Mapping and Applications of Microsatellite and AFLP Markers in Barley (*Hordeum vulgare*).

A Thesis Submitted to the
Faculty of Graduate Studies by

Tamara L. Franzmann

In Partial Fulfillment of the Requirements
for the Degree of Master of Science

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MAPPING AND APPLICATIONS OF MICROSATELLITE AND AFLP MARKERS IN BARLEY *(HORDEUM VULGARE)*.

BY

TAMARA L. FRANZMANN

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

Master of Science

ABSTRACT

The objectives of this study were to map both microsatellite and AFLP markers onto segregating barley populations, apply a core set of microsatellite markers to differentiate registered barley lines and to trace the inheritance of chromosomal segments based on pedigree.

A total of twenty new microsatellite markers were added to two existing Harrington x TR306 barley genetic maps. Further sources of microsatellite markers were examined, including wheat microsatellites and probing genomic DNA for repeats, but no additional microsatellite markers were identified.

By using eleven AFLP combinations, 189 polymorphic loci were identified and 138 were mapped onto an existing Shyri x Galena genetic map. The additional markers added 1469 cM to the map and a direct correlation was noticed between the number of AFLP markers added to a chromosome and the increase in chromosome length. Several different mapping experiments were conducted to determine the effects of different classes of markers on the Shyri x Galena map. Mapping of the new AFLP loci increased the overall map length regardless of the marker class excluded from the genetic map.

Two applications of microsatellite markers were applied to barley cultivars. First, a set of nine microsatellite markers, found on five chromosomes, were used to distinguish twenty registered barley cultivars. Secondly, twenty-five microsatellite markers were also screened on 12 barley cultivars and on their parental lines to trace the inheritance of chromosomal segments.

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I. INTRODUCTION

Barley (*Hordeum vulgare*) is the fourth most important crop in the world following wheat, maize and rice (Bass 1993). It is produced in all regions of the world for malt or animal feed. In the 2000 Canadian crop year, barley was produced on 10 million acres (Canadian Wheat Board 2000). There are several breeding programs dedicated to barley improvement and molecular markers are becoming an important tool for the breeders.

In barley, molecular markers have been used for varietal fingerprinting (Russell et al. 1997), production of genetic maps (Becker and Heun 1995; Becker et al. 1995; Giese et al. 1994; Graner et al. 1991; and Liu et al. 1996), marker assisted selection (Chen et al. 1994; Bauer et al. 1997), and DNA fingerprinting and genetic diversity (Saghai Maroof et al. 1994).

High density, robust genetic maps are essential for genomic studies. They can be developed through analysis of restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and microsatellites markers.

There are advantages and disadvantages to each method. Microsatellite and RFLP markers are codominant, while RAPD and AFLP markers are dominant. RFLP markers are labour intensive and time consuming, microsatellite primers are initially time consuming to develop because of the specific sequence information required. RAPD and AFLP markers require no prior sequence information. AFLP markers generate the greatest number of loci per primer pair

but microsatellite markers detect the greatest number of alleles per primer pair (Powell et al. 1996).

II. LITERATURE REVIEW

Barley

Economic Importance and Distribution

Hordeum vulgare L., cultivated barley, is the fourth most important crop in the world following wheat, maize and rice (Bass 1993) and is produced in all regions of the world. It belongs to the tribe *Triticeae* in the grass family *Poaceae*. Its origins have been dated by carbon dating to approximately 7900 BC in Iran and Chinese beers were brewed over 4000 years ago from barley, millet and maize (Bass 1993). There are both summer and winter forms that can either be two-rowed or six-rowed types according to spike morphology.

Settlers to the Selkirk region first introduced barley to Western Canada in 1812. In 1929, the first registered varieties of barley, Velvet and Trebi, were released in western Canada (Harrison 1950). Since then there have been several successful varieties, the most notable being the two-row malting variety, Harrington, which captured approximately 45% of the 10 million seeded barley acres in the 2000 crop season (Canadian Wheat Board 2000).

Barley grain has two main uses. The grain can either be turned into malt, which is then used to produce beer and whiskey, or is it used for animal feed. Barley is also a valuable crop for research because it is a diploid organism. It has been widely used as for the development of genetic maps and identification of genes that may be common in cereal crops (Kasha 1996).

The Barley Genome

H.vulgare is a self-pollinating diploid with 2n = 2x = 14 and the genome is approximately 5.3 x 10^9 base pairs. In barley, a gene density of one gene every 123-212 kilobases can be expected if the genes are spaced equidistantly (Panstruga et al. 1998).

Barley Breeding

H.vulgare is a self-pollinating species and therefore all offspring are almost genetically identical to the parent plant. They are not completely identical due to spontaneous mutations and because barley can have an outcrossing frequency of up to 0.5% (Poehlman and Sleper 1995). This level of outcrossing is not sufficient to introduce enough variation in a breeding program so breeders use controlled crosses to introduce desired variation. Following crossing, desirable genotypes may be selected through pedigree selection, bulk-population, single-seed descent or doubled haploid production until an acceptable level of homozygosity is reached (Poehlman and Sleper 1995).

These procedures may take upwards of ten generations before a desirable line is produced. Registration of a barley variety may take an additional five to ten years because several generations must be tested for agronomic traits including yield, disease resistance and, if applicable, malt quality in locations across western

Canada (Bass 1993). Molecular biology may be helpful in reducing this time frame if screening for some traits can be done in the laboratory.

Genetic Markers

A genetic marker is a heritable character that is easily discerned (Lui 1998). It can be used for identification of an individual or a cultivar through DNA fingerprinting, trait tagging and genetic mapping. The most common genetic markers include morphological, protein and DNA markers. DNA markers include restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphisms (AFLP), randomly amplified polymorphic DNA (RAPD) and microsatellites (simple sequence repeats). The marker can be a gene of a known function, a DNA fragment of unknown function or a heritable phenotype (Liu 1998).

Allelic Variation

All marker systems are dependent upon allelic variation, which is defined as the alternate forms of a gene or a genetic marker at a locus (Liu 1998). Different alleles at a locus may result in different expressed phenotypes. By identifying the alleles associated with the phenotypic differences, individuals can be selected based on their variation at a locus.

Morphological Markers

Morphological markers may also be heritable phenotypes. Plants are selected based on traits such as yield, lodging resistance and pest resistance. This approach can be successful, provided there is sufficient expression of the genotype. Phenotypic selections are subjective, they are often influenced by environmental conditions and they are labour intensive (Russell et al. 1997).

Phenotype is often a result of genotype by environmental interactions. If the environmental conditions are not favourable, full expression of the phenotype may not be observed, therefore more accurate selection of traits may be achieved through genotypic indicators than phenotypic indicators (Russell et al. 1997). However, genotypic markers may be difficult to develop for complex traits and phenotypic markers may be more practical in a breeding program.

Isozymes

An isozyme is an enzyme that is catalytically and structurally similar to another enzyme within the same organism (Strickberger 1985). Isozyme analysis involves producing a distinct electrophoretic pattern of a protein in a starch or polyacrylamide gel. It relies on size and charge differences between proteins. While isozymes are informative there is not a sufficient number available to be applicable to breeding programs and this has restricted their use for trait screening (Edwards et al. 1992).

Restriction Fragment Length Polymorphism (RFLP)

The use of restriction fragment length polymorphisms (RFLP) as genetic markers was proposed by Botstein et al. (1980). RFLPs use restriction enzymes to identify a target sequence and to cut the DNA at that site. A restriction enzyme that normally cuts at a site will not cut if there has been a mutation in the sequence or if the site has been lost through recombination. This will result in different numbers and sizes of fragments produced depending on the individual being screened. RFLPs are detected through electrophoresis and Southern blot hybridization (Southern 1975) after restriction of genomic DNA with restriction enzymes.

The first locus identified using RFLP analysis was the $^{G}\gamma$-, $^{A}\gamma$-, δ-, and β-globin locus found in humans (Jeffreys et al. 1979). RFLPs were also used to map the first gene, Duchenne Muscular Dystrophy also present in humans (Murry et al. 1982). Genetic maps have been developed using RFLPs for crops including barley (Heun et al. 1991; Graner et al. 1991), maize (Helentjaris 1987), rice (McCouch et al. 1988) and wheat (Chao et al. 1989). In addition to genetic mapping, RFLP's can be used to determine genetic diversity (Miller and Tanksley 1990), locate quantitative trait loci (QTL) (Burr et al. 1988) and for marker assisted breeding (Young and Tanksley 1989).

The advantage of the RFLP marker system is that it is a codominant marker system. It can detect heterozygous individuals as distinct from either possible homozygous state at the same locus. However, the RFLP system is a time consuming, labour intensive process requiring large quantities (μg scale) of

relatively pure DNA (Waugh and Powell 1992). The codominant information that is generated is highly informative but the quantity of information is not practical to apply the technology to large scale breeding programs.

Polymerase Chain Reaction (PCR)

The development of the polymerase chain reaction (PCR) by Mullis (1986) greatly changed the field of genetics. PCR-based technologies are faster, less labour intensive and cheaper than RFLP. PCR allows the amplification of a specific locus from a small quantity (ng scale) of DNA. The PCR reaction involves a series of three basic stages. First, DNA is denatured into single stranded form, secondly primers are annealed to the DNA and finally the primers are extended to make a copy of the DNA.

The specificity of the PCR reaction is dependent on annealing temperature, $MgCl_2$ concentration and requires primers, deoxynucleotides for primer extension and *Thermus aquaticus* DNA polymerase for catalysing the reaction.

Randomly Amplified Polymorphic DNA (RAPD)

The randomly amplified polymorphic DNA (RAPD) technique was developed by Williams et al. (1990). In this PCR reaction, a single, arbitrary primer of approximately ten nucleotides is used to amplify DNA. It is based on the probability that a given DNA sequence, complementary to the primer, will occur in the genome

on opposite DNA strands, in opposite orientations within a distance amplifiable by PCR (Waugh and Powell 1992). The resulting banding pattern may be a single band or it may be more complex with several bands. This multiple pattern results when the primers can bind to more than one location in the genome (Waugh and Powell 1992).

Unlike RFLPs, RAPD markers are generally dominant markers. Codominant RAPD markers are possible when there is a change in the length between the two binding sites but not the loss of a site (Yu et al. 1993). The genotypic scoring of a dominant marker is based on the presence or absence of an amplification product (Williams et al. 1990). However, the presence of an amplified DNA segment cannot distinguish heterozygotes from homozygotes. Alternatively, a product may not be amplified in every individual. The absence of a band may be due to failure of the PCR, like other methods, or due to sequence differences. Sequence differences may result from either a change in the sequence of the primer binding site (a point mutation) or from a change in the target sequence which prevents amplification including large deletions, insertions or inversions (Waugh and Powell 1992).

RAPD markers have the advantage of being easy and relatively inexpensive to perform with minimal labour. DNA can be less pure than for RFLP and no sequence information is required to develop the primers.

Amplified Fragment Length Polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP) is a PCR-based marker system developed by Vos et al. (1995). As shown in Figure 1, DNA is restricted with *Eco*RI and *Mse*I to generate restriction fragments then adapters are ligated to the ends of the restriction fragments. A two step selection reduces the number of amplified fragments to approximately 50-100 fragments for each AFLP reaction (Vos et al. 1995). The number of resulting fragments depends on the complexity of the genome and the number of selective nucleotides used. An advantage of AFLP is that it can be applied to genomic DNA of any source without an initial investment in cloning or sequencing (Vos et al. 1995).

High quality DNA is required to ensure a complete restriction digest as partial digestions may result in bands that are interpreted as false polymorphisms. The DNA banding pattern is not affected by DNA concentration, the same banding pattern has been obtained from template DNA ranging from 2.5 pg to 25 ng (Vos et al. 1995).

The polymorphic, amplified fragments generated by AFLP are inherited in a Mendelian fashion and correspond to unique genome positions (Vos et al. 1995). Each fragment is uniquely characterized by its size and the primers used for amplification. AFLP markers can be validly used as landmarks in genetic and physical maps because they correspond to unique genome positions (Vos et al. 1995).

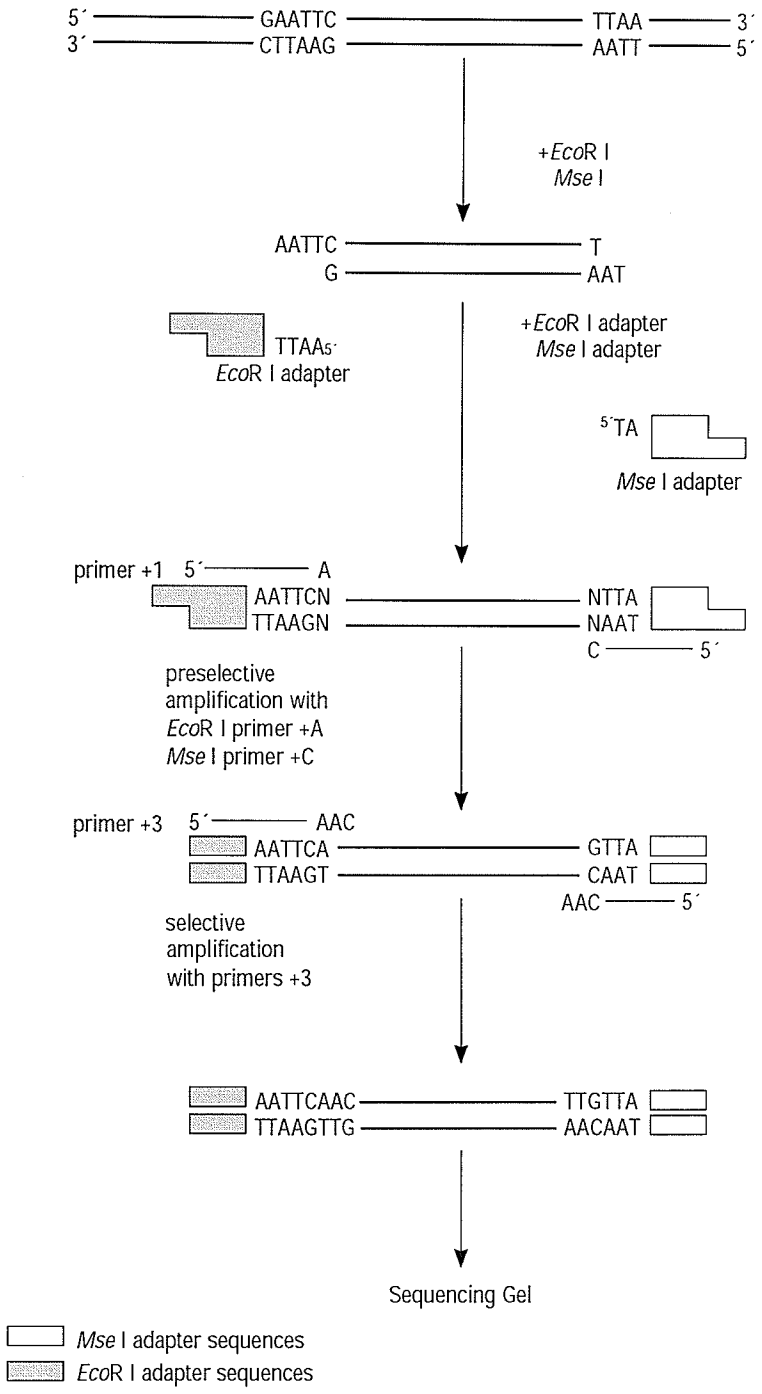

Figure 1: Illustration of the AFLP procedure, including restriction of the genomic DNA, ligation of adapters, pre- and selective amplification. (Source: Lin and Kou 1995).

AFLP markers have been successfully used for developing genetic maps, identifying loci and DNA fingerprinting. Genetic maps have been produced in many crops including barley (Becker et al. 1995), rice (Mackill et al. 1996), and soybeans (Keim et al. 1997). AFLP fingerprinting was originally described by Vos et al. (1995) using *Arabidopsis thaliana*, tomato, maize, and yeast and has since been applied to several species including wild bean collections, (Tohme et al. 1996) and lettuce (Hill et al. 1996).

AFLP fragments have been cloned and developed into allele specific amplicons in crops such as rice (Cho et al. 1996), and the poplar tree (Cervera et al. 1996). The AFLP marker system has been used to develop phylogenetic relationships and study genetic diversity in lentils (Sharma et al. 1996), pea (Lu et al. 1996), soybean (Maughan et al. 1996) and wheat (Barrett and Kidwell 1998).

AFLP is a generally a dominant marker system resulting in the presence or absence of restriction fragments rather than length differences (Vos et al. 1995). However codominant AFLP markers are also possible (Keim et al. 1997). While microsatellites may detect a greater degree of heterozygosity between accessions, AFLP will detect a greater number of polymorphic markers per primer pair than microsatellites (Powell et al. 1996).

Microsatellites

Microsatellites, also referred to as simple-sequence repeats (SSR), are short tandem repeats of a few nucleotides (2-6 bp) found spaced throughout a genome (Hamada et al. 1984). They are detected through a PCR reaction using specific primers, based on the sequences flanking the repeat. Microsatellites are ideal genetic markers as they have been closely linked to genes (Hamada et al. 1984) and are inherited in a codominant and Mendelian fashion (Morgante and Olivieri 1993).

Microsatellites were first used in human genetics as markers for disease diagnosis (Fu et al. 1991) and genetic mapping (Beckmann et al. 1993). Microsatellites have since been applied to many crops including wheat, maize, soybeans, rice and barley for genetic mapping and as genetic markers. In human genetics the most common repeat is $(CA)_n$ (Weber 1990) but the $(AT)_n$ repeat is the most common repeat found in plants (Taramino and Tingey 1996). A survey of thirty-four different species revealed that microsatellites occur every 50 Kb on average and are most commonly found in introns or in 3' or 5' untranslated regions (Morgante and Olivieri 1993). There are three forms of microsatellites: perfect (a run of repeats), imperfect (a run of repeats that is interrupted by one or more bases) and compound (a run of two or more sets of repeats) (Morgante and Olivieri 1993).

Even though there is a maximum of two alleles per locus in any diploid individual there may be many alleles at a given locus within a species. Amplification

of 28 and 37 alleles for two different loci has been demonstrated in barley (Saghai Maroof et al. 1994). There could potentially be as many alleles as there are repeats.

Microsatellites are ubiquitous in plant genomes and are more polymorphic than RFLP (Morgante and Olivieri 1993), RAPD (Powell et al. 1996) and AFLP markers (Powell et al. 1996; Mackill et al. 1996). While AFLP resulted in the greatest number of markers per primer pair, the microsatellite markers had the most diversity at a given locus (Powell et al. 1996). This makes microsatellite markers useful in species with low levels of polymorphism. Mapping populations and breeding programs are dependent on the detection of polymorphisms between the parents. In self pollinating species, such as barley and wheat, the polymorphism rate is low. This low rate makes mapping genes and identification of variation between lines difficult.

The function and origin of microsatellites is unknown but they appear to be neutral because the varying repeat sizes have no effect on the organism (Saghai Maroof et al. 1994). They also appear to be subject to natural selection if the repeat occurs in the coding region of a gene (Saghai Maroof et al. 1994). The observed polymorphisms are currently thought to have arisen from unequal crossing over or from slippage during replication (Taramino and Tingey 1996).

The primary disadvantage associated with microsatellites is the relatively high cost and time required for the development of primers as compared to RAPDs or AFLP. The sequences upstream and downstream of the microsatellite must be known and primers designed within this sequence. RFLP, RAPD and AFLP marker

systems have the advantage of not requiring this advanced knowledge of the sequence (Lee et al. 1995).

Microsatellite Development

Microsatellites and their associated flanking sequences can be identified by searching published databases, screening DNA libraries or by using previously developed microsatellite primers from a related species (Brown et al.1996).

Primers that have been previously developed in related species require little optimization and are an inexpensive option. Genomic regions may be conserved between species and the primers may be cross-applicable (Brown et al. 1996; Moore et al. 1991).

Public databases, such as GenBank, contain large amounts of sequence information obtained from laboratories involved in genomics and/or sequencing projects. These databases can be screened for di-, tri-, tetra- and other nucleotide repeats. After a microsatellite sequence is identified, the associated flanking sequences must also be available to design the forward and reverse primers (Brown et al. 1996).

If screening public databases and primers from related species do not provide sufficient markers, then genome screening may be used to develop microsatellite primers (Brown et al. 1996). A genomic library must be created and screened with synthetic oligonucleotides to identify clones containing repeat

sequences, the clones are then sequenced and primers are developed in the regions flanking the repeat sequence (Ma et al. 1996).

Marker Applications

RFLP, AFLP, RAPD or microsatellite markers may be used for genetic mapping, DNA fingerprinting, marker assisted selection, genetic diversity studies and trait tagging.

Genetic Mapping

A genetic map is defined as a linear arrangement of genes or genetic markers based on recombination events (Liu 1998). Genetic maps consisting of closely spaced DNA markers are useful for genomic analysis. DNA markers that are linked to a trait of interest can be used for gene cloning, medical diagnostics and for trait introgression in plant and animal breeding (Tanksley et al. 1989). Morgan and Sturtevant (1913) developed the first genetic map using *Drosophila melanogaster* (Strickberger 1985). Since then maps have been produced for many species including, *E. coli* (Blattner et al. 1997), cattle (Bishop et al. 1994), humans (Donis-Keller et al. 1987; Human Genome Project 2000), and *Arabidopsis* (Arabidopsis Genome Initiative 2000). Genetic maps for barley have been produced by RFLPs (Graner et al. 1991; Heun et al. 1991), AFLPs (Qi et al. 1998; Becker et al. 1995) microsatellites (Liu et al. 1996) and RAPDs (Giese et al. 1994).

Genetic maps are also referred to as linkage maps and are constructed based on observed recombination frequencies between loci. Physical maps are different from genetic maps because they are not constructed based on recombination frequencies but rather on the actual distance in base pairs between sites (Stracken and Read 1996).

Mapping Populations

A mapping population is required to produce a genetic map. Mapping populations cannot be created for genetic mapping of the human genome. Populations for human genetics must be identified in large families or in highly related groups. In other species, such as plants, it is possible to use controlled crosses, in addition to natural crosses, to obtain mapping populations. To facilitate location of polymorphic loci, crosses are made between plants with a large degree of genetic distance. Segregating populations may include F_2 or BC_1 progenies, F_3 or single seed decent.

The doubled haploid population is a common population used for barley genetic studies. The primary advantage of doubled haploids is that homozygous plants can be produced from heterozygous parents in a single generation (Poehlman and Sleper 1995). Doubled haploids can be used to produce homozygous lines in months instead of years using traditional breeding (Morrison and Evans 1988).

In cereals, doubled haploid plants may be produced through a wide cross and chromosome elimination, anther culture or microspore culture. A wide cross with *Hordeum bulbosom*, first described by Lange (1971) is a common choice for *H. vulgare*. Once cell division begins, the *H. bulbosom* chromosomes are eliminated. The remaining *H. vulgare* chromosome complement is doubled using colchicine to produce a homozygous, diploid plant. Crosses with *Zea mays* are also used but they are less efficient than with *H. bulbosom* and some maize DNA introgression has been detected (Chen et al. 1991).

Alternatively anther or microspore culture of pollen can be used to produce double haploid plants. The procedure involves harvesting anthers or microspores from a flower and culturing them on media. An embryo or callus will develop which will eventually give rise to a haploid plantlet. The plantlet is treated with colchicine to double the chromosomes and produce a doubled haploid plant. The difference between microspore culture and anther culture is that for microspore culture, the microspores have been dissected out of the anther.

There is concern that double haploids are not genetically stable but analysis at the karyotype, protein and DNA levels revealed no evidence of genetic change regardless of the barley genotype used to produce doubled haploids by microspore culture (Finnie et al. 1991). However there has been evidence of differential transmission of alleles from one parent of a barley cross used for RFLP mapping that responded better to in vitro culture (Graner et al. 1991).

MAPMAKER 3.0

MAPMAKER 3.0 is a computer package for constructing genetic linkage maps using simultaneous multipoint analysis of any number of loci (Lander et al. 1987). It can analyze codominant, dominant or recessive traits from F_2-type pedigrees or codominant traits from CEPH pedigrees (Lincoln et al. 1992). CEPH pedigrees are pedigrees of large human families and the information pertaining to them is gathered, maintained and distributed by the Centre d'Etude du Polymorphisme Humain in Paris, France (Watson et al. 1996).

Genetic maps are based on recombination frequency. Recombination frequency is the percentage of segregating progeny that are recombinants for a pair of linked loci. The recombination frequency gives an estimate of the distance between two loci in a chromosome based on the assumption that the probability of crossover is proportional to the distance between loci (Lui 1998). From the estimated recombination frequencies, the most probable order of markers along a chromosome is calculated.

There are several steps required to construct a genetic map. The first step is a pairwise linkage analysis for all possible two-locus combinations. The "two-point" command performs the pairwise linkage analysis by computing all two-point recombination fractions and maximum LOD scores between all pairs of loci (Lincoln et al. 1992). A LOD score, or logarithm of the odds, is the likelihood that two loci are linked. It is the ratio between the probability that two loci are unlinked and the probability that they are linked (Watson et al. 1996).

The second step is to group the markers into different linkage groups. The "assign" command will divide the loci into linkage groups based on the "two-point" results. If the number of markers used and the genome coverage is high, the number of linkage groups will equal the number of chromosomes.

The next step is to order the markers in a linkage group. The "three-point" command is used after linkage groups have been defined to calculate the likelihoods of all three-point crosses for the linkage group. The three-point analysis excludes the majority of unlikely orders by calculating the map distances and likelihoods for all three possible orders of linked triples of markers (Lincoln et al. 1992). After unlikely orders have been removed the final order of the linkage group is determined by the "order" command.

The "order" command estimates the multipoint recombination fractions among the adjacent loci. The "order" command identifies a subset of markers that are more likely than any other and maps the remaining markers into this subset one at a time. The likelihood for different possible marker orders is calculated and the order that maximizes the likelihood is accepted (Lincoln et al. 1992).

Finally after an order has been determined, the "ripple" command is used to confirm the map order. It permutes through the order of the neighbouring markers and compares the likelihoods of all resulting maps and will suggest any alternatives to the "order" generated map (Lincoln et al. 1992).

Certain parameters can be user defined including the minimum number of lines that need to be informative for each marker, the maximum distance between

any two loci, the minimum LOD score, and whether the Haldane or Kosambi mapping function is used. The Kosambi function will account for interference, or the negative effect of one crossover on the occurrence of crossovers nearby but the Haldane function assumes no interference (Lincoln et al. 1992).

Marker Assisted Selection

Marker assisted selection (MAS) is a selection method using markers that are closely linked to a gene for introgression of genes of interest (Hospital et al. 1992). Markers can be used to target a specific gene, known as marker assisted foreground selection (Hospital and Charcosset 1997) or they can be dispersed throughout the genome to recover the recurrent parent more efficiently, known as marker assisted background selection (Melchinger 1990).

MAS allows for the selection of traits based on genotype, before phenotypic evaluation is possible and for an easier evaluation of traits that are difficult to score phenotypically. MAS can increase the efficiency of breeding programs by identifying the genes a plant possesses the number of lines sent to field trials can be reduced. Also, for backcrossing programs, breeders can screen for the recurrent parent at early stages (seed or early leaf) rather than growing the plant out to maturity allowing selection of the most desirable individuals and thereby reducing the time for variety registration.

Markers used for MAS can be for single gene traits (Bauer et al. 1997) or multiple gene traits (Steffenson et al. 1996; Chen et al. 1994). Developing markers

for multiple gene traits is more difficult than single gene traits because if environmental conditions influence the phenotypic data it will be difficult to correlate it to the mapping data.

Genetic Diversity

The identification of genetic diversity in germplasm may assist breeders to continue to incorporate novel traits into plants. This diversity can be detected by identifying the level of variation at each locus across cultivars. While all marker systems can be used to detect diversity, microsatellite markers are the markers of choice for diversity studies because of the large number of alleles that can be detected at a locus and their distribution throughout genome (Bruford and Wayne 1993; Russell et al. 1997).

DNA Chips and Microarrays

The recently developed DNA chip and microarray technologies allow for rapid, simultaneous analysis of thousands of polymorphisms in a single experiment. (Schena 1999). Microarrays were initally used for monitoring gene expression but have been expanded to include mutation detection, polymorphism analysis, mapping and evolutionary studies (Schena 1999). Microarrays and DNA chips are based on hybridization of DNA to a chip and fluorescent labelling and detection. A complete genome or a series of different alleles for one or several genes can be

bound to the chip which is then screened with fluorescently labelled probes. While microarrays and DNA chips can be easily automated and they generate large amounts of information, there is a large cost associated with initial chip development.

DNA Fingerprinting

Jeffreys and colleagues first developed DNA fingerprinting, or DNA typing, as a paternity identification technique in 1985 (Dodd 1985). DNA fingerprinting involves the display of a unique set of DNA fragments for a specific DNA sample that allows it to be differentiated from other DNA samples (Vos et al. 1995). It has been used for varietal differentiation in wheat (Pecchioni et al. 1996; Lee et al. 1995), barley (Saghai Maroof et al. 1994), rice (Yu and Nguyen 1994) and rye (Iqbal and Rayburn 1994).

Breeder's Rights and Quality Assurance

There is a need to reliably distinguish varieties of crop plants and establish their purity (Russell et al. 1997). A number of factors have driven the need for markers to differentiate varieties including certified seed, plant variety rights, germplasm protection, customer tolerances and specialty markets. There are specialty markets that cater to specific consumer requests including specific

cultivars of barley for malting, specialty oil canola, wheat with specific proteins or non-genetically modified food products.

DNA fingerprinting can be used to differentiate cultivars to ensure a shipment is of a required cultivar. Specific DNA markers can be used to screen seed lots to ensure that it is either free from or contains a particular gene.

Synteny Mapping

Synteny mapping is the mapping of regions of ancestral DNA to reconstruct a modern genome and it is based on the theory that gene order may remain constant across species over great evolutionary distances (Moore et al. 1995). In spite of evolutionary changes in basic chromosome number and DNA per genome among the *Triticum*, *Hordeum*, *Avena*, *Oryza* and *Zea* genera, it is apparent that sizeable regions of ancestral DNA have been conserved across species (Moore 1995). The rearrangement of chromosomal blocks may allow for the reconstitution of the ancestral chromosomes (Moore et al. 1995). This data will also provide information on the organization of the physical chromosome relative to sequences and gene distribution. Synteny mapping can also be used to follow the transfer of chromosomal segments along a pedigree.

Synteny mapping has been used in the field of human genetics. Genes found in mice, and pigs have been used to identify similar genes in humans (Rettenberger et al. 1995; Ehrlich et al. 1997). The smaller size of the mice, and pig genomes allows for more rapid identification of genes.

Comparative analysis of progenitor rice, maize and domesticated panicoids has lead to inferences on the genome structure of progenitor maize (Wilson et al. 1999). By isolating blocks that were similar between rice, maize and panicoids, the progenitor maize genome was reconstructed.

Mapping Microsatellite Markers in Barley

(Harrington x TR306 cross)

ABSTRACT

There are relatively few microsatellite markers currently available for barley. This is mainly due to the high cost and time commitment required for initial primer development. Seventy-nine publicly available microsatellites were screened on the Harrington x TR306 population and twenty were added to two Harrington x TR306 genetic maps. Two additional methods were also examined in an attempt to increase the number of microsatellite loci. These included cross application of wheat microsatellite markers and screening of a barley genomic library for repeats for development of new primer pairs. Neither of these methods yielded additional microsatellite markers.

INTRODUCTION

Microsatellites are defined as tandem repeats of a few base pairs and are found throughout the genomes of plants, animals and humans (Litt and Lutty 1989; Weising et al. 1989). Microsatellites have been used extensively in human genetics. They have been linked to traits of interest, including disease genes (Christensen et al. 1998; Weiler et al. 1998) and have been used in the construction of human linkage maps (Weissenbach et al. 1992). They have also been used for isolating loci of interest, population and evolutionary studies, fingerprinting and pedigree analysis.

The dinucleotide repeat, $(CA)_n$, is the most common repeat in mammals (Weber 1990) whereas the $(AT)_n$ repeat is most commonly found in plants (Taramino and Tingey 1996). Microsatellites are spaced throughout the genome. An estimate from rice estimates the frequency of two specific dinucleotides, $(GA)_n$ and $(GT)_n$ to be spaced every 225 Kb and 450 Kb respectively (Wu and Tanksley 1993). A different estimate based on the screening of 34 different plant species predicts at least one dinucleotide or trinucleotide repeat every 50 kilobases (Morgante and Olivieri 1993).

Microsatellites are non-coding segments of DNA and to date have no known function. Even though they may not have a specific function, they can affect the coding sequence. The expansion of a dinucleotide or trinucleotide microsatellite repeat within a coding sequence can disrupt the reading frame and has been linked

to diseases such as fragile-X syndrome (Fu et al. 1991) and myotonic dystrophy (Brook et al. 1992) in humans.

While microsatellites have been used extensively in human genetics, the use of microsatellites has just recently become common in plant genetics. This is due to the large cost of primer development and because primers must be developed for every species. A small number of primers developed in related species may be cross applicable between species and may be an inexpensive option (Brown et al. 1996; Moore et al. 1991).

Microsatellites make excellent genetic markers because they are codominant, polyallelic, simple to use and inexpensive after initial primer development. Variation in the number of repeats is detected by PCR amplification using primers located in the unique flanking DNA and electrophoresis.

In self-fertilizing species, the level of variation within the genome is lower than cross-pollinated species and little variation can be detected with RFLPs, RAPDs or isozymes (Graner et al. 1990). An alternative system is microsatellite markers, which are capable of detecting the greatest amount of polymorphism over any marker system (Powell et al. 1996).

While a diploid organism such as barley can only have two alleles per locus, within a population there may be many different alleles for that locus. A microsatellite locus may have as many different alleles as there are repeats (Saghai Maroof et al. 1994). This polyallelism generates more information per marker than a marker system that detects only the presence or an absence of an allele.

There are only a small number of publicly available microsatellites for barley because of the cost associated with developing them but the available markers were screened on the parental lines Harrington and TR306 and a doubled haploid population (Harrington x TR306) (Kasha and Kleinhofs 1994). Harrington x TR306 is a doubled haploid population currently used by the North American Barley Genome Project for the identification of agronomic traits of importance to breeders.

There are two genetic maps for the Harrington x TR306 cross and the polymorphic microsatellite markers were mapped onto both maps. The first map was developed by D. Mather et al. (1994) and has 127 markers, the second map was developed by A. Kleinhofs et al. (1995) and has 190 markers.

Recognizing that there are only a small number of microsatellite primers available, two alternatives were examined in an attempt to increase the number of primers. The first alternative was to screen a genomic library of the cultivar Harrington to identify repeats and then develop primer pairs flanking the repeats. The second alternative was to screen a set of wheat microsatellite primers that were available in the laboratory. It has been suggested that microsatellite markers may be cross-applicable between related species such as wheat and barley (Saghai Maroof et al. 1994). The set of wheat microsatellites was screened on Harrington and TR306 to identify any markers that would be cross-applicable and could be mapped on the Harrington x TR306 map.

MATERIAL AND METHODS

Mapping Existing Microsatellite Markers

Genomic DNA from the Harrington x TR306 doubled haploid barley population was provided by Dr. Diane Mather of the North American Barley Genome Project. The population consisted of 120 individuals and the 94 individuals with the greatest quantities of available DNA were selected as the mapping population.

There were two sets of microsatellite markers available for mapping. The first set of microsatellites was obtained from Dr. Peter Langridge at the University of Adelaide, Austrailia. The second set was obtained from Dr. Luke Ramsey at the Scottish Crop Research Institute, Dundee, Scotland. All microsatellite primers were used in a PCR reaction with 30 ng genomic DNA, 0.8 mM dNTPs, 1.5 mM $MgCl_2$, 1x PCR buffer (Perkin Elmer), 1unit Taq Polymerase (Gibco BRL), 10 pmol each forward and reverse primer to a final volume of 25 μL. All PCR reactions were carried out in an MJ Research thermocycler, with one of four cycling conditions seen in Table 1. Microsatellite primer sequences for the 79 pairs screened in this experiment and 9 others used in later experiments and their associated PCR cycling program are in Appendix A.

After cycling was complete, an 8 M urea stop solution with 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol and 0.40% (w/v) orangeG was added to the PCR reaction to a 5 M final concentration. The samples were denatured at 90^0C for 10 minutes, placed on ice and loaded onto a 6% (w/v) polyacrylamide gel (19:1 acrylamide/bis-acrylamide).

Table 1: The four PCR cycling conditions used for the amplification of the barley microsatellite primers.

Conditions	PCR1	PCR2
Initial denature	10 minutes at 95^{0}C	5 minutes at 95^{0}C
Cycles of: Denaturing Annealing Touchdown Extension	18 cycles 1 minute at 94^{0}C 30 seconds at 64^{0}C 1^{0}C decrease every second cycle 1 minute at 72^{0}C	18 cycles 1 minute at 94^{0}C 30 seconds at 69^{0}C 1^{0}C decrease every second cycle 1 minute at 72^{0}C
Cycles of: Denaturing Annealing Extension	30 cycles 1 minute at 94^{0}C 1 minute at 55^{0}C 1 minute at 72^{0}C	20 cycles 1 minute at 94^{0}C 1 minute at 60^{0}C 1 minute at 72^{0}C
Final Extension	10 minutes at 72^{0}C	5 minutes at 72^{0}C

Conditions	PCR3	PCR4
Initial denature	5 minutes at 95^{0}C	5 minutes at 95^{0}C
Cycles of: Denaturing Annealing Extension	30 cycles 2 minutes at 94^{0}C 1 minute at 55^{0}C 1 minute at 72^{0}C	40 cycles 1 minute at 94^{0}C 1 minute at 60^{0}C 1 minute at 72^{0}C
Final Extension	10 minutes at 72^{0}C	10 minutes at 72^{0}C

The gel was run for two hours at 85 watts in a 1x Tris-Boric acid-EDTA (pH 7.5) (TBE) buffer. The resulting DNA fragments were visualized using the Silver Sequence™ silver staining kit (Promega). Two individuals independently scored the gels. Markers with the same allele as the Harrington parent were designated as "A" and markers exhibiting the TR306 parent allele were designated as "B". An example of a gel with polymorphisms between the parents and the segregation of the parental alleles in the cross can be seen in Figure 2.

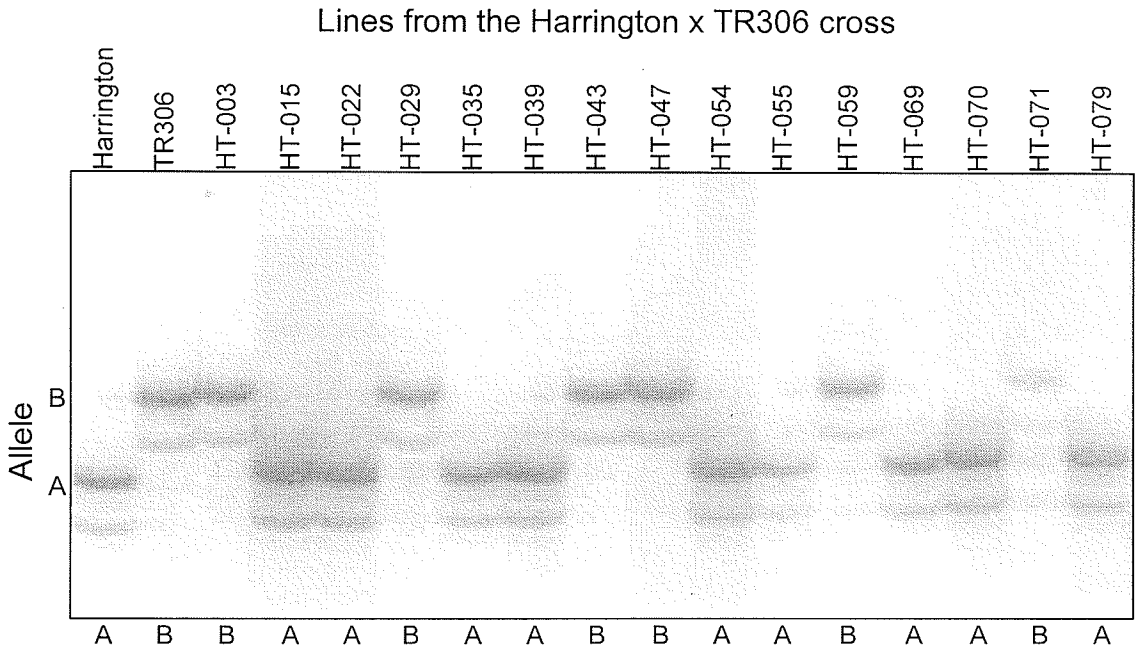

Figure 2: Amplification profile of parental lines and fifteen doubled haploid lines from the Harrington x TR306 population using the microsatellite marker HVM62. The allele associated with the Harrington parent is designated as the "A" allele. The allele associated with the TR306 parent is designated as the "B" allele. The score assigned to each of the lines is shown below the image.

Genetic Mapping

Microsatellites were mapped onto both the D. Mather and A. Kleinhofs base maps using MAPMAKER 3.0 (Whitehead Institute), the mapping data associated with each respective map, and the new microsatellite data. The data was combined into a file and coded as codominant markers. For each chromosome two RFLP markers that had previously been mapped to the Harrington x TR306 map were chosen as anchors, or reference points as seen in Table 2. RFLP markers were selected because RFLP markers are generally the first markers mapped onto a genetic map and subsequent markers have been mapped with reference to them. All other markers, including previously mapped and new microsatellite markers were ordered against these anchors. Mapping was performed under the following criteria; a minimum LOD of 4.0, a maximum distance of 40 cM, a minimum of 70 individuals with data for each marker and the Kosambi function was used to obtain centimorgan (cM) values from the recombination values.

A two-point analysis identified putative linkage groups with the "two point" and "assign" commands. Three-point analysis, with the "three-point" command, removed any unlikely orders in the linkage groups and the "order" command was used to generate a map by placing the markers one at a time within the linkage groups by multi-point analysis. The "ripple" command was used to confirm the results generated by the "order" command.

Table 2: RFLP markers selected as anchors for the mapping of new microsatellite markers. Listed by original Mather or Kleinhofs genetic map and chromosome number.

Mather Genetic Map	
Marker	Chromosome
aHor2	1H
AtpbA	1H
ABG058	2H
ABG619	2H
ABG471	3H
ABG607	3H
ABG709	3H
ABG714	4H
ABG715	4H
ABG366	4H
Dor5	5H
BCD298	5H
MWG652a	6H
MWG934	6H
Prx1A	7H
ABC310B	7H

Kleinhofs Genetic Map	
Marker	Chromsome
aHor2	1H
AtpB	1H
ABG058	2H
ABG619	2H
ABC252	2H
ABG471	3H
MWG847	3H
ABG709	3H
TubA1	4H
ABG366	4H
Dor5	5H
BCD298	5H
MWG652	6H
MWG934	6H
BCD129	7H
ABC310B	7H

Wheat Microsatellites

A set of sixty-four wheat microsatellites was screened on the Harrington x TR306 population. The PCR reactions were made to a final volume of 25 uL with 1x PCR buffer, 0.8 mM dNTPs, 1.5 mM $MgCl_2$, 10 pmol each forward and reverse primer, 1 unit Taq polymerase (Gibco/BRL) and 30 ng genomic DNA. PCR cycling

conditions were as follows, 95^{0}C for 5 minutes, 30 cycles of 95^{0}C for 1 minute, 55^{0}C for 1 minute, 72^{0}C for 1 minute and a final extension of 72^{0}C for 10 minutes. The PCR products were analyzed on a 6% polyacrylamide gel as described previously.

Microsatellite Development

To examine the process of microsatellite development, genomic DNA from the barley cultivar Harrington was digested with *Pst*I and *Eco*RI. The digested DNA was electrophoresed overnight on a 0.9% agarose gel containing 0.5 μg/mL ethidium bromide in a Tris-borate-EDTA (TBE) 1x buffer at 20 volts. Fragments within the 500-1000 bp range were excised under 254 nm UV light from the agarose gels. The DNA was purified from the agarose using Supelco GelElute™ columns.

The cloning vector that was used was pUC19. The plasmid was cut with *Pst*I and *Eco*RI. The digested vector and excised Harrington DNA were combined in an equimolar ratio and ligated at 15^{0}C overnight. One microliter of the ligated DNA and vector were electroporated at 2.2 kV with 20 uL of DH10B *E.coli* cells. The transformants were plated onto LB plates with X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside). Stabs were taken of the white colonies and stored in 384 well plates with LB storage media. A total of 1152 colonies were selected. The 384 well plates were replica-plated onto LB with ampicillin petri dishes that were overlaid with uncharged nylon membranes. The replica plates were grown at 37^{0}C for 10 hours until colonies developed on the membrane.

Before hybridizing the probes to the membrane, the DNA was released from the colonies and fixed to the membranes. This was done by first treating the membranes with a 10% sodium dodecyl sulfate (SDS) solution to rupture the cells and release the DNA. A 0.5N NaOH and 1.5M NaCl solution was used to denature the DNA. A 0.5M Tris-HCl (pH 7.5), 1.5M NaCl and 0.001M EDTA solution was used to neutralize the membrane. Two washes followed, the first with 2x saline-sodium citrate (SSC) and 0.1% SDS and the second with 2x SSC. The membrane was baked at 80^0C for two hours and stored at 4^0C in plastic wrap.

The hybridization reaction involved a pre-hybridization step to reduce the amount of non-specific binding. This pre-hybridization was done using a 50% formamide, 5x SSC, 0.2% SDS, 0.1% Sarkosyl, 2% (w/v) Blocking reagent (Boehringer Mannheim) and 0.1 mg Salmon sperm DNA. The membranes and solution were placed at 40^0C for 2-4 hours.

Following pre-hybridization, the membranes were probed with 20 μg of four different repeat sequences, $(AT)_{15}$, $(GA)_{15}$, $(TCT)_5$ and $(AAC)_5$, in hybridization tubes at 37^0C overnight.

Visualization of the membrane was done with DIG-labeling according to the DIG system for nucleic acid analysis (Boehringer Mannheim, Laval, PQ). The DIG labeled membrane was exposed to Kodak X-OMAT AR film for two hours and developed as per Kodak instructions.

RESULTS

A total of twenty of the seventy-nine available microsatellite primer pairs were mapped onto both Harrington x TR306 maps. Four other microsatellites, BMAC298, BMAC167, BMAC30 and BMAC31, were also segregating between the parents but they could not be assigned to a linkage group. The twenty mapped microsatellite markers and the associated chromosome can be seen in Table 3.

Table 3: The twenty mapped microsatellite markers listed by the chromosome to which they mapped on both the D. Mather and A. Kleinhofs Harrington x TR306 maps.

Chromosome	Markers
2H	HVM26, HVBKASI, HVCSG
3H	HVM70, HVM62, EBMAC541, BMAC13
4H	HVM40, HVSCABG, BMAC299, HVM3, HVM68, WMS6
5H	HVDNH7, HVM6
6H	HVM65
7H	HVWAXY, HVCMA, BMAC64, HVM49

The genetic maps can be seen in Figures 3 through 16 with the new microsatellite loci in boldface text. The microsatellites increased the length of the D. Mather map from 1269.1 cM to 1351.2 cM and the A. Kleinhofs map from 1254.1 cM to 1306.3 cM, a difference of 82.1 cM and 52.2 cM respectively. There was an average 11.7 cM increase per chromosome for the D. Mather map and 7.5 cM for the A. Kleinhofs map.

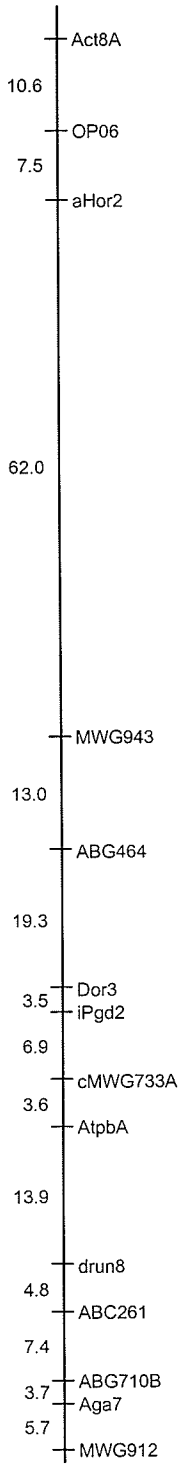

Figure 3: Genetic map of Harrington x TR306 chromosome 1H using the D.Mather base map and no additional microsatellite loci.

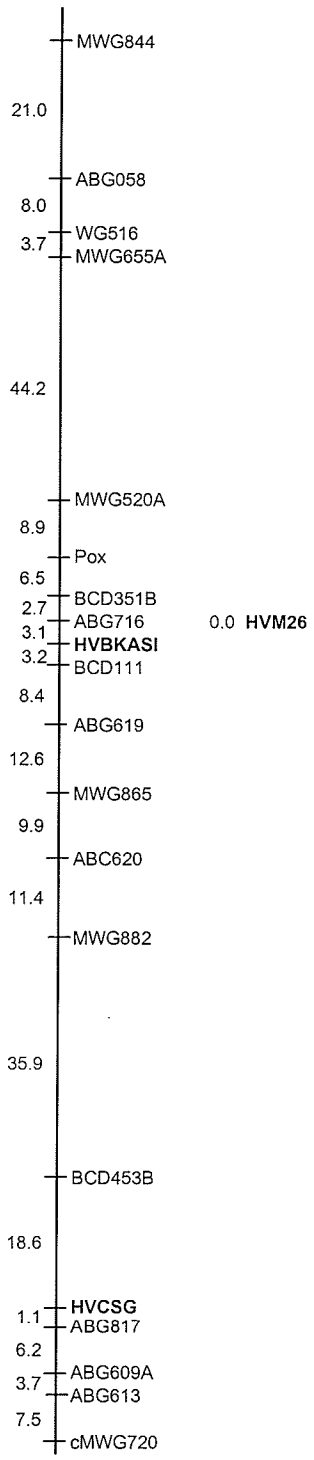

Figure 4: Genetic map of Harrington x TR306 chromosome 2H using the D.Mather base map and three additional microsatellite loci.

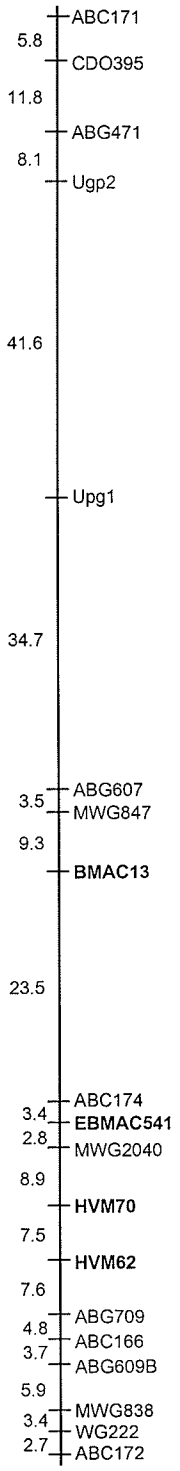

Figure 5: Genetic map of Harrington x TR306 chromosome 3H using the D.Mather base map and four additional microsatellite loci.

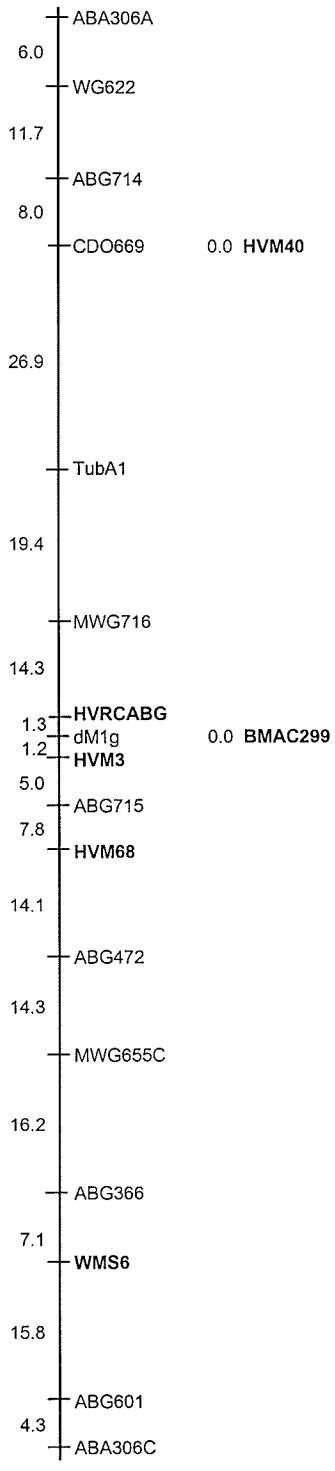

Figure 6: Genetic map of Harrington x TR306 chromosome 4H using the D.Mather base map and six additional microsatellite loci.

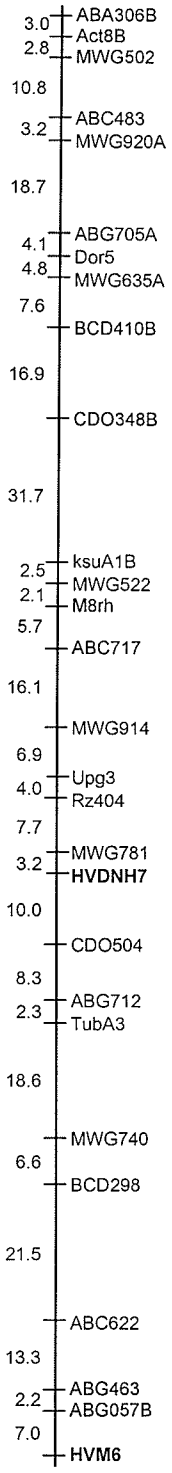

Figure 7: Genetic map of Harrington x TR306 chromosome 5H using the D.Mather base map and two additional microsatellite loci.

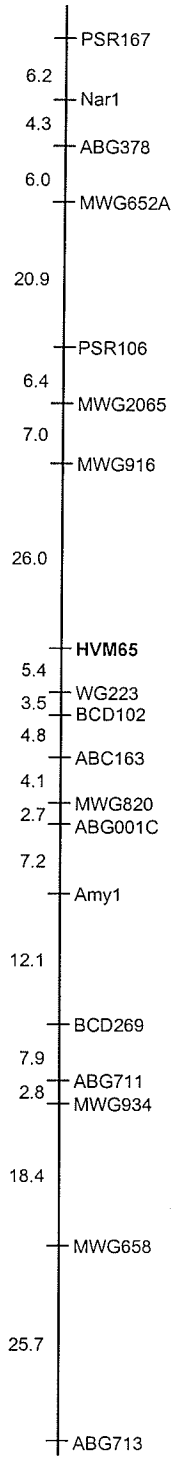

Figure 8: Genetic map of Harrington x TR306 chromosome 6H using the D.Mather base map and one additional microsatellite locus.

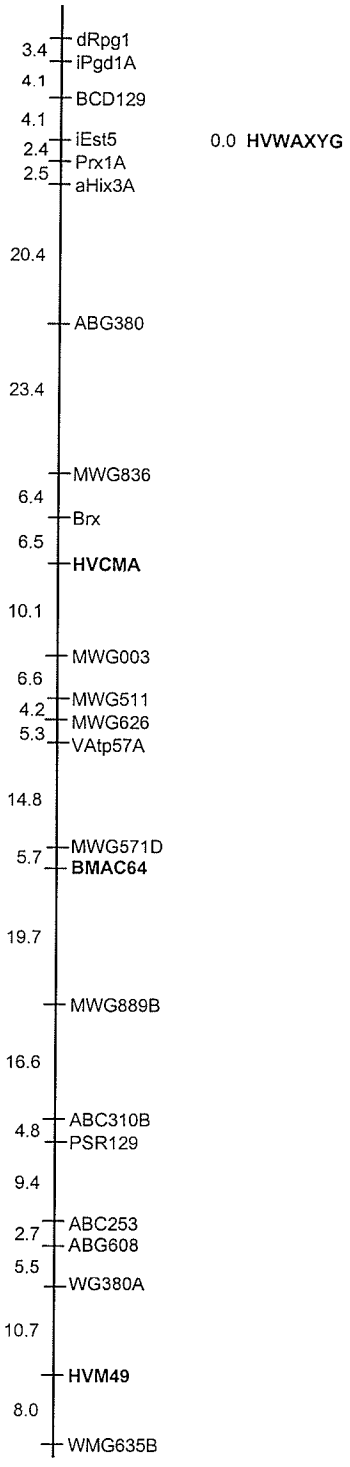

Figure 9: Genetic map of Harrington x TR306 chromosome 7H using the D.Mather base map and four additional microsatellite loci.

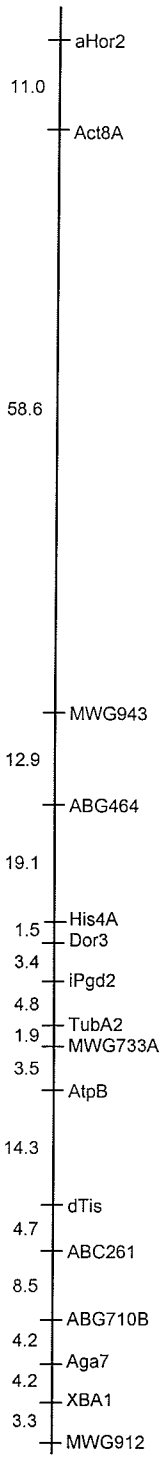

Figure 10: Genetic map of Harrington x TR306 chromosome 1H using the A Kleinhofs base map and no additional microsatellite loci.

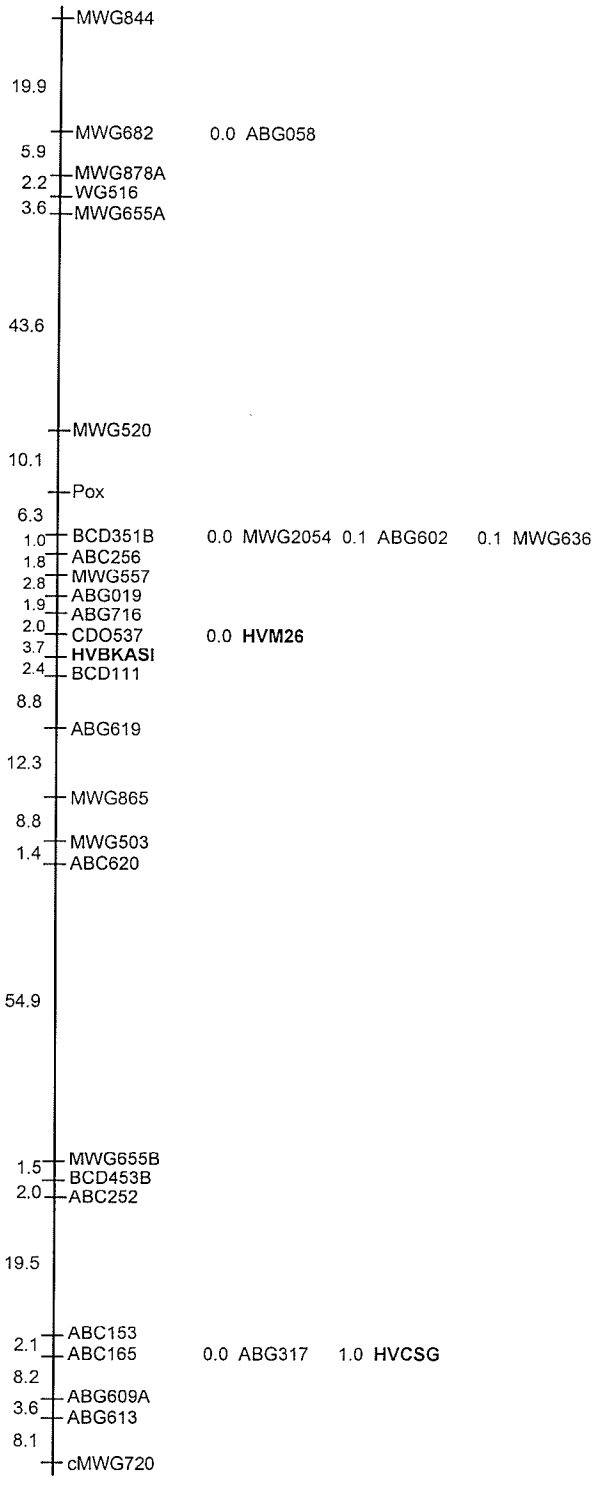

Figure 11: Genetic map of Harrington x TR306 chromosome 2H using the A Kleinhofs base map and three additional microsatellite loci.

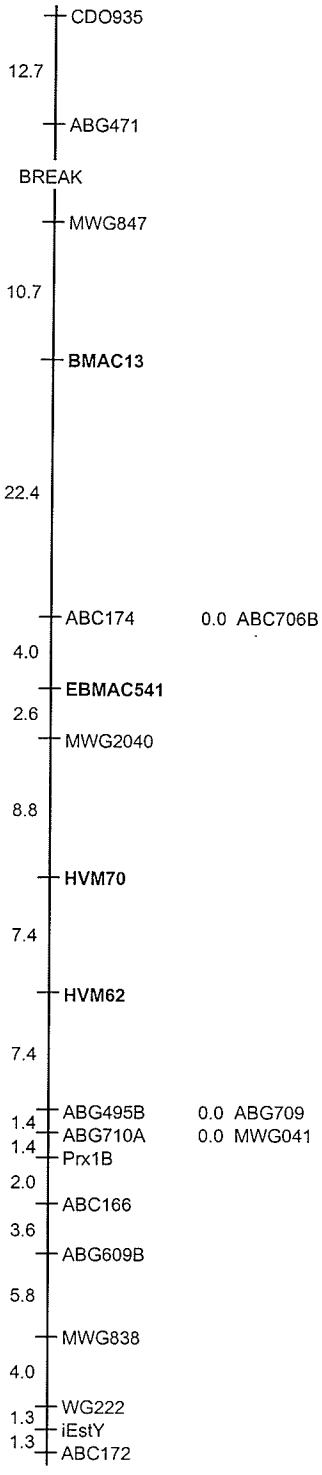

Figure 12: Genetic map of Harrington x TR306 chromosome 3H using the A Kleinhofs base map and four additional microsatellite loci.

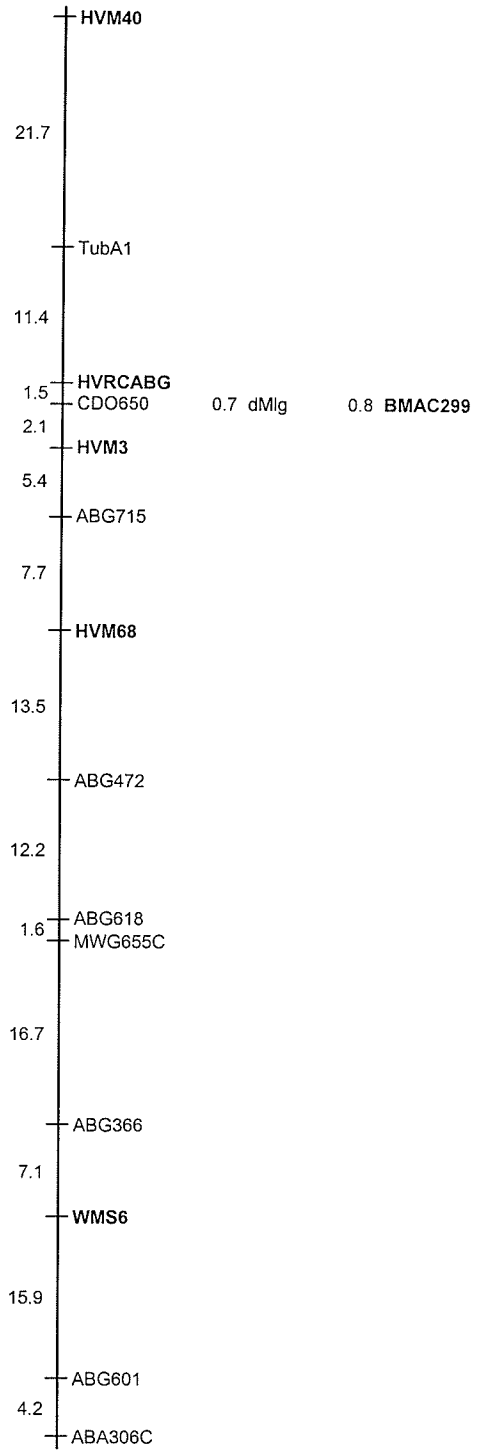

Figure 13: Genetic map of Harrington x TR306 chromosome 4H using the A Kleinhofs base map and six additional microsatellite loci.

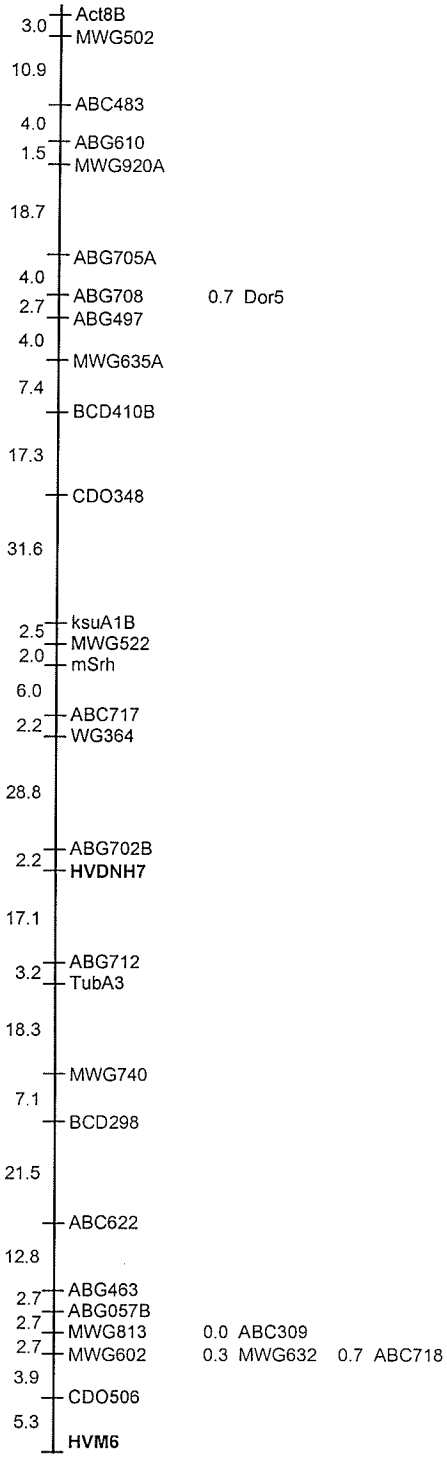

Figure 14: Genetic map of Harrington x TR306 chromosome 5H using the A Kleinhofs base map and two additional microsatellite loci.

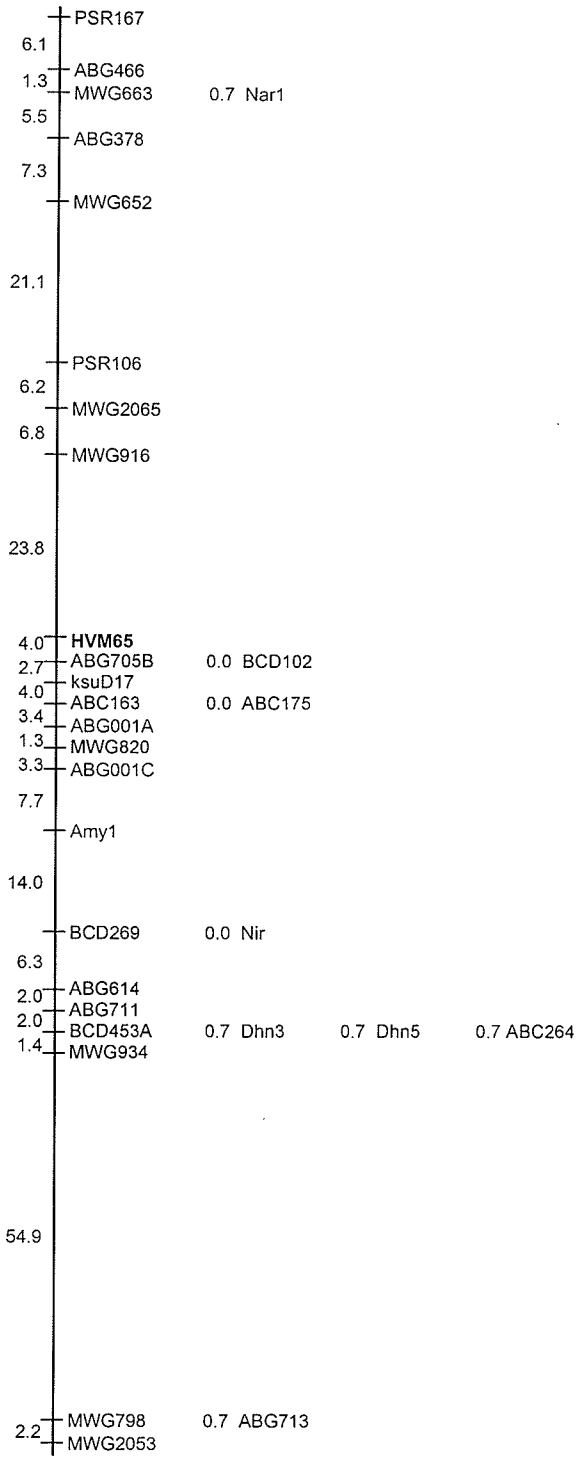

Figure 15: Genetic map of Harrington x TR306 chromosome 6H using the A Kleinhofs base map and one additional microsatellite locus.

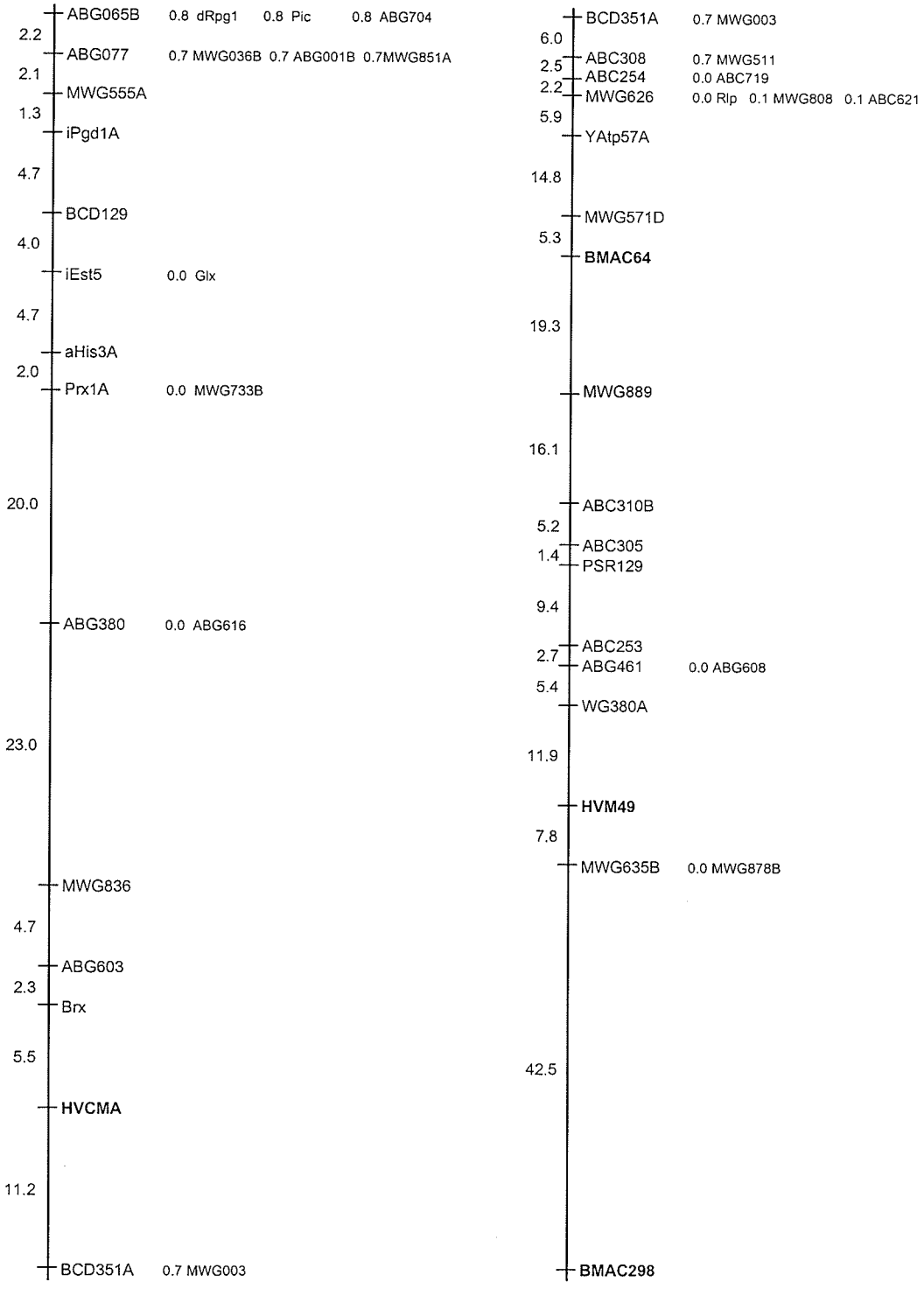

Figure 16: Genetic map of Harrington x TR306 chromosome 7H using the A Kleinhofs base map and four additional microsatellite loci.

The mapping of the new microsatellite primers was completed twice under the same conditions and identical results were obtained both times. Of the seventy-nine microsatellite primer pairs that were available forty-five produced a monomorphic band between the parents and could not be used for mapping and ten did not produce an amplification product with either parent.

The screening of sixty-four wheat microsatellites available in the lab for markers that were cross applicable in barley resulted in twenty-eight of the sixty-four markers producing an amplification product and the remaining markers did not produce any product. The resulting fragments that were produced were monomorphic, indicating that the same allele was present in both parents and the markers cannot be used for genetic mapping.

The screening of 1152 clones from the Harrington *Eco*RI/*Pst*I library with $(AT)_{15}$, $(GA)_{15}$, $(TCT)_5$ and $(AAC)_5$ did not produce a positive signals with any of these four probes.

DISCUSSION

The microsatellite markers mapped onto six of the seven chromosomes. No microsatellites mapped to chromosome 1H. The markers that did map onto the population were spread throughout the genome and were not clustered in one region. The additional microsatellites resulted in an increase of 52.2 cM for the A. Kleinhofs map and 82.1 cM for the D. Mather map, with 34.7 cM being added to

chromosome 3H. The majority of markers mapped along the chromosome arms and were not added to the ends of the chromosomes.

The additional microsatellite markers were successfully added to the existing maps for barley and they did not disrupt the marker orders or the distances between the existing markers.

The high number of monomorphic banding patterns, 45 out of 79 markers, or 57%, indicates the high degree of similarity between the parental lines and the need for larger sets of microsatellite markers for effective genome coverage.

While there have been some instances of cross applicability of microsatellite markers between wheat and barley (Saghai Maroof et al. 1994), the wheat microsatellite primers yielded no additional polymorphisms. All resulting fragments were monomorphic and could not be mapped onto the Harrington x TR306 map.

A complete set of microsatellite primers is available for human genetic studies and projects are underway to develop microsatellite primers for all major crops. In barley there were only a small number of publicly available barley microsatellite primer pairs available for use in mapping studies and all pairs were exhausted. A small-scale experiment was undertaken to understand the primer development process and to attempt to locate a few microsatellites.

Microsatellite primers have been developed through several different methods. Databases such as GenBank contain large numbers of searchable sequence information for a large number of species. The sequences can be searched for repeats along with the flanking sequences. Primers are then designed

in the flanking sequences. The disadvantage to this approach is that the sequences are not a random representation of the whole genome.

Another approach to developing primers for microsatellites is by probing libraries for clones that contain repeats and sequencing the clone to obtain the repeat and the flanking sequences. Microsatellites are expensive to develop through library screens because of the time and the sequence information required for primer development.

The methylation-sensitive restriction enzyme *Pst*I was selected to digest regions of the genome that were not methylated. Methylation has been associated with the absence of transcription (Lewin 2000) and regions around the centromere are thought to have lower transcription rates. The methylated regions around the centromere would not be digested by *Pst*I allowing fragments to be selected from the middle to the ends of the chromosome arms. There are gaps in these regions of the genetic maps and it would be of interest to identify additional loci in these regions.

In related experiments, Struss and Plieske (1998) prepared clones in a similar manner and probed them with $(GA)_{15}$ and $(GT)_{15}$ resulting in 1.2% and 0.7% of clones containing repetitive sequence. The $(GT)_{15}$ probe was used and eight positive signals could have been expected. However, no clones containing repeat sequences were identified.

Further studies of larger libraries would be required to identify repeat sequences as the library may not have been representative of the genome. These

repeat sequences are present in the genome as some of the available primers are designed for these repeats.

Incorporation of AFLP markers into the Shyri x Galena genetic map

ABSTRACT

In this study the AFLP marker system was used to increase the marker density of the Shyri x Galena barley cross. A total of 138 AFLP markers were added to the map, resulting in an increase of the map length from 1243.8 cM to 2712.9 cM. A strong correlation was identified between the number of AFLP markers added to a chromosome and the increase in length.

In addition to mapping new AFLP markers into the existing Shyri x Galena map, different mapping experiments were performed by eliminating three different classes of markers. An increase in map length was observed regardless of the marker class removed and the marker order did remain consistent.

INTRODUCTION

There is an increasing demand for information regarding genomics, gene structure and location, gene interactions and an understanding of the genetic relationships between and within species. A reliable genetic map is essential for genomic studies and several methods are available for producing maps: isozymes, AFLP, RFLP, RAPD and microsatellites. The AFLP system is widely employed in plant genetic mapping programs, as it is relatively cheap to perform and rapidly generates a large number of unique genetic markers.

The amplified fragment length polymorphism (AFLP) protocol was developed by Vos et al. (1995). AFLP analysis detects the presence or absence of restriction fragments rather than length differences like RFLP. The fragments are produced through restriction digestion and PCR amplification. These amplified fragments are inherited in a predictable, Mendelian fashion, are found throughout the genome and can therefore be used to generate linkage maps (Vos et al. 1995).

New AFLP markers were generated on a doubled haploid barley mapping population, Shyri x Galena, with previously mapped AFLP, RFLP and microsatellite markers. The additional markers were added to the map to increase marker density and increase marker coverage on the chromosome arms.

In addition to mapping newly generated AFLP markers, it was also of interest to determine how these AFLP markers affected the Shyri x Galena map when mapped with different marker classes from the original mapping data. There were three different markers classes: RAPD, microsatellite and RFLP. It had previously

been noticed that the addition of AFLP markers to an existing Proctor x Nudinka barley RFLP map resulted in an increase from 1096 cM to 1873 cM (Becker et al. 1995).

MATERIALS AND METHODS

AFLP Amplification and Detection

Dr. Patrick Hayes of Oregon State University, Corvallis, Oregon provided genomic DNA from 150 individuals of the Shyri x Galena barley doubled haploid population. Eighty-five lines were selected for mapping based on the quantity of DNA available. A total of eleven AFLP primer combinations were applied to the Shyri x Galena population as described by van Eck et al. (1995) using a Gibco BRL AFLP® kit. The genomic DNA was restricted with *Eco*RI and *Mse*I for two hours at 37^0C and the enzymes were inactivated at 70^0C for 15 minutes. *Eco*RI and *Mse*I adapters containing sticky ends were ligated to the fragments. A preamplification step was performed using an aliquot of the ligation mixture.

The primer used for preamplification was specific to three DNA segments; the newly ligated adapter, the remaining bases of the restriction site on the fragment and to a single base on the 3' end. The preamplification cycling conditions were as follows: 20 cycles of 94^0C for 30 seconds, 56^0C for 60 seconds and 72^0C for 60 seconds and they were performed in an MJ Research thermocycler. The preamplification step was diluted 10:1 and a selective amplification was performed. The primers used in the selective round of amplification were identical to the

preamplification except that three nucleotides were added to the 3' end of the primer instead of one nucleotide. The cycling conditions for the selective amplification were as follows: ten cycles of Touchdown PCR (Don et al. 1991), 94^0C for 60 seconds, 65^0C - 56^0C for 60 seconds, with a 1^0C decrease each cycle, and 72^0C for 90 seconds, followed by 23 cycles of 94^0C for 30 seconds, 56^0C for 30 seconds and 72^0C for 60 seconds.

After completion of the selective amplification an 8 M urea stop solution with 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol and 0.40% (w/v) orangeG was added to the PCR reaction to a 5 M final concentration. The samples were denatured at 90^0C for 10 minutes, placed on ice and loaded onto a 6% (w/v) polyacrylamide gel (19:1 acrylamide/bis-acrylamide). The pGEM (GibcoBLR) standard marker was also loaded for sizing the bands. The gel was run for two and a half hours at 85 watts in a 1x Tris-Boric acid EDTA (pH 7.5) (TBE) buffer. Following electrophoresis, gels were silver stained using the Silver Sequence™, silver staining procedure (Promega) and two individuals independently scored the polymorphisms.

The migration distances of the polymorphic bands were measured and converted into base pair sizes. The polymorphisms were assigned marker names corresponding to the three nucleotides of the selective primer at the 3' end of the common *Mse*I or *Eco*RI primer. The designators for the selective primers are shown in Table 4. The assigned marker name consists of a two-digit code for each of the *Eco*RI and *Mse*I selective primers. One *Eco*RI and one *Mse*I primer are combined

resulting in a marker name. The name is immediately followed by a lowercase letter that designates the polymorphism, for example E32M59a.These codes do not indicate the size of the amplified fragment. Appendix B lists the AFLP markers along with their associated fragment sizes.

Table 4: Standard nomenclature for naming AFLP markers based on the three selective nucleotides at the 3' end of the *Eco*RI or *Mse*I common primers. A code is assigned to each selective primer and the marker name is the combined *Eco*RI and *MseI* codes.

*Eco*RI selective primer	*Eco*RI code	*Mse*I selective primer	*Mse*I code	Marker Name
ACA	E35	CTC	M60	E35M60
ACC	E36	CCA	M51	E36M51
AAC	E32	CCG	M53	E32M53
AAC	E32	CTA	M59	E32M59
AAC	E32	CTC	M60	E32M60
AAG	E33	CGA	M55	E33M55
AAG	E33	CTC	M60	E33M60
ACA	E35	CTG	M61	E35M61
ACA	E35	CTA	M59	E35M59
AAC	E32	CCA	M51	E32M51
ACC	E36	CTA	M59	E36M59

(Source: Qi and Lindhout 1997)

Mapping New AFLP Markers

A Chi square test (θ= 0.01) was performed on the complete set of 189 AFLP markers to remove markers that did not fit a 1:1 segregation ratio. The 159 new AFLP markers were mapped onto the existing Shyri x Galena map provided by Dr. Patrick Hayes using MAPMAKER 3.0 under the following conditions: a minimum LOD of 4.0, a maximum linkage distance of 40 cM and a minimum of 70 individuals with data for the marker. The Kosambi function was used to convert recombination values to cM distances.

Mapping Experiments

In addition to mapping the newly generated AFLP makers onto the existing Shyri x Galena genetic map, four different mapping experiments were run with MAPMAKER 3.0. The newly generated AFLP data were mapped with different combinations of the original Shyri x Galena mapping data to determine the effects of the different marker types on the genetic map as seen in Table 5. The original mapping data was composed of AFLP, microsatellite, and RFLP markers. A list of markers from the original Shyri x Galena mapping data excluded for each mapping experiment is found in Appendix C.

Table 5: The four different mapping experiments on the Shyri x Galena mapping population using different combinations of the original markers plus the new AFLP markers.

Experiment	Mapping Data Included	Mapping Data Excluded
SG	Original AFLP data Original microsatellite data Original RFLP data	New AFLP data
SGaf	Original microsatellite data Original RFLP data New AFLP data	Original AFLP data
SGmic	Original AFLP data Original RFLP data New AFLP data	Original microsatellite data
SGr	Original AFLP Original microsatellite data New AFLP data	Original RFLP data

The mapping experiments were completed under the following conditions using MAPMAKER 3.0. A minimum LOD of 4.0, a maximum distance of 50 cM, a

minimum of 70 individuals with data for each marker and the Kosambi function was used to obtain centimorgan (cM) values from the recombination values.

A two-point analysis identified the linkage groups with the "two point" and "assign" commands. Three-point analysis, with the "three-point" command, removed any unlikely orders in the linkage groups and the "order" command was used to generate a map by placing the markers one at a time within the linkage groups by multi-point analysis. The "ripple" command was used to confirm the results generated by the "order" command.

RESULTS

Mapping New AFLP Markers

A total of 189 markers were generated using eleven AFLP combinations. A Chi square test removed 30 markers (15.9%) that did not segregate in the expected 1:1 ratio. The remaining 159 markers were mapped onto the Shyri x Galena base map as shown in Figures 17 to 23. The new AFLP loci are in boldface text. A total of 138 of the 159 AFLP markers were successfully mapped. The remaining markers were not linked to any existing linkage groups. The AFLP markers mapped to every chromosome and filled in many gaps present in the original map. Of the new AFLP loci, 17%, 17%, 10%, 6%, 15%, 17% and 18% mapped to chromosomes 1H, 2H, 3H, 4H, 5H, 6H and 7H respectively.

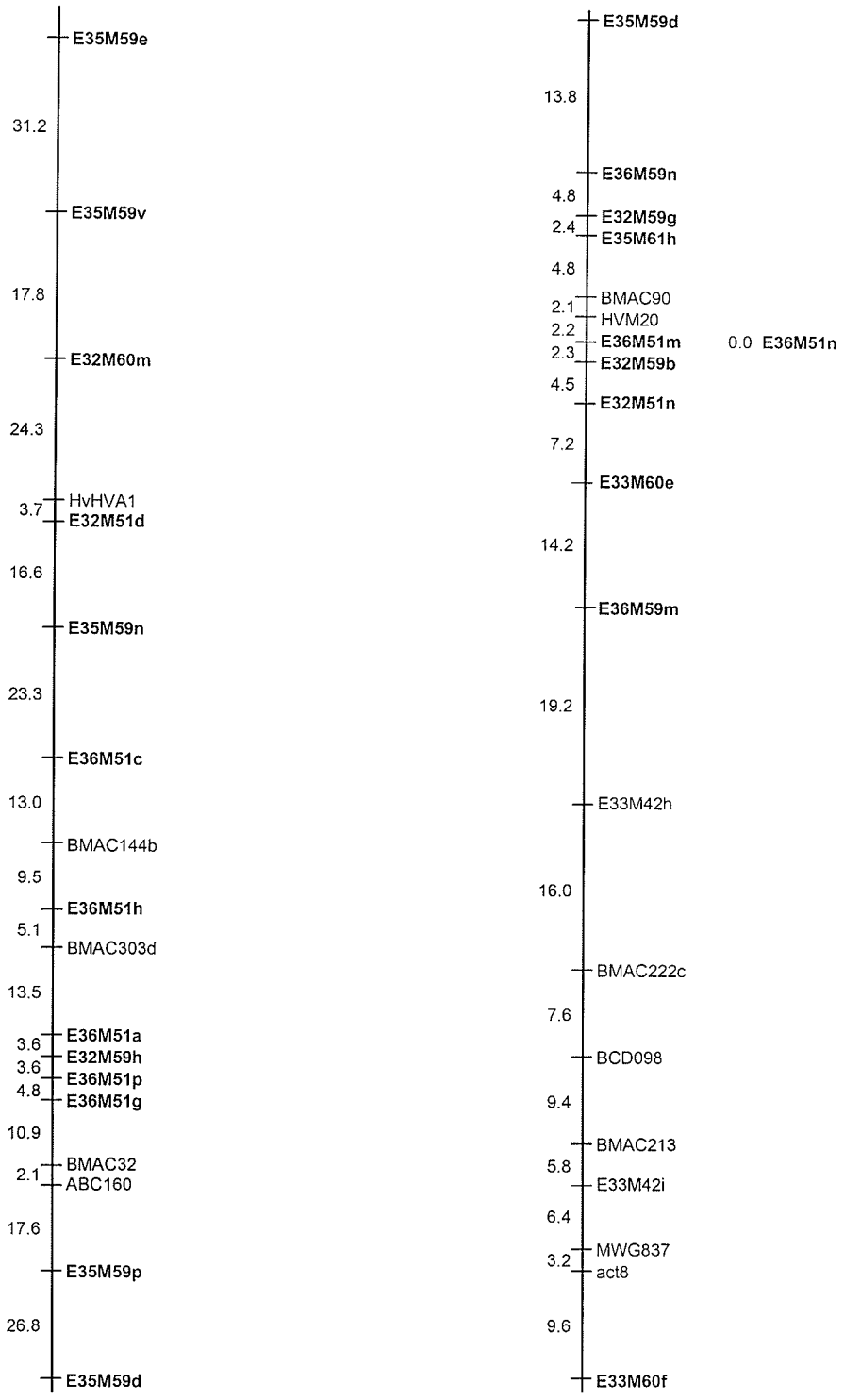

Figure 17: Genetic map of Shyri x Galena chromosome 1H with new AFLP markers and all original markers

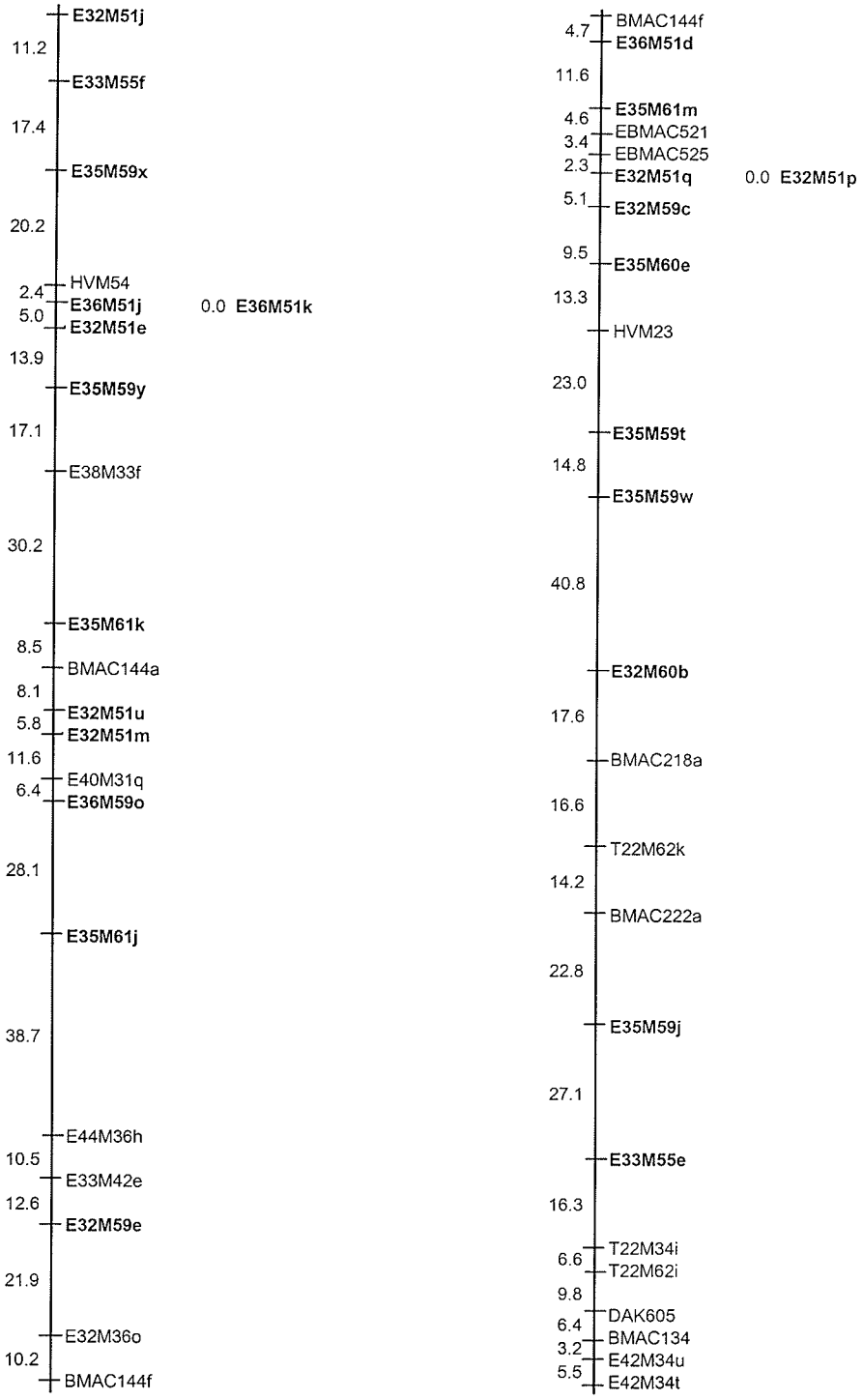

Figure 18: Genetic map of Shyri x Galena chromosome 2H with new AFLP markers and all original markers

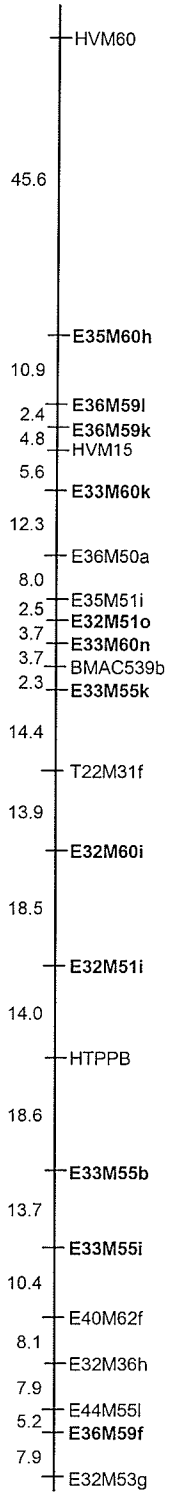

Figure 19: Genetic map of Shyri x Galena chromosome 3H with new AFLP markers and all original markers

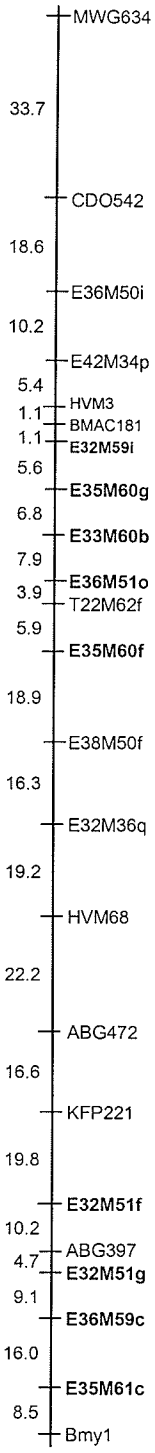

Figure 20: Genetic map of Shyri x Galena chromosome 4H with new AFLP markers and all original markers

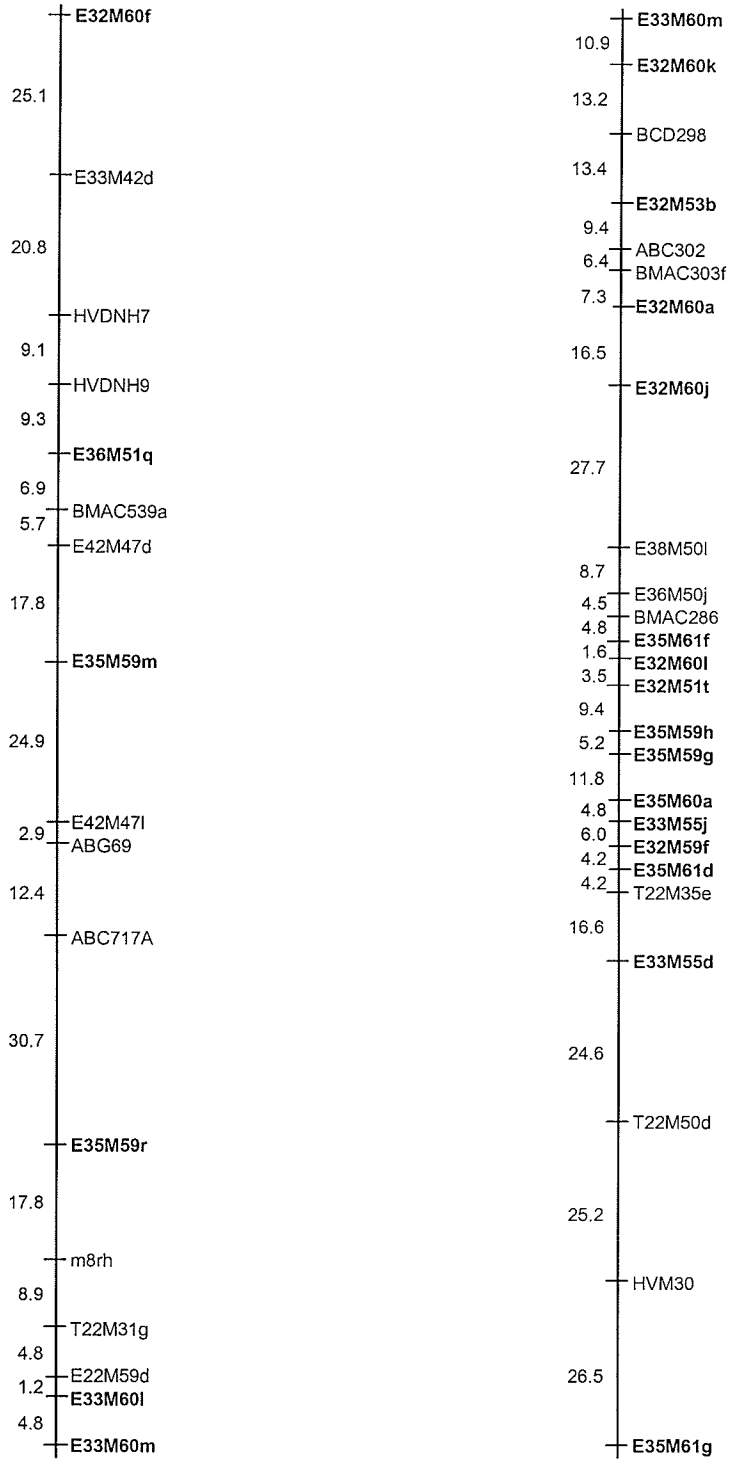

Figure 21: Genetic map of Shyri x Galena chromosome 5H with new AFLP markers and all original markers

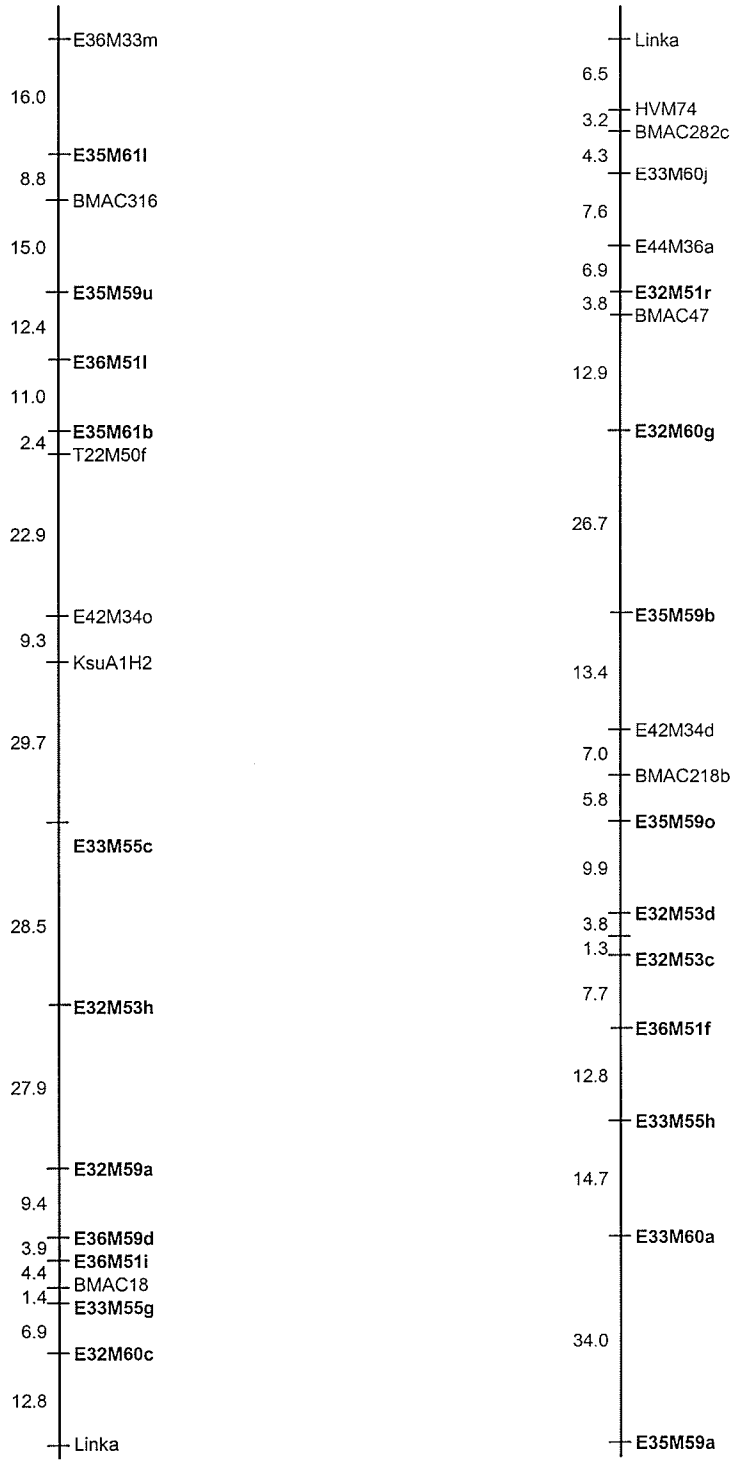

Figure 22: Genetic map of Shyri x Galena chromosome 6H with new AFLP markers and all original markers

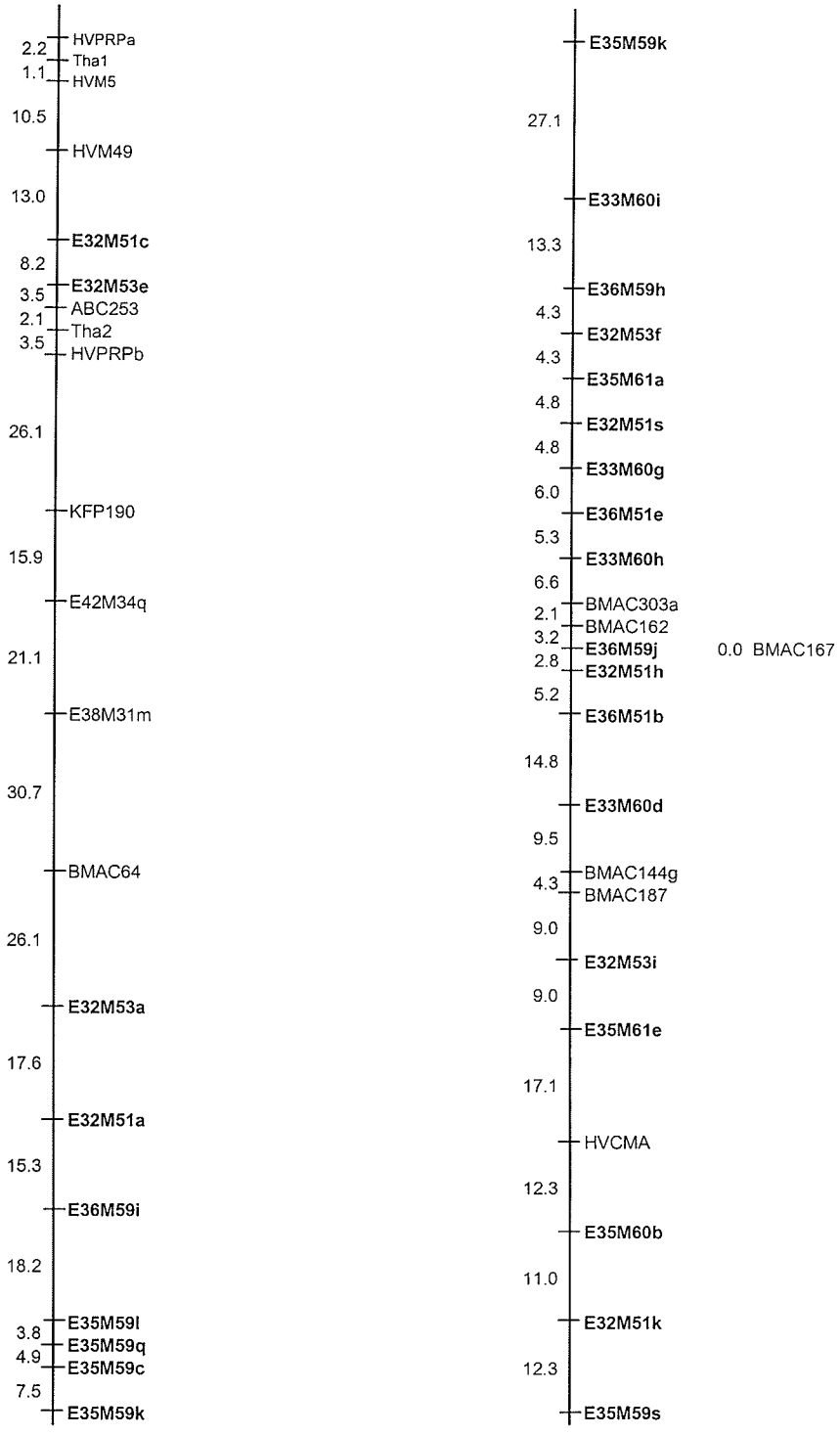

Figure 23: Genetic map of Shyri x Galena chromosome 7H with new AFLP markers and all original markers

The order of the original markers was maintained with the addition of the new AFLP markers. There were a few exceptions where the marker order of two or more markers was reversed from the original marker order upon the addition of the AFLP markers as seen in Table 6.

Table 6: Marker orders that were reversed between the original Shyri x Galena genetic map and the map with the new AFLP markers, listed by chromosome.

Chromosome	Marker 1	Marker 2	Marker 3
2H	EBMAC521	EBMAC525	HVM23
2H	BMAC144a	E40M31q	
3H	E32M36h	E44M55l	
5H	HVDNH7	HVDNH9	
6H	BMAC 18	Linka	
7H	Tha1	HVPRPb	

The addition of AFLP markers to the Shyri x Galena base map resulted in the stretching of the map from 1243.8 to 2712.9 cM, an increase of 118%. Comparing these two maps showed a strong positive correlation between the increase in length of the chromosomes and the number of AFLP markers that were added ($r = 0.93$). Of the increase, 321 cM (22%) was added to the map at the ends of chromosomes.

Mapping Experiments

The genetic maps for the mapping experiments can be seen in Figures 24 through 54. They are arranged first by chromosome then in the following order: (1) SG, (2) SGaf, (3) SGmic, and (4) SGr.

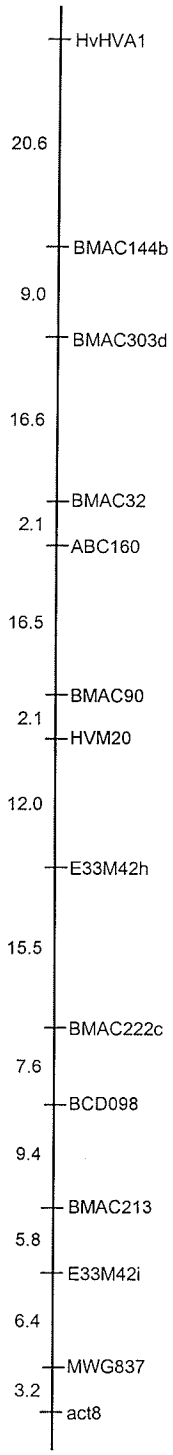

Figure 24: Genetic map of Shyri x Galena chromosome 1H with only the original markers

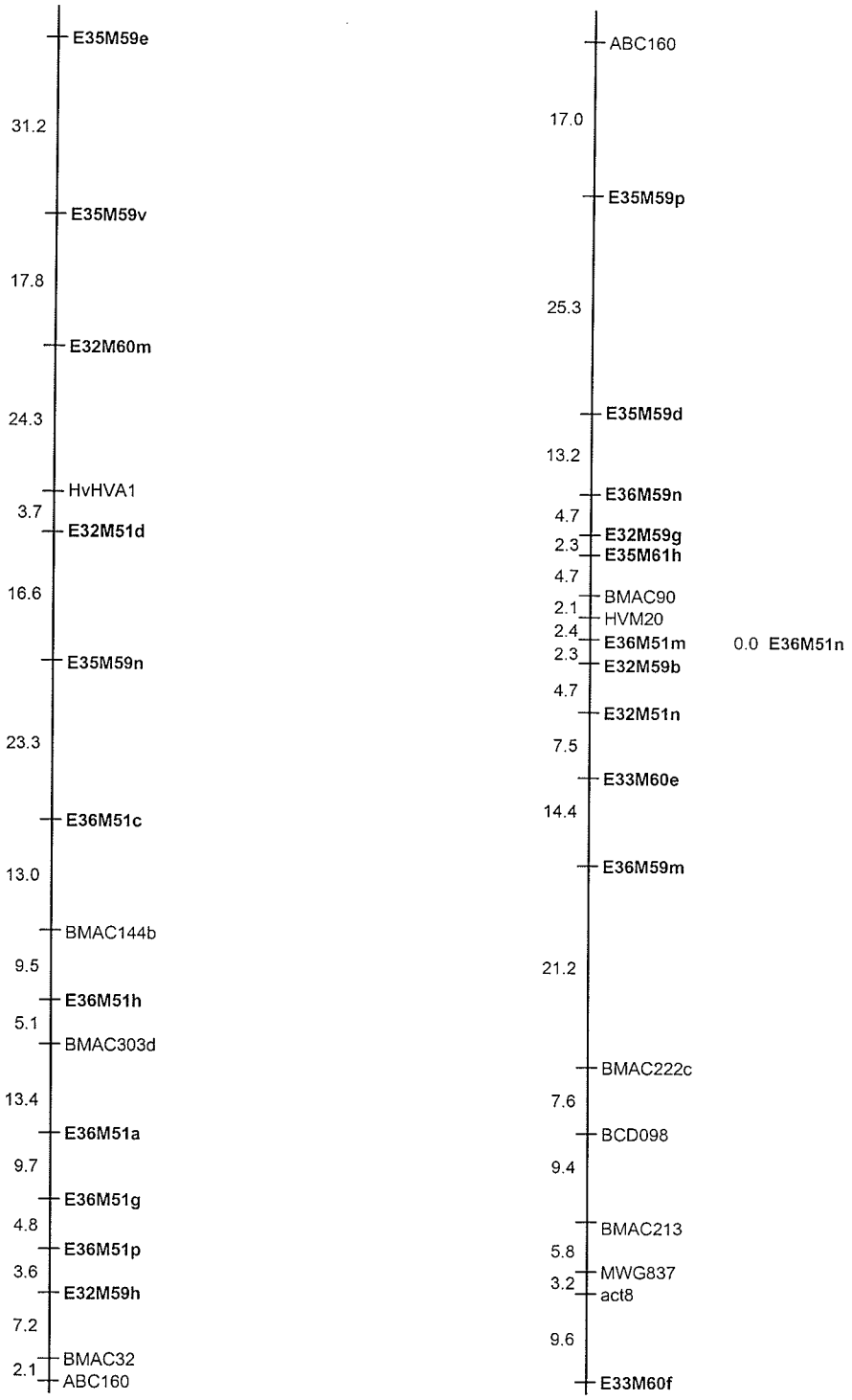

Figure 25: Genetic map of Shyri x Galena chromosome 1H with new AFLP markers and original markers but without the original AFLP markers.

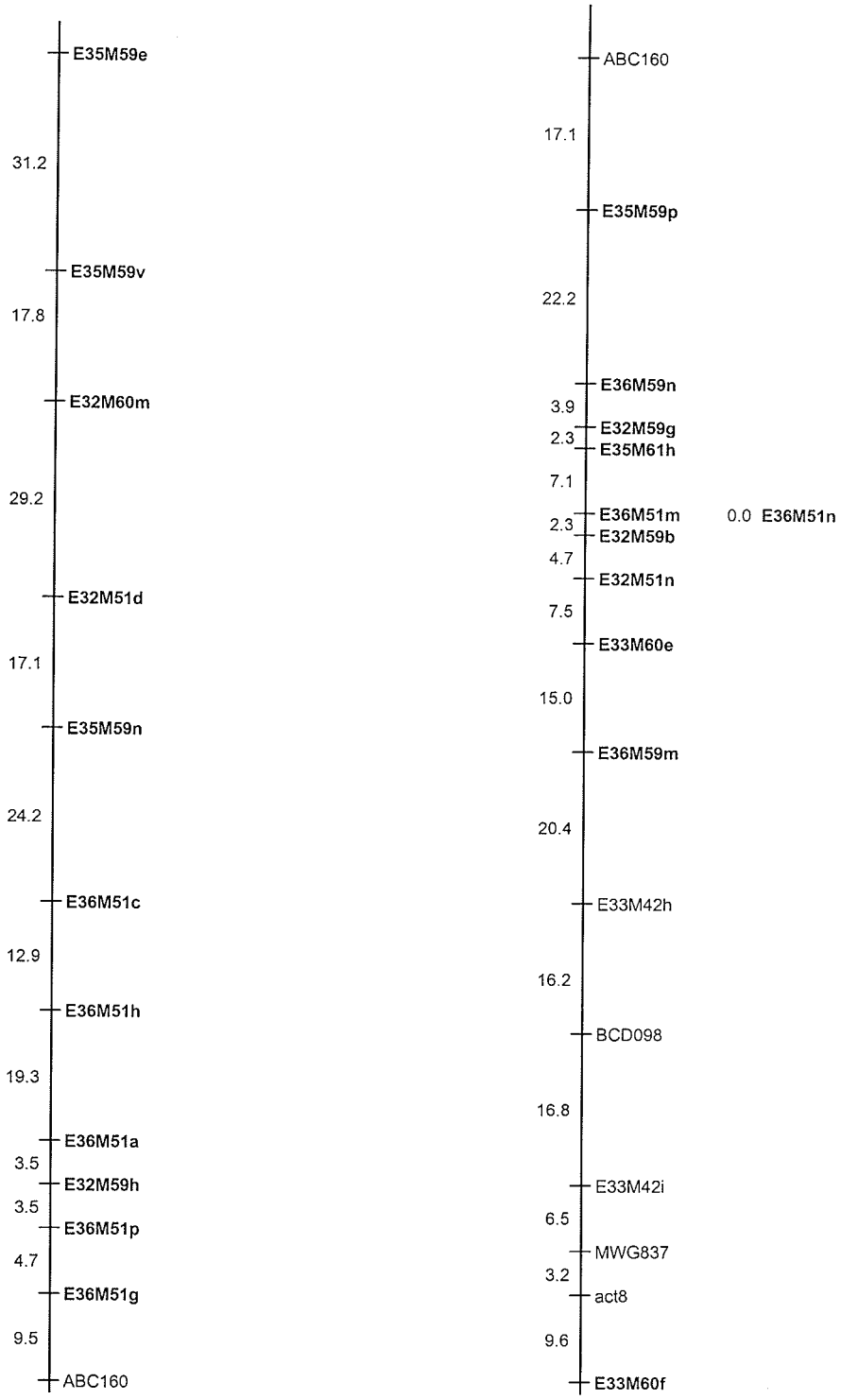

Figure 26: Genetic map of Shyri x Galena chromosome 1H with new AFLP markers and original markers but without the original microsatellite markers.

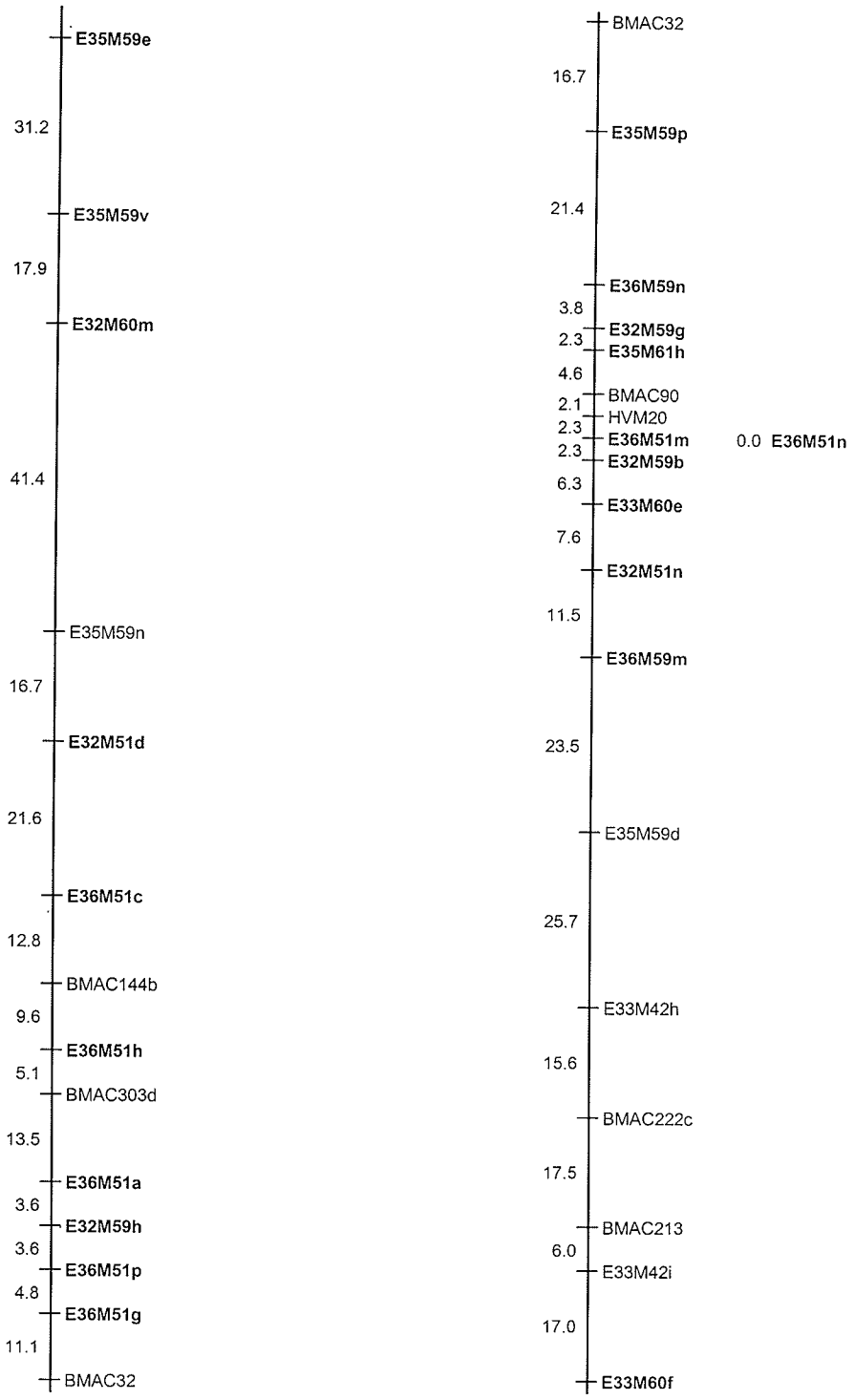

Figure 27: Genetic map of Shyri x Galena chromosome 1H with new AFLP markers and original markers but without the original RFLP markers.

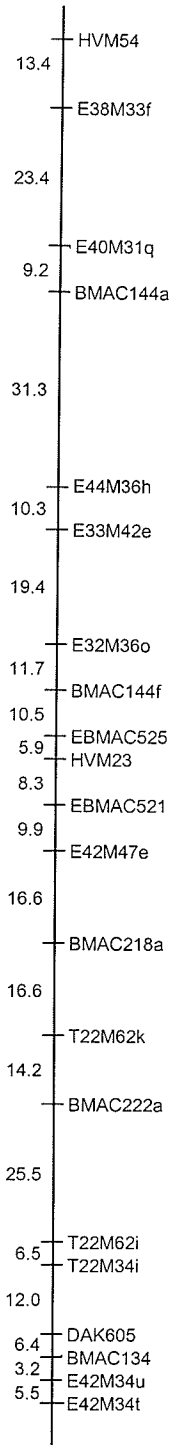

Figure 28: Genetic map of Shyri x Galena chromosome 2H with only the original markers

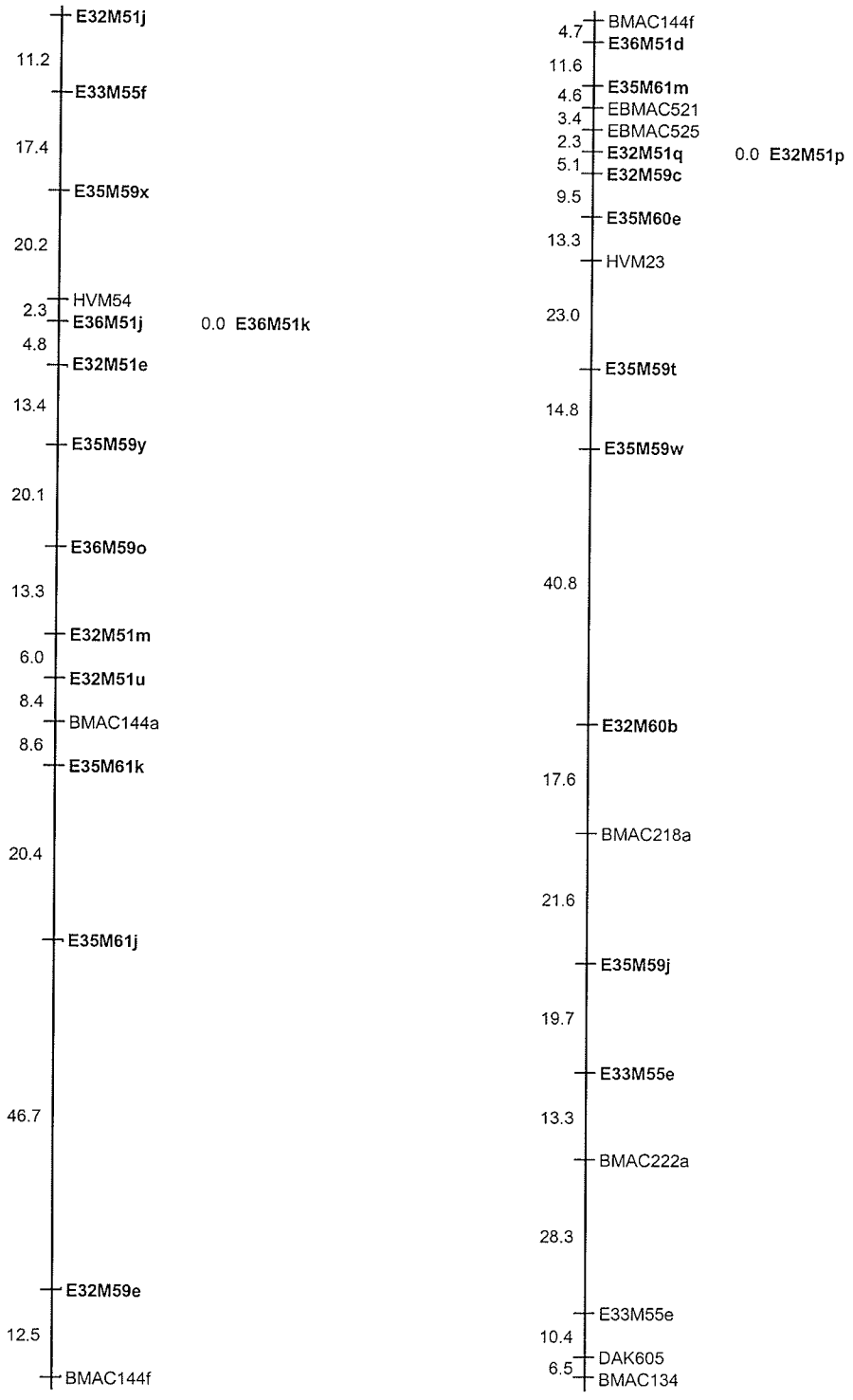

Figure 29: Genetic map of Shyri x Galena chromosome 2H with new AFLP markers and original markers but without the original AFLP markers.

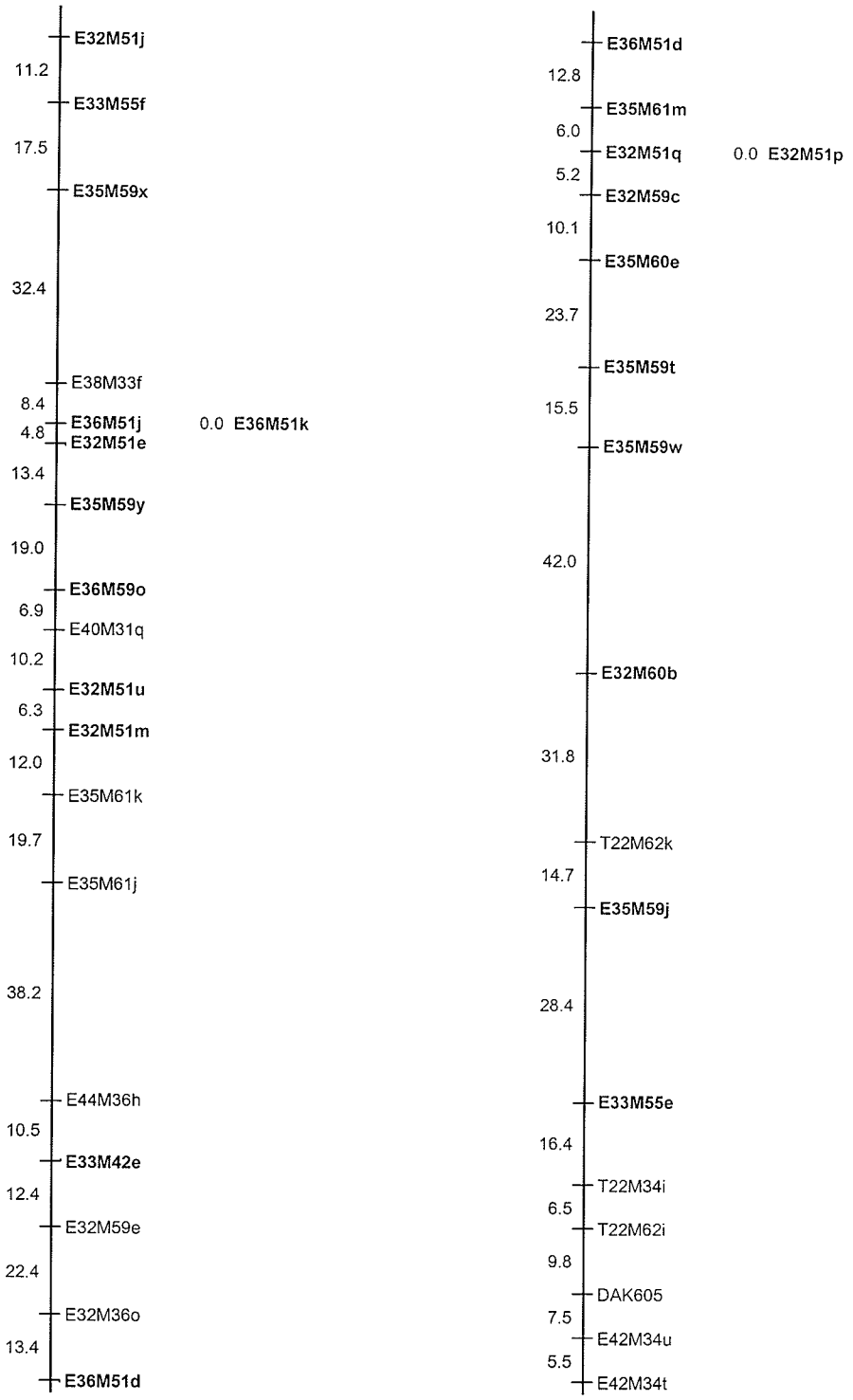

Figure 30: Genetic map of Shyri x Galena chromosome 2H with new AFLP markers and original markers but without the original microsatellite markers.

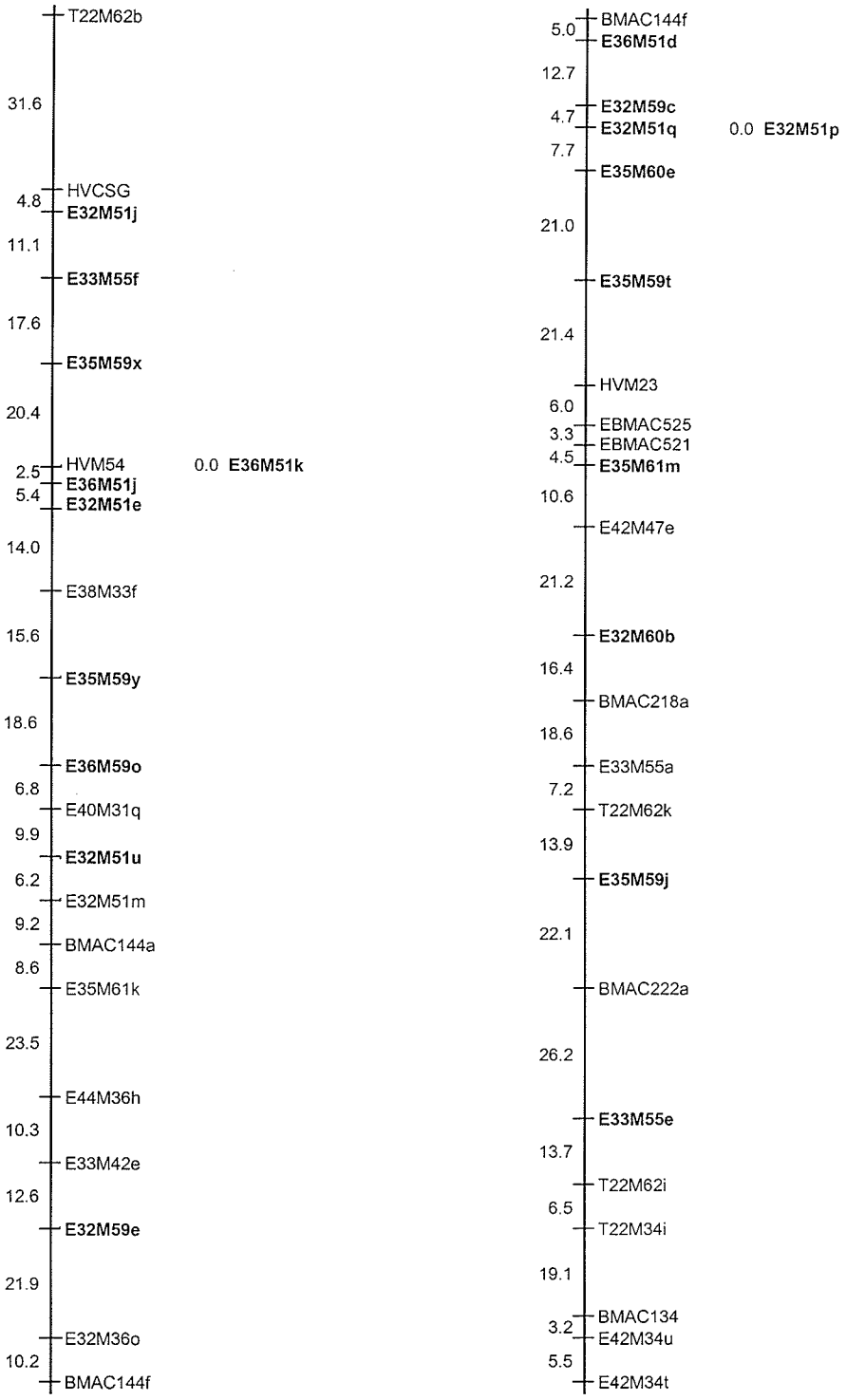

Figure 31: Genetic map of Shyri x Galena chromosome 2H with new AFLP markers and original markers but without the original RFLP markers.

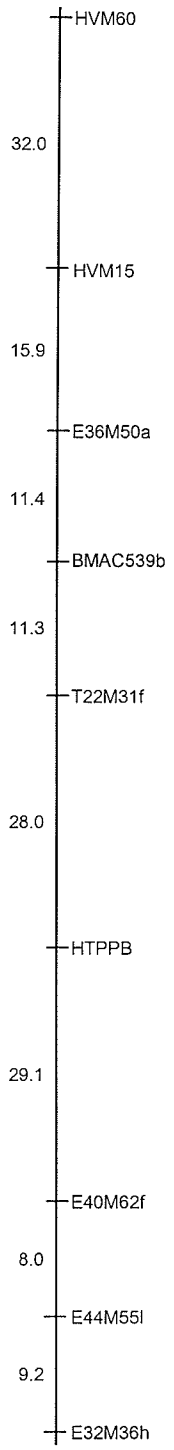

Figure 32: Genetic map of Shyri x Galena chromosome 3H with only the original markers.

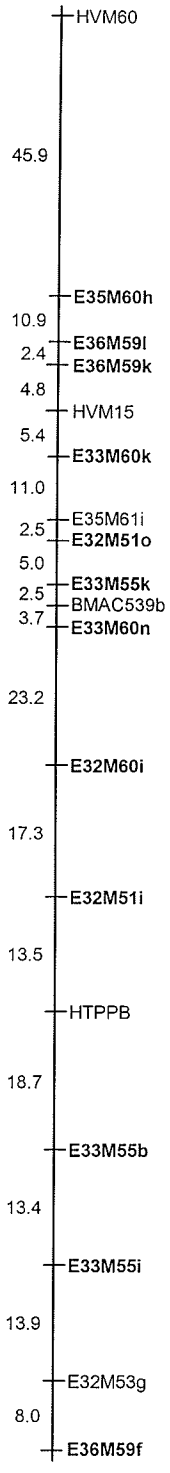

Figure 33: Genetic map of Shyri x Galena chromosome 3H with new AFLP markers and original markers but without the original AFLP markers.

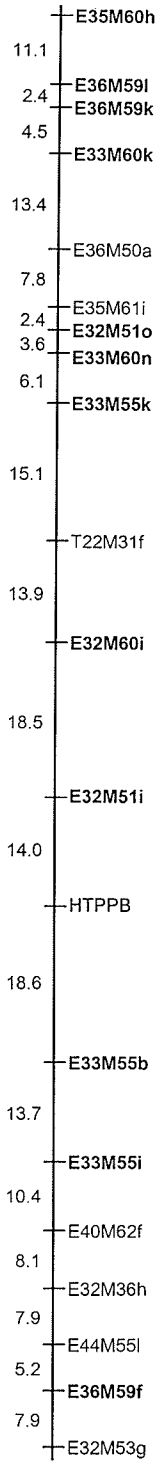

Figure 34: Genetic map of Shyri x Galena chromosome 3H with new AFLP markers and original markers but without the original microsatellite markers.

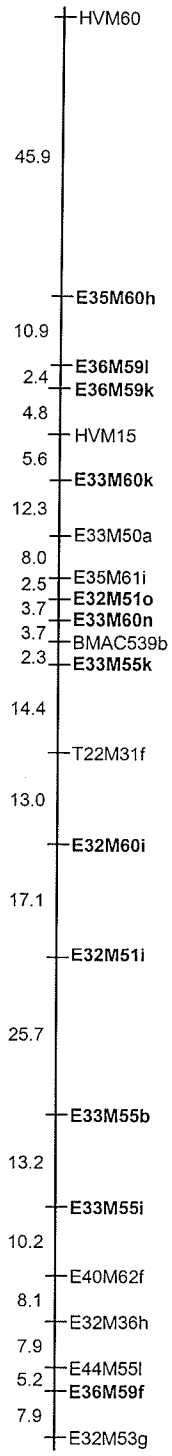

Figure 35: Genetic map of Shyri x Galena chromosome 3H with new AFLP markers and original markers but without the original RFLP markers.

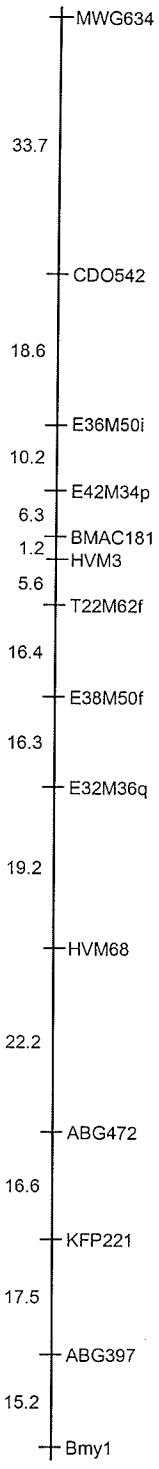

Figure 36: Genetic map of Shyri x Galena chromosome 4H with only the original markers

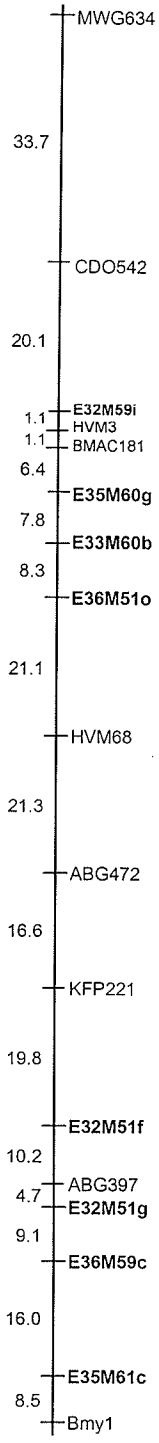

Figure 37: Genetic map of Shyri x Galena chromosome 4H with new AFLP markers and original markers but without the original AFLP markers.

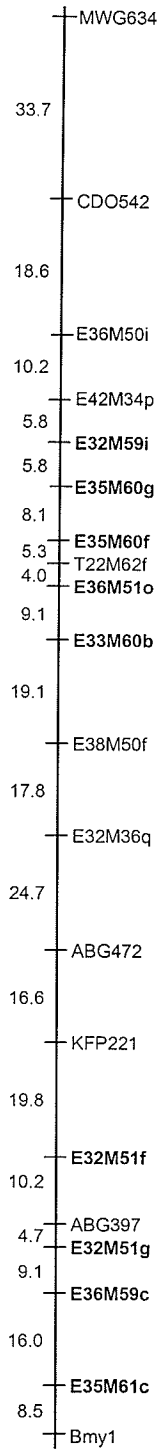

Figure 38: Genetic map of Shyri x Galena chromosome 4H with new AFLP markers and original markers but without the original microsatellite markers.

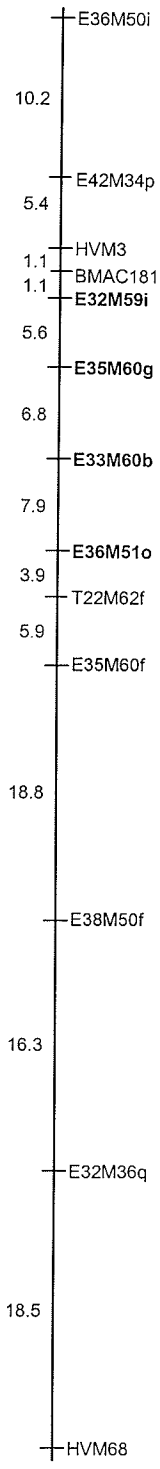

Figure 39: Genetic map of Shyri x Galena chromosome 4H with new AFLP markers and original markers but without the original RFLP markers.

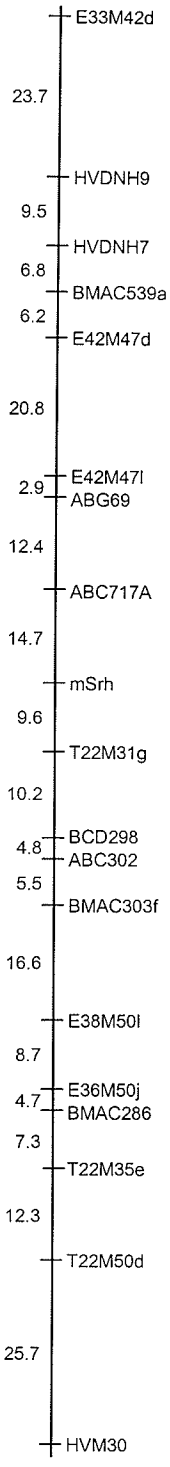

Figure 40: Genetic map of Shyri x Galena chromosome 5H with only the original markers.

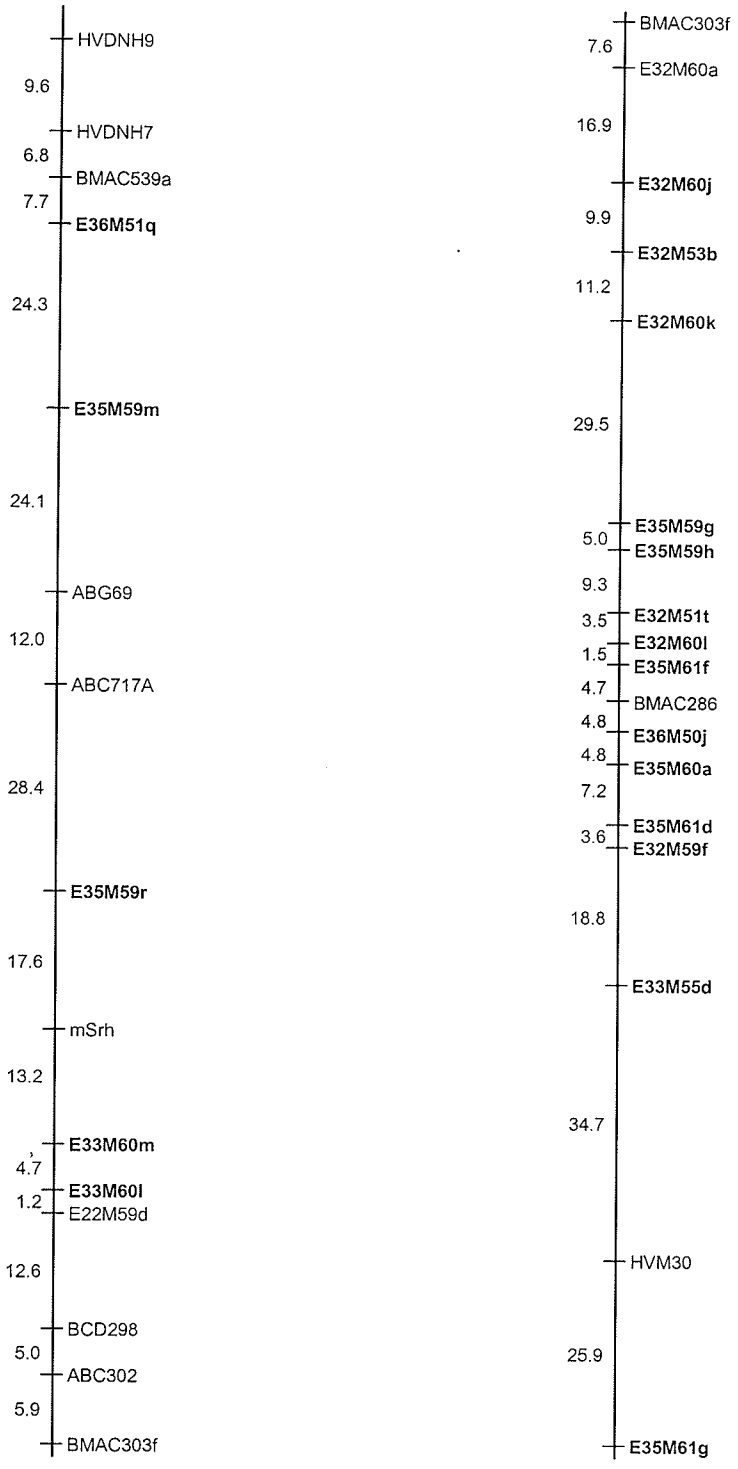

Figure 41: Genetic map of Shyri x Galena chromosome 5H with new AFLP markers and original markers but without the original AFLP markers.

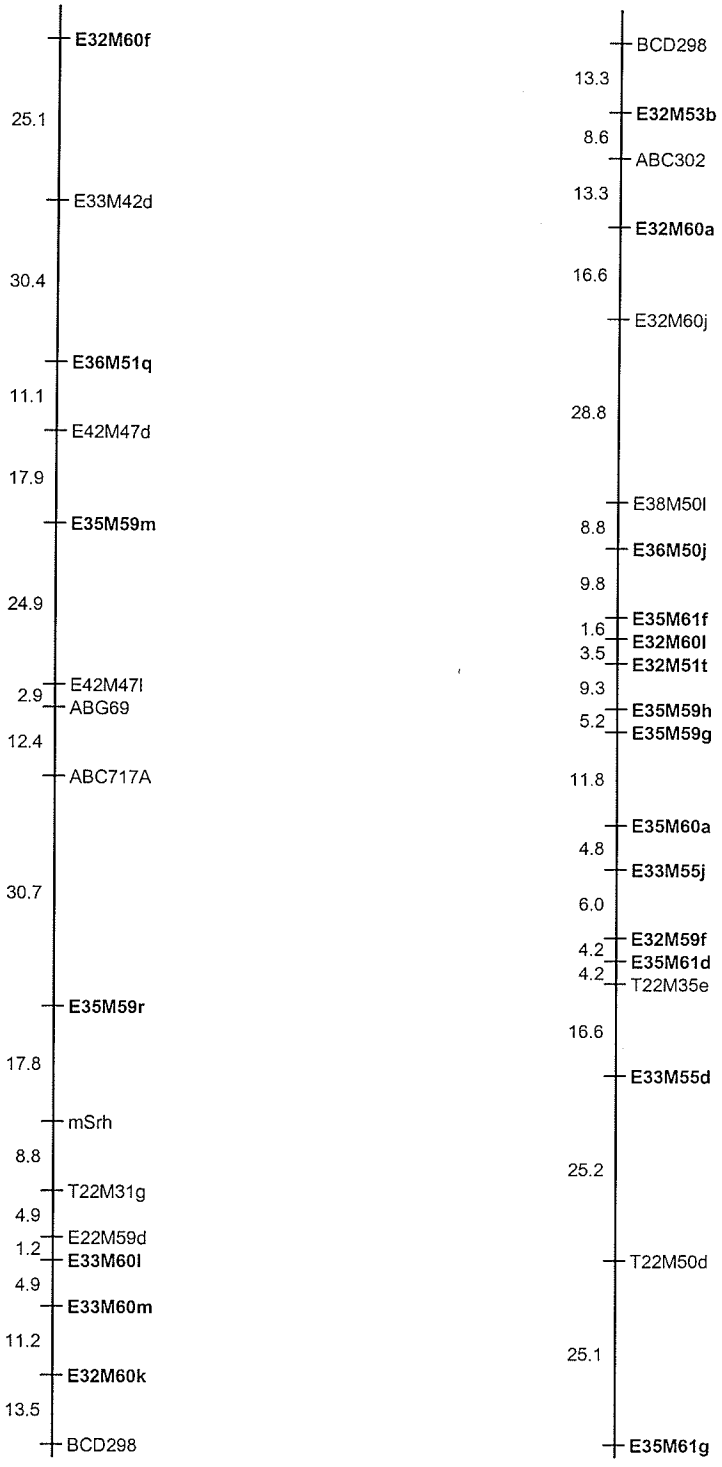

Figure 42: Genetic map of Shyri x Galena chromosome 5H with new AFLP markers and original markers but without the original microsatellite markers.

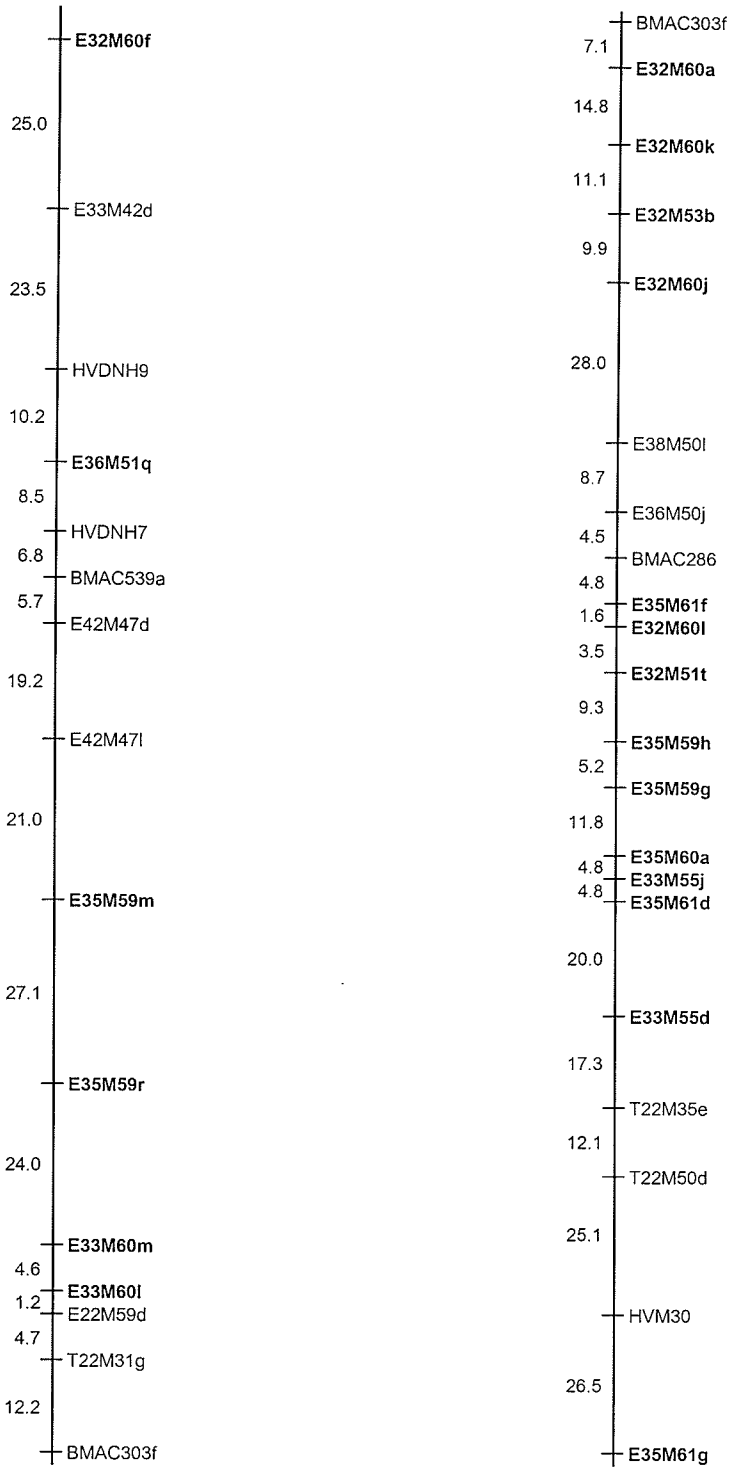

Figure 43: Genetic map of Shyri x Galena chromosome 5H with new AFLP markers and original markers but without the original RFLP markers.

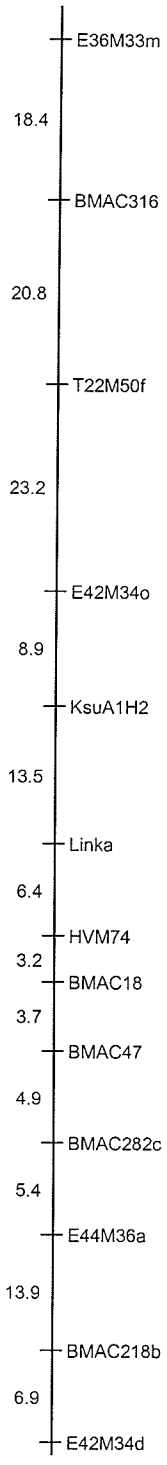

Figure 44: Genetic map of Shyri x Galena chromosome 6H with only the original markers.

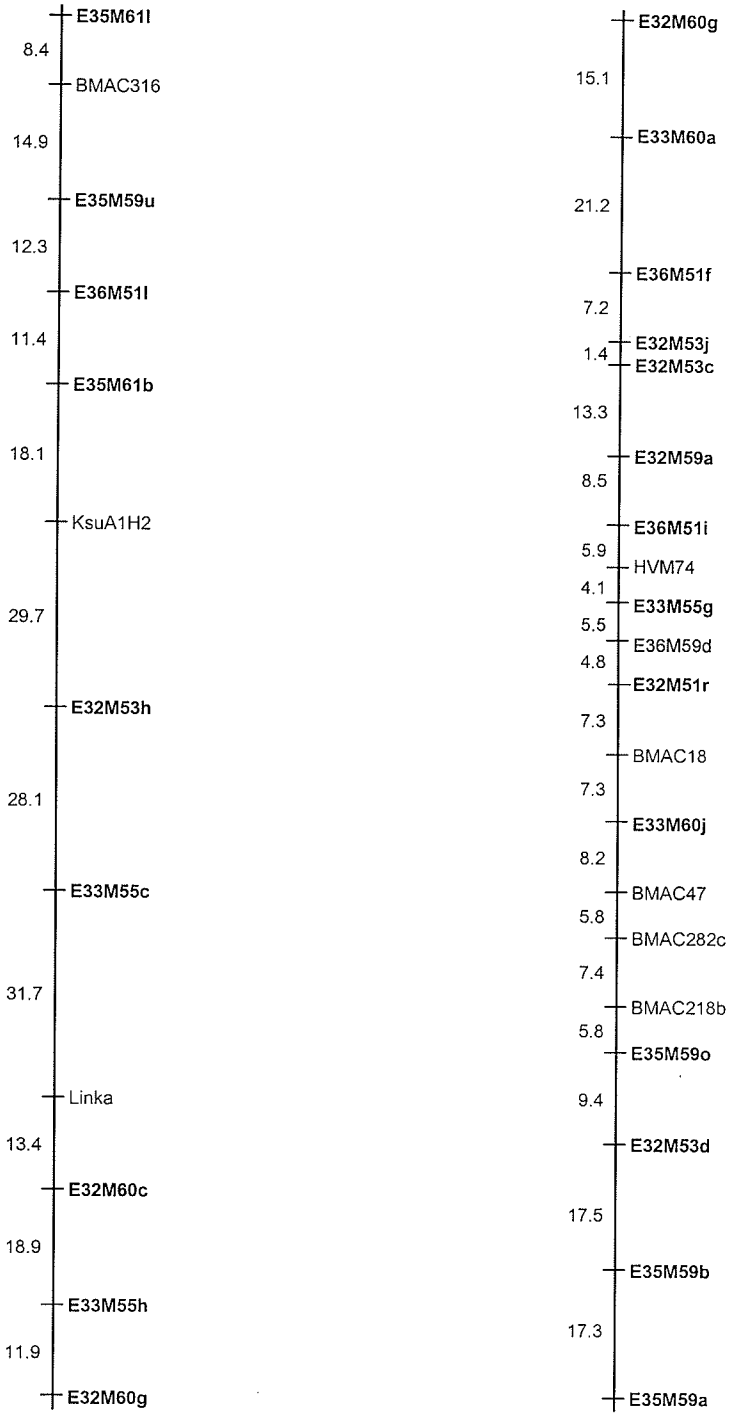

Figure 45: First possible genetic map of Shyri x Galena chromosome 6H with the new AFLP markers and original markers but without the original AFLP markers.

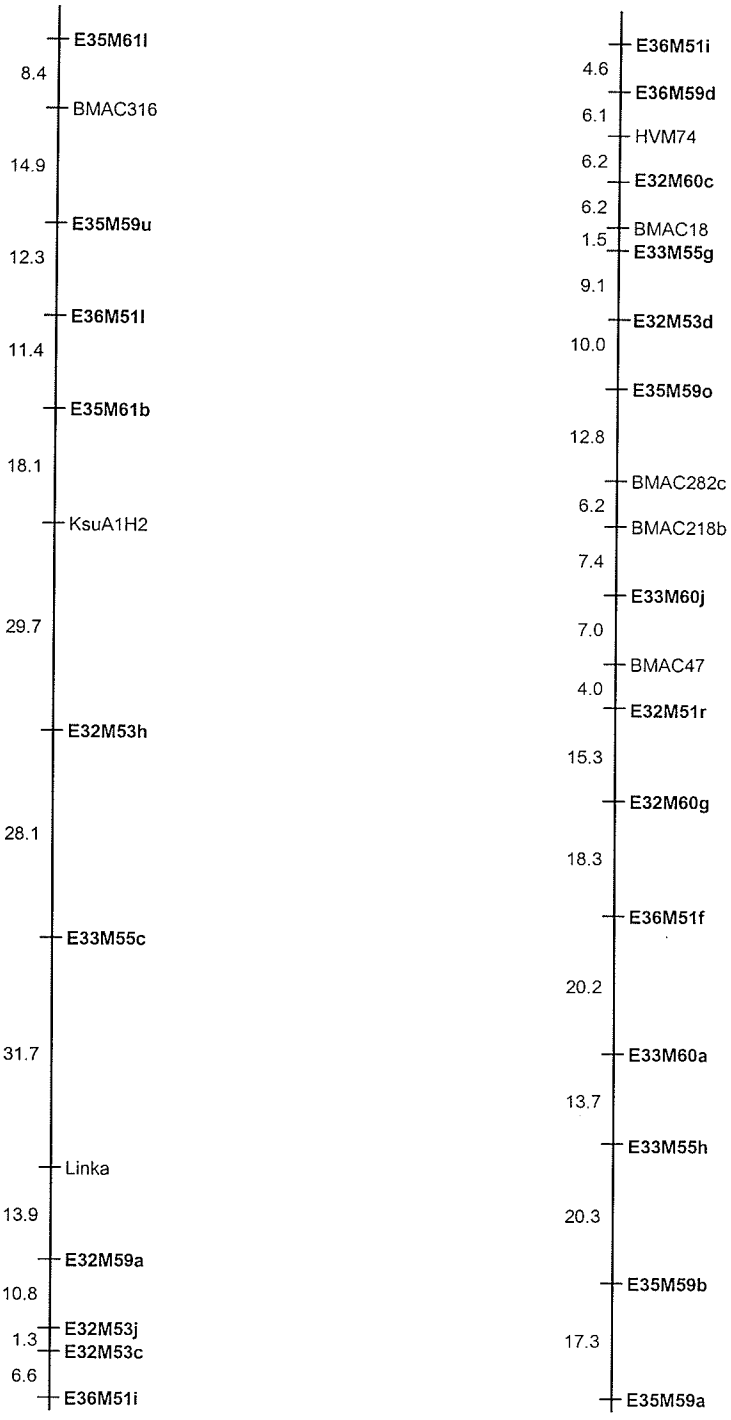

Figure 46: Second possible genetic map of Shyri x Galena chromosome 6H with the new AFLP markers and original markers but without the original AFLP markers.

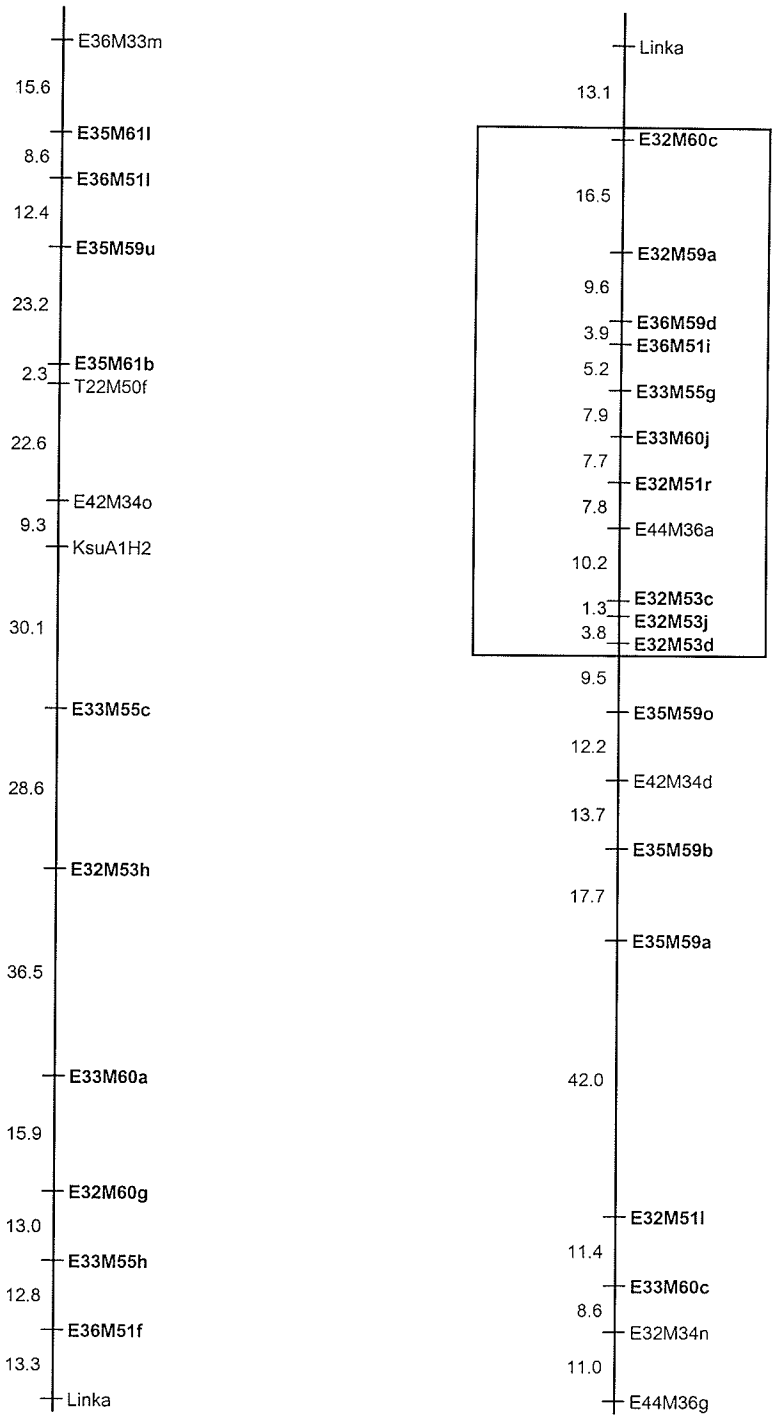

Figure 47: First possible genetic map of Shyri x Galena chromosome 6H with the new AFLP markers and original markers but without the original microsatellite markers. The region with the variable marker order is outlined.

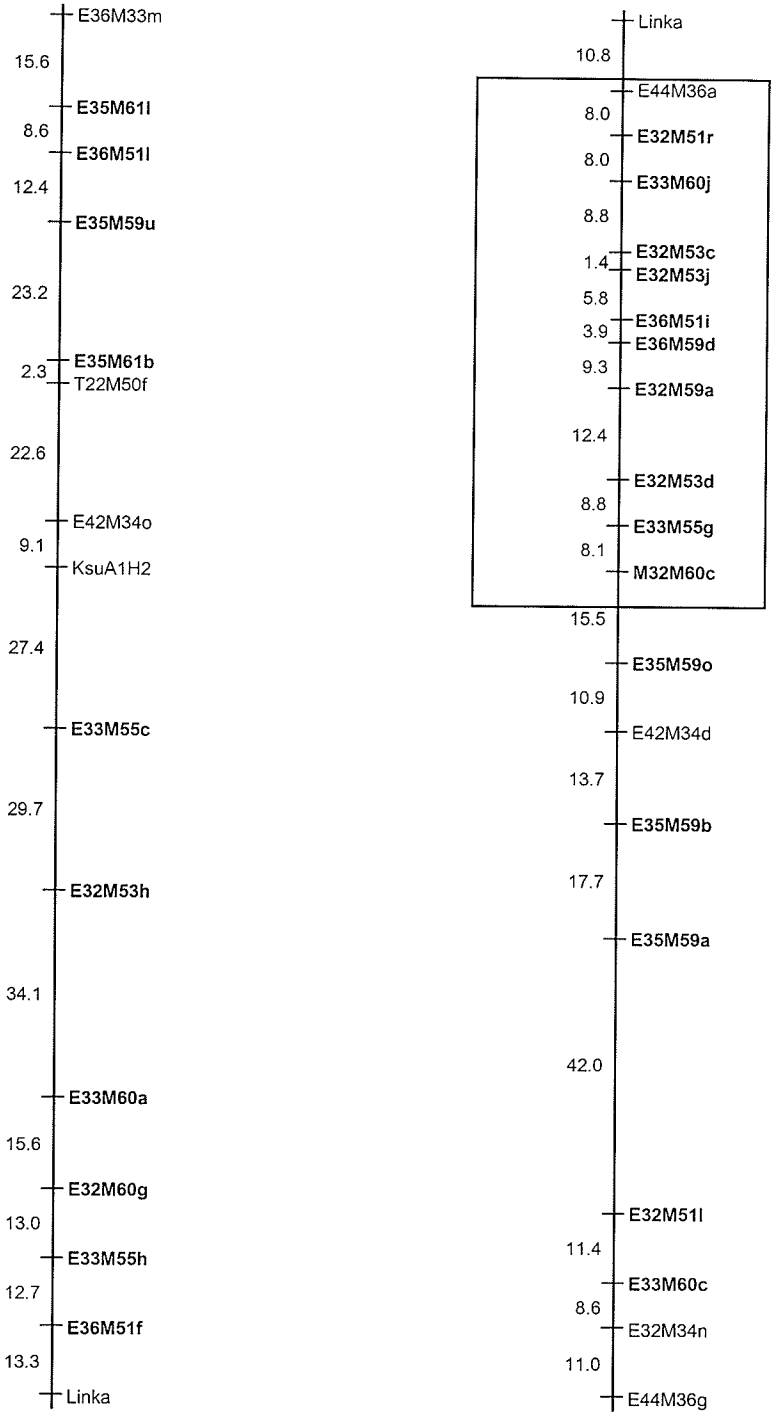

Figure 48: Second possible genetic map of Shyri x Galena chromosome 6H with the new AFLP markers and original markers but without the original microsatellite markers. The region with the variable marker order is outlined.

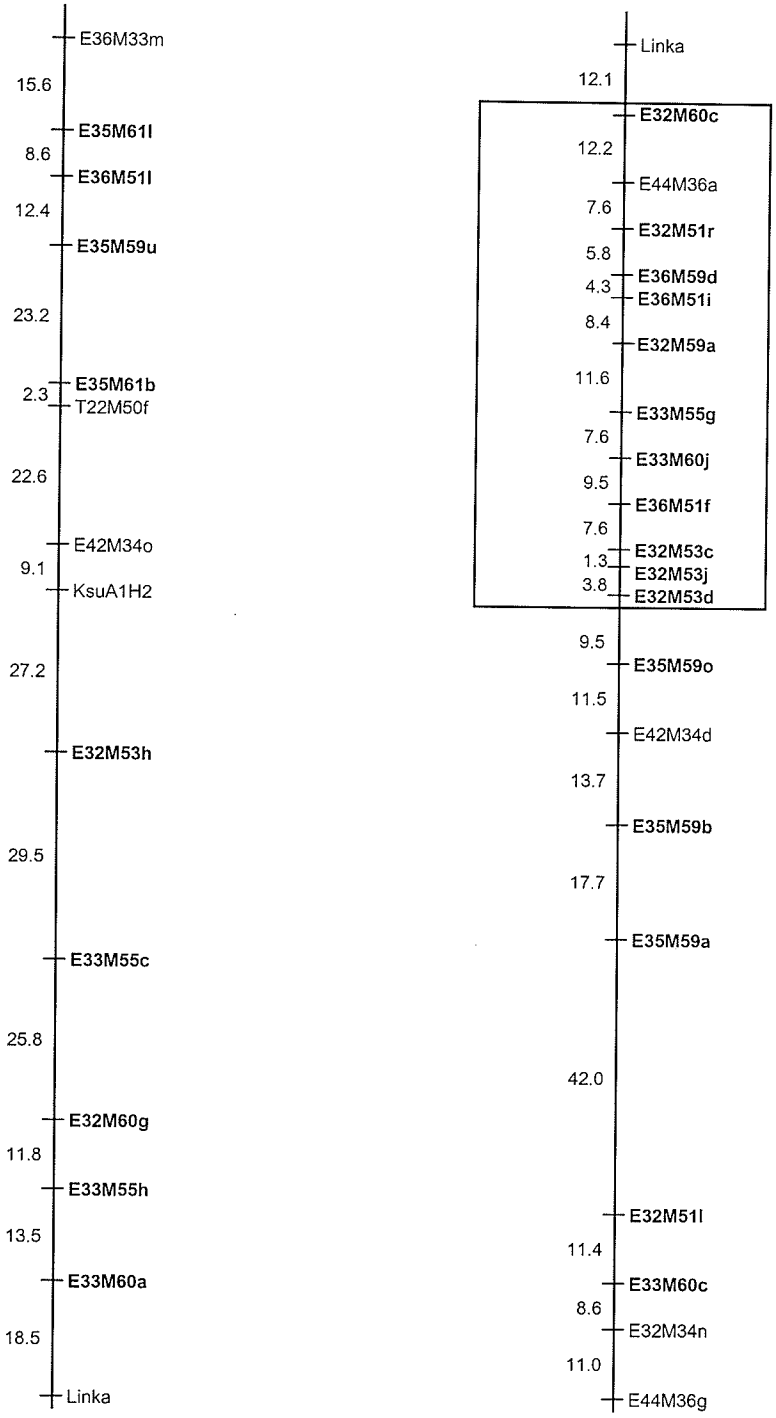

Figure 49: Third possible genetic map of Shyri x Galena chromosome 6H with the new AFLP markers and original markers but without the original microsatellite markers. The region with the variable marker order is outlined.

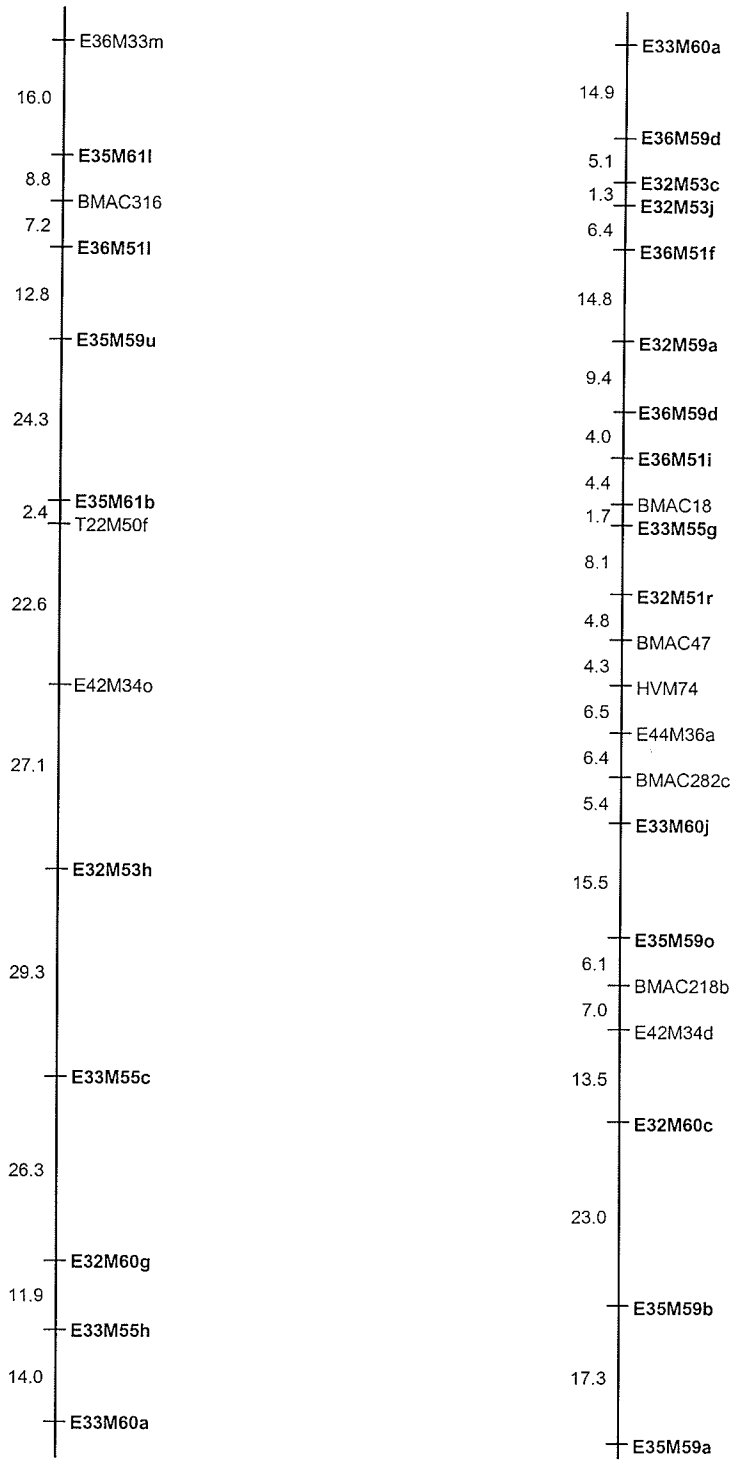

Figure 50: Genetic map of Shyri x Galena chromosome 6H with new AFLP markers and original markers but without the original RFLP markers.

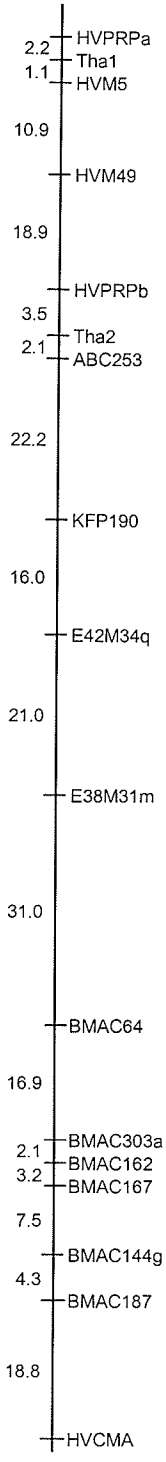

Figure 51: Genetic map of Shyri x Galena chromosome 7H with only the original markers.

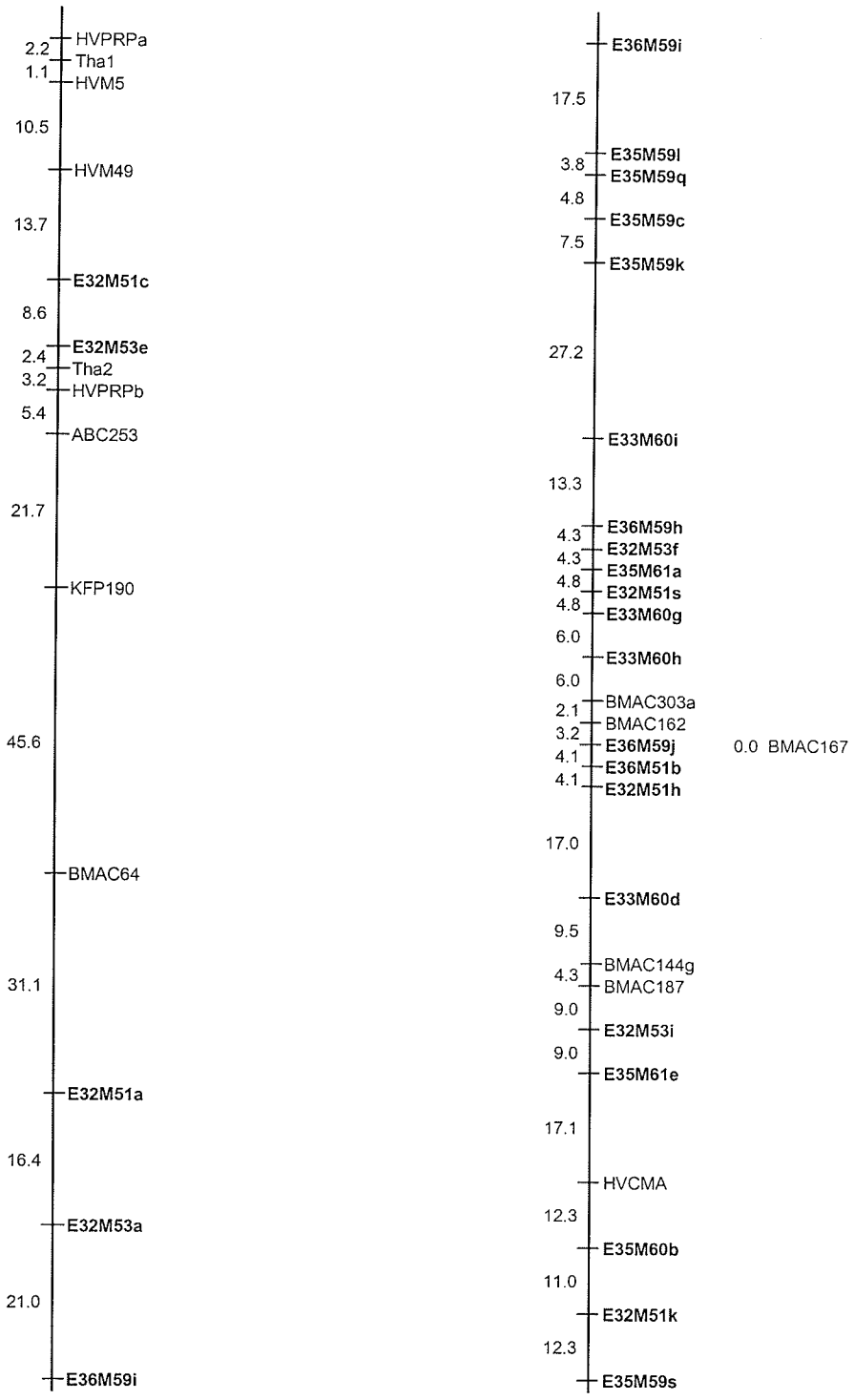

Figure 52: Genetic map of Shyri x Galena chromosome 7H with new AFLP markers and original markers but without the original AFLP markers.

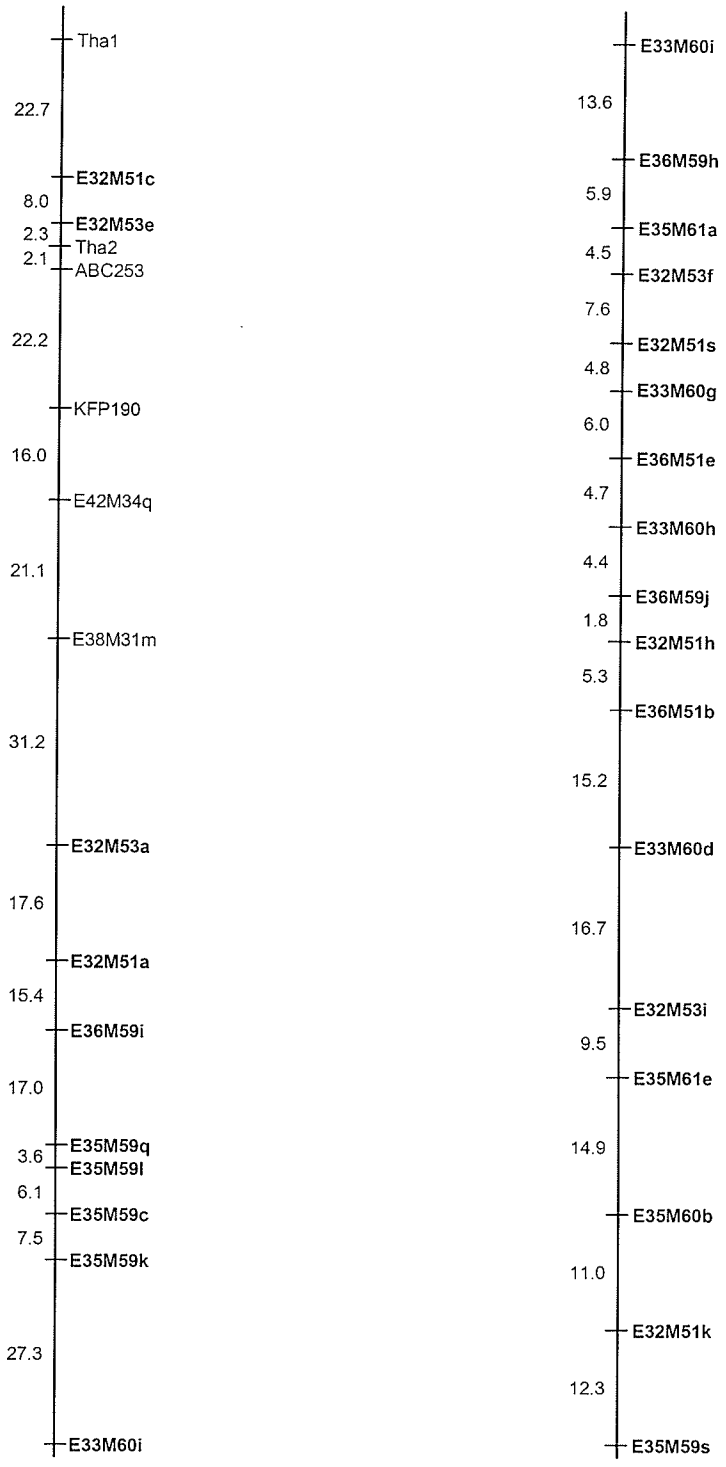

Figure 53: Genetic map of Shyri x Galena chromosome 7H with new AFLP markers and original markers but without the original microsatellite markers.

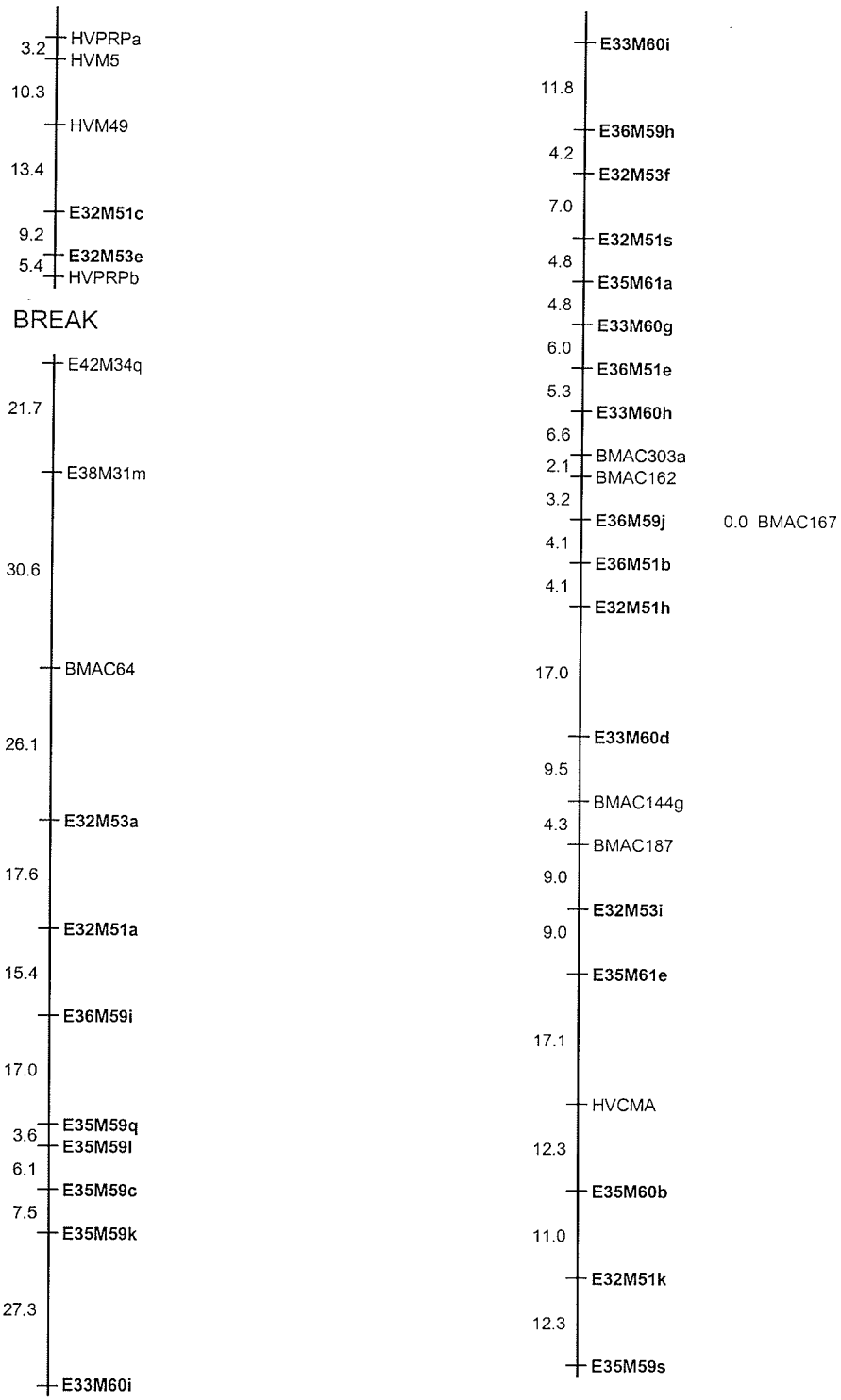

Figure 54: Genetic map of Shyri x Galena chromosome 7H with new AFLP markers and original markers but without the original RFLP markers.

The mapping experiments resulted in consistent marker orders in all experiments for 6 of the 7 chromosomes. Chromosome 6H did not produce consistent genetic maps between experiments or between repeated mapping of the SGmic and SGaf experiments. The SGmic mapping experiment produced three possible maps for chromosome 6H, all nearly identical in map length at 457.3 cM, 455.7 cM and 437.3 cM but the marker order varied in a 76 cM section of the chromosome between E35M59o and Linka. On either side of this variable region, marker order was consistent between multiple runs of MAPMAKER 3.0. The SGaf mapping experiment produced two possible maps for chromosome 6H that were also almost identical in length at 371.8 cM and 373.4 cM. There was a conserved region between the two maps of 185.5 cM.

The mapping experiments increased the total map length from 1243.9 cM to 2423.8 cM in the SGr experiment and to approximately 2353.2 cM in the SGaf experiment and 2496.8 cM in the SGmic experiment. The SGaf and SGmic total lengths use the average lengths of chromosome 6H from the multiple maps produced by each of the mapping experiments.

The chromosome lengths generated by the four mapping experiments can be seen in Table 7 as well as the chromosome lengths generated from mapping the original mapping data plus the new AFLP data (SGall).

Table 7: Total length of the chromosomes for each of the mapping experiments and the percent increase in chromosome length from the original Shyri x Galena map.

Experiment	Chromosome	Chromosome length from mapping experiment	Percent increase from original map
SG	1H	126.8 cM	0%
SGall	1H	362.8 cM	186%
SGaf	1H	342.7 cM	170%
SGmic	1H	327.7 cM	158%
SGr	1H	379.1 cM	199%
SG	2H	259.8 cM	0%
SGall	2H	559.0 cM	115%
SGaf	2H	455.8 cM	75%
SGmic	2H	494.6 cM	90%
SGr	2H	531.3 cM	105%
SG	3H	144.9 cM	0%
SGall	3H	234.4 cM	62%
SGaf	3H	202.1 cM	40%
SGmic	3H	184.6 cM	27%
SGr	3H	224.8 cM	55%
SG	4H	199.0 cM	0%
SGall	4H	261.7 cM	32%
SGaf	4H	205.8 cM	3%
SGmic	4H	247.1 cM	24%
SGr	4H	101.5 cM	-49%
SG	5H	202.4 cM	0%
SGall	5H	469.5 cM	132%
SGaf	5H	372.0 cM	84%
SGmic	5H	434.4 cM	115%
SGr	5H	424.6 cM	110%
SG	6H	129.2 cM	0%
SGall	6H	405.0 cM	214%
SGaf map 1	6H	371.8 cM	187%
SGaf map 2	6H	373.4 cM	189%
SGmic map 1	6H	457.3 cM	254%
SGmic map 2	6H	455.7 cM	253%
SGmic map 3	6H	437.3 cM	239%
SGr	6H	382.6 cM	196%
SG	7H	181.7 cM	0%
SGall	7H	420.4 cM	131%
SGaf	7H	402.2 cM	121%
SGmic	7H	358.3 cM	97%
SGr	7H	379.9 cM[A]	109%

A = approximate distance because two linkage groups were formed when mapping this chromosome under these conditions and distance between the groups is unknown.

DISCUSSION

Mapping New AFLP Markers

The AFLP method for producing markers was very rapid and simple to perform after initial digestion and ligation steps were completed. Unlike microsatellites, which identify one locus per primer pair, AFLP identified 50-100 loci per primer combination and of these, between 9 and 21 were polymorphic depending on the primer combination.

The combinations selected for the Shyri x Galena cross were based on those that provided the greatest polymorphism in barley and other cereals as found previously in the laboratory. A single restriction, ligation and preamplification reaction were required to perform all eleven selective amplifications and 189 polymorphisms.

The completeness of the initial digestion will also affect the number and pattern of bands produced (Vos et al. 1995). If the digestion is not complete, the experiments are not repeatable. To verify that the digestion used for all reactions was complete, a second digestion was done and the E-AAC and M-CCA combination was reused for the selective amplification. The same banding pattern resulted from both restriction digests indicating that the original digestion and ligation reaction was reliable for subsequent selective amplifications.

As a control the original mapping data was remapped without the additional AFLP markers (experiment SG). The original map and the SG map were identical ensuring that the mapping conditions are correct.

The new AFLP markers were mapped into the existing Shyri x Galena while maintaining the original marker order with only a few exceptions. This demonstrated that the markers could be successfully mapped into the existing map. However, the additional markers significantly increased the map length. In another barley cross between Proctor and Nudinka, a similar result was obtained with the addition of AFLP markers (Becker et al. 1995). This map increased from 1096 cM to 1873 cM.

While some gaps on the map were filled with the addition of new AFLP markers, some gaps still remain. These regions of the chromosomes may be identical by descent in the lines and there is no recombination in this area.

AFLP markers generally mapped between existing markers and were not added onto the ends. In the SGall experiment, only 13% of the additional map length was added to the ends. This indicates that the AFLP markers were not tacked onto the ends of the linkage groups, rather they mapped within the linkage groups.

Mapping Experiments

The lengths of all chromosomes increased in all mapping experiments except chromosome 4H in the SGr mapping experiment. This is because the tip of the chromosome was removed with the absence of the RFLP markers. Chromosomes 3H and 4H had the smallest increases in length over all the experiments and chromosomes 1H and 5H had the largest increases in length.

As seen previously, the map produced with all original markers and the new

AFLP markers (SGall) resulted in a stretching of 1469 cM. The mapping experiments showed that removal of the original AFLP (SGaf), microsatellites (SGmic), and RFLP (SGr) markers from the mapping set had almost identical effects on the original map length with increases of 1109 cM, 1253 cM and 1180 cM respectively. The mapping experiments demonstrated that the removal of the different markers classes did not greatly affect the overal length of the SGall map but it was reduced by 360 cM, 216 cM and 289 cM.

The removal of the RFLP (SGr) markers had the greatest effect on a chromosome. By removing this marker class, chromosome 7H was broken into 2 linkage groups.

The marker orders were generally consistent for all chromosomes except chromosome 6H. Chromosome 1H and 5H had nearly identical marker orders between all experiments. Chromosome 2H had identical marker orders between all experiments except for a 40 cM region between markers E35M61k and E36M59o where three markers varied in order. Chromosome 4H had consistent marker orders but 52 cM were removed from the end when the RFLP markers were removed in the SGr mapping experiment. The marker orders for chromosome 7H were consistent between mapping experiments but comparisons of chromosome length could not be made between the original map and chromosome 7H when using the SGaf and SGr mapping conditions. This was because two linkage groups were formed and the distance separating the linkage groups is not known.

Chromosome 6H did not map consistently between mapping experiments.

The maps of the original mapping data and the original mapping data plus all new AFLP data had identical marker orders. However, SGmic, SGaf and SGr had large regions of inverted marker orders and the orders were not consistent between these three experiments or between the SG and SGall maps.

In general, AFLP markers may be used successfully to identify loci or traits and may be used to produce genetic maps. The AFLP markers could be mapped onto an existing map with little disruption to the marker order. However the AFLP markers greatly increased the map length.

Applications of Microsatellite Markers to *Hordeum vulgare*

ABSTRACT

Molecular markers are an important tool for plant breeding. Microsatellite markers are used for genetic mapping, trait tagging, and DNA fingerprinting. The ease of use of microsatellite markers makes them ideal for distinguishing crop varieties and for identifying the inheritance of chromosomal segments in a pedigree.

Two applications of microsatellites were examined, the fingerprinting of cultivars and tracing the inheritance of chromosomal segments. First, twenty registered malting barley cultivars were differentiated from one another with nine microsatellite markers. Secondly, microsatellite markers were used to try to trace the inheritance of chromosomal segments from parental lines to twelve registered cultivars.

INTRODUCTION

Microsatellites and other marker systems have been applied to crops for the development of genetic maps, trait tagging, marker-assisted breeding, and genetic diversity studies. Markers have been used for DNA fingerprinting to differentiate cultivars in crops such as barley (Russell et al. 1997), and wheat (Lee et al. 1995; Pecchioni et al. 1996), for tracing the inheritance of chromosomal segments (Moore 1995; Wilson et al. 1999), and for reconstructing pedigrees (Sefc et al. 1998).

Accurately distinguishing different varieties of crops is becoming increasingly important as more varieties of value-added crops are released to market. A single set of core microsatellites that is able to distinguish different barley varieties would be a useful diagnostic tool.

In addition to variety identification, microsatellites may be used for different methods of marker assisted breeding. One method that is currently being explored identifies the inheritance of chromosomal segments. This theory of breeding involves moving blocks of the genome around, such as a chromosome arm, instead of moving specific genes. This theory can also be used to identify ancestry through the development of pedigrees, lineage and phylogenetic trees (Moore et al. 1995).

MATERIALS AND METHODS

Cultivar Differentiation

DNA from twenty malting barley cultivars was available in the laboratory as listed in Table 8. The PCR reaction mixtures were 30 ng genomic DNA, 1x PCR buffer, 0.8mM dNTPs, 1.5 mM $MgCl_2$, 20 pmol each forward and reverse primer and 0.75 unit Taq DNA polymerase to a final volume of 15 μL. The PCR reactions were performed on an MJ Research thermocycler with one of four possible PCR cycling conditions listed in Table 9. Twenty-seven microsatellite markers listed in Table 10 were screened across the cultivars.

Table 8: Twenty registered malting barley cultivars used for differentiation by microsatellites.

AC Oxbow	BT215	Betzes	Centennial	Ellice
Jet	Hannachen	Harrington	Klages	Manley
Metcalfe	Norbert	Olli	Stein	Stratus
TR118	TR129	TR133	TR299	Velvon II

Table 9: Four PCR cycling conditions used for the amplification of the barley microsatellite primers.

Conditions	PCR1	PCR2
Initial denature	10 minutes at 95°C	5 minutes at 95°C
Cycles of Denaturing Annealing Touchdown Extension	18 cycles 1 minute at 94°C 30 seconds at 64°C 1°C decrease every second cycle 1 minute at 72°C	18 cycles 1 minute at 94°C 30 seconds at 69°C 1°C decrease every second cycle 1 minute at 72°C
Cycles of Denaturing Annealing Extension	30 cycles 1 minute at 94°C 1 minute at 55°C 1 minute at 72°C	20 cycles 1 minute at 94°C 1 minute at 60°C 1 minute at 72°C
Final Extension	10 minutes at 72°C	5 minutes at 72°C

Table 9: Continued

Conditions	PCR3	PCR4
Initial denature	5 minutes at 95^{0}C	5 minutes at 95^{0}C
Cycles of Denaturing Annealing Extension	30 cycles 2 minutes at 94^{0}C 1 minute at 55^{0}C 1 minute at 72^{0}C	40 cycles 1 minute at 94^{0}C 1 minute at 60^{0}C 1 minute at 72^{0}C
Final Extension	10 minutes at 72^{0}C	10 minutes at 72^{0}C

Table 10: The twenty-seven microsatellite markers used to screen the malting barley lines and the PCR cycling program used for each primer.

Marker	PCR program	Marker	PCR program	Marker	PCR program
HVM3	PCR3	HVM6	PCR1	HVM20	PCR1
HVM23	PCR2	HVM31	PCR1	HVM40	PCR1
HVM54	PCR1	HVM60	PCR1	HVM62	PCR1
HVDNH7	PCR4	HVDNH9	PCR4	HVBKASI	PCR4
HVCSG	PCR4	BMAG6	PCR3	BMAC13	PCR3
BMAC18	PCR2	BMAC31	PCR3	BMAC32	PCR2
BMAC64	PCR4	BMAC90	PCR4	BMAC167	PCR3
BMAC186	PCR4	BMAC224	PCR3	BMAC299	PCR2
EBMAC501	PCR4	EBMAC525	PCR3	EBMAC541	PCR3

After amplification, an 8 M urea stop solution with 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol and 0.40% (w/v) orangeG was added to the PCR reaction to a 5M final concentration. The samples were denatured at 90^{0}C for 10 minutes, placed on ice and loaded onto a 6% (w/v) polyacrylamide gel (19:1 acrylamide/bis-acrylamide). The pGEM (Gibco/BLR) standard marker was also loaded and was used for sizing the bands. The samples were electrophoresed for two and a half hours at 85 watts in a 1x Tris-Boric acid-EDTA (pH 7.5) (TBE) buffer. Following electrophoresis, gels were silver stained using the Silver Sequence® silver staining kit (Promega).

The migration distances of the bands were measured and converted into base pair sizes. A regression analysis was performed with the migration distances of the pGEM standard marker's known base pair size fragments and the migration distances of the microsatellites.

Synteny Mapping

Pedigree information or DNA was available for one or more parental lines for twelve of the registered cultivars, as seen in Table 11. The cultivars were screened with twenty-five microsatellite markers. The amplified alleles in the offspring were identified as being from one or both of the parents.

Table 11: List of registered cultivars and their parental lines used for synteny mapping.

Cultivar	Cross
BT215	Klages / RPB72-456[a]
Harrington	Klages / S72114[a]
Klages	Betzes / Domen[a]
Manley	Norbert / MT54-7143[a]
Metcalfe	AC Oxbow / Manley
Norbert	WPG M7118-792-13[a] / Klages
Stein	Norbert / MR54-7143[a]
CDC Stratus	Manley / ID81027[a]
TR118	Harrington / Maris Concorde[a] / WM795[a]
TR129	Nairn[a] / Manley
TR133	Manley / SM85221[a]
TR229	AC Oxbow / Manley

(from Therrien 1998 per comm)
a – DNA not available in laboratory

RESULTS

Cultivar Differentiation

The twenty cultivars could be distinguished from each other using a set of nine microsatellite markers. The base pair sizes for the alleles amplified by the microsatellite markers for each cultivar are in Table 12. Not all nine microsatellite markers are require to differentiate every cultivar, for example, Jet can be distinguished from every other cultivar by just using the markers BMAC32.

Synteny Mapping

The base pair sizes of the alleles amplified by each marker for cultivars and their associated parental lines can be seen in Figure 55. The markers are in the same order that they would be found along a chromosome. When the parents have been screened blocks of chromosomes can be traced down the pedigree. There were two different outcomes. The first is when DNA from one of the parents is not available, for example, Klages x RPB72-456 producing BT215 where no DNA was available for RPB72-456. If the amplified alleles were different between Klages and BT215, it could be assumed that BT215 inherited its allele from the other parent. If the allele was the same between Klages and BT215, it cannot be known if the other parent also carried the same allele. It could be possible that both parents have the same allele from a related ancestor.

The second outcome is when DNA from both parents was available, for example Oxbow x Manley producing Metcalfe. Here it is possible to determine from

Table 12: Base pair size of the allele amplified by the microsatellite loci for each of the twenty barley cultivers and lines. Chromosomes on which the markers are located are in brackets.

		Microsatellite Marker								
		BMAC32 (1H)	HVM54 (2H)	EBMAC525 (2H)	HVBKASI (2H)	HVSCG (2H)	EBMAC541 (3H)	HVM40 (4H)	BMAC299 (4H)	HVM6 (5H)
Cultivars	Centennial	219	156	152	202	199	108	148	200	178
	Norbert	219	156	152	202	205	108	166	196	176
	Stein	219	156	-	-	205	108	166	196	176
	Klages	219	164	152	202	205	108	-	-	176
	Manley	219	164	152	202	205	108	166	200	176
	TR133	219	164	152	202	205	108	166	196	176
	TR299	219	164	152	202	205	108	166	196	176
	Metcalfe	219	164	152	202	199	108	166	200	176
	Hannachen	219	164	152	205	205	111	148	-	180
	Betzes	219	164	152	205	199	111	166	-	180
	Oxbow	219	164	154	202	199	108	166	200	176
	Stratus	219	164	-	202	205	108	-	196	176
	TR118	219	164	-	202	205	108	166	196	180
	TR129	219	164	-	202	205	108	-	-	180
	Ellice	219	164	-	202	199	108	166	200	176
	Olli	219	162	158	205	205	108	148	200	180
	Velvon II	229	156	154	202	205	104	-	200	-
	Harrington	221	164	152	205	205	108	166	196	176
	BT215	221	156	-	202	205	108	-	196	176
	Jet	251	162	150	205	211	114	154	196	178

Note: Results with a "-" indicate that a data point is missing

Chromosome		Marker	Klages (P)	BT215 (C)	Manley (P)	Stratus (C)	AC Oxbow (P)	Manley (P)	Metcalfe (C)	Manley (P)	TR129 (C)
	1H	BMAC32	219	221	219	219	219	219	219	219	219
		BMAC90	222	220	222		222	222	222	222	
		HVM20	136	158	158	158	158	158	158	158	158
	2H	HVM54	164	156	164	164	164	164	164	164	164
		HVBKASI	202	202	202	202	202	202	202	202	202
		EBMAC525	152		152		154	152	152	152	
		HVM23	247	247	247	247	247	247	247	247	247
		HVSCG	205	205	205	205	199	205	199	205	205
	3H	BMAC13	163	161	163	163	163	163	163	163	163
		EBMAC541	111	111	108	108	108	108	108	108	111
		HVM62	260	260	268	268	268	268	268	268	260
		BMAC224	203	203	203	205	201	203	203	203	203
		BMAG6	177	173	177	177	177	177	177	177	173
		HVM60	122	122	122	122	122	122	122	122	118
	4H	HVM40			166		166	166	166	166	
		BMAC299		196	200	196	200	200	200	200	
		HVM3	140			140	134				140
		BMAC186	134	134	134	134	134	134	134	134	134
	5H	HVDNH9	146	146			146				146
		HVDNH7			180	180		180	180	180	
		HVM6	176	176	176	176	176	176	176	176	180
	6H	BMAC18	158	158	158	158	158	158		158	158
		HVM31	176	176	176	176	176	176	176	176	176
	7H	BMAC167	198	194	198		194	198	198	198	
		BMAC64		159	157	159	159	157	157	157	159

Figure 55: Twenty-five microsatellite markers on seven chromosomes used to screen twelve pedigrees. The markers are listed on the left side and the barley lines are listed along the top. The barley line is followed by a P or a C to indicate whether it is a parental line or a cultivar. The amplified allele for each line from a respective marker is indicated in base pairs.

Chromosome	Marker	Norbert (P)	Klages (P)	Manley (C)	Betzes (P)	Klages (C)	Norbert (P)	Stein (C)	Klages (P)	Norbert (C)
1H	BMAC32	219	219	219	219	219	219	219	219	219
	BMAC90	222	222	222		222	222		222	222
	HVM20	158	136	158		136	158	158	136	158
2H	HVM54	157	164	164	164	164	157	156	164	157
	HVBKASI	202	202	202	205	202	202		202	202
	EBMAC525	152	152	152	152	152	152		152	152
	HVM23	247	247	247	247	247	247	247	247	247
	HVSCG	205	205	205	199	205	205	205	205	205
3H	BMAC13	163	163	163	163	163	163	163	163	163
	EBMAC541	108	111	108	111	111	108	111	111	108
	HVM62	268	260	268	260	260	268	260	260	268
	BMAC224	201	203	203	203	203	201	203	203	201
	BMAG6	177	177	177	175	177	177	177	177	177
	HVM60	122	122	122	122	122	122	122	122	122
4H	HVM40	166		166	166		166	166		166
	BMAC299	196		200			196	196		196
	HVM3	140	140	140	138	140	140		140	140
	BMAC186	134	134	134	134	134	134	134	134	134
5H	HVDNH9	146	146	146		146	146	146	146	146
	HVDNH7			180	180					
	HVM6	176	176	176	180	176	176	176	176	176
6H	BMAC18	158	158	158	158	158	158		158	158
	HVM31	176	176	176	176	176	176	176	176	176
7H	BMAC167	194	198	198	192	198	194		198	194
	BMAC64	147	157	157	147		147	159		147

Figure 55: continued

Chromosome	Marker	Klages (P)	Harrington (C)	Manley (P)	TR133 (C)	AC Oxbow (P)	Manley (P)	TR299 (C)	Harrington (P)	TR118 (C)
1H	BMAC32	219	221	219	219	219	219	219	221	219
	BMAC90	222		222	222	222	222	222		222
	HVM20	136	158	158	158	158	158	158	158	158
2H	HVM54	164	164	164	164	164	164	164	164	164
	HVBKASI	202	205	202	202	202	202	202	205	202
	EBMAC525	152	152	152	152	154	152	152	152	
	HVM23	247	247	247	247	247	247	247	247	247
	HVSCG	205	205	205	205	199	205	205	205	205
3H	BMAC13	163	163	163	163	163	163	163	163	163
	EBMAC541	111	111	108	111	108	108	108	111	111
	HVM62	260	260	268	260	268	268	268	260	260
	BMAC224	203	203	203	203	201	203	203	203	201
	BMAG6	177	177	177	177	177	177	177	177	
	HVM60	122	122	122	122	122	122	122	122	122
4H	HVM40		166	166	166	166	166	166	166	166
	BMAC299		196	200	196	200	200	200	196	196
	HVM3	140	138		134	134	140	140	138	140
	BMAC186	134	134	134	134	134	134	134	134	134
5H	HVDNH9	146	146			146		146	146	148
	HVDNH7			180	180		180			
	HVM6	176	176	176	176	176	176	176	176	180
6H	BMAC18	158	158	158	158	158	158	158	158	158
	HVM31	176	176	176	176	176	176	176	176	176
7H	BMAC167	198	194	198		194	198	198	194	
	BMAC64			157	159	159	157			159

Figure 55: continued

which parent the alleles came if there are different alleles between the parents. If the same allele is present in the parents and the offspring, it is not possible to determine from which parent the allele came.

It was also observed that there were no heterozygotes for any of the markers, either in the parental lines or in the cultivars. This is because the breeding lines are highly inbred and there may be little variation at the microsatellite loci.

DISCUSSION

Cultivar Differentiation

It was possible to differentiate all cultivars from each other with a set of nine different microsatellite markers. Varietial differentiation may be used by seed companies for quality assurance, in terminals for verification of shipments, identification of products in foodstuffs for consumer trust and it may be used in debates involving intellectual property and protected traits.

The PCR reactions were electrophoresed on a polyacrylamide gel and not on agarose gels. Acrylamide gels can provide a high degree of resolution between alleles that may differ by only a few base pairs. The acrylamide system would work well for applications that could allow 8-10 hours for results, including PCR reaction. It may not be suitable in terminals or grain elevators where results are required immediately. Agarose screening would not reduce the time frame when the screening involves resolving differences of a few base pairs. The high-resolution agarose, MetaPhor® (FMC), would still require 5-6 hours plus the PCR reaction. An

alternative to screening microsatellite markers on agarose gels would be to first identify single nucleotide polymorphism (SNP) that could be used to differentiate the cultivars. The SNPs could then be used in a TAQMAN® reaction to screen the cultivars without the use of electrophoresis.

Synteny Mapping

Tracing the progression of the chromosomal segments was possible for the cultivars that had pedigree information available for both parents. This is not unexpected, as the offspring must receive its chromosomes from one parent or the other. When two different markers on the same chromosome amplified alleles from the same parent, the chromosomal segment between the markers may all be from that parent. In this way it may be possible to determine the chromosomal segments that arise from the different parents.

As genomics increases the knowledge of the genetic makeup of different plants it may be found that certain genes may cluster together. If a plant exhibits several genes of interest that are clustered together, screening for the chromosomal segment could be done in the offspring, thereby eliminating a separate test for each gene. As seen in Figure 56, four quantitative trait loci (QTL) map to chromosome 6H. Three of these, alpha amylase, diastatic power and extract viscosity, map closely along the chromosome. Two markers, one on each side of this region could be used to screen for the presence of this chromosomal segment.

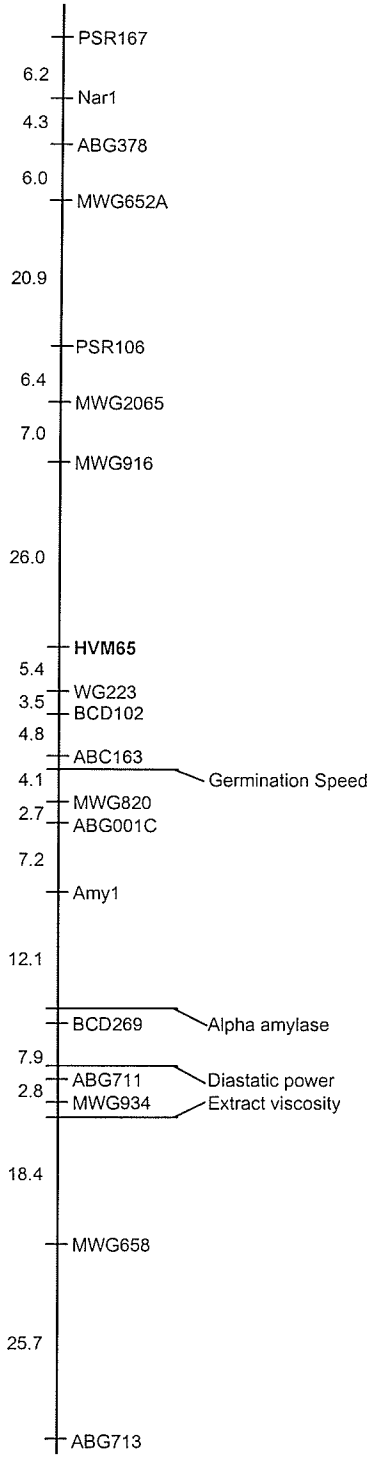

Figure 56: Genetic map of Harrington x TR306 chromosome 6H using the D.Mather base map, microsatellite locus HVM65 and four QTL loci. (Souce of QTL loci: Mather et al. 1997)

It is likely that breeders continually select certain chromosomal regions because they contribute significantly to some quality trait or adaptability of the cultivar. These regions could be identified by the analysis described here and would enable more specific searches for variation in adapted cultivars or wild germplasm.

This screening approach is feasible only if it is more efficient and easier than current procedures. Primer pairs were selected that amplified under the same PCR conditions and pairs were used that could be loaded multiple times on a polyacrylamide gel, reducing time, labour and expenses. This study would require additional screening with more microsatellites on each chromosome.

CONCLUSIONS

The purpose of this project was to map both microsatellite and AFLP markers onto existing barley genetic maps and to examine applications of microsatellite markers.

The mapping of microsatellites involved mapping twenty additional microsatellite markers onto two different Harrington x TR306 genetic maps. The markers were distributed along the chromosomes and mapped to all chromosomes except chromosome 1H. While some of the markers mapped on top of or very close to existing markers, many filled in gaps along the chromosome. The markers that filled in gaps many be useful in identifying sources of variation that were previously undetected.

An additional 59 markers were available but of these 45 were monomorphic between the two parents and ten other markers did not produce an amplification product and could not be mapped. The remaining four markers were polymorphic but could not be assigned to a linkage group.

To try to increase the number of microsatellite markers available two other sources were examined. The first was a screening of wheat microsatellite markers. While there was some amplification, no polymorphic markers were identified. The second method was the development of a genomic library and probing the library with simple repeats to locate repeat sequences. It was thought that if repeat sequences could be identified and sequenced, new primer pairs could be developed, however no positive clones were identified.

This study demonstrated that larger numbers of microsatellite markers are required when developing genetic maps for barley because of the high degree of similarity between lines. It also demonstrated that while microsatellites from related species may amplify loci, they are not all cross-applicable and that the development of microsatellite markers requires a much larger scale genomic screen.

From 11 different AFLP combinations, 159 markers were identified that could be mapped into an existing Shyri x Galena genetic map. The addition of these markers increased the map length from 1243.8 cM to 2712.9 cM, or by 1469 cM. The majority of the markers mapped between existing markers along the length of the chromosomes and 321 cM (22%) of the increase was added to the ends of chromosomes.

To examine the effects on the map when different marker classes were utilized, a series of mapping experiments were performed. These experiments involved mapping the new AFLP markers with different combinations of the original mapping data. It was noticed that the map length increased regardless of the marker class that was removed from the mapping file.

While the AFLP markers did successfully map onto the map without disruption of the marker order they resulted in a large increase in the map length. The removal of different marker classes did not demonstrate that the AFLP markers mapped more efficiently with one class than another.

By using a core set of nine microsatellite markers, it was possible to distinguish twenty registered cultivars from one another. In addition it was

possible to trace the inheritance of chromosomal segments from parental lines to cultivars.

The applications of markers are broad and of the two utilized here the differentiation of cultivars was easy and rapid to perform and could be applied to larger sets of lines. The synteny mapping demonstrated how future breeding of plants may utilize markers to trace the inheritance of chromosomal segments. This study was very small and would require much larger sets of markers and more complete pedigrees to be applied to a breeding program.

APPENDIX

APPENDIX A

LIST OF BARLEY MICROSATELLITE PRIMERS, PCR CONDITIONS, EXPECTED SIZES, AND REPEAT MOTIFS.

Primer	PCR program	F sequence	R sequence	Size (bp)	Repeat
HVM02	PCR1	CAGGTGTCTAGTGGGTGCCT	TTACATCCAAGGAGCAATCCC	201	(GA)11
HVM03	PCR3	ACACCTTCCCAGGACAATCCATTG	AGCACGCAGAGCACCGAAAAAGTC	188	(AT)29
HVM04	PCR3	AGAGCAACTACCAGTCCAATGGCA	GTCGAAGGAGAAGCGGCCCTGGTA	198	(A)9
HVM05	PCR3	AACGACGTCGCCACACAC	AGGAACGAAGGGAGTATTAAGCAG	202	(GT)6(AT)16
HVM06	PCR1	CATGAATGAATGATTGGTTTTG	CGCATCCGATGTATGAGTAA	175	(GA)9
HVM07	PCR3	ATGTAGCGGAAAAATACCATCAT	CCTAGCTAGTCGTGAGCTACCTG	174	(AT)7
HVM09	PCR3	CTTCGACACCATCACCCAG	ACCAAAATCGCATCGAACAT	221	(TCT)5
HVM11	PCR3	CCGGTCGGTGCAGAGAG	AAATGAAAGCTAAATGGGCGATAT	181	(GGA)3(GGA)(GAA)2
HVM13	PCR1	AGTAGCTATGTGTTTGGATCGC	CATCAAGGGCATCCTCATG	249	(GA)12
HVM14	PCR1	CGATCAAGGACATTTGGGTAAT	AACTCTTCGGGTTCAACCAATA	158	(CA)11
HVM15	PCR1	TCTAACCACGGCGTCCT	CGTGACTGGAACCCTGC	166	(GA)8
HVM20	PCR1	CTCCACGAATCTCTGCACAA	CACCGCCTCCTCTTTCAC	151	(GA)19
HVM22	PCR1	TTTTGGGGGATGCCTACATA	TTTCAAATGGTTGGATTGGA	167	(AC)13
HVM23	PCR2	TCGGTGAAGAAATACGAGGC	TCTTTGTGACCTACCGGTCC	246	(GA)9
HVM26	PCR2	GGCTATCACATTTGGTACCATC	GCATGTGTAGGTGTTGGTGG	206	(CA)11
HVM27	PCR1	GGTCGGTTCCCGGTAGTG	TCCTGATCCAGAGCCACC	192	(GA)14
HVM30	PCR2	AGTGGGGAATGAGAGAATGG	TGCTTGTGGGTCATCACAC	150	(CA)8
HVM31	PCR1	CGGTTTCTGGTTGCTTGG	CGAAGGTCTCAGGCTTCATG	163	(AC)9
HVM33	PCR1	ATATTAAAAAAGGTGGAAAGCC	CACGCCCTCTCCCTAGAT	157	(CA)7
HVM34	PCR2	ACCATGTTGCGTGTTGCTT	CGGTTCGAAATCGAGTGG	222	(GA)10
HVM36	PCR1	TCCAGCCGAACAATTTCTTG	AGTACTCCGACACCACGTCC	114	(GA)3
HVM40	PCR1	CGATTCCCCTTTTCCCAC	ATTCTCCGCCGTCCACTC	160	(GA)6(GT)4(GA)7
HVM43	PCR1	GGATTTTCTCAAGAACACTT	GCGTGAGTGCATAACATT	239	(CA)9
HVM44	PCR1	AATCTCAGGTTCGTGGGCA	CCACGGAGACCACCTCACTT	114	(GA)8
HVM49	PCR1	CTCTATAGGCACGAAAATTCC	TTGCACATATCTCTCTGTCACA	105	(CA)12
HVM50	PCR3	TGAAGAAGCCCTCCGTATTC	TGCTCAAGGCTCTAGGCTGA	213	(GA)9
HVM51	PCR1	TCTAAATTACCTTCCCAGCCA	AAGCAGACATGTAGGAGGTCA	151	(GA)3(GGGA)3(GA)8
HVM54	PCR1	AACCCAGTAACACCGTCCTG	AGTTCCCTGACCCGATGTC	159	(GA)14
HVM60	PCR1	CAATGATGCGGTGAACTTTG	CCTCGGATCTATGGGTCCTT	115	(AG)11(GA)22
HVM62	PCR1	TCGCGACCAGACGAGAAG	AGCTAGCCGACGACGCAC	251	(GA)11
HVM63	PCR1	CGCGCAAGCATGAATACTC	ACTCACAAGTGGCGCGTAC	124	(GA)9
HVM64	PCR1	GATGTGAAGGCTGCCTG	ACACGCCCTATTACCCAGTG	253	(GA)4(GT)7(CT)2(GT)4(GA)8
HVM65	PCR1	AGACATCCAAAAAATGAACCA	TGGTAACTTGTCCCCCAAAG	129	(GA)10
HVM67	PCR1	GTCGGGCTCCATTGCTCT	CCGGTACCCAGTGACGAC	116	(GA)11
HVM68	PCR1	AGGACCGGATGTTCATAACG	CAAATCTTCCAGCGAGGCT	204	(GA)22
HVM70	PCR3	CCGCCGATGACCTTCTC	ACCCACGACCTATGGCAC	154	(CA)8
HVM74	PCR3	AGGAAGTCATTGCGTGAG	TGATCAAGAATGATAACATGG	162	(GA)13
HVM77	PCR1	GAAATTTGGTGTATGATGGTT	CAAATCTTAAATCTCTCTGTTT	199	(CA)7

Primer	PCR program	F sequence	R sequence	Size (bp)	Repeat
HVACL1	PCR3	TTTGGAATTATTCTGTGGGACC	GGGATTCAATCAAGTATTCGGA	150	(AT)7
HVADH1	PCR4	GAATTCTCATGAGGGATGCTTC	CAACTGAAACTCATGGCAT	206	(TGC)7
HVBARE1	PCR4	GGTTTCCGGAATGGTCCGGAAACGAA	TCTTGGGCTTGCCCACAAGCCCACTA	214	(G)16
HVBDG	PCR3	GAGAGAGAAAGAGAATGGCAGG	AAAAAACTGCACCCAATCACTT	145	(CT)6
HVBKASI	PCR4	ATTGGCGTGACCGATATTTATGTTCA	CAAAACTGCAGCTAAGCAGGGGAACA	197	(C)10(A)11
HVCMA	PCR4	GCCTCGGTTTGGACATATAAAG	GTAAAGCAAATGTTGAGCAACG	141	(AT)9
HVCSG	PCR4	CACTTGCCTACCTCGATATAGTTTGC	GTGGATTCCATGCATGCAATATGTGG	196	(CA)4(C)17
HVDHN7	PCR4	TTAGGGCTACGGTTCAGATGTT	ACGTTGTTCTTCGCTGCTG	177	(AAC)5
HVDHN9	PCR4	CATGGACAAGATCAAGGAGAAG	CCCATTATTTATCTGTAGGAACGC	128	(AC)6
HVGINRE	PCR3	GAGTTGCAACAACAACAGGGTA	GTCATGCAGATGATGCAGATCT	136	(TG)6(G)14
HVLEU	PCR4	TTGGAAGTGTACAGCAATGGAG	TGAAAGGCCCCACAAGATAG	166	(AAAT)7
HVPRP1B	PCR2	ATAACTACCAGATCACCTTCTGCC	GGAACGAAGGGAGTATTAAGCA	167	(GT)6(AT)15
HVRCABG	PCR3	ACACCTTCCCAGGACAATCC	CAGAGCACCGAAAAAGTCTGTA	182	(AT)29
HVSIP1A	PCR4	AACAATGTGTACGTCCACGC	ATTCCACACAAGCAACATTCAG	149	(CTT)5
HVWAXYG	PCR4	TCCAATGGCATCTACAGGACGGCCAA	GCAGGTTGAGCTGCGCAAAGTCGTCG	205	(AT)9
WMC1E8	PCR3	TCATTCGTTGCAGATACACCAC	TCAATGCCCTTGTTTCTGACCT	197	(AC)24
WMS6	PCR4	CGTATCACCTCCTAGCTAAACTAG	AGCCTTATCATGACCCTACCTT	205	(GA)40
BMAC18	PCR2	GTCCTTTACGCATGAACCGT	ACATACGCCAGACTCGTGTG	138	(AC)11
BMAC29	PCR4	ATCCAGCGATTCAAACACAAC	CTTCTCACTGACCGACTTATACCA	169	(AC)20
BMAC30	PCR2	CCCAATCGGAGTTACAGATG	GCCTCTCTGAGAARFFATC	155	(AC)22
BMAC31	PCR3	AGAGAAAGAGAAATGTCACCA	ATACATCCATGTGAGGGC	175	(AC)28
BMAC32	PCR2	CCATCAAAGTCCGGCTAG	GTCGGGCCTCATACTGAC	215	(AC)14
BMAC35	PCR1	TCTCATCATTTTTTTGGGTGG	TGTGACCAAATACAAGAGGCC	168	(AT)10(AC)29
BMAC40	PCR3	AGCCCGATCAGATTTACG	TTCTCCCTTTGGTCCTTG	236	(AC)20
BMAC64	PCR4	CTGCAGGTTTCAGGAAGG	AGATGCCCGCAAAGAGTT	153	(AC)21
BMAC67	PCR3	AACGTACGAGCTCTTTTTCTA	ATGCCAACTGCTTGTTTAG	171	(AC)18
BMAC90	PCR4	ACATCAACCCTCCTGCTC	CCGCACATAGTGGTTACATC	221	(AC)20
BMAC93	PCR3	CGTTTGGGACGTATCAAT	GGGAGTCTTGAGCCTACTG	151	(AC)24
BMAC134	PCR3	CCAACTGAGTCGATCTCG	CTTCGTTGCTTCTCTACCTT	148	(AC)28
BMAC162	PCR4	CATGTGTTGAAATCAGTTTTG	CCCTCTCTCTCTCTCTCTCTC	187	(AC)15(CT)16
BMAC167	PCR3	CATTTCCACTTCAAAATATCC	CCAAAGTTTGAGTGCAGAC	184	(AC)20
BMAC186	PCR4	GTTGTTACGTGTGTGTCTGC	CGCAACCTTACATCAACAC	129	(AC)7
BMAC187	PCR4	GCTCTCTCTCAGAAAAATGAA	GAATTATTCTAGGGCTGTGAA	177	(AC)13(AT)9
BMAC222	PCR3	ATTTGAATGTCCAACAGAATC	GGGACTAAAGCCCCTTAC	165	(AC)23
BMAC224	PCR3	GCATATATACCACCCTTGGT	ATTCTTGATGGCTATAGCTTG	166	(AC)5/(AC)5
BMAC273	PCR3	ACAAAGCTCGTGGTACGT	AGGGAGTATTTCACCCTTG	186	(AC)20(AG)20
BMAC298	PCR2	ACAACCACACAAGGTTGTC	TTCCAGTTTCAGTCTCTTCAT	190	(AC)11
BMAC299	PCR2	ATAGATGGTAATCATAAGAACAGG	ACTATGACTAGTCGTTTCCACA	181	(AC)15

Primer	PCR program	F sequence	R sequence	Size (bp)	Repeat
BMAC403	PCR2	ATAGCTGGATATGCAACTTGAGC	TACATCCAGTCGTTTTCGCA	201	(AC)18
BMAG3	PCR3	GATCAAAGAGAACATGCGAT	GTAGTTCAGCATAGACCTACAGG	149	(AG)28
BMAG4	PCR3	GTTTCCCATGCGACGTTC	CGGATGACGATTGATAGGTGT	166	(AG)14
BMAG6	PCR3	TTAAACCCCCCCCCTCTAG	TGCAGTTACTATCGCTGATTTAGC	174	(AG)17
BMAG7	PCR3	TGAAGGAAGAATAAACAACCAACA	TCCCCTATTATAGTGACGGTGTG	185	(AG)16(AC)16
BMAG10	PCR2	AGTAGTTCACCCTTGGGGCT	AGCACGTGATACATCAAGAACG	161	(CT)19
BMAG13	PCR3	AAGGGGAATCAAAATGGGAG	TCGAATAGGTCTCCGAAGAAA	155	(CT21)
BMAG14	PCR2	GCAGGGGTTGAACATCTCAT	CACAGGGAAACAGCTATGACC	228	(CT)15
EBMAC501	PCR4	ACTTAAGTGCCATGCAAAG	AGGGACAAAAATGGCTAAG	151	(AC)13
EBMAC525	PCR3	TGACAGTGTCTCCAGTAATGA	GTTTGTTCTTTTGATTTTGTTG	149	(AC)14
EBMAC540	PCR3	TGTGTAGGTGTAGATGGTGTG	TTGTCACACTAACATAGAGAAATG	114	(AC)8
EBMAC541	PCR3	ACGGATCTACTTTAGCTAGCA	AAACAACCCCACACAATC	106	(AC)9

APPENDIX B

AFLP MARKERS GENERATED BY ELEVEN COMBINATIONS OF *MseI AND EcoRI* PRIMERS AND THEIR ASSOCIATED BASE PAIR SIZES

Combination	Marker Name	Size (base pairs)
E-AAC + M-CCA	*E32M51a	1095
	*E32M51b	622
	*E32M51c	544
	*E32M51d	493
	*E32M51e	488
	*E32M51f	431
	*E32M51g	422
	*E32M51h	409
	*E32M51i	381
	*E32M51j	374
	*E32M51k	284
	*E32M51l	275
	*E32M51m	270
	*E32M51n	209
	*E32M51o	173
	*E32M51p	149
	*E32M51q	148
	*E32M51r	147
	*E32M51s	144
	*E32M51t	118
	*E32M51u	266
E-AAC + M-CCG	*E32M53a	505
	*E32M53b	409
	*E32M53c	290
	*E32M53d	233
	*E32M53e	216
	*E32M53f	886
	*E32M53g	127
	*E32M53h	110
	*E32M53i	95
	*E32M53j	76
E-AAC + M-CTA	*E32M59a	942
	*E32M59b	718
	*E32M59c	450
	*E32M59d	413
	*E32M59e	405
	*E32M59f	268
	*E32M59g	225
	*E32M59h	192
	*E32M59i	175
E-AAC + M-CTC	*E32M60a	763
	*E32M60b	482
	*E32M60c	460
	*E32M60d	431
	*E32M60e	396
	*E32M60f	381
	*E32M60g	371
	*E32M60h	332
	*E32M60i	252
	*E32M60j	230
	*E32M60k	227
	*E32M60l	189
	*E32M60m	84
E-AAG + M-CGA	*E33M55a	886
	*E33M55b	763
	*E33M55c	611
	*E33M55d	544
	*E33M55e	371
	*E33M55f	321
	*E33M55g	310
	*E33M55h	306
	*E33M55i	177
	*E33M55j	175
	*E33M55k	96
E-AAG + M-CTC	*E33M60a	886
	*E33M60b	643
	*E33M60c	622
	*E33M60d	436
	*E33M60e	413
	*E33M60f	374
	*E33M60g	317
	*E33M60h	310
	*E33M60i	300
	*E33M60j	230
	*E33M60k	216
	*E33M60l	173
	*E33M60m	157
	*E33M60n	200
	*E33M60o	83
	*E33M60p	80
	*E33M60q	70
E-ACA + M-CTA	*E35M59a	1000
	*E35M59b	942
	*E35M59c	740
	*E35M59d	622
	*E35M59e	544
	*E35M59f	471
	*E35M59g	385
	*E35M59h	378
	*E35M59i	364
	*E35M59j	353
	*E35M59k	338
	*E35M59l	306
	*E35M59m	284

Combination	Marker Name	Size (base pairs)
E-ACA + M-CTA	*E35M59n	257
	*E35M59o	230
	*E35M59p	218
	*E35M59q	208
	*E35M59r	196
	*E35M59s	182
	*E35M59t	153
	*E35M59u	137
	*E35M59v	135
	*E35M59w	126
	*E35M59x	124
	*E35M59y	101
E-ACA + M-CTC	*E35M60a	632
	*E35M60b	562
	*E35M60c	460
	*E35M60d	427
	*E35M60e	418
	*E35M60f	364
	*E35M60g	373
	*E35M60h	198
E-ACA + M-CTG	*E35M61a	482
	*E35M61b	360
	*E35M61c	350
	*E35M61d	332
	*E35M61e	327
	*E35M61f	281
	*E35M61g	205
	*E35M61h	194
	*E35M61i	148
	*E35M61j	137
	*E35M61k	136
	*E35M61l	125
	*E35M61m	120
E-ACC + M-CCA	*E36M51a	763
	*E36M51b	740
	*E36M51c	422
	*E36M51d	413
	*E36M51e	348
	*E36M51f	300
	*E36M51g	262
	*E36M51h	222
	*E36M51i	175
	*E36M51j	166
	*E36M51k	165
	*E36M51l	158
	*E36M51m	123
	*E36M51n	105

Combination	Marker Name	Size (base pairs)
E-ACC + M-CCA	*E36M51o	92
	*E36M51p	264
	*E36M51q	82
E-ACC + M-CTA	*E36M59a	601
	*E36M59b	581
	*E36M59c	544
	*E36M59d	465
	*E36M59e	455
	*E36M59f	385
	*E36M59g	371
	*E36M59h	343
	*E36M59i	308
	*E36M59j	306
	*E36M59k	268
	*E36M59l	266
	*E36M59m	216
	*E36M59n	214
	*E36M59o	171

APPENDIX C

LIST OF MARKERS EXCLUDED FROM THE MAPPING DATA FOR EACH OF THE MAPPING EXPERIMENTS ON THE SHYRI x GALENA POPULATION

Table 1 : List of original AFLP markers excluded for mapping experiment SGaf: new AFLP markers and original RFLP and microsatellite markers.

Chromosome	Marker
1H	E33M42i
	E33M42h
2H	E42M34t
	E42M34u
	T22M34i
	T22M62i
	T22M62k
	E42M47e
	E32M36o
	E33M42e
	E44M36h
	E40M31q
	E38M33f
3H	E32M36h
	E44M55l
	E40M62f
	T22M31f
	E36M50a
4H	E36M50i
	E42M34p
	T22M62f
	E38M50f
	E32M36q
	T22M34g
5H	T22M50d
	T22M35e
	E36M50j
	E38M50l
	T22M31g
	E42M47l
	E42M47d
	E33M42d
6H	E36M33m
	T22M50f
	E42M34o
	E44M36a
	E42M34d
7H	T22M35h
	E38M31m
	E42M34q

Table 2 : List of original microsatellite markers excluded for mapping experiment SGmic : new AFLP markers and original RFLP and AFLP markers.

Chromosome	Marker
1H	BMAC213
	BMAC222c
	HVM20
	BMAC90
	BMAC32
	BMAC303d
	BMAC144b
	HvHVA1
2H	BMAC134
	BMAC222a
	BMAC218a
	EBMAC521
	HVM23
	EBMAC525
	BMAC144f
	BMAC144a
	HVM54
3H	EBMAC539b
	HVM15
	HVM60
4H	BMAC181
	HVM3
	HVM68
5H	HVM30
	BMAC286
	BMAC303f
	EBMAC539a
	HVDHN7
	HVDHN9
6H	BMAC316
	HVM74
	BMAC18
	BMAC47
	BMAC282c
	BMAC218b

Chromosome	Marker
7H	HVCMA
	BMAC144g
	BMAC187
	BMAC167
	BMAC162
	BMAC303a
	BMAC64
	HVPRPb
	HVM49
	HVM5
	HVPRPa

Table 3 : List of original RFLP markers excluded for mapping experiment SGr: new AFLP markers and original microsatellite and AFLP markers.

1H	act8
	MWG837
	BCD098
	ABC160
2H	DAK605
3H	HTPPB
4H	MWG634
	CDO542
	ABG472
	KFP221
	ABG397
	Bmy1
5H	ABC302
	BCD298
	mSrh
	ABC717A
	ABG69
6H	KsuA1H2
	Linka
7H	KFP190
	ABC253
	Tha2
	Tha1

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