasomic (different chromosome)	Aaaa(♀)xAaaa(♂)	1:1(♀)x1:1(♂)	4 (both) :5 (344bp): 2 (342bp) :1 (absent)
			bBbb(♀)xbbbb(♂)	1:1(♀)x0:1(♂)	

Absent=1:1. The expected Absent value should be 0, which is incompatible with the result (1/4 Absent) from the gel picture. If these 2 copies of band 342bp are both on the non-homologous chromosomes of chromosome E, no matter whether it is in tetrasomic or disomic inheritance, the expected gamete combination should all contain band 342bp, which is also not compatible with ¼ Absent. This possibility can be excluded too. The results would be the same if 3 copies of band 342bp on mother plant.

In conclusion, only 1 copy of both bands 344bp and 342bp can be located on the same allele A (see table 9), but disomic and tetrasomic inheritance cannot be distinguished based on the segregation ratio calculation for these two bands (see table 8).

Table 9. The position of band 344bp and band 342bp on the 'haplotype' of the chromosomes

	Mother					Father			
Allele	A	B	C	D		E	F	G	H
344bp	X					X			
342bp	X								
338bp									
324bp									
293bp									
273bp									

X stands for the fragments is present.

Bands 344bp+338bp combination

According to the fragment pattern observed on the gel pictures and the first possibility of second step calculation, the progeny segregation ration is Present (344bp+338bp):Present (344bp):Present (338bp):Absent = 34:68:42:2≈1:2:1:0, (see table 5), which means the expected gamete combination of mother plant should not include the situation that both bands are Absent, which is possible if, in disomic inheritance, 1 copy of band 342bp is on the homologous chromosome of A, i.e., chromosome B.

Assuming tetrasomic inheritance and 1 copy of band 338bp on either chromosome B, C, or D, the expected Absent value in the progeny should be 1/12, which is not compatible with the true value of 0, the similar condition is this 338bp co-exist with 344bp on the allele A. If 2 copies of 338bp, then the expected gamete combination should be Present (344bp+338bp): Present (338bp): Present (344bp)=2:3:1, which means that the expected progeny segregation ratio should be (344bp+338bp): Present (338bp): Present (344bp)= 7:3:2, which is different from the value of 1:2:1.

Table 10. The possible expected segregation comparison between disomic and tetrasomic inheritance based on combination of bands 344bp and 338bp

Band	Copy number	Mode of inheritance	Gamete pattern	Gamete segregation ratio (Present:Absent)	Expected Progeny segregation ratio (Present:Absent)
344bp+338bp	1+1	Disomic (same chromosome)	Aaaa(♀)xAaaa(♂)	1:1(♀)x1:1(♂)	2 (both) :1 (344bp): 0 (342bp) :1 (absent)
			Cccc(♀)xcccc(♂)	1:1(♀)x0:1(♂)	
		Disomic (non-homologous)	Aaaa(♀)xAaaa(♂)	1:1(♀)x1:1(♂)	3 (both) :3 (344bp):1 (342bp) :1 (absent)
			ccCc(♀)xcccc(♂)	1:1(♀)x0:1(♂)	
		Disomic (homologous)	Aaaa(♀)xAaaa(♂)	1:1(♀)x1:1(♂)	1 (both) :2 (344bp):1 (342bp) :0 (absent)
			cCcc(♀)xcccc(♂)	1:1(♀)x0:1(♂)	
		Tetrasomic (same chromosome)	Aaaa(♀)xAaaa(♂)	1:1(♀)x1:1(♂)	2 (both) :1 (344bp): 0 (342bp) :1 (absent)
			Cccc(♀)xcccc(♂)	1:1(♀)x0:1(♂)	
		Tetrasomic (different chromosome)	Aaaa(♀)xAaaa(♂)	1:1(♀)x1:1(♂)	4 (both) :5 (344bp): 2 (342bp) :1 (absent)
			cCcc(♀)xcccc(♂)	1:1(♀)x0:1(♂)	

In conclusion of this step, tetrasomic could be excluded based on the result above, and this copy of band 338bp is on B. This disomic result can be proved in the following steps (see table 11).

Table 11. The position of 3 bands on the 'haplotype' of the chromosomes

	Mother					Father			
Allele	A	B	C	D		E	F	G	H
344bp	X					X			
342bp	X								
338bp		X							
324bp									
293bp									
273bp									

Bands 342bp+338bp combination

In table 3, we have found these two bands didn't segregate independently, otherwise the expected segregation would be 3:3:1:1. Based on the results of last step, if tetrasomic inheritance, the expected gamete combination would be Present (342+338): Present (342bp): Present (338bp): Absent = 1:2:2:1, the progeny follows the same expected segregation ratio. But the observed segregation in the progeny is Present (342+338): Present (342bp): Present (338bp): Absent = 13: 56: 63: 14, even the value of Present (342+338) and Absent is higher than the theoretical value 0:1:1:0, it is still not sufficient to support tetrasomic inheritance, but the reason for these two higher values is not clear. Because the p-value of segregation 1:2:2:1 is smaller than segregation 0:1:1:0 (see table 5). The major part of the progeny (81.5%) appear to follow the Present (342bp): Present (338bp)=1:1 segregation ratio, which may point at disomic inheritance, but part of the plants (18.5%) do not fit into it. The same with bands 342+324bp combination, these 18.5% plants that may not follow disomic inheritance under bands 342+338bp combination, are almost the same plants with the combination of bands 342+324bp except 1 progeny.

Bands 344bp+324bp combination

The calculation and result of this combination are similar with 344bp+338bp (see table 10). The progeny segregation ratio is Present (344bp+324bp): Present (344bp): Present (324bp) 36:66:41≈1:2:1, 0.5<P-value(df=2)<0.7 (see table 5). Back to the observed data on the gel, bands 338bp and 324bp almost co-segregate all the time, only 1 plant showed segregate pattern. So, this 324bp should be on the same chromosome B (see table 12).

Table 12. The relative position of 4 bands on the 'haplotype' of the chromosomes

	Mother					Father			
Allele	A	B	C	D		E	F	G	H
344bp	X					X			
342bp	X								
338bp		X							
324bp		X							
293bp									
273bp									

Bands 344bp+273bp combination

The condition is compatible with 344bp+342bp. following the same calculation steps with disomic inheritance, based on these calculations above, the copy number of 273bp should be 1.

If band 273bp locates on the chromosome B and forms a pair with A in disomic inheritance, then the expected gamete combination ratio should be Present (344bp): Present (273bp)= 1:1, and the expected progeny segregation ratio would be Present (344bp+273bp): Present (344bp): Present (273bp): Absent = 1:2:1:0, which is incompatible with the value Present (344bp+273bp): Present (344bp): Present (273bp): Absent = 70: 32: 5: 39≈ 2:1:0:1 observed in the population (see table 5).

If band 273 is on the non-homologous allele C or D, then the expected progeny segregation ratio would be Present (344bp+273bp): Present (344bp): Present (273bp): Absent = 3:3:1:1, which is also different from the measured ratio.

If band 273bp is on A, the expected progeny segregation ratio should be Present (344bp+273bp): Present (344bp): Present (273bp): Absent = 2:1:0:1. The observed values do not significantly differ from this ratio (see table 5), so most likely band 273bp should co-segregate with 344bp and is located on chromosome A (see table 13). Five plants don't fit into this haplotype, account for 3.4% of the population, but they are included in the 18.5% of the plants that don't fit in this haplotype mentioned above (bands 342+338bp combination). Until this step of calculation, the haplotype of bands from female plant has been constructed. The reason for these plants incompatible with this haplotype could be due to recombination, or intermediate inheritance, etc. which would be discussed later.

Table 13. The relative position of 5 bands on the 'haplotype' of the chromosomes

	Mother					Father			
Allele	A	B	C	D		E	F	G	H
344bp	X					X			
342bp	X								
338bp		X							
324bp		X							
293bp									
273bp	X								

Bands 293bp

As mentioned above, band 293bp from father plant, both parents share band 344bp. probably, the chromosome inheritance of both parents is different, based on the calculation above, and mother plant is disomic inheritance for sure.

So firstly, assuming father plant is tetrasomic inheritance, following the same steps of the calculation above, the copy number of the band 293bp is still 1. Band 293bp is not on A. After these calculations, the expected progeny segregation ratio is Present (344bp+293bp): Present (344bp): Present (293bp): Absent =4:3:2:1, which is incompatible with the result Present (344bp+293bp): Present (344bp): Present (293bp): Absent = 35:67:26:18 observed in the population (see table 5).

If it is disomic inheritance, there are two possibilities: band 293bp is on the homologous chromosome (F) or on a chromosome of the other pair (G or H). The expected progeny segregation ratio value of these two possibilities are Present (344bp+293bp): Present (344bp): Present (293bp): Absent = 1:2:1:0 and Present (344bp+293bp): Present (344bp): Present (293bp): Absent = 3:3:1:1 (see table 3), but neither these two values are compatible with the values observed in the population. But based on these results, the fact that band 293bp is on the homologous allele of allele A can be excluded, otherwise the value of Absent would be 0. Whether father plant follows disomic inheritance or tetrasomic inheritance needs to be confirmed in a further step by Joinmap 4.0, so band 293 would be neglected temporally.

Based on the results of combination of these two bands, the "haplotype" was established. The gamete segregation pattern could be like this (separating the bands on the gel across chromosomes, to help interpretation of the gels but not intended to depict the relative location of the bands on the chromosomes in any way)(figure 2). Then, to check if the progeny segregation ratio does fit this hypothesis haplotype, it will be tested by comparing different combination of 4 bands and 5 bands.

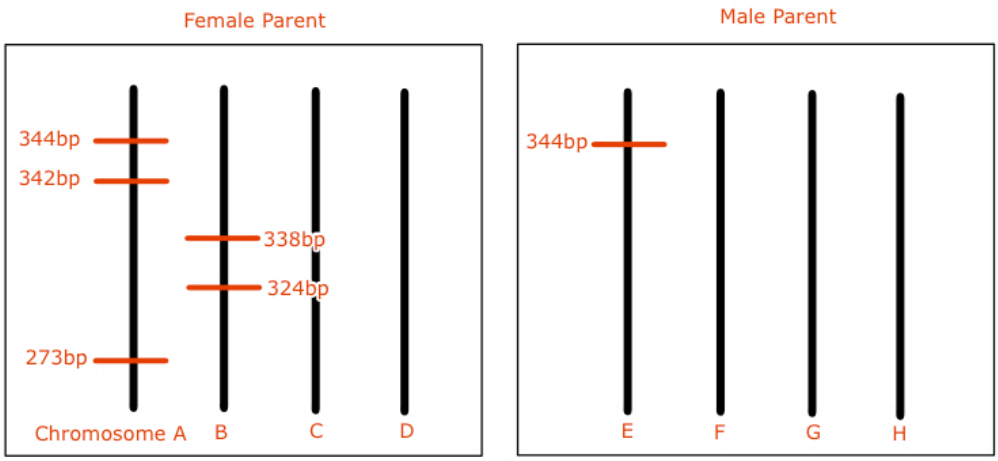


Figure 2. Demonstration of the haplotype of the 5 bands

Chromosomes A and B, C and D, E and F, G and H are homologous.

The results showed that 76.7% of progeny are compatible with this hypothesis gamete segregation, which follow 56:35:21=2:1:1 (see table 14), and Fit-test χ^2= 3.02<3.84$_{(0.05\ \ df=2)}$. Meanwhile, around 24% of the progeny doesn't fit this segregation. If excluded band 273bp (see table 11), then 80.4% of progeny will follow this hypothesis gamete segregation. χ^2= 2.786765<5.99$_{(0.05\ df=2).}$ These two tables indicating that the patterns of offspring without fitting the haplotype were. Bands 338 and 324bp from chromosome B almost always co-segregate, expect pattern No. 11 (table 15).

Table 14. The number of plants with different patterns of 5 bands

Band (bp)	Random assortment of 5 bands (32 possible combinations)																														
Pattern No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
344	X	X		X	X			X	X		X	X	X				X			X		X	X					X	X	X		
342	X	X	X	X	X		X	X	X	X			X	X				X			X			X					X		X	
338	X	X	X		X	X	X					X		X	X		X		X					X			X	X	X	X	
324	X	X	X	X		X	X	X		X	X				X	X		X		X						X		X		X	
273	X		X	X		X			X	X	X	X				X			X		X	X				X	X	X		X	
Number of plants	4	7	0	0	0	3	3	0	56	0	1	0	0	0	35	0	0	0	0	0	0	6	5	0	0	0	0	4	0	21	0	1

Gray shading denotes the number of plants following the hypothesis disomic inheritance.

Table 15. The number of plants with different patterns of 4 bands

Band (bp)	Random assortment of 4 bands (16 combinations)															
Pattern No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
344	X	X		X	X	X			X		X	X				
342	X	X	X	X		X	X			X			X			
338	X	X	X		X		X	X	X					X		
324	X		X	X	X			X		X	X				X	
Number of plants	11	0	3	0	25	56	0	38	0	0	1	11	0	1	0	2

Gray shading denotes the number of plants following the hypothesis disomic inheritance.

Due to insufficient bands from pollen donator plants, and doesn't fit in this haplotype, to analyze if this bands if fit in either linkage group. Assuming disomic inheritance, the data including band 297bp from father plant could be handled by Joinmap 4.0. Combining with the data set compromising 258 markers investigated by Boucoirain et al., (in preparation), a linkage map was constructed (figure 3-A). Band 297bp was excluded into 1 separate group by the software, and it cannot be mapped into any existing linkage group. Here the 4 bands were grouped into 1 linkage group, one with other markers derived from female parent P540. As depicted in figure3-A, the shortest distance among these 4 bands is 0.8cM. Band 344bp was present in both parents. The band from female plant cannot be distinguished from male plant, hence the software excluded band 344 to compute the female (P540) linkage map. If exclude the 258 markers from female plant, then these 6 bands were divided into 2 linkage groups, band 297 still be categorized separately (figure 3-B). The first two linkage maps were computed according to that all 146 progenies follow disomic inheritance. But probably not all the progeny follow the disomic inheritance, and Joinmap software is designed for diploid species. It means that this software can compute only 76.7% of the progeny. In case of these progenies didn't follow disomic inheritance, we also performed the data analysis by Joinmap without these 24.3% of progeny. After exclude 24.3% progenies those don't fit in this haplotype, the distance among these 5 bands declined (figure 3-C). Bands 342 and 324 located on the same position, as they didn't co-segregate, which means that these two bands located on the same position of homologous chromosomes A and B.

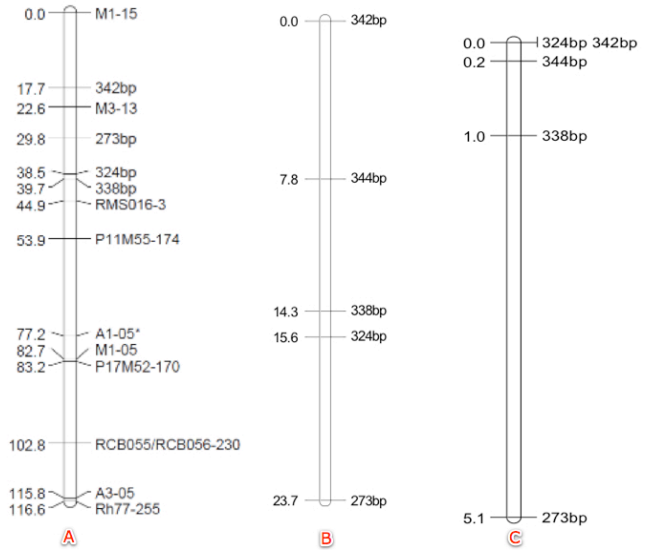


Figure 3. Genetic linkage map of tetraploid rose progeny of parents P540 and P867.

According to Biber et al. (2010), 7 segregating Rdr1 paralogues outside of this contig from *R. multiflora* hybrid 88/124-46 population, were mapped onto chromosome 1 from spanning genetic interval is genetically restricted to 18.5 cM. In addition, this Rd1LRR primer was designed from a highly conserved part of the LRR region of 9 Rdr1 paralogues in 1 diploid crossing population, spanning 300Kb. Probably these five fragments are five paralogs of Rdr1 (figure 3-B).

2.4 Discussion

Meiosis is an obligatory process for all sexually reproducing organisms. Strict homologous chromosome pairing is displayed by both diploids and diploidized allopolyploids (Sybenga, 1975). In this research, we distinguish allopolyploidy from autopolyploidy by parentage, more accurately, by mother plant. And according to the statistical analysis of K5 tetraploid roses population with the multi-bands and multi-locus marker Rd1LRR, suggesting that the genotype of around 77% of progeny is consistent with disomic inheritance. But there are around 23% of the offspring, which didn't fit in this haplotype. It is probably caused by several reasons. For instances, recombination between homologous chromosomes, but also possible for recombination between non-homologous chromosomes during formation of quadrivalent, etc.

Due to lacking enough genetic background information, if the parents derived from diploid crossing then duplication or from two tetraploid cultivars crossing is unclear. Based on the expectation discussed above and less efficient bands pattern from father plants, it is only possible to interpret the female plant polyploidy origin in this research. Back to this question, if the parents derived from diploid crossing,

one possibility is that AC or AD, BC or BD from two different diploid species (based on figure 2), namely of AC crossed with BD or AD crossed with BC.

And one possible polyploidy origin is through the mating of plants producing 2n pollen grains or 2n. The other possibility to form polyploidy is through duplication of the genome (Wu et al., 2001). But the second possibility can be eliminated, because commonly it leads to the genotype like AA, BB, and our calculation suggesting each marker is only 1 copy. To this chromosome, if the similarity between two grandparents is relatively large, then the mother plant is very likely to form all or mostly bivalents during meiosis. Cifuentes et al (2010) indicated that the proportion of trivalent or quadrivalent caused by misparing of homeologous chromosomes in the newly formed allopolyploids species is depend on the discrimination between multiple sets of homologous chromosomes. If back to the 23% of offspring bands pattern (table 14), all the patterns can be explained by recombination between homologous chromosomes. For instances, pattern No.1 and 2 can be caused by crossover or recombination during meiosis between chromosome A and its homologous chromosome B. And the recombination ratio of target locus also depends on its position from the centromere. After the recombination, two possible chromosome segregations would generate two types of gametes. However, recombination between homeologous chromosomes, also could lead to these progeny genotypes. Additionally, if the quadrivalents can be formed during meiosis is still unclear (figure 4). Because going back to the gel pictures, many non-informative bands were shared between parental plants, and also among progenies. If four chromosomes ABCD all contain these bands, it is possible to indicate AB/CD, these two sets of chromosomes share some similarity. Then depend on the level of similarity between these chromosomes; quadrivalent may be formed, which could lead to more complex gametes genotypes during meiosis. Hence, through testing more markers outside this specific locus would unravel and make sure if all the recombinations are caused by homologous chromosomes.

Another possibility is still following disomic inheritance, namely of chromosome A and B segregate, same with chromosome C and D, and lead to normal gametes formation. Some other chromosomes generate unbalanced aneuploidy, which could lead to reduced fertility and even lethality of gametes. While misparing may involve homoeologous associations, it may also include paralogous associations, both of which lead to complicated modes of inheritance, chromosomal rearrangement, and aneuploidy (Gaeta and Pires, 2010). For instance, in *Brassica napus* allohaploids, most bivalents observed during meiosis show allosyndesis (involving pairing of homoeologous chromosome), while up to 30% indicate autosyndesis (involving chromosomes within the same parental genome that presumably contain duplicated regions) (Nicolas et al., 2007). While Cifuentes et al (2010) observed that repeated polyploidy resulted in different levels of crossover suppression between homoeologs in *B. napus* allhaploids through analyzing the genetic diversity *PrBn* locus, which was shown to determine the meiotic behavior in this 363 allohaploids produced from 29 accessions. Based on the stimulation autotetraploid Arabidopsis model, Comber et al (2010) found that the inheritance model can shift from tetrasomic to disomic inheritance after several generations. However, how many

generation it takes to breed this mother plant, which is not clear. But there are approximately 77% offspring of this K5 population showing disomic inheritance for sure.

Additionally, there is another extreme in allopolyploids, the inheritance may represent that of an autopolyploid, in which there is no discrimination between the multiple sets of homologous chromosomes (Gaeta and Pires, 2010; Cifuentes et al., 2010). However, if two sets of homologous chromosome are very similar or even identical, most of the offspring should show tetrasomic inheritance. The allopolyploidy will be formed when it's sufficient of differentiation to prevent homoelogous pairing, or in some exceptional cases, some well differentiated chromosomes behave as in an allopolyploid, whereas other sets are not well differentiated and behave as in an autopolyploid (Sybenga, 1996). Even so, there will be no such 77% of the progeny following disomic inheritance. Meanwhile, if all the mother plants are identical or even different location with microenvironment variation in the greenhouse, all could influence the segregation during meiosis. Cifuentes et al (2010) found that in some cases, the number of univalent determined for different allohpaloids *B. napus* isolated from the same accession differed depending on their spatial locations in the greenhouse. In addition, if these mother plants were obtained through sexually reproduction, then it has some possibilities to got different identical genotype in mother plants, which may generate different segregation pattern. Otherwise all the mother plants were reproduced by vegetative production, and all the mother plants genotype should be identical, except chimeras.

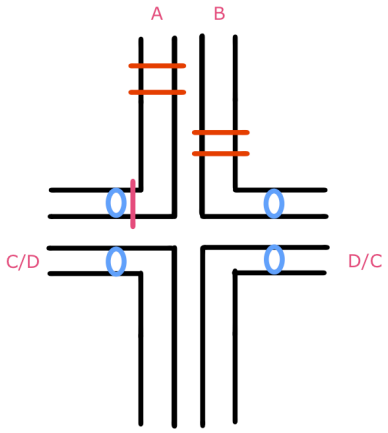


Figure 4. Sample meiosis pattern of an allopolyploid quadrivalent in mother plants. Blue circles represent centromere.

Compare to the allpolyploids, autopolyploids more often exhibit multivalent that mostly lead to tetrasomic inheritance, similar with allopolyploid species. It has been reported that the homologous chromosomes may pair in any combination, forming univalents, bivalents, trivalents or quadrivalents in newly formed tetraploid plants (Synbenga, 1975). But the equal chance to pair with the other 3 chromosomes in

autotetraploid can explain these 77% offspring genotype is highly doubted. And also, based on the calculation above, this hypothesis has been excluded. Even in autopolyploidy species, the occurrence of bivalent pairing is higher than multivalent (Ramsey and Schemske, 2002). However, if multivalent formed, the double reduction could just happen. But in this study, it can be eliminated. Because it cannot explain the 24% progeny that don't follow disomic inheritance, based on the bands pattern. If the double reduction happens, two chromatids of a single chromosome can pass to the same gamete, which can produce the gamete type AA, BB, CC, DD if tetraploid species (Wu et al. 2001). It means that only pattern no 9, 15, 30 in table 10, those number would be inflected. But this phenomenon is not responsible for the recombination between these five bands. It can only increase the ratio of the progeny consisted with disomic inheritance. Due to the polyacrylamide gel, even ABI is hard to score the copy number of the bands; the double reduction phenomenon cannot be seen on the gels. In addition, this phenomenon is only formed in autopolyploids, theoretically the frequency of double reduction in autopolyploid ranging from 0 to 0.3 (Fisher, 1947; Haynes and Douches, 1993; Wu et al., 2005).

Commonly, it can be estimated when constructing the general polyploidy model in outcrossing polyploidy species. Until now, no real general polyploidy model has been constructed for analyzing gene segregation in all the polyploidy species. Mostly these models are established based on pseudo backcrossing populations. These populations don't have the selfing incompatibility problems, and the chromosomes from parents can be distinguished, for instance, abcd and efgh (Wu et al. 2001; Wu et al., 2005; Stift et al. 2008; Comber et al. 2009). In our research, these simulation models cannot be used for analyzing the inheritance mode of K5 population, because the chromosomes cannot be distinguished at the beginning. In addition, if the parents are autopolyploid or allopolyploid, which is not clear too. Therefore, according to the segregation observed on the gel, to construct the haplotype first, allow us to be able to identify the inheritance mode of K5 tetraploid rose population.

Looking back to the data observed on the gels, according to our calculation, band 293bp can be aligned into this haplotype, and neither disomic inheritance nor tetrasomic inheritance. Due to lacking of enough bands from male plant, we can't make sure male plant follow disomic or tetrasomic inheritance. Therefore whether male plants are autotetraploid or allopolypoid, which need to be confirmed by other multi locus markers.

For the female plants, all the bands from mother plants except for band 344bp, the recombination among these four bands should be caused by the mother plants' gametes formation at meiosis. If the female plants are autotetraploid, then it is very unlikely to produce almost 80% progeny that follow the same chromosome segregation mode of allotetraploid plants. It is highly possible that the female plant is allopolyploid rose cultivars. The reason for 20% recombination in this population still need to be confirmed by more markers on this chromosome in further. If it was caused by the homoeologous recombination, then the aneuploidy progeny is very likely to appear in this population, and the ploidy level of this population has not

been tested before. Or if all the progeny's ploidy levels are identical, then it is caused by the recombination between homologous chromosomes. However, if only one multilocus primer on 1 chromosome could stand for the whole 7 sets of chromosome in this population still needs to be confirmed by utilizing more multilocus markers. Therefore, confirm the ploidy level and adding more multilocus markers will help to identify and confirm the chromosome inheritance mode of this tetraploid roses population.

In addition, for linkage mapping, Joinmap 4.0 construct the linkage map based on the genetic recombination, and designed for diploid species, the distance between these bands became shorter after exclude the progeny that may not follow disomic inheritance (figure 3-C). However, as we discussed above, the recombination can be caused by recombination between homologous or homoeologous chromosome. Therefore, these 20% progeny can't be called that they don't follow disomic inheritance according to the fact that they don't fit into this haplotype. Because even these progeny can be explained by disomic inheritance as mentioned above.

This software can only handle the calculation among homologous chromosomes with disomic inheritance populations; otherwise tetrasomic and recombination between homoeologous would enlarge the actual genetic distance between markers. If the 23% of offspring are caused by homologous recombination, then figure 3-2 will be the more accurate linkage map.

But whether it is better to study the inheritance with more markers that each has less allele in the future linkage map, this haplotype method could be not so efficient. Because if only take these markers with multilocus pattern to construct these linkage maps with less information, many information or gaps will exist on the linkage map. Therefore, one solution is that use multilocus markers and markers with less alleles to build two maps respectively, then combine these separated maps and reconstruct the four haplotypes of the linkage groups. Then more clear-cut linkage maps can be constructed, although this would be more time consuming work. But it could be an efficient way to identify and distinguish the haplotype of this tetraploid rose population and even in other polyploidy species.

2.5 Conclusion

Based on the calculation and analysis, K5 tetraploid rose population follow disomic inheritance, the female plant is very likely an allotetraploid rose cultivar; however, whether other sets of chromosomes all follow the same segregation still need to be confirmed by more multilocus markers. Meanwhile, the results suggest that the recombination is very likely caused by homoeologous or homologous recombination than tetrasomic inheritance, but still need to be confirmed by cytological analysis; due to self-incompatibility and great segregation of favorite traits of most rose cultivars, it is not a good choice to use the K5 rose population as a model plant to study if the rose population would shift from disomic to tetrasomic or not after several generation of self-crossing.

This statistical analysis of multilocus gene can be used for constructing the 'haplotype' of tetraploid species, even with unknown genetic background of polyploidy species, and if the primers are highly polymorphic, which could help to link enormous markers with different haplotype by combining other software. In addition, this method can help to find the progeny following disomic inheritance, so as to construct more accurate linkage map.

Chapter 3 Genetic differentiation research on two groups of roses

3.1 Introduction

Most plants population species show different levels of genetic structuring, which may be caused by a variety of non-mutually exclusive factors, like environmental barriers, historical processes. Because the genetic structure reflects the alleles exchange condition between populations, understanding gene flow and drift become to be the central to many research fields including population genetics and ecology, conservation biology and epidemiology (Francois and Nicolas, 2002). The application of molecular marker for identifying the phylogenetic relationship has been done successfully in roses and many other species (Esselink et al., 2002; Chistiakov et al., 2006). Since the development of polymerase chain reaction (PCR) in 1980s (Saiki et al., 1985), microsatellites have been widely used for investigating the genetic structure analysis and addressing questions in the evolutionary and conservation biology (Francois and Nicolas, 2002; Oddou-Muratorio et al., 2004; Andrea et al., 2005; Korkovelos et al., 2008; Smulders et al., 2009).

Genetic diversity is important for the maintenance of the variability and evolutionary of populations and species. Population can avoid fitness declines caused by environmental change through phenotypic plasticity, by migration to other localities, and by adaptation (Jump et al 2006; Rolf et al 2008). There are two principle forms of genetic diversity: neutral genetic variation and adaptive or selective genetic variation (Rolf et al 2006). The neutral genetic diversity (Fst) refers to a gene or a locus that has no (or almost no) effect on the fitness. The neutral molecular markers have been used for studying for current dispersal, gene flow, and migration for over two decades (Rolf et al 2008). The selective genetic variation commonly refers to a quantitative trait (Qst), rarely a gene. Commonly it is analyzed in quantitative genetic experiments under controlled and uniform environmental conditions (Rolf et al 2006). Several researches indicated that Fst at neutral microsatellites markers are not influenced by diversifying selection (selection heterogeneity) in the outcrossing populations (Charlesworth et al., 1997; Latta, 1998; Rolf et al 2006; Porcher et al 2006; Rolf et al 2008). According to Porcher et al (2006), only highly selfing species with strong linkage disequilibria among loci, their Fst at neutral microsatellites markers are influenced by selection heterogeneity. They found that in predominantly selfing species *Arabidopsis thaliana* that evolved during 8 generations, the genetic differentiation of neutral microsatellites is much larger under heterogeneous selection than under uniform selection.

To estimate the connection and patterns of gene flow among populations, extremely widely researches have been done rely on Fixation index (F_{ST}, F_{IS} F_{IT}) (Wright, 1951), a parameter describing the degree of genetic differentiation among population, which measures the mean proportion of total genetic variance contained

in a population (Olivier, 2003). The F_{IS} measures the homozygosity of alleles within individuals of sub-populations as compared to Hardy Weinberg expectation, also called inbreeding coefficient. It compares the average observed heterozygosity (Ho) of individuals in each subpopulation and Hardy Weinberg expected heterozygosity (He) for all subpopulations. The average difference between Ho and He is due to non-random mating. Another F_{IT} measures the homozygosity of individuals in the total population and thus measures the deviation from this Hardy Weinberg expectation. The three indices are connected by the famous equation $(1-F_{ST})= (1-F_{IS})(1-F_{ST})$ (de Meeûs and Goudet, 2007; Matthew, 2009).

The interpretation of F_{ST} values can be difficult task, for instances, same F_{ST} values can be obtained from different patterns of allele frequencies (Wright, 1978; Hartl and Clark, 1997). Two extreme values for F_{ST} are 1 and 0. A value of 1 stands for no diversity within subpopulations, the other extreme value 0 means that the samples within a panmictic (means random mating) unit (Francois and Nicolas, 2002). For further interpretation of F_{ST}, it has been indicated a value range from 0 to 0.05 stands for little genetic differentiation; between 0.05 and 0.15 means moderate differentiation; value from 0.15 to 0.25 means great differentiation, the value above 0.25 means very great genetic differentiation (Hartl and Clark, 1997). For the other indices F_{IS} and F_{IT}, value range from -1 to 1. If the value is negative, it means that Ho is larger than He, due to outbreed between subpopulations. If the value is positive, then more inbreeding between subpopulations (Gangbiao, 2009; Hoda et al., 2009).

Canadian garden roses are characterized by winter hardiness. The aim of Canadian garden roses breeding is combine high level of winter hardiness with disease resistance, long flowering season and appealing flower traits. The inheritance of winter hardiness study indicated that this trait is controlled by few genetic factors (Felicitas, 1979). Investigation of genetic differentiation among European garden roses, Canadian garden roses and cut roses will be useful for breeding new rose cultivars with improved traits.

Black spot is one of most serious and wide spread disease in roses. Since the dominant resistant gene Rdr1 was discovered in 1998, several molecular markers for the gene have been developed (Von Malek et al., 2000; Kaufmann et al., 2003; Terefe and Debener, 2010). Rd1LRR is developed from a highly conserved part of LRR region of 9 completely sequenced Rdr1 paralogues of black spot resistant *Rosa multiflora* genotype (Terefe and Debener, 2010). According to Terefe and Debener (2010), amplification of 34 rose cultivars resulted in 52 distinct fragments, representing a minimum of 52 different NBS-LRR genes. The high diversity for the number and allelic composition of the Rd1LRR provide an opportunity to investigate the differentiation of this black spot resistant gene *Rdr1* among different rose species, include wild species and cultivars, during modern rose breeding. In this study, we will use this Rd1LRR multilocus SSR marker to detect the genetic diversity among rose cultivars and wild rose species.

To be able to compare the genetic variation of European garden roses and Canadian garden roses, several neutral microsatellites markers with good polymorphism will be selected to determine the degree of differentiation among 3 groups of rose

cultivars. The F_{ST} at these neutral SSR markers can be directly used for analyzing the genetic variation and population differentiation among European garden roses, Canadian garden roses, and cut roses.

3.2 Material and method

3.2.1 Plant Material

56 roses varieties with various ploidy levels were selected, including 30 wild species from four sections *Caninae, Synstylae, Gallicanae, Pimpinellifoliae* (Victoria and Peter, 2009; Roberts et al., 2009) (Table 12.), and 26 modern rose cultivars. These 56 rose varieties will be used for analyzing the similarity and differentiation of this *Rdr1* paralogues based on the LRR conserved region. Genomic DNA isolation follows the same protocol with chapter 1.

Table 12. List of the ploidy level and categories of 56 cultivars

Number		Subgenus *Rosa*	Ploidy
P1	cv28	Rootstock	2N=6
P2	cv29	Rootstock	2N=6
P3	cv31	Cut roses	2N=4
P4	cv48	Cut roses	2N=4
P5	cv49	Cut roses	2N=4
P6	cv50	Cut roses	2N=4
P7	cv52	Cut roses	2N=4
P8	cv57	Cut roses	2N=4
P9	cv62	Cut roses	2N=4
P10	cv166	Cut roses	2N=4
P11	cv555	Cut roses	2N=4
P12	cv566	Cut roses	2N=4
P13	RS59	Diploid rose cultivar	2N=2
P14	RS60	Diploid rose cultivar	2N=2
P15	RS55	Diploid rose cultivar	2N=2
P16	RS92	Diploid rose cultivar	2N=2
P17	RS18	Diploid rose cultivar	2N=2
P18	B2.05	Diploid rose cultivar	2N=2
P19	RS436	Diploid rose cultivar	2N=2

P20	RS444	Diploid rose cultivar	2N=2
P21	RS442	Diploid rose cultivar	2N=2
P22	RS447	Diploid rose cultivar	2N=2
P23	117	Diploid crossing parents	2N=2
P24	119	Diploid crossing parents	2N=2
P25	P867	Tetraploid crossing parents	2N=4
P26	P540	Tetraploid crossing parents	2N=4
P27	*R. canina*	Section *Caninae*	2N=5X
P28	*R. villosa mollis*	Section *Caninae*	2N=3,4X
P29	*R. inodora*	Unknown	2N=6X
P30	*R. dumalis*	Section *Caninae*	2N=5X
P31	*R. sherardii*	Section *Caninae*	2N=4,5,6X
P32	*R. caesia*	Section *Caninae*	2N=5X
P33	*R. canina*	Section *Caninae*	2N=5X
P34	*R. corymbifera*	Section *Caninae*	2N=5X
P35	*R. corymbifera*	Section *Caninae*	2N=5X
P36	*R. arvensis*	Section *Synstylae*	2N=2X
P37	*R. arvensis*	Section *Synstylae*	2N=2X
P38	*R. gallica*	Section *Gallicanae*	2N=4X
P39	*R. glauca*	Section *Caninae*	2N=4X
P40	*R. glauca*	Section *Caninae*	2N=4X
P41	*R. jundzillii*	Section *Caninae*	2N=6X
P42	*R. jundzillii*	Section *Caninae*	2N=6X
P43	*R. tomentella*	Section *Caninae*	2N=5X
P44	*R. subcollina*	Section *Caninae*	2N=5X
P45	*R. elliptica*	Section *Caninae*	2n=5X
P46	*R. tomentella*	Section *Caninae*	2N=5X
P47	*R. canina*	Section *Caninae*	2N=5X
P48	*R. micrantha*	Section *Caninae*	
P49	*R. spinosissima*	Section *Pimpinellifoliae*	2n=4x
P50	*Rosa x irregularis*	Unknown	
P51	*R. tomentella*	Section *Caninae*	2N=4,5X
P52	*R. corymbifera*	Section *Caninae*	2N=5X
P53	*R. rubiginosa*	Section *Caninae*	2N=5X

P54	*R. damascena*	Section *Gallicanae* (unclear)	2n=4x
P55	*R. damascena*	Section *Gallicanae* (unclear)	2n=4x
P56	*R. damascena*	Section *Gallicanae* (unclear)	2n=4x

Three groups of rose cultivars (Appendix I), including Canadian garden roses (17 cultivars contain Parkland and Explorer series), European garden roses (93 cultivars), and cut roses (11 cultivars), were selected for analyzing the genetic differentiation (F_{ST}) using neutral genetic markers (SSRs).

3.2.2 Microsatellite markers

Rd1LRR primer developed by Debener (Terefe and Debener, 2010) was used for PCR amplification of these rose cultivar and wild species (56 cultivars) (Table 12).

According to the test results of the polymorphism quality of SSR primer pairs, 14 SSR primer pairs (Table 13) with good polymorphism were used to generate SSR markers from 150 primer pairs (Appendix II) investigated by Debener (Tested by Paul Arens), and the genomic DNA isolation and PCR protocol follow the same procedure with K5 tetraploid rose population.

Table 13 List of primer pair used for PCR amplification.

Marker	Motive	Primer sequences (5'-3')	Expected product length (bp)	Allele No.	Ann. Temp. (°C)
RMS003	GA	F: TGGGAAAGGGAAAGCAACA R: AAGGTAGGCAGAAGTGACAGACAT	151	10/11	50
RMS008	GA	F: TCTCTGCGACAAAAACAAACACT R: CCATGAAGCGGCGGAGAGGA	176	7	53
RMS015	GA	F: TAATGTAGGCAGATATAAAGGAGT R: GCAGCTGCACAACAAGGAA	185	10/11	50
RMS017	AT&GT	F: AGGTCCCGTTATTTCAGG R: AGTTGGCTTATGGCTTTTT	246	8	50
RMS029	GA	F: GGATAAAACCAACGGGACAGACTC R: TCCGACACCATCCCTCCTACATAA	201	7	55
RMS034	GA	F: GCTTCTCGGTCTCGTGCTCTC R: CTCCCGCTCAAATCAATAAATCTC	136	10	55
RMS047	GA	F: GCTCCCTCAATTTCCACTCA R: ACCAACCCAATTCGCTCAT	98	8	50
RMS062	GA&GT	F: GCGAACGGCATTTACTTGT R: GGTTGTTCTGGGTGGTTTTT	189	11	50

RMS080	GT	F: GCTTTCAAAGATGGGAAACCT R: TTGGTATCACATTTACTCTCATTGC	213	9	50
RMS082	2xGA	F: AACAACACACGCGGAATATG R: TGCAGTTGGAGTTGGAGTTG	113	7/8	55
RMS085	GA	F: ATGCCCATGACTATCTTGCC R: TCCAAGATGAAGAATTGCGG	204	-	55
RMS088	GA	F: TCCTGATTCGTATCATCCACTG R: GAAGGCCTCAAGGTTCCTCT	207	10	55
RMS097	GA&GT	F: ATCTGGCTGAACACCACACA R: CATGCTAACTCTCCATGTTCCA	163	5	55
RMS112	AT&GT	F: CAAGGATACCAGTCGGAGAGA R: AGAAATGGACAGCTCCGAAA	227	5	55

The PCR amplified products were diluted in 1:10 in the loading buffer and electrophoresis on the 6.5% polyacrylamide gel by using LI-COR 4200 analyzer and LI-COR 4300 analyzer. The presence of SSR alleles was scored allele by allele; no attempt was done to score the allele dosage.

Genetic differentiation statistical analysis

Statistical analysis was carried by two softwares: SPAGeDi 1.3 (Spatial Pattern Analysis of Genetic Diversity)(Hardy and Vekemans, 2002) for population genetic analysis and NTSYS 2.02 (Rohlf, 1998) for clustering analysis based on molecular data with the unweighted pair group method using arithmetic average (UPGMA). The genetic distance among these cultivars was assessed by a PCO plot analysis. SPAGeDi 1.3 can handle the data from the plants with various ploidy levels, the genetic differentiation (F_{ST}) across regions (European and Canada) was also calculated by this software.

3.3 Results

3.3.1 Genetic variation among rose cultivars and wild rose species based on the Rd1LRR

PCR analysis with Rd1LRR in 30 wild species (lane 27-56) and 26 rose cultivars (lane 1-26) with different ploidy levels resulted in 82 clear and informative fragments (see figure 4.).

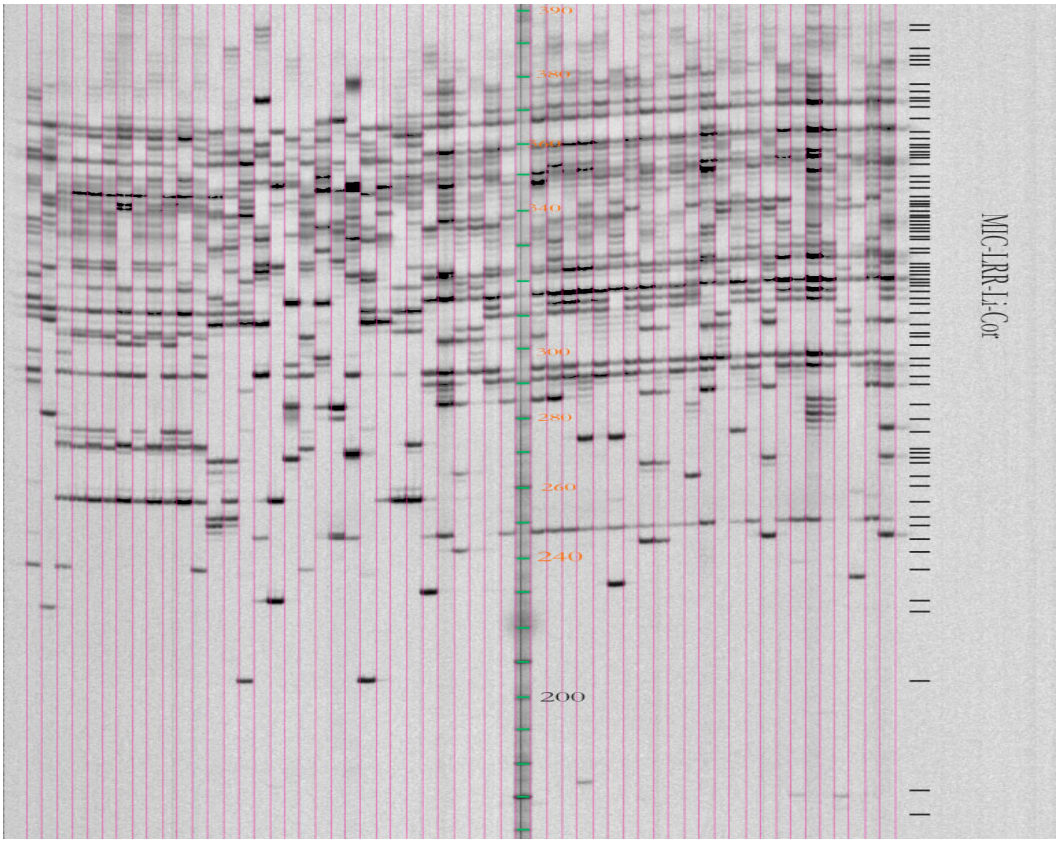


Figure 4. Polyacrylamide gels of Rd1LRR fragment patterns for 56 rose cultivars. Lanes1 and 2 are rootstocks, lanes 3 to 12 are tetraploid cut roses, lanes 13 to 22 are diploid roses varieties, lanes 23 and P24 are diploid crossing parents of 1 rose population, lanes 25 to 26 are tetraploid crossing parents of K5 rose population, lanes 27 to 56 are wild species (Table 12).

The UPGMA tree was generated based on the data on 82 informative bands. The 56 rose samples were divided into 2 main clusters at 0.72 similarities coefficient based on the SSR data (figure 5.). The wild species (P27-56) and 1 rootstock cultivar were

placed in 1 main cluster. The rest of the rose cultivars with different ploidy level (Table 12) were separated into another one. In the first cluster, the highest genetic similarity coefficient (0.99) among the entire 56 samples was P39 (*R. glauca*, 2n=4x) and P46 (*R. tomentella*, 2n=5x), P37 (*R. arvensis*, 2n=2x) and P42 (*R. jundzillii*, 2n=6x). Taking 0.75 as a threshold, the second main cluster was divided into 3 sub-clusters, in which tetraploid crossing parents P25 (P867), P26 (P540) and tetraploid cut roses clustered together. The diploid crossing parents P23 (117), P24 (119) were placed with diploid rose varieties, but 1 rootstock variety (P2 2n=6x) was also included into this sub-cluster, and the similarity coefficient between P2 and P24 is more than the one between P23 and P24. The third sub-cluster contained 4 diploid varieties (P16, 18, 20, 22), but these are quite dissimilar.

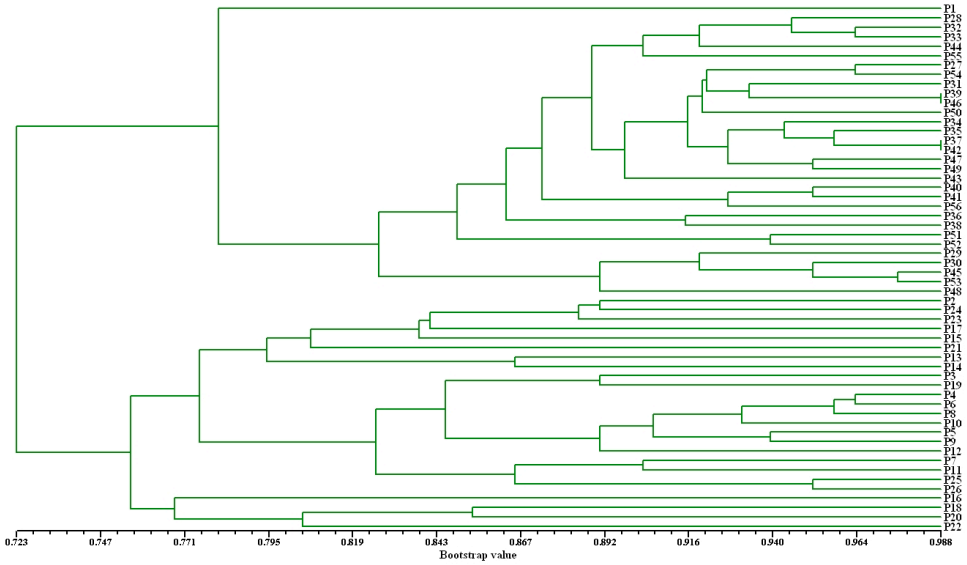


Figure 5. Cluster analysis (UPGMA) of rose varieties based on 82 bands amplified from the Rd1LRR primer. Numbers P1-P2 are rootstocks, P3-P12 are tetraploid cut roses, P13-P22 are diploid roses varieties, P23-P24 are diploid crossing parents of 1 rose population, P25-P26 are tetraploid crossing parents of 1 rose population, P27-P56 are wild species with different ploidy levels.

3.3.2 Genetic differentiation among 3 groups of rose cultivars: European garden roses, Canadian garden roses, and cut roses.

PCR analysis with these 14 primers, of which 11 primers were amplified, except for primers RMS080, RMS088, RMS127, different numbers and sizes of bands obtained from these 11 primers (table 14). They were scored with consensus numbers. Of 11 amplified primers, primer RMS017 showed the most diverse bands pattern, and the range of fragment size is also the largest among these 11 pairs of primers, is from 209bp to 278bp. Genetic differentiation (F_{ST}) among different groups was calculated by SPAGeDi 1.3 (table 15).

Table 14 List of amplified primer pairs.

Marker	Motive	Fragment length (bp)	No. of allele
RMS003	GA	142-210	24
RMS008	GA	147-192	22
RMS015	GA	137-232	29
RMS017	AT&GT	209-278	32
RMS029	GA	200-234	16
RMS034	GA	122-189	31
RMS047	GA	115-150	20
RMS062	GA&GT	155-201	24
RMS082	2xGA	108-147	16
RMS085	GA	191-257	21
RMS097	GA&GT	164-200	13

According to the F_{ST} value calculated by SPAGeDi, it had been found that only Two F_{ST} values from the average value of all loci are larger than 0.05. Two F_{ST} values were closely to 0.05. The results suggested that genetic differentiation between European garden roses and Cut roses is moderate differentiation based on the interpretation of F_{ST} value by Wright and Hartl & Clark. However, the genetic differentiation in garden roses as a function of geographical distance is 0.0169, indicating only a small amount of genetic differentiation between European garden roses and Canada rose. In addition, two subcategory of Canadian garden roses genetic differentiation was estimated at 0.0125. All F_{IS} and F_{IT} value calculated by SPAGeDi are negative (table 16).

Table 15. Genetic differentiation (Fst value) of 3 groups of cultivars

Group	3 cato (European +Canadian+ Cut Rose)	2 cato (European+ Cut Rose)	2 cato (Canadian+ Cut Rose)	2 cato (European+ Canadian garden roses)	2 cato (Canadian Parkland+ Canadian Explorer)
			F_{ST} value		
ALL LOCI	0.0449	0.0844	0.0576	0.0169	0.0125
Primer 3	0.0503	0.0858	0.0373	0.0315	-0.0209
Primer 8	0.0501	0.1189	0.1149	0.0143	0.0046
Primer 15	0.0242	0.0554	0.0409	0.0034	-0.0171
Primer 17	0.0209	0.0529	0.0371	0.0038	0.0068
Primer 29	0.0707	0.1232	0.0295	0.0421	0.0485
Primer 34	0.0072	0.0166	0.0079	0.0027	-0.0077
Primer 47	0.0435	0.0935	0.064	0.012	-0.0056
Primer 62	0.0216	0.0518	0.0515	0.0022	0.0086
Primer 82	0.0715	0.1221	0.119	0.0043	0.0666
Primer 85	0.0686	0.1002	0.0833	0.0348	0.0468
Primer 97	0.0721	0.1124	0.0406	0.0395	0.0106

Table 16. Fixation indices (F_{IS}, F_{IT}) for each locus and 3 groups of rose cultivars.

	3 cato (European +Canadian+ Cut Rose)		2 cato (European+ Cut Rose)		2 cato (Canadian+ Cut Rose)		2 cato (European+ Canadian garden roses)		2 cato (Canadian Parkland+ Canadian Explorer)	
Locus	Fit	Fis	Fit	Fis	Fit	Fis	Fit	Fis	Fit	Fis
ALL LOCI	-0.1374	-0.1909	-0.0965	-0.1975	-0.0743	-0.1399	-0.1772	-0.1974	-0.145	-0.1595
Primer3	-0.0875	-0.1451	-0.0461	-0.1443	-0.1084	-0.1514	-0.1085	-0.1445	-0.1622	-0.1385
Primer8	-0.1429	-0.2032	-0.0662	-0.2101	-0.0259	-0.1591	-0.189	-0.2063	-0.1606	-0.1659
Primer15	-0.1046	-0.132	-0.0669	-0.1296	-0.0851	-0.1315	-0.1304	-0.1343	-0.1577	-0.1382
Primer17	-0.087	-0.1102	-0.056	-0.115	-0.0382	-0.0782	-0.1092	-0.1133	-0.0802	-0.0876
Primer29	-0.1954	-0.2863	-0.1542	-0.3164	-0.1087	-0.1423	-0.2513	-0.3062	-0.1218	-0.179
Primer34	-0.1426	-0.1508	-0.1366	-0.1558	-0.0675	-0.076	-0.1568	-0.1599	-0.119	-0.1104
Primer47	-0.1594	-0.2121	-0.111	-0.2257	-0.0629	-0.1356	-0.2052	-0.2198	-0.1439	-0.1375
Primer62	-0.0716	-0.0953	-0.04	-0.0968	-0.0372	-0.0934	-0.092	-0.0944	-0.0813	-0.0907
Primer82	-0.1515	-0.2402	-0.0803	-0.2306	-0.0629	-0.2065	-0.253	-0.2584	-0.2829	-0.3745
Pirmer85	-0.1138	-0.1958	-0.0829	-0.2035	-0.0529	-0.1486	-0.1576	-0.1994	-0.1088	-0.1633
Primer97	-0.2852	-0.385	-0.2535	-0.4123	-0.1852	-0.2353	-0.3445	-0.3998	-0.205	-0.2178

The genetic distance matrices among 121 rose cultivars calculated by SPAGeDi. It was modified into dissimilarity matrices then was imported in NTSYS 2.02 to do Principal coordinates analysis (PCO). 3D PCO analysis of the main variation among the 11 neutral SSR markers showed the same result with genetic differentiation calculated by SPAGeDi (figure 6). In this figure, cut rose group was separated clearly from European garden rose group and Canadian garden roses group. Although the major plot indicated the genetic differentiation between European garden roses and Canadian garden roses is small, there were still some exceptions. For instance, 2 European garden roses cultivars 19 and 88, 3 Canadian garden roses (explorer series) cultivars 2, 5 and 8 located outside the major dots area. It can be observed clearly on the 2D PCO pictures (figure 7). The PCO analysis results were confirmed by software R (R Development Core Team, 2005)(figure 8). In case of labeling mistake for these four cultivars, for instances, these four cultivars' genomic DNA probably extracted from rootstock, or data shifting mistake, we exclude these four and cut rose group, then computer data. Figure 9 and 10 show the sorted data of 2 groups of cultivars. Within same group, not all the Canadian garden roses plot together, same with the European garden rose. There is one relatively main plot shown in figure 9, but no clear cut between groups or even within groups were found to be grouped together, although the genetic distance between Canadian groups and European groups is smaller than the cut rose.

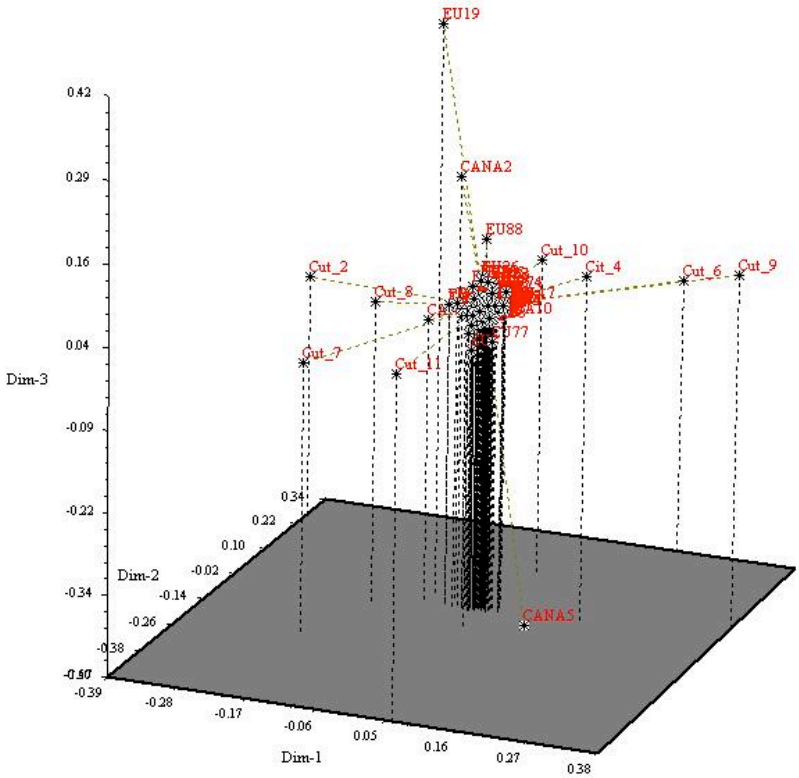
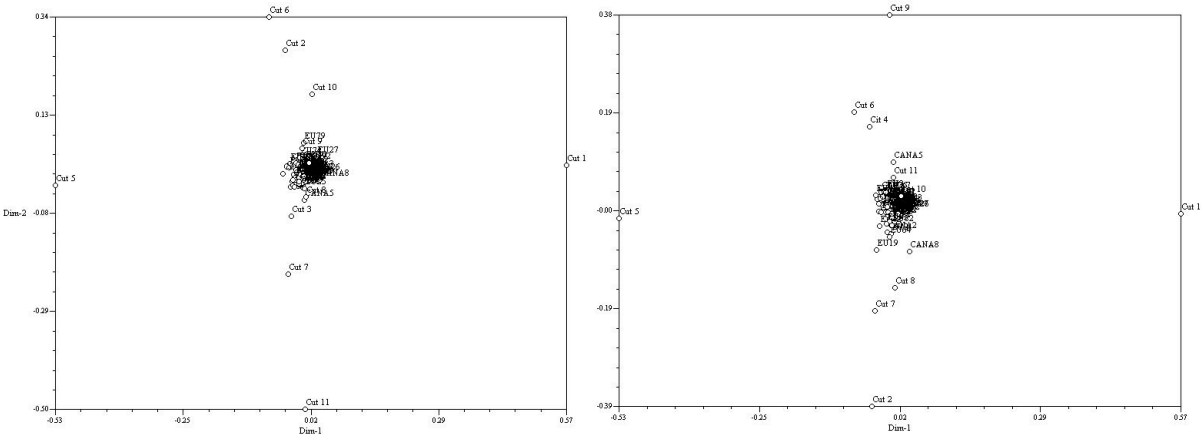


Figure 6 3D PCO analysis computed by NTSYS 2.02: Genetic similarity among European garden roses, Canadian garden roses and cut roses.

Cana: Canadian garden roses (explorer series)

CA: Canadian garden roses (Parkland series)

Eu: European garden roses cultivars



Cut: cut roses

Figure 7. 2D PCO analysis computed by NTSYS 2.02: Genetic similarity among European garden roses, Canadian garden roses and cut roses

Cana: Canadian garden roses (explorer series)

CA: Canadian garden roses (Parkland series)

Eu: European garden roses cultivars

Cut: cut roses

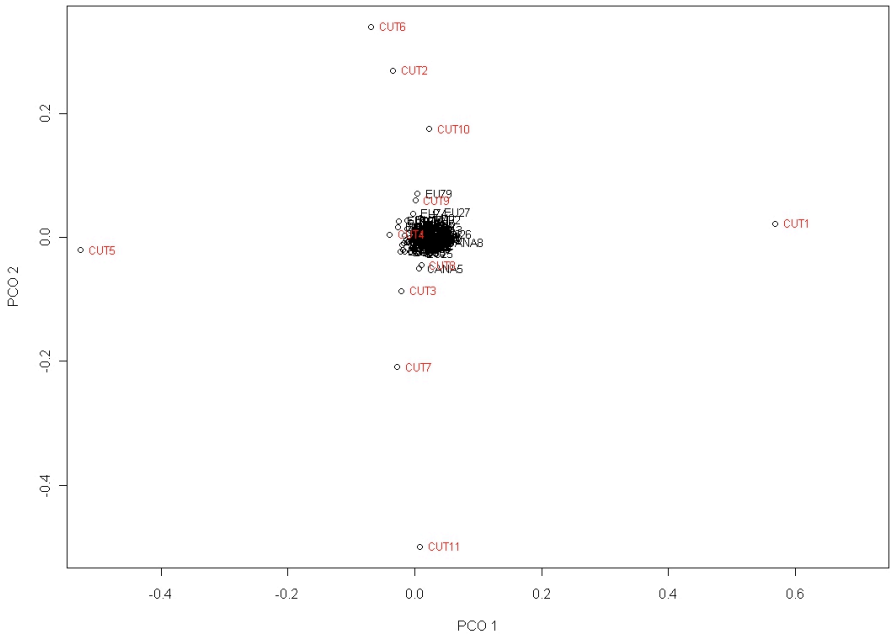


Figure 8. 2D PCO analysis of genetic differentiation among 3 groups of rose cultivars (computed by software R)

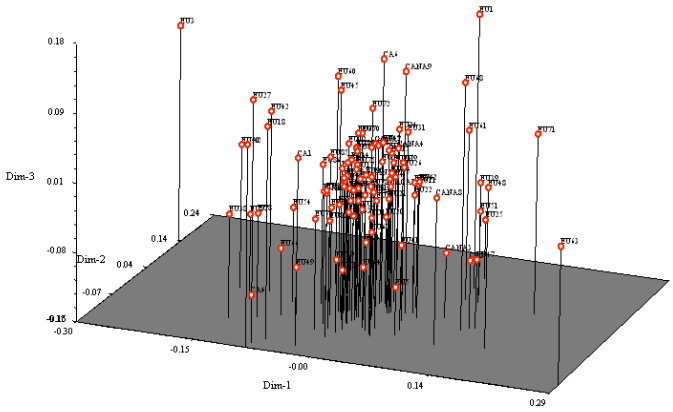


Figure 9. 3D PCO analysis of genetic differentiation among 3 groups of rose cultivars

Cana: Canadian garden roses (explorer series)

CA: Canadian garden roses (Parkland series)

Eu: European garden roses cultivars

However, Canadian roses also do not represent a clear separate part of the parameters space (see figure 10), it is very difficult to define if that the European roses "contain" Canadian roses or Canadian roses group occupy part of European roses. The result is probably due to small size of this Canadian rose groups or different breeding program all involved similar gene pool. In addition, if go back to the original data, the Canadian rose didn't show large different number of alleles with European roses cultivars. CANA9 showed lower alleles number than other Canadian roses cultivars in some locus, but it also didn't separate far from other rose cultivars, hence one can't expect that they share less effective number of alleles with other European roses.

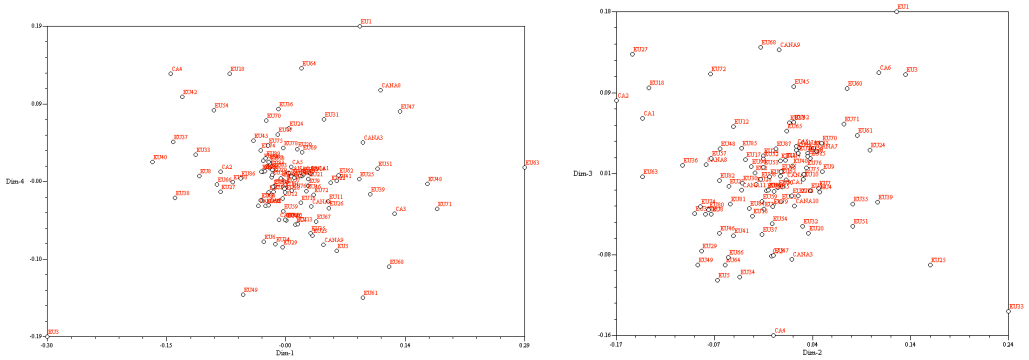


Figure 10. 2D PCO analysis of genetic differentiation among 3 groups of rose cultivars

Cana: Canadian garden roses (explorer series)

CA: Canadian garden roses (Parkland series)

Eu: European garden roses cultivars

3.4 Discussion

3.4.1 Compare genetic diversity of LRR region of Rdr1 paralogues among rose cultivars and wild rose species.

In dendrogram, only the rose cultivars and wild rose species were classified into 2 major clusters. The similarity among wild rose species and even within the same section of *Caninae*, the variability was detected (see figure 5). We found even same wild species from the same section can be grouped with the ones from different sections together into the same sub-clusters. For example, diploid wild species *R. arvensis* (P37) from section *Synstylae* is closely related to hexaploidy *R. jundzillii* from Section *Caninae*, these two wild rose species shared the same banding pattern of Rdr1 paralogues, which was also found between tetraploid R. glauca (P39) and pentaploid R. tomentella (P46). Meanwhile, the other hexaploidy *R. jundzillii* (P41) seems more similar with tetraploid species *R. glauca*. Similarly with *R. canina* (P47) and *R. spinosissima* (P49).

In addition, this *Rdr1* was found in diploid species *R. multiflora* belonging to section *Synstylae* (Debener et al., 1998), which could probably not be the only original resources for contribution to this resistant gene differentiation, if looking back to the breeding process of the modern cultivated rose, or the formation of this specific black spot resistant region involved in more other resources (Stewart, 1969). But within the other main cluster (non-wild species), it can be found that the sub-cluster contained relatively identity ploidy level. It is still need to be approved that whether the formation and differentiation of this specific black spot resistant gene could have yet done before the appear of modern rose cultivars, or involved many resources in further. However, it is very difficult to define the evolutionary history of this blackspot resistance through the whole modern rose breeding. Because the selection pressure may keep the locus intact in many species, and all progenies of these wild species without this resistant gene may extinct also. Hence in the natural populations, it is very hard to trace back the evolutionary history of this specific resistant gene. One solution is that based on neutral markers, to test the genetic relationships among wild species and cultivars, and then probably the evolutionary history of this resistant gene can be defined. But due to its highly diversifying patterns, *Rdr1* could be used for testing the segregation of Rdr1 gene cluster in different populations.

According to the similarity comparison between different ploidy level and sections of subgenus *Rosa*, it indicated a high genetic diversity in this Rdr1 gene family, which is consistent with the result from Tefere and Debener (2010). In the wild species, selection pressure by the environment and multiple pathogenic races of *Diplocarpon rosae* wolf probably lead to multiple paralogs of this Rdr1 gene. It has been found different black spot races in several countries (Debener, 1998; Uggla

Carlson-Nilsson; Whitake and Stan, 2009). However, in most wild species and modern rose cultivars are susceptible to this disease. And during the breeding of modern rose cultivars, human selection would also effect this Rdr1 gene paralogs combination, for instance, select cultivars from different gene pool to breed new cultivar and population. Therefore, ploidy level could be one reason to interpret the second cluster of modern cultivar, because after several years of human selection, they may gradually share relative more similar gene pool. But for wild species, they are under different environmental conditions, and after many generations natural selection, the unfavorable type could be eliminated. And also different isolates of pathogen in different local environments may also lead to various genotypes of wild rose species (Debener, 1998). Hence it is very difficult to analyze the evolutionary of this black spot resistant gene Rdr1 based on our set of cultivars. Together with the modern rose breeding information and using some neutral SSR marker may allow us to find the resistant gene's evolutionary information. But, the high diversity within the LRR region itself can provide sufficient variability for the creating new resistance specificities and for studying the genetic diversity among different cultivars.

3.4.2 Genetic differentiation among European garden roses, Canadian garden roses, and cut roses cultivars

Generally, the genetic differentiation F_{ST} value of 0.05 will be considered as the reasonably low; the structuring condition between populations would be interpreted as weak. While this F_{ST} value range could shift from different species (Francois and Nicolas, 2002), for instances, the populations of the European eels sampled from Iceland to North America (Wirth and Bernatchez, 2001). It may also not be the representative value range in *Rosa*. But according to the research on 734 hybrid tea rose varieties, the genetic differentiation among 17 breeding companies is F_{ST}=0.0056+/-0.0011 (Smulders et al., 2009). The result shows that these 17 breeding companies could select the same gene pool. Hence, this general value range mentioned above could be suitable for interpreting the genetic differentiation value in *Rosa*.

Compare with Canadian garden roses population, the F_{ST} is similar with the genetic differentiation value between 2 subpopulations (Parkland series and Explorer series) of Canadian garden roses. These two subpopulations grow in different regions of Canada, and the winter hardiness level is also not the same. Considering no mutation as a hypothesis, then the degree of genetic differentiation is related to the number of migrants, $F_{ST}=(1+4N_em_e)^{-1}$(Wright, 1931). Because Canadian rose and European roses are not natural population or wild species, in this case, the migrants indicate the numbers of genotypes that breeders have taken from other breeding programs to do their own program, or the number of their shared introgressions from same wild species. The negative values of FIS and FIT indicate that the loss of heterosigotes between Canadian and European roses groups may be due to

frequently gene flow or bred from similar wild species. The slightly different F_{ST} value between 0.0169 and 0.0125 indicated that two groups of cultivars have the similar gene pool. While the highly mutation rates of microsatellite markers during evolution, as the mutation cannot be neglected from the migration, so the F_{ST} will underestimate the real differentiation in the highly structured population (Wright, 1978), however, the high mutation rate of microsatellite itself has been proved not a problem, due to high heterozygosities, which reduce the random variation between loci (Beaumont and Nichols, 1996). Hence the real F_{ST} value between European garden roses and Cut rose cannot be lower or too much lower than the value 0.08 calculated by SPAGeDi based on the microsatellite data; it means the genetic differentiation between European garden roses population and Cut rose population, Canadian garden roses population and Cut rose population are moderate. From figure 9 and 10, we have found within the European group, it showed vague plot, and some of Canadian cultivars were also spread into this unclear cut plot. While the differentiation between European garden roses population and Canadian garden rose population is little according to the general interpretation of F_{ST} value. The results indicate that generally two groups of garden roses cultivars share similar gene pool. And if discard the mistake in scoring and labeling of the cultivars, these two European garden rose and two Canadian garden rose were very likely to be selected from different gene pool in case of labeling mistake of rootstock plants. And for cut rose group, probably they didn't share same gene pool with the other two groups. This result was consistent with PCO plot analysis. It is clear cut for the main plot and cut rose group.

3.5 Conclusion

The black resistant Rdr1 gene analogues show great variation between wild rose species and rose cultivars, even within each section or same species based on this specific region. For the estimation of the genetic diversity between rose cultivars and wild rose species and the highly diverse of the Rdr1 paralogues indicate that Rdr1 will be a powerful tool to analyze the genetic differentiation about this locus within *Rosaceae*.

According to the F test and PCO plot analysis, less genetic differentiation was found between Canadian garden roses and European garden roses, but also two European garden rose cultivars and two Canadian garden rose cultivar were found to have large genetic differentiation among 3 groups of cultivar, the results suggesting most of the Canadian garden rose and European garden rose share similar gene pool; in addition, through remodifying the data format of non neutral Rdr1 LRR multilocus marker, the general genetic differentiation (F_{ST}) among wild species, cut rose, and garden rose may also be comparable in further study. For cut rose group, the F_{ST} and PCO plot analysis result suggest that cut rose cultivars were generated by using different gene pool from the garden roses.

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Appendix I

3 groups of rose cultivars (European garden roses, Canadian garden roses, cut roses)

	Cultivar name	Population Catogery
1	Abraham Darby	European garden roses
2	Adelaide Hoodless	Canadian garden roses (Parkland Series)
3	Alan Titchmarsh	European garden roses
4	Alexander McKenzie	Canadian garden roses (Explorer Series)
5	Amber Queen	European garden roses
6	Amelia renassaince	European garden roses
7	Anna Purna	European garden roses
8	Apple blossom	European garden roses
9	Astrid Lingren	European garden roses
10	Betty Harkness	European garden roses
11	Buttercup	European garden roses
12	Caribia	European garden roses
13	Charles Darwin	European garden roses
14	Charlotte	European garden roses
15	Christopher Marlowe	European garden roses
16	City of London	European garden roses
17	Claire rose	European garden roses
18	Climbing Bonica	European garden roses
19	Compassion	European garden roses
20	Cream ambudance	European garden roses
21	Crown princess Margareta	European garden roses
22	Cygne noir	European garden roses

23	Cuthbert Grant	Canadian garden roses (Parkland Series)
24	David Thompson	Canadian garden roses (Explorer Series)
25	Desinger sunset	European garden roses
26	Diamond border	European garden roses
27	Double terrazza	European garden roses
28	Eglantyne	European garden roses
29	Escopade	European garden roses
30	Evelyn	European garden roses
31	Ferdinand Pitchard	European garden roses
32	FP/1	European garden roses
33	FP/2	European garden roses
34	FP/3	European garden roses
35	Gentle Hermione	European garden roses
36	Gertrude Jackyll	Canadian garden roses (Explorer Series)
37	Glowing pink	European garden roses
38	Graciously pink	European garden roses
39	Graham Thomas	European garden roses
40	Helene renaissance	European garden roses
41	Henrey Kelsey	Canadian garden roses (Explorer Series)
42	Heritage	European garden roses
43	Hope for humanity	Canadian garden roses (Parkland Series)
44	Irish hope	European garden roses
45	James Galway	European garden roses
46	Jens Munck	Canadian garden roses (Explorer Series)

47	Johan Franklin	Canadian garden roses (Explorer Series)
48	John Cabot	Canadian garden roses (Explorer Series)
49	John Davis	Canadian garden roses (Explorer Series)
50	JP Connel	Canadian garden roses (Explorer Series)
51	Kings Mac	European garden roses
52	L'aimant	European garden roses
53	Lavander dream	European garden roses
54	LD Braithwaite	European garden roses
55	Lemon coture	European garden roses
56	Leonardo da Vinci	European garden roses
57	Lilian Baylis	European garden roses
58	Madrigal	European garden roses
59	Margareth Merril	European garden roses
60	Majroire Marshall	European garden roses
61	Mary rose	European garden roses
62	Mayflower	European garden roses
63	Morden amaretto	Canadian garden roses (Parkland Series)
64	Morden centerial	Canadian garden roses (Parkland Series)
65	Mullard jubilee	European garden roses
66	Nadia renessaince	European garden roses
67	Nipper	European garden roses
68	Nostalgie	European garden roses
69	Orange terrazza	European garden roses
70	Othello	European garden roses

71	Papagena	European garden roses
72	Pat Austin	European garden roses
73	Patricia Kent	European garden roses
74	Pink terrazza	European garden roses
75	Pearl ambudance	European garden roses
76	Penny Lane	European garden roses
77	Perception	European garden roses
78	Perpetually yours	European garden roses
79	Peter Cottrel	European garden roses
80	Piccolo	European garden roses
81	Pink tiare	European garden roses
82	Princess Alexandra	European garden roses
83	Princess of Wales	European garden roses
84	Queen of Sweden	European garden roses
85	Samaritian	European garden roses
86	Sharifa Asma	European garden roses
87	Shephardess	European garden roses
88	Shorpshire lass	European garden roses
89	Snow goose	European garden roses
90	Snowdon	European garden roses
91	Songs of praise	European garden roses
92	St. Alban	European garden roses
93	Summer song	European garden roses
94	Sun hit	European garden roses
95	Sunset buolevard	European garden roses
96	Sweet dreams	European garden roses
97	Teasing Georgia	European garden roses
98	Therese Bugnet	Canadian garden roses

		(Explorer Series)
99	Tivoli	European garden roses
100	Velvet fragnance	European garden roses
101	Violet parfume	European garden roses
102	White lace	European garden roses
103	Wild Edric	European garden roses
104	Wildeve	European garden roses
105	William Baffin	Canadian garden roses (Explorer Series)
106	Winchester chatedrale	European garden roses
107	Winnipeg parks	Canadian garden roses (Parkland Series)
108	Y	European garden roses
109	unknow1	European garden roses
112	refcv11	Cut rose
113	refcv12	Cut rose
114	refcv14	Cut rose
115	refcv15	Cut rose
116	refcv16	Cut rose
117	refcv21	Cut rose
118	refcv22	Cut rose
119	refcv24	Cut rose
120	refcv28	Cut rose
121	refcv29	Cut rose
122	refcv30	Cut rose

Appendix II.

List of Primers from Thomas Debener

Name	Motive	Product	Tm	Primer F* 5'->3'	Tm	Primer R 5'->3'
RMS001	GT&GC	242	57. 1	TTCAAAATTGCTGCCCCCTTAG	44. 8	TACCAGTTGAGTGAGAAATAGTT
RMS002	GA	138	36. 5	AATAATTTTTCTTTTGGTA	36. 6	GATTTGTTTTCACTATTCA
RMS003	GA	151	52. 9	TGGGAAAGGGAAAGCAACA	53. 0	AAGGTAGGCAGAAGTGACAGACAT
RMS004	GT&AT	143	55.0	CAGGCCAAGGAAGAGGTAAGTAAA	55.7	CGTATGCGCGTGTAGGAAGG
RMS005	GA	143	53.1	CTACCGGTGACCAGTGACGA	51.9	ATTTTGCCCTCTCCCTTTGT
RMS006	GT&GA	114	53.0	ACCGGTCTCATCTTTCCATTG	52.2	GTAGGTCGGTCCGTCTGTCA
RMS007	GA	171	48.4	TCTTTCCGACTCCGACAA	54.8	TATGCCATTCAGACTCTCCAACAC
RMS008	GA	176	53.4	TCTCTGCGACAAAAACAAACACT	61.9	CCATGAAGCGGCGGAGAGGA
RMS009	CT&GT	145	47.3	ATTGGCAAAAGATTCTCCTAC	46.5	ACTTGGTAATTTCGAGCATAA
RMS010	GA	105	61.2	GGTTGGGGGAAATTGAAGCAGAGA	58.9	TCTTTTCTTCTACAAACCCCAACCAAC
RMS011	GT	190	47.9	TAGAAACGACCAATAAAAGAGG	48.0	TAACGAAACATCATCAATAGCA
RMS012	GT	141	48.8	ATAGAAAAATAGAGGGGGTGTG	46.4	GATCGAAAAGTGGTCAAAATA
RMS013	GA	208	57.8	GCCTTAGCCGGGGTTTTCAA	45.6	GATCAATACCGAACTAACAAAG
RMS014	GA	124	56.1	TATTCTTTCTTCCCACCGACGAC	56.2	CCTCACTGCCAACCCAACTGT
RMS015	GA	185	46.5	TAATGTAGGCAGATATAAAGGAGT	52.1	GCAGCTGCACAACAAGGAA
RMS016	GA	121	55.1	GGCCTGGACCTTTCTCATTTG	56.9	AACCGCTGCTGCTTTCATTTTT
RMS017	AT&GT	246	46.2	AGGTCCCGTTATTTCAGG	46.2	AGTTGGCTTATGGCTTTTT
RMS018	GT	125	46.4	TTTTGGGTGGGTAAGTTTT	48.0	TTGGCCAATAAGGAAGACA
RMS019	GA	104	57.1	ACCGTTTCCATTACCCTTTCACC	57.1	CGTCGGCCATGGATTTTTGTA
RMS020	GA	239	59.9	AGGCGCCCATGCAAAATCAA	47.9	TTCCTAACGCAAACTATGTAAAT
RMS021	GA	188	50.9	AATTCCCTCTTACCCAAAACAC	55.2	CCGGCGAAGTCCCCTATG
RMS022	GA	170	51.3	AAGAAGATAAATTAGGGGGAAAAA	52.6	GCGCGAACATATTGATTGGT
RMS023	GT	170	42.4	TTTGCTATTAATTACAGATGAA	51.3	TAAACAATATAAATGGGGGAGTAAAT
RMS024	AT&GT	200	44.0	ACTACTGTAAAATATGAAAAATCC	50.5	GTAGTAGCGGTTGCAAGAAAATA
RMS025	AT/GT	167	33.4	TAATGTAAGCTAACTAATCT	47.1	TTTTAAATTTTCGGTGGAGA
RMS026	GT	129	38.8	ATAGATATGTTTGGGTTCA	39.3	AATGTCAGGTTTTGTTATG

RMS027	AT&GT	189	47.7	ACCGTTGTGCTTATCAGGA	48.2	ATTGGTGGTGCTTTTACATTAC
RMS028	AT&GT	237	50.0	TAGGCAAGACCATGAACCAG	49.9	TGTGCCTGTTTGCTTGTGTA
RMS029	GA	201	56.80	GGATAAAACCAACGGGACAGACTC	58.3	TCCGACACCATCCCTCCTACATAA
RMS030	GA	201	46.2	GATAAATTTCAAGGCGAGAG	50.9	AAAAGATGAACGACCCAAATAAT
RMS031	GA	202	40.5	TATATTAAAGAACAAGTGAGAAC	43.1	GTGGCTATCGAAAAACAA
RMS032	AT&GT	193	40.7	AGAAACCAACCTTAGCAT	44.1	AACCATCCATATTTCAGTCA
RMS033	GA	203	60.0	CAAGAGATGTCGGAAAAGCAGGAAG	59.9	TGCACACCCAAATTTACAAACCACAT
RMS034	GA	136	55.6	GCTTCTCGGTCTCGTGCTCTC	55.2	CTCCCGCTCAAATCAATAAATCTC
RMS035	GA	229	58.3	CCTCCTTGGCAGCCTTTTCATT	56.1	ATCGGCTATCCACATCGTCTACAC
RMS036	GA	235	56.4	CTCGCGGCCCAAATAACAAT	55.9	TTGCCCTTACATTTTCTCTACTCCATA
RMS037	GA	228	59.9	AACCTCGGAGCCGCATTTCAC	52.1	AGTTTTCCTCGCCAGATAAGC
RMS038	GA	115	50.3	GTGATAAGAGCAAAACAAGATGG	53.8	CTCGCGGAAGCCTCAAAA
RMS039	2xGA	124	52.1	GCTGCTTTCTCCAATCAACAA	52.1	CAGCTCAGCAAAGGGGACTA
RMS040	GT	143	46.6	AACCCCAAACTTCCTAAACT	45.7	TCTGTATCTACTGTGGCTAACC
RMS041	GA	249	49.2	TTAACCCAAAGCACCAAAAT	48.5	ACCTTCACCGATGTATCACC
RMS042	AT&GT	181	55.4	GCATGGCCAGGCTCTTCAC	55.5	ATGCCAAACGTCTCAGTCAACC
RMS043	GA	215	52.6	GATCAAAGATGGGTTCTCCTCTC	54.6	AGGGGAATCTTTGAAAGTCGTTC
RMS044	AT	204	49.6	ACCGATGGATGGCAATAAC	49.7	ATACAGGACATAAACGGCTACC
RMS045	AT&GT&AT	233	40.0	GAAAATAAGGACATCATCTAC	41.4	GGTGCCTCCATTATTTAC&GA
RMS046	AT&GT	247	45.0	AAAGGATTGCTGGATGTG	42.4	TATTCGCGTGGACTCTAT
RMS047	GA	98	51.6	GCTCCCTCAATTTCCACTCA	51.7	ACCAACCCAATTCGCTCAT
RMS048	GA&AT	197	41.8	ATAAGTATGAAAAAGTAAAATGAT	44.0	GTATACTAGAAAAACAAAACTGGT
RMS049	AT&GT	178	39.9	AAAAATACAACCGAAAAA	52.6	CCAACCCGTCAAGGCTAAA
RMS050	AT&GA	169	43.1	TAAGCCTAAGAAAAACTCATT	48.6	CAGCCGTCAGATTCACTTG
RMS051	GT	215	46.5	AGTAGACTGTCCTCCATTTAGC	50.9	ATACCATCAGAGAAGAGACGACAC
RMS052	GA	224	59.8	TTAGCCGTTAATTGAGTCGACAACCT	57.0	TGATGAACCCAATAGAATGAAAACACGA
RMS053	GA	160	56.9	GGCGGTAGCTAGTGACTGGAATCT	55.4	CCCTTACCCTTACCCCTTTGTTAC
RMS054	AT&GA	239	48.8	CTGGGAGGAGAACTCTGTCA	48.7	TAGCTTATTAGTCTGCATTGATGA
RMS055	GA	192	53.4	TGATCACAAGAGCTTTTCAAGTTTAG	53.4	AGTTAGGCGCATGTACAAGAAAAT

RMS056	GA	133	36.7	TGTGTAGATTAGCATTCC	35.2	GATCTAGGATGATTCAATA
RMS057	GAA/GA	174	63.4	CGAGGTGGGTAAGGGCGAACAAAG	63.5	CCCATCCAAAGCGAGACGACGAC
RMS058	GT	143	50.6	CAACCCCTGAAGCCTGAA	47.4	TTTGTAACCCATTTGACCATA
RMS059	AT&GT	126	42.6	ACAGTCTTATAGTGGCTTCC	44.9	TACAGGGTTCTAATTGATACATAC
RMS060	GA	219	41.6	CATTCATTTGACTCTAAGGA	43.5	TATTCTGGTCTAAGCTATTGTAA
RMS061	GT	211	49.6	ATATCAGCCGTCCCATCAG	38.9	TTAGAAAATCCCAAACAT
RMS062	GA&GT	189	50.4	GCGAACGGCATTTACTTGT	50.5	GGTTGTTCTGGGTGGTTTTT
RMS063	GAA	90	60.4	CCACCGCCCACAATCACAATG	59.9	GCTCTGCGGAGTGGGAATGGT
RMS064	GA,GT	227	43.7	TTTTTGCAATATGTGAAGC	50.3	GATTGGTCAACCGATATGTAGAA
RMS065	GA	111	42.2	TATAGCTCGGTAGATTCAAA	56.2	CCAGACTGCCCCCAACTCATA
RMS066	GA	198	48.8	TCCACCCACAGACCACAG	49.5	AAGCTCCCTACGATTTCACTC
RMS067	GA	169	50.2	CAATCTGCAATCCGAATCC	47.5	ATGGTGAAAAACAGAAATACTACA
RMS068	GA	199	52.8	GTGCGCTTTCTGCTCCATT	51.8	CATTTTGTCCTACGTTTTCACTTC
RMS069	GT&GA	232	53.0	TCGGAGATTAAGAGTGAGGTGAGT	56.9	GTGCCCACTTACCCAAACCATC
RMS070	GA	173	45.2	TGCCTCTCGATACAAACC	54.0	AATAAGAACCAATACCCCGAAGAG
RMS071	GT	90	44.4	GTTAGCATCTGGCACATTAT	46.3	AGTTCCTTGACCAGCAGAG
RMS072	GA	110	46.3	TTAGCTCAAGAATTCATCAAAG	51.9	TCCAAACCGAGCTAAGAAAACT
RMS073	AT&GT/GA	156	46.0	AAACCCCTTTTATGTAGAAGTAG	45.5	TAAAACATGAAATTATAACAATAGTGA
RMS074	AT&GT	237	51.5	GCTTCTATCCACAGTTTCACCTC	51.0	TTCATGTCAACGCTTCTGTAATAG
RMS075	AT&GT	237	54.4	GCCCGTAAAAGCCCGTAAA	48.3	TTGGTCAACCGATATGTAGAAT
RMS076	GA	180	48.9	TGGATGCAAACACCTACAAA	58.1	CGTCGCCGGCATTCGTC
RMS077	GA&GT	154	60.375	AGGTGAACATGGGCCAACTA	57.436	TCAAAGAATGAGTGCCTACTAAGA
RMS078	GT	112	59.585	CCATTCCAAAGTTGCACGTA	60.49	CTCTACTGCCAGCAACCACA
RMS079	GA	182	59.502	CCGGTATGGAGAGGAATGAG	59.841	GCAATTATCCTTGACAGAACCC
RMS080	GT	213	59.585	GCTTTCAAAGATGGGAAACCT	59.470	TTGGTATCACATTTACTCTCATTGC
RMS081	GT&GA	164	57.402	TTTGACACACACACACAAACAT	59.784	GACTGAGAAACAAGTCCGTCCT
RMS082	2xGA	113	59.469	AACAACACACGCGGAATATG	59.873	TGCAGTTGGAGTTGGAGTTG
RMS083	GT	90	60.837	GACGTCCGCACTTTAGCAAC	61.720	AGGTCCTCAGCATAGACGGC
RMS084	GT	185	59.893	GGGAGTCTCAAGAGCTACCGT	58.787	CTTCATGTAAGCCACTGGACA

RMS085	GA	204	59.923	ATGCCCATGACTATCTTGCC	61.110	TCCAAGATGAAGAATTGCGG
RMS086	GA	150	60.195	TTCTGTTTCATCTGGCCTCC	59.700	GTTCGTAGATTCAGGTCGGC
RMS087	GA	229	60.328	GCCCAACTATTCCTCCCACT	60.454	CCCACAGTTGTCCAACACAA
RMS088	GA	207	59.955	TCCTGATTCGTATCATCCACTG	59.817	GAAGGCCTCAAGGTTCCTCT
RMS089	AT&GT	161	59.107	TTCTTATTGTTGGTTTGGAAGAAA	59.394	TCAATAGTGAGGTGCGAGGA
RMS090	GT&GC	204	59.837	TGTGTGTGTATCCATGGCCT	60.80	ATCTGCAATGACAATGGCAA
RMS091	GA&GT	207	59.513	GATCAGGGTGAATACCGAGC	59.589	GCCACTCTTCTCTGTCCTCAA
RMS092	AT&GT	208	59.546	TGAAATGAGAGACCAATTCCAA	58.762	ATCAAGTGAGCCGATGGAG
RMS093	GA	116	60.301	CGTTCTCGTTGTTGTCATCG	60.540	CCCTCTCTCTCCAGTCACGA
RMS094	GA	175	59.918	TCCTATCCACACCGACATCA	60.125	TCACAAATACCTTCCACTCGC
RMS095	GA	163	59.649	CCAATCTCCTCAACTCCCAG	59.730	TCAGGGCTTCTAAAGCTTGC
RMS096	AT&GT&AT	203	59.485	TGACCAATATGACAGAGAACCAA	58.143	TGATAGCCTTACATATGGAAACATT
RMS097	GA&GT	163	60.162	ATCTGGCTGAACACCACACA	60.132	CATGCTAACTCTCCATGTTCCA
RMS098	GT/GA	172	59.790	CACGTCCCATTCCAGAATTT	59.943	CCCTCAATGGAGAGCAAGAG
RMS099	GA	166	60.88	GGTCTGGTTCCTTGAGGTGA	60.96	CTCTCTCGTCCGAAAGCATC
RMS100	GT&AT	169	59.556	AGAGCTCCGCTCTGGATATG	59.911	AAGCCAAAGCTTACGTGCAT
RMS101	GA	133	59.291	GAAGAGACTGAAAGCTTGAAGGA	60.388	CTCCTCTCCACTCCTCACCA
RMS102	GT	170	59.891	AACTAAATGGTTGAGATGCCAAA	59.642	GGAATTTCGTTCCTTAAGCTAAGTT
RMS103	GT	193	59.960	ATTATGCGAACCAAACGAGG	60.214	TGGCAGCATTCTCCCTAAAC
RMS104	GA	209	57.11	CTAAAGCTTGAGCAAACAAATG	59.955	GGAGTATTGGCCGTAGGTGA
RMS105	GT&AT	189	58.857	TTGGTCTAATGCCCTATCCC	60.53	CCAGCCCTAGCCATAATTGA
RMS106	GA	189	58.100	CTCTCCCTCTCTGCATCAAA	59.982	CCTCTTCTCTGCAACCCAAG
RMS107	AT&GT	194	60.73	CGACCTTGAACTCGATGGAT	59.266	CATGAAAGTGGAGCTAGCTAAGAA
RMS108	GA	183	61.395	GATCGCCATGGCATGTAAAG	59.592	TTCTTCTAGTTTCCGGCTGC
RMS109	GT	115	59.625	TGCAAACCTAAATTCCACAGAA	60.12	TGGCCTCTACAGCTCCTGTT
RMS110	GT	194	59.673	TATGAGAATGAGCGTGTGGG	60.532	TTCCCTCTCATTCCTCTCCC
RMS111	GA	135	57.738	TTAGTCATCATCTTCAGTTATCAAGA	59.933	ATTCAATTGGCTTCACTGGGA
RMS112	AT&GT	227	59.294	CAAGGATACCAGTCGGAGAGA	59.813	AGAAATGGACAGCTCCGAAA
RMS113	GA	174	60.263	CATGGATTGCGTGTCTTCTG	59.955	GGCATCAGAAAGCTGAAAGG

RMS114	GA	224	60.134	AGTCGCATAACAGGACTGGG	59.894	TTGGGATTTCGGATAAGTCG
RMS115	GA	222	60.27	CGTGAAGACGCAAAGTCAAA	60.59	GGAGGAGAAGGAGGATTTGTG
RMS116	AT&GT	228	59.989	CACCCACTGGAATACTGGCT	58.724	CGACAAGCATGACCTGAAAT
RMS117	GA	199	59.950	TCTTCTTCTCTCACCGCCAT	60.74	GGCCGATTTGTTGACCTAGA
RMS118	(AT&)GT	168	59.75	TGGCTATGGGAAGAACATGA	59.545	TCAGACAAATAATGCGTTACCAA
RMS119	AT&GT	122	59.857	GCACGCACACATATATAACAACAA	59.807	GATATCCGCAGCCAAGAAAG
RMS120	GT	193	57.360	CAGTTGAAGAGAACCAAGGG	60.162	TGGTGGGTAGGGAAATGAAA
RMS121	GT	94	60.1	TCCTCTCCAAGACACAATATTCAA	60.999	GCCCTCTCTGCTCTCCCTAA
RMS122	GA	229	60.822	ATTCCACTTCCTCCTTCCCA	59.874	GGATTCTTTCCTCCTGACCC
RMS123	GA	167	59.128	AAACACTCTAAGGAGGTATTCCCTAA	59.137	CGAAGTCTCCCATGGTTTCT
RMS124	GT	107	57.353	TTTGTGGTCGTGTGTGTGTAT	58.149	AGGCACAAATACTATCCACCTG
RMS125	GA	160	60.589	AAGTGAAGACTGAGCGACCG	59.694	CTACTCCAATGTCCGCTTCC
RMS126	GT	210	59.822	AACGACCGCCTAGGAGAAA	58.48	TTGTTTCTGTTCGAATGGGT
RMS127	GA	220	59.967	TGCCTTTCTAGATTTGCTGGA	60.812	TAGTTGTTCGTCACCCACCC
RMS128	GA	230	60.16	AGCATCACGAGCACATTCAG	60.470	GCGAAGATTCACCCAATGAC
RMS129	GT	229	59.203	ACGTGCACACACTCACACAC	57.100	ACTGATGCAGTTTGCTCTGA
RMS130	GA	126	59.518	CAAATCAATCTGCAAACCCA	59.833	TTTGCGAATACCAGATGCAG
RMS131	GA	230	60.615	CGGCCAGAGATAACAGATGG	58.938	TGTTTGTTGCTTAACTACTACAACCTT
RMS132	GA	184	59.454	TGTGGTTATGAATTGCTGGTG	59.956	TTCAGTTTGGTTGAATGGGAG
RMS133	GA	124	59.731	TCTGCAACAATCAGCAGAAGA	59.901	ATTTCTGGCAAATCCGAATG
RMS134	GA	226	58.173	TGAGCTCAAGCAATATGCAA	58.817	GGCTGTCTCTGATTCCAGTATG
RMS135	GA	190	60.11	GACCGATTGGAGAGGAATGA	58.909	TTGCCTTTCTCCCTTCTGTT
RMS136	GA	114	57.218	GATCATGAGAGTCGCCAAA	59.939	AAGAGGCAGATATGGAGCGA
RMS137	GA	228	60.362	TGTACATGATGATGGGACGC	59.847	GGCAATTGCAAAGACAGTCA
RMS138	GA&andere	157	60.22	CTTCTGAGAGCCACACACCA	60.339	GCAAACACATCCCATCATCA
RMS139	GA	187	60.169	CAAGTATCTGCTCAGGCAAGC	60.218	CCATCACATTCGGCTCTTCT
RMS140	GT	123	59.792	CCAATAGCGATGCAATGAGA	59.52	TTGGCTACCACTAACCTCCC
RMS141	GT	202	58.624	ACAGAGACTTGACGCTGCAT	59.668	AGCGTGTGTAGCTAGGGAGC
RMS142	2xGA	186	60.255	TGGCCTCAACGTCTTCTACC	58.588	CCTGAAATATCCCTATGTCAGAAA

RMS143	GA	230	60.261	GTGGGAAGTGTGGGAACAAC	59.617	GCCTCATCCTGTCCATCTTC
RMS144	GT	202	57.412	TTTATCACTGTCACAAGGCATTA	59.661	GAGCTCCATGAGGTGTTTCC
RMS145	2xGA	122	60.397	TGCTCACTTACCCAGAAGCC	59.350	TCTCTCTCATTTCAAGAGTAAACCC
RMS146	GT	186	59.454	ACAAGGCATTCACCTTGGTT	58.253	TTTCTGGGCCTGCATAAATA
RMS147	AT&GT	191	59.583	CCAATCTCAATAACACCGAGC	59.767	TCTTTGTGCTGCTAATGCTCA
RMS148	GT	230	59.756	TTTAGCAGGCATTGGCACTAT	59.698	ACCTCCAGCACCAACTCCT
RMS149	AT&GT&AT	203	59.566	CGGTGTGTAGTTGATTCGGA	60.195	TCAAATTCTGGCCTCTGTCC
RMS150	GT	209	60.251	TGCTGCAGTATGATGCCAAT	59.55	TGGAAATCCTTTCCTTTCCTT