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Isolation of polymorphic microsatellite loci from the flea beetle *Phyllotreta nemorum*

Mapping of resistance genes

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Preface

The interaction between insects and their host plants is an interesting one. Plants defend themselves against insects and the insect which is not able to feed on the plant anymore co-evolves to be able to use the plant again as a host. These steps repeat themselves and seem to be a never ending arms race. One typical insect versus host plant interaction is that of the yellow striped flea beetle, a small beetle that has included *Barbarea vulgaris* in its host plant range. A lot of questions can be raised looking at this interaction. One of the questions relates to the modes of inheritance causing resistance. Segregation patterns suggest that autosomal as well as sex-linked loci are present (Nielsen, 1997). But it is unknown whether these loci originated as independent mutations, as homologs or that a fusion or translocation has taken place.

I have developed polymorphic microsatellite markers to investigate by means of gene mapping whether the resistance in a flea beetle population where the resistance gene is inherited autosomal is the same gene as in a flea beetle population where the resistance gene is inherited sex-linked. By doing this, I was able to combine two of my interests; population ecology and genetics (molecular ecology).

There are a few people that I would like to thank for their help and support during my MSc- thesis. At first I would like to thank my supervisors Peter de Jong, Kim Vermeer and Patrick Verbaarschot for their advise and the valuable comments on the report. I would also like to thank Patrick for his help in the laboratory and Jens Nielsen for providing the Y-linked beetles from Denmark.

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Abstract

The interaction of the flea beetle *Phyllotreta nemorum* and its host plant *Barbarea vulgaris* ssp. *arcuata* (Cruciferae)is an interesting one. This flea beetle seems to have evolved resistance genes (R-genes) which enables this beetle to include the normally toxic plant *Barbarea* in his host plant range. Segregation patterns suggest that this R-gene can be inherited sex-linked as well as autosomal. However, it is unknown whether these loci originated as independent mutations, as homologs or that a fusion or translocation has taken place.

During this study I have developed polymorphic microsatellite markers to investigate by means of gene mapping whether the resistance in a flea beetle population where the R-gene is inherited autosomal is caused by the same gene as in a flea beetle population where the resistance gene is inherited sex-linked.

A genomic DNA library has been used for the development of microsatellite markers. DNA sequences were checked for nucleotide repeats and primers were designed on the flanking regions. These primers were tested for polymorphism. Fifteen new polymorphic microsatellite markers have been developed. Due to time constrains these markers have not been used for creating a genetic linkage map, but only the ten already known microsatellite markers have been used.

For the creation of a genetic linkage map ten non mated male beetles with a Y-linked inheritance of the R-gene were each mated individually with a virgin susceptible beetle, resulting in offspring in which the R-gene segregates . The two lines that produced most offspring were used for creating a genetic linkage map. Another student made a genetic linkage map with a flea beetle line with autosomal inheritance of the R-gene.

A genetic linkage map could not be created with the Y-linked beetle line. There occurs no crossing over between the sex-chromosomes of *P.nemorum*. There for the R-gene on the Y-chromosome can not be mapped. It was also not possible to make a genetic linkage map of the autosomes of the flea beetle, the flea beetle has 15 chromosomes and only 7 microsatellite markers have been used in this study and all of these markers were not linked.

The newly developed polymorphic microsatellite markers together with the already existing markers can be used to create a genetic linkage map with the R-gene of a flea beetle population with autosomal inheritance of the R-gene. To create a more dense map, these markers can be combined with AFLP markers. It is also possible to use the microsatellite markers in population genetic studies.

1. Introduction

There are about 1 million insect species known of which the order Coleoptera represents the largest group. One of the largest families within this group is the Chrysomelidae or leaf beetles (Medeiros et al., 1996). As the name already implies, leaf beetles are insect herbivores, phytophagous insects, and frequently specialize on only a few plant species.

Between many phytophagous insects and plants a co-evolutionary arms race is going on. Plant species often contain secondary compounds that are toxic and/or deterrent to defend themselves. The phytophagous insect may in return evolve a new type of counter-adaptation to this secondary compound and will be adapted to utilize this plant. As a reaction the plant will evolve also a new secondary compound. This cycle can repeat itself continuously (Thompson, 1994).

There is a phytophagous insect, the yellow striped flea beetle, where this co-evolutionary arms race has probably just begun. This flea beetle has increased his host-plant range by including wintercress (Nielsen, 1996). The underlying genetic basis of this evolutionary change is largely unknown. Studies however did reveal the possession of major resistance genes in this flea beetle. Those major genes can be autosomally as well as sex-linked inherited (Nielsen, 1997). It is still unclear whether both modes of inheritance include the same gene and that translocation or fusion has taken place. Other possibilities are that these loci originated as independent mutations, or as homologs (Nielsen, 1997,1999).

With this study I tried to map the resistance gene with existing and newly developed microsatellite markers to detect whether in a population with Y-linked inheritance the resistance gene is the same one as the one present in populations with autosomal inheritance of the resistance gene.

1.1 Phyllotreta nemorum

The flea beetle *Phyllotreta nemorum* L. (Coleoptera: Chrysomelidae: Alticinae) is also known as the yellow striped flea beetle. This flea beetle is an oligophagous insect that feeds in particular on cruciferous host plants. The most common host plants in Denmark are charlock (*Sinapsis arvensis* L.), to a lesser extent the cultivated and wild radish (*Raphanus sativus* L. and *R. raphanistrum* L.) and wintercress (*Barbarea vulgaris*). The larvae are leaf miners and the adult beetles feed on the same plant (Nielsen, 1997; de Jong et al., 2000).

Phyllotreta nemorum

1.1.1 *Barbarea vulgaris* as host plant

The utilization of *B.vulgaris* ssp. *arcuata* (Opiz) Simkovics seems to be an expansion of the host plant range since less than 20% of the known *B.vulgaris* are attacked by *P.nemorum*. *B. vulgaris* is a polymorphic plant, as it exists in two forms. The pubescent form (P-type) is suitable for all flea beetles, the glabrous form, the G-type (the form studied in the present work, and therefore in the remainder of this report referred to as *Barbarea*) is unsuitable for most flea beetles (Nielsen, 1996). The flea beetle is also polymorphic as some genotypes are susceptible to the defense mechanisms in the G-type, whereas others are resistant (de Jong & Nielsen, 1999). The two plant forms have a different glucosinolate content, but this is not correlated to resistance (Agerbirk et al., 2001). The resistance to *P.nemorum* seems to be due to the occurrence of a triterpenoid saponin in *Barbarea* (Shinoda et al., 2001). There are four populations of *P.nemorum* known which utilize *Barbarea* within the study region (Nielsen & de Jong, 2005).

1.1.2 R-gene

The ability of *P.nemorum* to utilize *Barbarea* is controlled by major, dominant resistance genes (R-genes)(de Jong & Nielsen 1999). Most beetles found on *Barbarea* are homozygous (RR) for the ability to use *Barbarea*, whereas resistant beetles found on other host plants are usually heterozygous (Rr) (de Jong & Nielsen, 1999).

A study of Nielsen (1997) showed with segregation patterns that this R-gene can be inherited sex-linked (X-linked as well as Y-linked), but also beetles with autosomal loci are present (de Jong et al,. 2000). Nielsen (unpublished) discovered in a flea beetle population from Switzerland sex-linked beetles that produced offspring where the resistance did no longer involve sex-linked loci, but autosomal loci. This suggests that it is unlikely that the same mutation has occurred independently at the sex-chromosomes and autosomes. The Y-linked gene may have originated as an autosomal gene and a part of that autosome has translocated to the Y-chromosome. Another possible explanation is a fusion of the Y-chromosome with a pair of autosomes where the resistance genes are located on (Nielsen, 1997,1999).

1.2 Population genetics

1.2.1 Co-evolution

Thompson (2001) describes co-evolution as a reciprocal evolutionary change in interacting species driven by natural selection. When an attempt was made to organize all information known about ecological, genetic, and phylogenetic structures of real species into a hierarchical framework for coevolutionary analysis the geographic mosaic theory of co-evolution was developed (Thompson, 2005). The general structure of the geographic mosaic theory of co-evolution contains three hypotheses and predictions (Thompson, 2001, 2005).

The first hypothesis suggests geographic selection mosaics. These selection mosaics favour different ways of evolution in different populations. As a consequence of this hypothesis, populations will develop different traits shaped by interactions.

The second hypothesis suggests the existence of co-evolutionary hotspots. At these hotspots reciprocal selection is occurring. The co-evolutionary hotspots are embedded in a broader matrix of co-evolutionary coldspots where local selection is non-reciprocal or where no other interacting species is occurring (*see figure 1*). The prediction of this hypothesis suggests that traits of interacting species will be well matched in some communities, where their fitness increases, but in some communities this will result in a local mal adaptation because of mismatched traits.

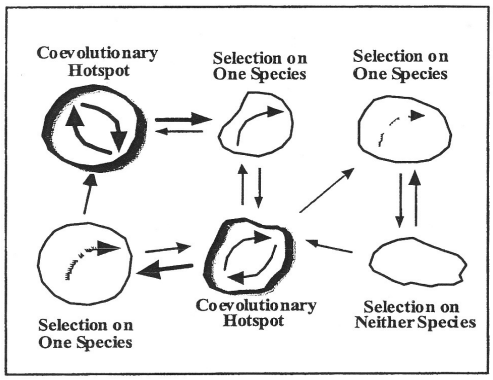

Figure 1. Hypothetical example of a complex geographic mosaic in a coevolving interaction. Arrows within circles indicate natural selection on one or both interacting species, Different arrows directions in different circles represent different (co)evolutionary trajectories. Arrows between communities indicate gene flow (Taken from Thompson, 2001).

The last hypothesis involves a continual geographic remixing of the range of coevolving traits. The genetic structure of co-evolving species can change through new mutations, gene flow, random genetic drift and extinction of local populations (Thompson, 2001, 2005). Just few coevolved traits will actually spread across all populations to become fixed traits.

Among flea beetle populations living on different host plants also a geographic mosaic of interactions is present (Nielsen & de Jong, 2005). R-genes are produced in high frequencies in populations living on *Barbarea*. From here they can disperse to neighbouring populations living on other host plants. On *Barbarea* there will be selection against the r-allele, as the genotype rr cannot survive on this plant.

1.2.2 Local adaptation

Local adaptation is an important concept in evolutionary processes, because co-evolution starts with the co-evolutionary dynamics of locally interacting species. Ongoing local co-evolutionary adaptation is the starting point for these dynamics, as it creates the basic template for the geographic mosaic of co-evolution (Thompson, 2005).
Local adaptation occurs when local selection favours a particular genotype that is best adapted to the local environment. This genotype offers the highest fitness and will be transmitted to the next generation (de Jong & Nielsen, 2002).

To what degree populations are locally adapted to their environment depends on the relative rates of local selection and gene flow between populations (Peterson & Denno,1998). In some studies, it seems that local adaptation involved in host plant specialization of phytophagous insects is governed by negative pleiotropic gene action. This implies that genes that lead to better performance on one host are unlikely to improve performance on an alternative host as well (Mopper, 1996).

1.2.3 Population structure

Population genetic models usually assume that populations are homogeneous units in which mating is random. But in real life this is not always the case. Populations often develop an internal structure, created by the physical properties of an environment and the biological characteristics of the species. This structure is called population structure (Nunney, 2001).

There are two kinds of population structure that can influence evolutionary change: genetic structure and proximity structure. The first one can be defined as the non-random distribution of genotypes in space and time. The second structure can be defined by the size and composition of the group of neighbours that influence an individual's fitness (Nunney, 2001). Proximity structure occurs on a small scale, and usually only interactions with neighbours are involved. This is in contrast with genetic structure where complete populations are involved. Genetic structure can affect local adaptation and can create the possibility for the development of co-adapted gene complexes (Nunney, 2001).

P.nemorum has most likely developed a genetic structure. The host plants of this flea beetle are patchily distributed and a study showed a limited genetic differentiation between (sub)populations**.** This limitation may influence the spread of the resistance genes to *Barbarea* (de Jong et al ,2001; de Jong & Nielsen, 2002).

1.2.4 Co-adapted gene complexes

It is impressive that on *Barbarea* mainly homozygous resistant flea beetles occur. Large negative pleiotropic effects are very often accompanied with the possession of genes with major phenotypic effect, but there seems to be no evidence for trade-offs associated with the possession of resistance genes (de Jong & Nielsen, 2002). An absence of trade-offs would also suggest rapid spread of R-genes across host plants. Studies, however, could not support this spread of genes (de Jong & Nielsen, 2002). An explanation for this phenomenon can be the presence of co-adapted gene complexes. The high production rate of flea beetles that are homozygous resistant on *Barbarea* makes it possible to select modifiers to counteract the negative fitness effect associated with the possession of the resistant allele. These modifiers can coadapt with the resistance genes and might get linked or fixed (de Jong & Nielsen, 2002). The co-adapted gene complex can break up by hybridization between subpopulations using different host plants.

1.3 Molecular markers

Molecular markers can be used in ecology for a wide range of applications. Molecular markers can, for example, be used to estimate genetic diversity in populations, it can be used in behavioural ecology, but it can also be used to find genes that cause a disease. In this research molecular markers are used to construct a linkage map.

Molecular markers can be divided into two groups; co-dominant markers and dominant markers. Co-dominant markers can identify all of the alleles that are present at a particular marker-locus. Dominant markers can reveal only a single dominant allele (Freeland, 2005). In this study a co-dominant marker has been used, namely microsatellite markers. The development of co-dominant markers is more time consuming and expensive than the development of dominant markers, but microsatellite markers give more precise results. Moreover, the microsatellite marker is highly variable and selectively neutral, characteristics that make it possible for this marker to differentiate between closely related organisms, such as flea beetles in different subpopulations. In this research, molecular markers have been created for further flea beetle research, for example for the development of linkage maps and for the study of population genetics.

1.3.1 Microsatellite markers

Very often genomes contain areas where specific nucleotide sequences are repeated in tandem. They are arranged head-to-tail without interruption by any other base or motif (Goldstein & Slötterer, 1999). The number of times these sequences repeat themselves can be highly variable. These repeats are called variable number of tandem repeats (VNTRs). There are two kinds of VNTRs; minisatellites and microsatellites (Windelspecht, 2007).

Microsatellites or SSR's (simple sequence repeats) are sequences made up of a single sequence motif, no more than six bases long, that is tandemly repeated up to several hundred times (Goldstein & Slötterer, 1999; Windelspecht, 2007). Microsatellites are abundant and randomly spaced in the genomes of a wide variety of species, which makes them easy to use. Microsatellites are selectively neutral. Therefore mutations on microsatellites do not influence the fitness of the studied organism (Goldstein & Slötterer, 1999). Microsatellite markers are often used for DNA fingerprinting, paternity testing, gene flow studies and for the construction of linkage maps. Before this thesis, ten microsatellite markers had already been developed for *P. nemorum*, see table 1.

Table 1 Polymorphic microsatellite markers for *Phyllotreta nemorum* (Verbaarschot et al, 2007)

Locus (GenBank ID)	Repeated motif †	Primer sequences (5'–3')	Size range	Amplification conditions (T_a/no. of cycles)‡	No. of alleles (Effective no. of alleles)	H_O H_E
PnA03 (DQ507809)	$(CA)_{10}$	F: HEX-CAACGAGCAATCGATACAATTCG	205–225	55/35	11	0.93
		R: ACATTCTGCGCCGAGATTGG			(6.07)	0.84
PnA04 (DQ507810)	$(GCA)_6$	F: 6-FAM-AATTACGAGAGCAACATGTCGG	174–180	57.5/35	3	0.31
		R: ACTGTTGCTGTTGGGTTTGC			(1.63)	0.39
PnBB08 (DQ507811)	$(CAA)_4$ $N_{12}(CAA)_5N_{22}(CAA)_5$	F: HEX-CCTGATCCATTACCAGAACCTCC	215–281	62/35	13	0.64
		R: ATGGTGACTGTGACTGTGAGATGG			(5.22)	0.81
PnAB12 (DQ507812)	$(GCT)_{10}(GTT)_2$ $(GCT)_2GTT(GCT)_4$	F: NED-GAGATTGAGACGATTGCTGGG	176–218	62/35	14	0.75
		R: CTCAACAGTTGCATTACCAGC			(5.67)	0.83
PnD04 (DQ507813)	$(GCT)_7$	F: HEX-CTCGAGCTTGACTCACTACTGC	136–160	55/35	7	0.68
		R: CCAGTTCCAGTGATTCGAGC			(3.13)	0.68
PnD06 (DQ507814)	$(CAG)_7$	F: 6-FAM-CTTCTTCAGCAGCCTGATGG	157–184	50/32	9	0.79
		R: ATCACGTTCGGCACCACCTG			(4.00)	0.75
PnD09 (DQ507815)	$(GCA)_4N_{26}$ $(GCA)_6$	F: 6-FAM-GCTCCAACTACACCAAACTCG	167–193	55/30	10	0.77
		R: TCGCGTACCCGTAATAGTGG			(4.47)	0.78
PnE11 (DQ507816)	$(GCA)_5N_3$ $(GCA)_8$	F: HEX-GTACAGTCATGTCTTGGAACGC	211–247	57.5/35	12	0.89
		R: CTCGATTGCGTAGTAGCCGG			(5.48)	0.82
PnH09 (DQ507817)	$(GCG)_6$	F: NED-CGTGAGGCTTGTAGTATTTGG	162–165	55/30	2	0.10
		R: CTACCATCCGATGATGAACG			(1.13)	0.11
PnH12 (DQ507818)	$(CCG)_5$	F: NED-AACCCTGGACGCCTATTCTGC	140–167	55/30	9	0.32
		R: CGCGAATTACGGTTATCAGG			(1.47)	0.32
†The number after 'N' denotes the number of nucleotides in between repeat sequences.						
‡PCR cycle program: 1 cycle 3 min at 94 °C, no. of cycles (30 s at 94 °C, 45 s at T_a °C, 1 min at 72 °C) 10 min at 72 °C.						

1.3.2 Mapping with molecular markers

In this study microsatellite markers were used to generate a genetic linkage map. This map will show the different locations of the microsatellite markers and the R-gene in the genome. This outcome can be compared with a linkage map of an autosomal flea beetle line, to see whether there is one autosomal R-gene present that has fused to the Y chromosome or that multiple R-loci are involved in the resistance of *P.nemorum*. When in both cases the same microsatellite marker is linked to the R-gene, the same gene is involved.

For each combination of microsatellite markers the chance of recombination will be calculated. Recombination frequencies are based on crossing over during prophase of meiosis I. This will be a measurement of the relative distance in genetic map units (m.u.) between the markers. Map units are also called centiMorgans (cM). One m.u. represents having a 1 % chance of recombination of the markers by crossing over. The closer a marker and the resistance gene are located on a chromosome, the less chance that recombination takes place and the tighter the linkage is between them. By calculating recombination frequencies of the microsatellites and the R-gene, it is possible to identify the relative position of the resistance gene on a chromosome (Hartl & Jones, 1999).

1.4 Problems and aims

It is unclear whether the R-gene in a population with autosomal and sex-linked inheritance actually involves one and the same gene. It is assumed that the same gene is causing the resistance. Therefore the aim of this study is to detect whether the resistance in both populations is caused by the same gene.

When comparing a genetic map of a Y-linked population with an autosomal resistance population it will be possible to see whether the same marker is linked with the R-gene in both populations. More markers are developed to obtain a denser map and to prevent a situation where no markers are linked and therefore no crossing over between the markers can take place. These new markers can also be used in further studies.

2. Material and methods

This section is split up into two sub chapters, the first part (§ 2.1) deals with the flea beetles used, the second part (§ 2.2) deals with the molecular work used in this study.

2.1 Animals

Flea beetles originating from three different locations were used in this study: Taastrup, Kværkeby and beetles from Ejby.

Beetles from Taastrup do not survive on G-type plants and are therefore susceptible (ST; Susceptible Taastrup) with the genotype rr. The ST beetles used in this study have been kept under laboratory conditions for more than fifteen generations. More information on origin and maintenance of this ST line can be found in a previous study by de Jong and others (2000).

Some beetles from Ejby can survive on G-type plants and are therefore resistant. The resistance gene in these beetles is probably linked to the Y-chromosome. Flea beetles have the same sex determining system as humans where females are XX and males are XY, so only males in this line can possess the R-gene. The line of beetles where the R-gene is probably linked to the Y-chromosome will be referred to as the Y-linked line. This line has been kept under laboratory conditions for more than fifteen generations. The Y-linked line is being maintained by repeated backcrossing of Y-linked males with ST females to create a near-isogenic line (Nielsen, 1999).

2.1.1 Crosses

Ten non mated male Y-linked beetles were each mated individually with a virgin ST beetle, resulting in offspring in which the R-gene segregates (*see figure 2*). From these ten crosses, the two crosses that produced most offspring were used to create the actual linkage map. Beetles from the other lines have been used for the development of new microsatellite markers. The unfed offspring (F1) were separated after emergence. After the gender was determined of these adult beetles, they were put individually in plastic vials, closed with a plastic lid. This lid had a central hole which was closed with cotton wool. Each individual was given two leaf discs (diameter of 19 mm) of *B. vulgaris* ssp. *arcuata* G-type leaves to perform a bioassay. After three days the total amount of plant material eaten was determined. Animals that did eat a significant amount of the leaf disks were determined as resistant beetles and the ones that did not eat or only took a few nibbles were determined as susceptible. The gender of the beetles served as a second control for genotyping the Y-linked line as only males should inherit the resistance gene. Together with Y-linked beetles also some susceptible (ST) beetles were put into a bioassay. They acted as a control group to check whether the *Barbarea* was toxic. After the bio-assay the Y-linked beetles were stored at - 20° C for further use. Another student made crosses with flea beetles with autosomal inheritance.

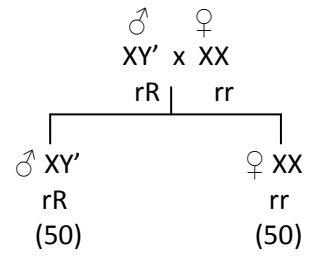

Figure 2. Diagram of the cross between the beetles. XY' are y-linked males, XX are the susceptible ST female beetles. Expected frequencies of offspring are given in parentheses.

2.2 Development of microsatellite markers and creating a genetic linkage map

In 2007 Patrick Verbaarschot developed primer pairs for 10 microsatellite markers using a tri-nucleotide enriched genomic library (Verbaarschot et al, 2007). A genomic library contains DNA fragments created using restriction enzymes and the process of cloning. The restriction enzymes subdivide the genome into small fragments that are small enough to study. These fragments are cloned into bacterial cells using a plasmid vector. The bacteria are then diluted and placed on a nutrient rich medium and will divide on the medium producing small colonies of genetically identical cells. Using a probe, a short sequence of nucleotides, colonies were screened for repeats of interest. Out of 1920 clones 337 were positive, they possessed a microsatellite, and 96 of those positive clones were sequenced. Primer pairs for 27 microsatellite repeats were designed, of which 15 primer pairs amplified a polymorphic fragment in *P.nemorum* (see appendix 2).

In this study the same enriched library has been used to develop more primer pairs for microsatellite markers.

To check the sequences for nucleotide repeats a software package for sequence analysis and data management, Vector NTI Advance 10 (Invitrogen), has been used. Forty-seven new useful microsatellites (candidates) were found. Each candidate was checked to make sure that 1) the repetitive elements were positioned not too close to the flanking region, 2) the flanking regions were long enough to design primers on, 3) the amplified sequence contained at least four repeats and 4) the amplified fragments ranged more or less between 100-300 bp. The candidates were also checked for identical inserts. Twenty-seven sequences were useful for further processing. For each of these sequences a primer pair was created amplifying the repeat using Vector NTI Advance 10. The primers were ordered at M.W.G. Biotech (www.mwg-biotech.com) and tested with flea beetle DNA. Initially an annealing temperature of 50° C was used for the different primer pairs, but not all primers worked well with this annealing temperature. Therefore in some cases a PCR gradient was set up to determine the optimum temperature of the primer set (see appendix 4, figure 4). Some PCR products gave bands on the agarose gel larger than expected. These PCR products were gel-purified and sequenced (MWG-Boitech) to make sure the primer combinations amplified the sequence as found using Vector NTI Advance 10. If they produced the right sequence, they were included in the further process. The primer sets that performed well on the Agarose gel were put on a Metaphor Agarose gel (Lonza). This gel has a high resolution that makes it possible to distinguish between polymorphic and monomorphic microsatellites (see appendix 4, figure 5). For this purpose, 12 different DNA samples were used, namely four different beetles from the Y-linked line, the Kv line and the Try line.

Due to time constrains the polymorphic markers could not be processed further by myself. Instead of using the new markers along with the already known microsatellite markers, only the ten known markers (Verbaarschot et al. 2007) were used in the further analysis below.

DNA extracted from two crosses of Y-linked beetles with ST females was used to perform PCR reactions with different microsatellite markers. After the PCR reaction the products were purified using sephadex loaded Multiscreen plates (Millipore, cat. nr. MSHVN4510). Two or three PCR products of each sample were combined and run on an ABI PRISM 3700 sequencer. Allele sizes were estimated using Gene Marker (Softgenetics).

In appendix 1 a more precise description of the methods is given.

3. Results

3.1 Rearing of beetles and bio-assays

In total 9 lines of flea beetles were set up and labeled from A to I. In total 200 flea beetles were reared. Beetle lines C en G had the largest number of offspring and were used for further processing. The other lines were stored at –20° C or used for the development of microsatellite markers. Beetles with a doubtful bio-assay outcome were tested again, those who were also doubtful the second time were listed as “unsure” and taken out of the experiment. An overview of the beetles is given in table 2.

Table 2. *Barbarea* resistance bio-assay of 9 lines of *P.nemorum*.

Beetle line	*Female/susceptible*	*Male/resistant*	*Total*	*Unsure*
A	1	7	8	0
B	2	3	5	0
C	**26**	**14**	**40**	**2**
D	13	14	27	1
E	13	15	28	2
F	3	6	9	1
G	**21**	**32**	**53**	**4**
H	3	6	9	4
L	10	11	21	1
Total	92	108	200	15

3.2 Development of microsatellite markers

Out of the 27 clones that matched the criteria (the repetitive elements were positioned not too close to the flanking region, the flanking regions were long enough to design primers on, the microsatellite contained at least four repeats and the amplified fragments ranged more or less between 100-300 bp and they had distinct inserts) 15 were polymorphic. These 15 were further tested using labeled forward primers.

Figure 3 represents a small part of the sequence of a clone that has been checked to match the criteria mentioned above. This clone contains the repetitive element AC (8 repeats). At both sides of the repeat enough the flanking region is long enough to develop primers on.

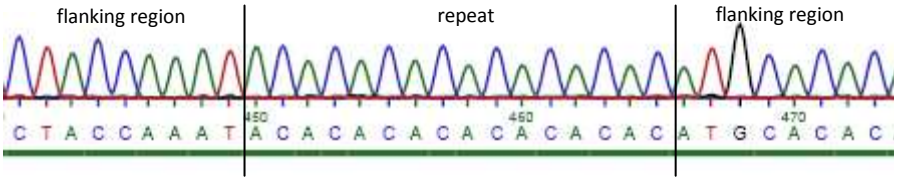

Figure 3. Example of a small part of a DNA sequence from marker A06 containing a repeat (AC)8. The numbers represent the position of the base pairs. This repeat is thus 16bp long ranging from position 450 – 465. The DNA at both sides of the repeat can function as flanking region.

Primers did not always work well at an annealing temperature of 50° C, so sometimes a PCR gradient was set up to determine the optimal annealing temperature. The products can be seen on an agarose gel (see figure 4). In this specific case, the amount of side product (vague smeared bands at 50 bp) disappears with increasing annealing temperature. The optimal annealing temperature can be found at the last well at 57° C . At this temperature hardly any side products are visible and the amount of product is high.

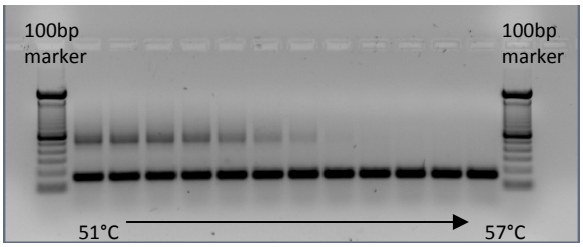

Figure 4. PCR with temperature gradient on an agarose gel (marker D06). In this case with increasing temperature the amount of product also increases as the side products disappear. An annealing temperature of 57°C would be optimal for this specific primer.

The 27 clones that were checked for polymorphism were put on a Metaphor Agarose gel. Polymorphic primers are needed, because they can reveal genetic differences between the beetles. Figure 5 shows the result of marker G06. Three different populations represented by four beetles have been used. This marker is polymorphic as several double bands or differences in lengths are visible.

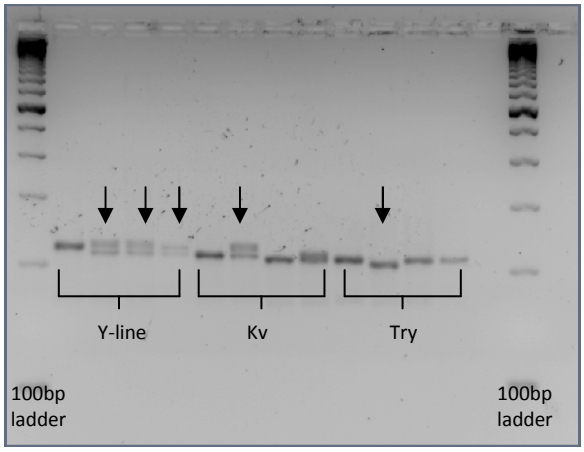

Figure 5. Metaphor Agarose gel showing polymorphic bands (arrows) of marker G06. Twelve beetles from three different populations have been used.

In total fifteen new polymorphic microsatellite markers were found (see table 3).
An overview of the positive clones and an overview of all primer pairs developed can be found in respectively appendix 2 and 3. Due to time constrains the new markers could not be included in the genetic linkage map.

3.3 Genetic linkage map

The results of the allele size estimation of the two beetle lines (line C en G) can be found in appendix 4. Unfortunately it was not possible to make any genetic linkage map.

Table 3. Newly developed microsatellite markers for the flea beetle Phyllotreta nemorum. F=forward, R=reverse. FAM, NED and HEX are fluorescent labels.

Locus	Repeated motif	Primer sequences 5' – 3'	A. temp	Size range
PnA06 fw1rv1	(AC)8	F: FAM-CCAGAAATGTGCATCGTACA R: GTTTGGTCTTTGTGATGGACAAGG	60	200
PnA10	(CGA)5	F: NED-ACTCACGCCCGAATCGCTTC R: GTTTAGAATGGACATGGTCGGCGG	57	135
PnB04_121	(GAC)5	F: HEX-CTTCGACCAACGGCCAGCCG R: GTTTCGTCACGGTTCGAACTGGGCGC	53	141
PnB06 fw2rv2	(GAC)30	F: HEX-CTGCGATTATGAATGAATGG R: GTTTTCGTCGCTGTCTTAGGC	55	343
PnB11_311	(CGT)4 en (TCG)4	F: 6-FAM-CTTCCGAAATAATCGTCTTC R: GTTTAATCTGGAGACGATGATGAC	57	142
PnC05_11	(TAAA)5	F: HEX-TCACCAAATTGTGACATGTACC R: GTTTACCATAAACGCACTGTTGA	57	151
PnC10 fw2rv2	(GAC)5	F: NED-GGGCAACGAAACAAACGACGGTACC R: CGTTTGAGTACCGCTGCCGG	50	213
PnD01	(GCC)5 en (TGC)8	F: 6FAM-AACTTTGCATCGAATTGTGC R: GTTTGTCTTGGAACGCTAATCGC	50	377
PnD02	(CAG)5	F: HEX-ATCAGCAGGCCATGCAGCAG R: GTTTGCGTTGTAGCCGGATTTTG	57	147
PnD10 fw1rv1	(CAG)7	F: NED-ATCCCCAGCTTTCTCTTGGAATAA R: GTTTAATATTTGCGGCTGTATGGC	50	309
PnD11	(CTG)4 en (CTG)7	F: NED-ATCACGTTCGGCACCACCTG R: GTTTCTTCAGCAGCCTGATGGGC	53	171
PnE08_61	(CAG)4	F: 6-FAM-GCAGCAGGTCGAGGCGACTG R: GTTTATTCGCCACCGTACCGTTCG	50	151
PnE10	(TGC)7 en (GCT)4	F: NED-TTTGAAAACTATTGCCCATC R: GTTTATGTCTTGGAACGCTAATCG	50	196
PnF03 fw2rv2	(TGC)11	F: FAM-AAATCCTTCAAAGGCTAAGCCAGC R: CAACAGGTTCAGCAGCAACG	55	345
PnG06	(GCC)8	F: HEX-CGTGGAATTTGTCAACACTTG R: GTTTGAGGACTTAGGTTTTGGTATAG	50	216

4. Discussion

A genetic map is based on recombination frequencies. These recombination frequencies are based on crossing over during prophase of meiosis I. Crossing over between sex chromosomes is thought not to occur in beetles (Smith & Virkki, 1978). Segregation patterns in this study also support this theory. No female resistant offspring is found when crossing susceptible females with Y-linked males. Based on this information it seems to be logic that no genetic linkage map could be created with the R-gene linked to a marker.

With no crossing over between sex chromosomes, it would still be possible to create a genetic linkage map with only the relative positions of the different markers on the autosomes. It would then still be possible to compare this map with the map with the autosomally inherited R-gene. If there would be any differences, this could suggest that something has changed on the autosomes of the Y-linked beetles, for example a fusion or translocation of a part of a chromosome. Unfortunately this map could not be created as *P.nemorum* has 15 chromosomes and only 7 markers were eventually used to create a linkage map. All markers were also not linked. To create a dense map, more microsatellite markers should be developed, but this will be quite expensive and time consuming. Another way to get a denser map is to combine all the SSR's with AFLP markers of the flea beetle (Breuker et al., 2005). AFLP markers are not co-dominant, but they can also give more information about the position of the resistance gene.

When looking at the beetle lines, some unexpected results were seen. In the crosses made with the beetles from the y-linked line, every female should be susceptible and every male resistant, as the gene will only be passed to the next generation from father to son. But during the bio-assays not all the results were that simple and clear. Some males (3%) did not eat from *Barbarea* and some females (4%) did eat from the plant. That some males did not eat can be explained rather simply. They might not be hungry and therefore did not eat. Beetles can survive without food for several days and the bio-assays took only 3 days. The female beetles that did eat are harder to explain. One explanation could be the lack of toxic components in *Barbarea*. *Barbarea* plants under sub-optimal circumstances, when for example not enough water is being supplied, produce less secondary compounds than when conditions are optimal. When this is the case and the plant is less toxic, more female beetles could eat from the plant, but only a few did. In order to be sure that the toxicity of the plant is sufficient to resist ST beetles, all biotic and abiotic factors should remain constant, which was not always the case during this study.

Another problem involved the Y-linked resistant beetles and the ST females. The Y-linked beetles lived a lot shorter and the ST females had fewer offspring than expected. Earlier studies revealed that there seem to be no trade-offs for the possession of the R-gene (Nielsen, 1999), but this study suggests a shorter lifespan.

The Y-linked line was maintained by backcrossing Y-linked males with ST females. The ST females used in this study and for the maintenance of the Y-linked line had been kept under laboratory conditions for more than fifteen generations. It could be that due to inbreeding the ST females had a lower fitness which resulted in fewer offspring and in less vital offspring (Y-linked line).

In the future it might be helpful to have a lot more microsatellite markers. These markers can be used to create a dense genetic linkage map of the autosomal beetles. The markers present today can already be used for population genetic studies. These markers can also be combined with AFLP's. With cytogenetics also more about the genetic background of the resistance in *Phyllotreta nemorum* can be revealed and not only in populations with autosomal inheritance of the R-gene.

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Appendix 1. Molecular work

DNA extraction

Three different DNA extraction kits have been used. The E.Z.N.A.™ Insect DNA Isolation Kit was used to extract the DNA from beetles used for testing the primers. The other two kits have been used for the extraction of DNA from beetles used for creating a linkage map.

Puregene Genomic DNA Purification Kit

The mouse tail protocol was used from this kit to extract flea beetle DNA.
A flea beetle was put into a 1.5ml microfuge tube, put into liquid nitrogen and crushed with a pestle. 300 µl Cell Lysis Solution was added together with 1.5 µl Proteinase K Solution (20 µl/ml) and mixed by inverting the tube 25 times.
The solution was incubated at 55° C for 30 minutes. To the lysate 1.5 µl RNase A Solution was added and mixed by inverting the tube 25 time and incubated for 15-60 minutes.
The sample was cooled by placing it on ice for 1 minute. To the cell lysate 100 µl Protein Precipitation Solution was added and this mixture was vortexed at high speed for 20 seconds. The tubes were centrifuged for 3 minutes at 1400 rpm.
The supernatant was poured into a clean 1.5 ml microfuge tube together with 300 µl 100% Isopropanol. The sample was mixed by inverting the tube gently 50 times. The tube was centrifuged for 1 minute at 1400 rpm and the supernatant was poured off. 300 µl 70% Ethanol was added and the tube was inverted to wash the DNA pellet. The tube was centrifuged for 1 minute at 1400 rpm and the ethanol was poured off. The tube was inverted and drained on clean absorbent paper and allowed to dry for 5-10 minutes.
After drying 50 µl DNA Hydration Solution was added. The sample was incubated for 1 hour at 65° C to rehydrate the DNA. Periodically the tube was tapped. The samples were stored at 4° C.

E.Z.N.A.™ Insect DNA Isolation Kit

Each beetle was put into a microcentrifuge tube and placed in liquid nitrogen. With a microtube pestle the beetle was subsequently pulverized to powder. 350 µl Buffer CTL and 25 µl Proteinase K (20mg/ml) was added and briefly vortexed. The sample was incubated at 60° C for half an hour. To the lystae 350 µl chloroform:isoamyl alcohol (24:1) was added and vortexed. The mix was centrifuged for 2 minutes at 10.000 x g. The upper aqueous phase was put into a clean microcentrifuge tube. To this tube one volume of Buffer CBL was added followed by 2 µl RNase A and vortexed at high speed for 15 seconds. After this the sample was incubated at 70° C for 10 minutes. After one volume of absolute ethanol was added and mixed, 750 µl of the mixture was applied to an HiBind® DNA column assembled in a 2 ml collection tube. The tube was centrifuged at 10.000 x g for 1 minute and the flow-through liquid had been discarded.
The column was placed back into the same collection tube and the remaining mixture was applied into the column and centrifuged.
The column was placed into a new collection tube and washed by adding 500 µl HB Buffer. The tube was centrifuged at 10.000 x g for 30 seconds. The flow-through was discarded and the column was placed into a new collection tube. 700 µl DNA Wash Buffer diluted with absolute ethanol was added and centrifuged at 10.000 x g for 1 minute. This step was repeated twice. After this step the column was reinserted into the collection tube and centrifuged at 10.000 x g for 2 minutes to remove all traces of ethanol.
The column was then placed into a clean 1.5 ml microcentrifuge tube and 50 µl of Elution Buffer preheated to 60° C - 70° C was added directly onto the HiBind matrix. The buffer was allowed to soak for 2 minutes after which it was centrifuged at 10.000 x g for 1 minute. The DNA samples were stored at 4° C.

QIAGEN DNeasy ® Blood & Tissue Kit

Each beetle was put into a microcentrifuge tube and placed in liquid nitrogen. With a microtube pestle the beetle was pulverized to powder. 180 µl of Buffer ATL and 20 µl proteinase K was added. The samples were mixed thoroughly by vortexing and incubated at 56° C for 1 hour. After being incubated the samples were vortexed and 200 µl Buffer AL was added. Immediately after being mixed by vortexing 200 µl ethanol was added and mixed again by vortexing.
The obtained mixture was pipetted into a DNeasy Mini spin column placed in a 2 ml collection tube. The samples were centrifuged at 8000 rpm for 1 minute. 500 µl Buffer AW1 was added and the samples were centrifuged again at 8000 rpm for 1 minute. The flow-through was discarded and the spin column was placed into a new 2 ml collection tube. For the second wash step 500 µl Buffer AW2 was added and the samples were centrifuged at 14000 rpm for 3 minutes to dry the column and to make sure all residual ethanol was removed. The spin column was placed into a clean 1.5 ml microcentrifuge tube and 100 µl Buffer AE was added directly onto the membrane. Finally it was incubated at room temperature for a few minutes and centrifuged at 8000 rpm for 1 minute to elute the DNA. The DNA samples were stored at 4° C.

PCR

PCR (polymerase chain reaction) was used to amplify specific DNA sequences, the sequences containing the microsatellite marker. In general the PCR mixture was first heated more than 90° C denaturing the DNA strands. Secondly the mixture was allowed to cool down to a temperature around 50° C for annealing; primers could bind to their complementary sequence. Finally, the temperature was raised again to around 70° C allowing *Taq* polymerase to synthesize the new DNA strand between the two primers. After this the cycle was finished and the mixture can undergo a new identical cycle increasing the number of copies of the target sequence (Windelspecht, 2007). Gel electrophoresis was used to separate the different DNA fragments and ethidium bromide was used as a staining agent to visualize the fragments (see paragraph Gel electrophoresis).

Primer PCR

The PCR mix with newly tested primers and flea beetle DNA consisted per sample of 5.0 µl Buffer 5x, 0.5 µl dNTP (200µM), 1.5 µl 1.5 mM $MgCl_2$, 1.0 µl 500 nM of the tested forward and reverse primer, 0.125 µl GoTaq, 13.875 µl nuclease free water and 2.0 µl flea beetle DNA.

The cycling procedure profile for the PCR reaction on a DNA Engine Peltier Thermal Cycler (Bio-Rad) was 94° C for 3 minutes, 40 cycles of 94° C for 1 minute, 50° C for 1 minute, 72° C for 1 minute and a final extension period of 72° for 5 minutes.

12 µl of PCR mix was run on an Agarose gel (see Paragraph XX) at 70V for about 1 hour. When the PCR mix was used in combination with a methaphor Agarose gel, 7 µl PCR mix was used and run for 3 hours at 80V.

Plasmid PCR
The PCR mix containing plasmids consisted of 5.0 µl Buffer 5x, 0.5 µl 200µM dNTP, 1.5 µl 1.5 mM $MgCl_2$, 0.5 µl500 nM M13 forward and reverse primer, 0.125 µl GoTaq, 16.375 µl nuclease free water and 0.5 µl template.
7 µl of PCR mix was run on a gel at 70V for about 1 hour.

The cycling procedure profile for the PCR reaction was 94° C for 3 minutes, 40 cycles of 94° C for 1 minute, 50° C for 1 minute, 72° C for 1 minute and a final extension period of 72° C for 5 minutes.

Gradient PCR
The annealing temperature ranged from 51° C to 57° C. The cycling profile is the same as for the other PCR reactions.

Gel electrophoresis

Gel electrophoresis was used to separate the different DNA fragments created during the PCR. The procedure is based on the principle that when exposed to an electric field negatively charged molecules will move toward the cathode, positive terminal, and positively charged molecules will move toward the anode, negative end. Agarose is used as a matrix to separate the molecules. Large fragments will be slowed down by the matrix and smaller fragments will pass easily through the pores of the matrix. A gel with a high percentage of Agarose will take long to run, but will provide a greater resolution of the fragments. Ethidium bromide is used as a staining agent to visualize the fragments. The first and last well on every gel is loaded with a molecular weight marker consisting of DNA fragments of known size to determine the size of the fragments (Windelspecht, 2007).

Agarose gel
Agarose gels used for the PCR reactions with beetle DNA and newly developed primers were 1,5% agarose gels with 1x TAE (40mM Tris, 20mM acetate, l mM EDTA, pH 8.3)
and 3,5 µl ethidium bromide (10 mg/ml).

Agarose gels used for PCR reaction with DNA inserts in plasmids were 1 % agarose gels with 1x TAE (40mM Tris, 20mM acetate, l mM EDTA, pH 8.3) and 3,5 µl ethidiumbromide per 100 ml.

Methaphor Agarose gel
The methaphor Agarose gel used was a 3.5 % gel with 1x TAE (40mM Tris, 20mM acetate, l mM EDTA, pH 8.3) and 3,5 µl ethidiumbromide per 100 ml. The gel was made using the Metaphor Agarose Product protocol by Lonza (www.lonza.com).

PCR product purification

In order to know precisely what products were produced during the PCR reaction, some samples have been purified and sequenced.
At first the PCR product was purified and sequenced. The results were not very satisfying; the number of positive clones was very small and the products were too small to analyze. Therefore a second attempt was made by purifying the PCR product from an agarose gel. In both procedures the QIAquick PCR Purification Kit from QIAGEN was used.

Purification of PCR product directly

In order to purify the PCR product, five times the volume of the PCR sample of Buffer BP was added and mixed. This sample was applied to a QIAqiuck spin column inserted in a 2ml collection tube and centrifuged for 1 minute. The flow-through was discarded and 750 µl Buffer PB was added to wash the solution and it was centrifuged for 1 minute. After the flow-through was discarded, the column was centrifuged again for 1 minute at maximum speed to completely remove residuals of ethanol. At last the column was placed in a clean microcentifuge tube and 30 µl of Buffer PE was added to elute the DNA. It was left standing for 1 minute before it was centrifuged.

Purification of PCR product from agarose gel

The DNA fragments were excised from the Agarose gel with a scalpel and put into a clean 1.5 ml tube. Three times the volume of the gel slice Buffer QG was added. To completely dissolve the gel slice the tubes were incubated at 50° for 10 minutes and vortexed every 2-3 minutes to mix. One gel volumes of isopropanol was added and mixed. The solution was applied to a QIAquick spin column and centrifuged for 1 minute. After the flow-through was discarded 0.5 ml of Buffer QG was added to the column and again centrifuged for 1 minute. In the last step 30 µl of Elution Buffer was added to the centre of the QIAquick membrane and it was soaked for 10 minutes instead of 1 minute as suggested in the protocol. The yield seems to be higher with a longer soaking time. After 10 minutes the column was centrifuged for 1 minute, in order to dissolve the DNA in the membrane.

Cloning

Two different methods were used for the ligation reaction. The first method using a pGEM-T vector did not result in sufficient separate colonies to pick from. Therefore a second method was used using GeneJET™ PCR cloning Kit from Fermentas. This method was less time consuming and gave better results.

Using pGEM-T vector ligation reaction from Promega

Ligation

The ligation reaction was set up by adding 5 µl 2x Rapid Ligation Buffer, 1 µl pGEM-T Easy Vector, 3 µl insert and 1 µl T4 DNA ligase (3 weiss units/µl) together.
The samples were stored at 4° C overnight.

Transformation

2 µl of ligation product and 20 µl of competent cells (XL2-blue, Stratagene) were mixed together and were put on ice for 30 minutes. The cells were heat shocked for 30 minutes exactly in a water bath of 42° C. After that the tubes were returned on ice for 2 minutes. 970 µl of SOC medium (2 g Bacto-Tryptone, 0.5 g yeast extract, 1 ml 1 M NaCl, 0.25 ml 1 M KCl, 2 ml 1 M Mg^{+}, 2 ml glucose all dissolved in 970 ml H_20) was added to the tubes and were incubated for one hour at 37° C while shaking at 225 rpm. 100 µl of each transformation was plated out and incubated overnight at 37° C.

AXI/LB plates have been used for this procedure. They contained 1.5 % agar, 25 µl ampicilline 100mg/ml, 50 µl X-gal 20mg/ml and 25 µl /100mM IPTG.

Plasmids which contained the insert turn out as white colonies; plasmids with no insert left the β-galactosidase gene intact causing the colonies to turn blue on AXI-plates. White colonies were picked from the plate with a sterile wooden tooth pick and put into an autoclaved tube with 3.5 ml LB medium (10g/l Tryptone, 5 g/l yeast extract and 5 g/l NaCl) with 3.5 µl ampicillin (100mg/ml). Finally the tubes were incubated overnight at 37° C while shaking at 225 rpm.

Using CloneJET™ PCR Cloning Kit from Fermentas

The sticky end protocol has been used.

For the blunting reaction each sample contained 10 µl buffer 2x, 3 µl of insert, 4 µl nuclease free water and 1 µl DNA blunting enzyme. The mixture was incubated at 70° C for 5 minutes and after that briefly chilled on ice.

Ligation
For the ligation reaction 1 µl of pJet 1.2/blunt cloning vector (50ng/ µl) and 1 µl T4 DNA ligase (5u/ µl) was added to the blunting reaction mixture. The ligation mixture was incubated for 5 minutes at room temperature.

Transformation
2 µl of ligation product and 20 µl of competent cells (XL2-blue, Stratagene) were mixed together and put on ice for 30 minutes. The cells were heat shocked for 30 minutes exactly in a water bath of 42° C. After that the tubes were returned on ice for 2 minutes. 970 µl of SOC medium was added to the tubes and were incubated for one hour at 37° C while shaking at 225 rpm. 100 µl of each transformation product was plated out and incubated overnight at 37° C.

LB ampicilline plates have been used for plating the ligation mixture. One liter of mixture contained 15 g Bacto Agar, 10 g Bacto Tryptone, 5 g Bacto Yeast Extract, 5 g NaCl all dissolved in 1 l water. Finally 1000 µl of ampicilline was added.

Only plasmids which contained an insert could grow on the specific plates used. Two colonies of each plate were touched with a sterile wooden tooth pick and put into an autoclaved tube with 3.5 ml LB medium containing 3.5 µl ampicilline (100mg/ml). Finally the tubes were incubated overnight at 37° C while shaking at 225 rpm.

Allele size estimation

Each used PCR sample contained one microsatellite marker (overview markers; see table 3). The samples were purified using a sephadex 96 well plate. This plate was first centrifuged at 910 rpm for 5 minutes. 20 µl of mix was put onto the plate, the mix consisted of 14µl of water and 2 µl of each (x3) PCR product. The plate was centrifuged at 910rpm for 5 minutes.

The final plate used for allele size estimation contained 10µl formamide/sizer solution and 1 µl of the purified PCR mixture (3 PCR products of each sample). The formamide/sizer consisted of 1 ml formamide and 15 µl sizer (see table 4). Plates were sealed with aluminum foil and briefly centrifuged.

Table 4. Internal sizers

is83	GTTTCTCCAGCTTTTGTTCCCTTTAGT
is117	GTTTCACTAGTTCTAGAGCGGCCG
is154	GTTTATAAGCTTGATATCGAATTCCTGC
is175	GTTTCCCCTCGAGGTCGACGGTATC
is209	GTTTGACTCACTATAGGGCGAATTGG
is272	GTTTGGTAACGCCAGGGTTTTCCCAGTC
is291	GTTTCTGCAAGGCGATTAAGTTGGGTAA
is328	GTTTCCTCTTCGCTATTACGCCAG
is366	GTTTGCCATTCAGGCTGCGCAACTG
is400	GTTTCGCTTAATGCGCCGCTACAG
is500	GTTTGCGAACGTGGCGAGAAAGGAAGG

Size estimation was done at Plant Research International using an ABI PRISM 3700 automated sequencer. Subsequently the PCR products were analysed using Genescan and Genotyper software (Applied Biosystems).

Appendix 2. Positive clones

Table of clones containing useful repeats. The clone name refers to position on the 96-wells plate. Some clones contained the same insert, for example A09 and A12. Only the first ones have been used in further processing.

	Clone			
1	A02			
2	A06			
3	A09	A12		
4	A10	A11	B02	C03
5	B03	B05	C07	C12
6	B04			
7	B06	B07	C08	
8	B11			
9	C05			
10	C10			
11	D01	D05		
12	D02	E09	E11	F02
13	D10			
14	D11			
15	E06			
16	E08			
17	E10			
18	F03			
19	F05			
20	F07			
21	F10			
22	F12	G08	H02	
23	G03			
24	G05			
25	G06			
26	G07			
27	G09	H03		
28	H01	H06		
29	H05			
30	H11			

Appendix 3. Overview primer pairs

Overview of all designed microsatellite primer pairs. The ones that showed polymorphic bands on a Metaphor Agarose gel after PCR can be used as a microsatellite marker.

	bp	forward	reverse	repeat	polymorfic
A02	109	GGGTGTTTGAGAGAAAGGGTG	CACGGACTGGCTCAATCTGC	(CA)4	No
A06	149	AAGGTCTTTGTGATGGACAAG	CCTCTTGCAATTTTTTTTCG	(AC)8	Yes
A09	119	AACCATGGACGCTAATAGAC	GTCGTGGGTTTTTTTTTTAC	(CGT)4	No
A10	119	AGAATGGACATGGTCGGCGG	ACTCACGCCCGAATCGCTTC	(CGA)5	Yes
B04_101	159	CGTCAGCCGGGAACCACAGG	CGGCTGGCCGTTGGTCGAAG	(GAC)5	No
B04_121	118	CGTCACGGTTCGAACTGGGCGC	CTTCGACCAACGGCCAGCCG	(GAC)5	Yes
B06	239	CTGTCTTAGGCGGTTTAACC	ATGGAAGCATTGTACGGAAG	(GAC)30	Yes
B11-111	99	CTTTCCCCCCCTTCTCTGCTCGTTC	ACCTCCACGGGCTGCGGTTG	(GTC)4	No
B11_311	89	AATCTGGAGACGATGATGAC	CTTCCGAAATAATCGTCTTC	(CGT)4 en (TCG)4	Yes
C05_11	367	TACCATAAACGCACTGTTGA	TCACCAAATTGTGACATGTACC	(TAAA)5	Yes
C05_631	99	GTGCAAATTGTCGAGACGGC	GATGTCATCCAGGTCCGTATC	(TCG)7	No
C10	79	TTAGGCACCGGAGTCACTGAC	AGTACGCGAGAGCGCTGATG	(GAC)5	Yes
D01	348	TGTCTTGGAACGCTAATCGC	AACTTTGCATCGAATTGTGC	(GCC)5 en (TGC)8	Yes
D02	139	TGCGTTGTAGCCGGATTTTG	ATCAGCAGGCCATGCAGCAG	(CAG)5	Yes
D10	99	TCTTCTTGTTGTTGTCCTGC	GCTGCTCGATTTAATCTGAC	(CAG)7	Yes
D11	129	TCTTCAGCAGCCTGATGGGC	ATCACGTTCGGCACCACCTG	(CTG)4 en (CTG)7	Yes
E06	169	TGAACCAACAGCAACAACAG	GTCGTTTCTTGTTGCTGCTG	(TGC)7 en (TGC)4	No
E08_221	129	TCACCACTGACCCGTTGCTG	AAATCGCACCAGCAGCATCAC	(CAG)4	No
E08_61	109	ATTCGCCACCGTACCGTTCG	GCAGCAGGTCGAGGCGACTG	(CAG)7	Yes
E10	149	ATGTCTTGGAACGCTAATCG	TTTGAAAACTATTGCCCATC	(TGC)7 en (GCT)4	Yes
F03	119	TGCAGCAACAACAACAGCGC	TCACGCGTAACAGGTGCTGG	(TGC)11	Yes
F05	179	AATCGGTCAACCAGCAATTC	AAGGACCAAAGAGATCAAAAAC	(TGC)4 en (TGC)4	No
G03_201	99	TCGATGAACAGGCCTCCGTG	TCTTGGGGAGGCTCGAGAAG		No
G03_372	88	CGCCGTTGTACCAGCACTATTTG	ATCGTACGGCTGTCGCATGC		No
G05	99	CACGTTATTAGGCATAGGCG	ATCAACGATTTACACAACCG	(CCG)4	No
G06	199	TTGAGGACTTAGGTTTTGGTATAG	CGTGGAATTTGTCAACACTTG	(GCC)8	Yes
G07	109	AGCCAGGAGGCGTTCATGAC	ATCCTTATCTTTTTTATCGGGATCC	(CGG)4	No

Appendix 4. Overview allele size estimations

Allele size estimations of the C-line. PF: Parent Female, PM: Parent Male, M: Male, F:Female.

	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B
Title line:	D09		A03		H12		D06		BB08		AB12		A04		D04		E11	
C-lijn P F 40	188	191	217	219	155	155	172	172	000	000	197	212	177	177	154	154	221	224
C-lijn PM 41	176	191	217	219	155	155	172	172	251	254	197	209	177	177	154	154	221	224
C-lijn M39	188	191	217	219	155	155	172	172	251	254	197	212	177	177	154	154	224	224
C-lijn M38	176	188	217	219	155	155	172	172	251	254	209	212	177	177	154	154	221	224
C-lijn M37	188	191	219	219	155	155	172	172	251	254	197	197	177	177	154	154	221	224
C-lijn M36	176	191	217	219	155	155	172	172	000	000	197	209	177	177	154	154	221	221
C-lijn M35	176	191	217	217	155	155	172	172	000	000	197	212	177	177	154	154	224	224
C-lijn M34	191	191	217	219	155	155	172	172	251	254	197	212	177	177	154	154	221	221
C-lijn M33	176	191	217	219	155	155	172	172	000	000	209	212	000	000	000	000	000	000
C-lijn M32	191	191	217	219	155	155	172	172	000	000	197	197	177	177	154	154	224	224
C-lijn M31	188	191	000	000	155	155	172	172	000	000	197	209	177	177	154	154	221	224
C-lijn M30	188	191	219	219	155	155	172	172	251	254	197	197	177	177	154	154	221	224
C-lijn M29	188	191	217	219	155	155	172	172	251	254	209	212	177	177	154	154	221	224
C-lijn M28	188	191	217	219	155	155	172	172	251	254	197	212	177	177	154	154	221	224
C-lijn M27	176	188	219	219	155	155	172	172	251	254	209	212	177	177	154	154	224	224
C-lijn M26	176	191	217	217	155	155	172	172	251	254	197	197	177	177	154	154	221	221
C-lijn M25	176	188	217	217	155	155	172	172	251	254	197	209	177	177	154	154	221	224
C-lijn F24	188	191	217	217	155	155	172	172	000	000	197	212	177	177	154	154	221	221
C-lijn F23	191	191	217	219	155	155	172	172	000	000	209	212	177	177	154	154	224	224
C-lijn F22	188	191	217	219	155	155	172	172	251	254	197	197	177	177	154	154	221	224
C-lijn F21	000	000	000	000	000	000	172	172	000	000	197	197	177	177	154	154	221	224
C-lijn F20	176	191	217	219	155	155	172	172	000	000	197	197	177	177	154	154	221	224
C-lijn F19	191	191	217	217	155	155	172	172	251	254	197	212	000	000	000	000	000	000
C-lijn F18	176	188	217	219	155	155	172	172	000	000	197	209	177	177	154	154	224	224
C-lijn F17	188	191	217	219	155	155	172	172	251	254	197	197	000	000	000	000	000	000
C-lijn F16	191	191	217	217	155	155	172	172	251	254	197	209	177	177	154	154	221	221
C-lijn F15	000	000	217	219	155	155	000	000	000	000	197	197	177	177	154	154	224	224
C-lijn F14	191	191	217	219	155	155	172	172	000	000	197	212	177	177	154	154	224	224

C-lijn F13	176	191	219	219	155	155	172	172	251	254	197	212	177	177	154	154	221	221
C-lijn F12	000	000	000	000	000	000	172	172	251	254	197	209	177	177	154	154	221	224
C-lijn F11	176	188	217	219	155	155	172	172	000	000	197	197	177	177	154	154	221	224
C-lijn F10	191	191	217	217	155	155	172	172	000	000	209	212	177	177	154	154	224	224
C-lijn F9	176	188	217	219	155	155	172	172	251	254	209	212	177	177	154	154	221	221
C-lijn F8	188	191	217	219	155	155	172	172	000	000	197	209	000	000	000	000	000	000
C-lijn F7	191	191	217	217	155	155	172	172	251	254	197	197	177	177	154	154	221	221
C-lijn F6	191	191	217	219	155	155	172	172	251	254	197	197	177	177	154	154	221	224
C-lijn F5	188	191	217	219	155	155	172	172	000	000	197	212	177	177	154	154	221	224
C-lijn F4	188	191	217	219	155	155	172	172	000	000	197	212	000	000	000	000	000	000
C-lijn F3	191	191	217	219	155	155	172	172	251	254	197	209	177	177	154	154	221	224
C-lijn F2	000	000	000	000	000	000	172	172	251	254	209	212	177	177	154	154	224	224
C-lijn F1	000	000	000	000	000	000	000	000	000	000	209	212	177	177	154	154	221	221

Allele size estimations of the G-line. PF: Parent Female, PM: Parent Male, M: Male, F:Female.

Population	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B
Title line:	D09		A03		H12		D06		BB08		AB12		A04		D04		E11		H12	
G-lijn PF47	176	191	217	219	155	155	172	172	0	0	197	212	177	177	154	154	221	224	162	165
G-lijn PM48	176	188	217	219	155	161	172	172	0	0	197	203	177	177	154	154	224	224	162	165
G-lijn M46	176	176	217	219	155	155	172	172	0	0	203	212	177	177	154	154	221	224	165	165
G-lijn M45	176	176	217	217	155	161	172	172	251	254	203	212	177	177	154	154	224	224	162	162
G-lijn M44	176	176	217	219	155	161	172	172	251	254	197	212	177	177	154	154	221	224	165	165
G-lijn M43	176	176	217	219	155	161	172	172	0	0	197	212	177	177	154	154	224	224	165	165
G-lijn M42	188	191	217	219	155	161	172	172	0	0	197	197	177	177	154	154	224	224	165	165
G-lijn M41	188	191	217	219	155	161	172	172	0	0	197	197	177	177	154	154	224	224	162	165
G-lijn M40	176	191	217	219	155	155	172	172	251	254	203	212	177	177	154	154	224	224	162	162
G-lijn M39	176	191	217	219	155	155	172	172	251	254	197	212	177	177	154	154	224	224	162	165
G-lijn M38	176	188	217	219	155	155	172	172	0	0	197	212	177	177	154	154	221	224	162	162
G-lijn M37	176	176	217	217	155	155	172	172	251	254	197	203	177	177	154	154	224	224	165	165
G-lijn M36	188	191	217	217	155	155	172	172	0	0	197	197	177	177	154	154	221	224	162	162
G-lijn M35	176	191	217	217	155	161	172	172	0	0	203	212	177	177	154	154	221	224	162	162
G-lijn M34	176	188	217	219	155	161	172	172	0	0	197	203	177	177	154	154	221	224	162	165
G-lijn M33	176	176	217	219	155	161	172	172	0	0	203	212	177	177	154	154	224	224	165	165
G-lijn M32	176	188	217	219	155	161	172	172	0	0	197	212	177	177	154	154	221	224	165	165
G-lijn M31	176	176	217	219	155	155	172	172	0	0	0	0	177	177	154	154	221	224	162	165
G-lijn M30	176	176	217	217	155	155	172	172	251	254	197	203	177	177	154	154	224	224	162	165
G-lijn M29	188	191	219	219	155	161	172	172	0	0	203	212	177	177	154	154	221	224	162	162
G-lijn M28	176	176	217	219	155	155	172	172	0	0	197	203	177	177	154	154	221	224	162	165
G-lijn F25	188	191	217	217	155	161	172	172	251	254	197	212	177	177	154	154	0	0	0	0
G-lijn F24	176	176	217	219	155	161	172	172	0	0	197	203	177	177	154	154	221	224	165	165
G-lijn F23	176	191	217	219	155	161	172	172	251	254	197	203	177	177	154	154	221	224	165	165
G-lijn F22	176	188	219	219	155	155	172	172	251	254	197	197	177	177	154	154	224	224	162	165
G-lijn F21	176	176	217	219	155	155	172	172	0	0	203	212	177	177	154	154	224	224	165	165
G-lijn F20	0	0	217	219	155	155	172	172	0	0	197	212	177	177	154	154	221	224	162	165
G-lijn F19	0	0	217	219	155	155	172	172	0	0	197	197	177	177	154	154	224	224	162	165
G-lijn F18	0	0	217	219	155	161	172	172	251	254	197	212	177	177	154	154	221	224	162	162
G-lijn F17	0	0	217	219	155	161	172	172	251	254	197	212	177	177	154	154	224	224	162	165

G-lijn F16	0	0	217	217	155	155	172	172	0	0	197	197	177	177	154	154	224	224	162	165
G-lijn F15	0	0	219	219	155	155	172	172	251	254	197	212	177	177	154	154	224	224	162	162
G-lijn F14	0	0	219	219	155	155	172	172	251	254	203	212	177	177	154	154	221	224	162	162
G-lijn F13	0	0	219	219	155	155	172	172	0	0	197	197	177	177	154	154	224	224	165	165
G-lijn F12	0	0	219	219	155	155	172	172	0	0	203	212	177	177	154	154	224	224	162	165
G-lijn F11	0	0	217	219	155	155	172	172	251	254	197	212	177	177	154	154	221	224	162	162
G-lijn F10	0	0	217	217	155	161	172	172	251	254	197	212	177	177	154	154	221	224	162	162
G-lijn F9	0	0	219	219	155	155	172	172	251	254	197	197	177	177	154	154	224	224	162	165
G-lijn F8	0	0	217	219	155	155	172	172	251	254	197	212	177	177	154	154	224	224	162	165
G-lijn F7	0	0	219	219	155	161	172	172	252	254	203	212	177	177	154	154	221	224	162	162
G-lijn F6	0	0	217	219	155	161	172	172	0	0	203	212	177	177	154	154	224	224	162	165
G-lijn F5	0	0	217	219	155	155	172	172	0	0	203	212	177	177	154	154	224	224	162	165
G-lijn F4	188	191	217	217	155	161	172	172	0	0	197	197	177	177	154	154	221	224	162	165
G-lijn F3	176	176	219	219	155	161	172	172	251	254	197	203	177	177	154	154	221	224	165	165
G-lijn F2	0	0	217	217	155	155	172	172	0	0	197	212	177	177	154	154	221	224	162	165
G-lijn F1	0	0	217	219	155	161	172	172	0	0	203	212	177	177	154	154	224	224	165	165