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SSR BASED GENETIC AND PHYSICAL MAPPING OF THE SHORT ARM OF RYE CHROMOSOME 1 (1RS)

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Abstract

Apart from the rye (Secale cereale L.) genome, the short arm of rye chromosome 1 (1RS) is present in triticale and in hundreds of wheat varieties world wide as the 1BL.1RS wheat-rye translocation. This chromosome contains several genes inducing resistance to biotic and abiotic stresses. Some authors also suggested on this chromosome arm the presence of genes enhancing yield and being responsible for better adaptation.

Genetic and physical maps are important tools for genome research but also for plant breeding. Simple sequence repeats (SSRs) are the preferred molecular markers for constructing genetic maps, because they are easy-to-use, reliable, and show co-dominant segregation.

So far, only few SSR markers were available for the rye genome in general, and for 1RS in particular. The aim of this study was to construct a new physical and genetic map of 1RS based on 119 newly developed 1RS specific SSR markers.

Using 12 1RS deletion lines 103 of these markers were physically mapped along the chromosome arm. In a mapping population of 192 F2 plants created by crossing three inbred lines of rye 22 of the markers could be genetically mapped.

Zusammenfassung

Roggen (Secale cereale L.) ist die wichtigste Getreideart für kühle Klimabedingungen und schwache Böden. Roggen ist Teil des Gattungsbastards Triticale und dient auch als Quelle für genetische Variabilität in der Weizenzüchtung.

Der kurze Schenkel des Chromosom 1 (1RS) entspricht ungefähr 5,6 % des Roggengenoms und ist durch die 1AL.1RS bzw 1BL.1RS Translokation in zahlreichen Weizensorten weltweit enthalten. 1RS enthält mehrere Resistenzgene gegen Mehltau, diverse Rostkrankheiten und Blattläuse. Weiters wird angenommen, dass das 1RS Chromosom zu einer Ertragssteigerung und einer besseren Umweltanpassung führen kann.

Im Vergleich zu anderen Getreidearten ist das Roggengenom noch relativ wenig erforscht.
Es wurden bereits mehrere genetische Karten mit diversen molekularen Markern entwickelt. Dabei sind allerdings nur wenige Mikrosatellitenmarker zur Anwendung gekommen.

Das Ziel dieser Arbeit war eine auf hauptsächlich auf Mikrosatellitenmarkern basierende genetische und physikalische Karte von 1RS zu entwickeln. Für die Kartierung standen 119 TSM (Tulln Secale Microsatellite) Marker, sowie 20 zum Teil bereits publizierte STS Marker zur Verfügung.

Für die physikalische Kartierung wurden zwölf Weizenlinien verwendet, die addiert zum Weizengenom, das 1R Chromosom mit jeweils unterschiedlich langen Deletionen des kurzen Schenkels enthielten. Solche Deletionschromosome erlauben eine gewisse physikalische Position eines Markers (Gens) entlang des Chromosoms zu bestimmen. Der Unterschied zwischen zweier aufeinander folgender Deletionslinien wird als Bin bezeichnet. Je Linie wurden zwei Pflanzen getrennt auf das Vorhandensein des Deletionschromosomes untersucht. In zwei Fällen wurden zwischen den beiden Pflanzen einer Linie unterschiedliche Deletionslängen gefunden, wodurch sich zusätzliche Bins ergaben. In Summe konnten 118 Marker 15 verschiedenen Bins zugeordnet werden. Eine deutliche Häufung der Marker nahe am Centromer war feststellbar.

Für die genetische Kartierung stellte Dr. B Hackauf (Groß Lüsewitz) drei Roggeninzuchtlinien, Sp3, Sp4 und Hy2, und zwei daraus resultierende F2-Populationen zur Verfügung. Für jede Population wurde aufgrund der Rekombinationshäufigkeiten eine Karte errechnet: Sp3 x Hy2 enthält 18 Marker und Sp4 x Hy2 besteht aus 15 Markern. Fünf Marker konnten in beiden Populationen kartiert werden und können zur Verbindung beider Karten genutzt werden. Die Verteilung der genetisch kartierten Marker mit deren Verteilung in den Bins entlang des Chromosoms stimmte recht gut überein. Alle genetisch kartierten Marker liegen im äußersten Drittel des Chromosomenschenkels. Die Positionen mehrerer bereits kartierter Marker stimmten mit ihrer Position in den neu entwickelten Karten überein.

Das Ergebnis dieser Arbeit ist die Präsentation der derzeit detailliertesten, auf SSR Markern basierenden Karten, des 1RS Chromosomenschenkels.

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1. Introduction

1.1. Rye (*Secale cereale* L.)

1.1.1. Origin and cultivation

The genus *Secale* belongs along with *Triticum* (wheat) and *Hordeum* (barley) to the tribe Triticeae. *Secale* is mostly composed of diploid species with 2n = 2x = 14 and the genome has been designated R. In some cultivated rye, such as *S. cereale*, tetraploid strains have been created. The progenitors of *S. cereale*, and cultivated rye species in general, are weedy ryes of Central and Southeast Asia which in turn originated from the introgression of *S. montanum* into *S. vavilovii* (Stutz 1972).

The first cultivation of rye started in Middle East Asia and the Black Sea region about 3000 years ago. The distribution of rye species, especially *S. cereale*, stretches from Northwest Europe to North Africa and from Central Asia to the Pacific coast. On the American continent rye can be found in the USA and South Canada as well as in the southern part of South America (Chikmawati et al., 2006).

Today rye is used as grain, hay, pasture, cover crop, green fodder and green manure. More than half of the annual harvest is used for baking dark bread, a staple food in northern Europe. According to the FAO (2007) the main growing regions are Germany, Russian Federation, Poland, Ukraine and the Baltic states. Even though rye is relatively low in yield compared to other cereals, it attracts attention due to its tolerance to biotic and abiotic stress. The distribution of rye reflects its good adaptation to cool temperate climate.

1.1.2. Botany and physiology

The morphology of rye is very similar to wheat, though it is generally taller. The long stem is used as photosynthetic area. Leaves, including stem and spikes, are of bluish colour and have a waxy surface. Spikelets are generally 2-flowered. Rye possesses an extensive deep-growing root system enabling the plant to withstand drought stress easier and resulting in a better soil structure. Therefore, rye can be used as green manure.

The optimal temperature range for germination of the seed is 25-31°C, though it can sink as low as 3-5°C. Rye is traditionally grown as a winter crop being sown as late as October. As a long-day plant, rye needs at least 14 hours of daylight accompanied by 5-10°C to develop flowers. Under short day conditions rye can stay vegetative for several years. This feature is used for either maintaining or multiplying genotypes by cloning under short day conditions.

1.1.3. Genetic aspects

In genetic research, rye plays a dual role. On the one hand rye is as an independent crop plant and on the other hand it acts as a donor species for wheat improvement programs.
The monoploid genome size (1Cx) of rye is estimated to be 7,917 Mbp, which is almost 40% larger than bread wheat and one of the largest genomes in temperate cereals (Doležel et al., 1998). The DNA amount (1C value) of *Secale cereale* L. cv. Imperial is 8,3pg (Bennett and Leitch, 1995). The major reason for such an immense genome size in rye lies in the presence of large amounts of repetitive DNA which are supposed to take up between 84% (Bartoš et al., 2008) and 92% (Flavell et al., 1974). According to Bartoš et al. (2008) approximately 36,000 genes are present in the whole rye genome.

Among the *Triticeae*, rye is the only outbreeding cereal species. Outcrossing, which maintains a high heterozygosity, is enforced through self-incompatibility. In rye self-fertilization is prevented by the complementary action of two genes, *S* and *Z*, which are located on chromosome arms 1RS and 2RL, respectively. The specific recognition response occurs if both alleles of a pollen grain are expressed in the tissue of the stigma (Hackauf and Wehling, 2005).

At present, more than 2050 markers for the rye genome are available. The vast majority (80%) are molecular markers developed in resent time. About 12% are biochemical markers and 8% are mapped morphological features. Most research focuses on chromosome 1R, followed by chromosome 6R, 5R, 2R, 4R, 7R, and 3R, respectively (Schlegel and Korzun, 2008) The special focus on 1R results from the presence of the 1RS translocation in hundreds of wheat varieties worldwide (Rabinovich, 1998). Approximately 9,807 rye sequences covering a total distance of 5Mbp are available. 90% of these are expressed sequence tags (ESTs) (Bartoš et al., 2008).

1.1.4. Wheat-rye introgression

The benefits of rye, such as its disease resistances, adaptation to poor soil condition and cold climate, are desired features in wheat breeding programmes. Various ways have been used to take advantage of these qualities and improve wheat varieties. One important aspect about rye chromatin in wheat is the lack of recombination between homoeologous chromosomes. This is prevented by an effective suppression system which is controlled by a gene called *Ph* on chromosome 5B in wheat. This suppression can be overcome by using *Ph* mutants. In the presence of *Ph* A, B, or D and rye chromatin do not recombine with each other. Therefore, introduced translocations or insertions will act as one completely linked block of genes (Graybosch, 2001).

Triticale is an allopolyploid hybrid of a wheat mother and a rye father. Hexaploid triticale derived from tetraploid wheat is composed of the homoeologous genomes AABBRR. Octoploid triticale (AABBDDRR) originating from hexaploid wheat is less common.

Other possibilities of wheat-rye introgressions are substitutions of either whole chromosomes or translocations of chromosome arms or smaller parts. These introgressions can either occur spontaneously or be induced. Rye chromosome 1 has experienced the 1R (B) substitution as well as translocations of the short and the long arm (Schlegel and Korzun, 1997; Rabinovich 1998). 2RL and 2RS have been introduced into the wheat genome and substituted the corresponding arms of chromosome 2B (2RS.2BL and 2BS.2RL). 2RL carries several disease resistance genes. In experiments 2RL carrying wheat lines showed reduced grain test weight and flour yield though only modest impacts on breadmaking qualities were reported. (Brunell et al 1999; Graybosch, 2001) Spontaneous translocations of 4R and 7R segments in wheat background have been reported (Zeller and Koller, 1981). Improvement in agronomic performance is expected of the 5RS.5DL translocation (Vahl et al., 2001). Rabinovich (1998) mentions a wheat line carrying a 6BS.6RL translocation to be resistant to powdery mildew (*Pm20*). Graybosch (2001) summarizes that all rye chromosomes carry genes which may be beneficial in wheat breeding programs.

1.2. Short arm of rye chromosome 1 (1RS)

1RS represents approximately 5.6% of the rye genome with a genome size of 441 Mbp (Bartoš et al., 2008). The distal part of the arm has a constriction, which is a nucleolus organizer region (NOR) and carries a satellite. However, in a wheat background the nucleolar

activity of the 1RS NOR is generally suppressed (Friebe et al., 1989). Cytological studies have shown the presence of large heterochromatic regions at the telomere of the chromosome (Baum and Appels 1991). Large terminal and subterminal C-bands are characteristic for 1RS (Friebe et al., 1989).

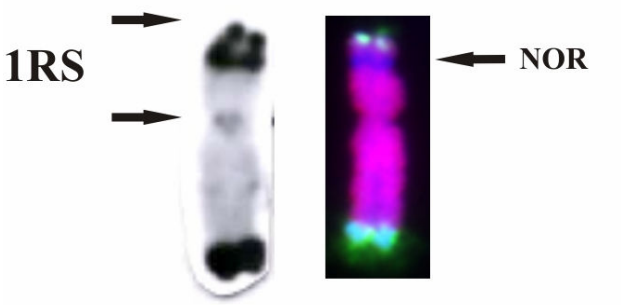

Fig. 1 1R chromosome; arrows indicate the short arm and the NOR. (Friebe et al., 2000; Tsuchida et al., 2008)

Through BAC end sequencing, Bartoš et al. (2008) presented the most detailed information about 1RS so far. It is estimated that 2000 genes are localized on 1RS. The major part of 1RS (at least 84%) consists of repetitive elements.

The dominating form of repetitive DNA in 1RS are retrotransposons (Class I elements), which propagated by an RNA intermediate (64%). They can be further distinguished into long terminal repeat (LTR) retrotransposons (62.8%) and non-LTR retrotransposons (1.5%). Class II elements, or DNA transposons occur less frequently (5%). Simple repeats take 0.2% and tandem repeats 0.3% of 1RS. The NOR explains the presence of almost 4.7% of sequences that are highly similar to ribosomal RNA genes (Bartoš et al., 2008).

1.2.1. 1RS translocation

The most frequent way to introduce rye chromatin into wheat varieties was the 1RS translocation (Baum and Apples, 1991). Hexaploid bread wheat consists of three homoeologous genomes, designated A, B and D and all forms of wheat-rye chromosomal translocations, 1AL.1RS, 1BL.1RS and 1DL.1RS, have been created.

1.2.2. Origins of 1RS translocations

Graybosch (2001) lists two 1AL.1RS translocations; both times 1RS was incorporated from the rye cultivar "Insave". The "Amigo translocation", in the wheat germplasm line "Amigo", carries resistance genes for powdery mildew (*Erysiphe graminis* DC, *Pm17*) and green bug (*Schizaphis graminum* Rond., *Gb2*) (Rabinovich, 1998). The second translocation also shows green bug resistance but its allele differs from that on 1AL.1RS "Amigo"(Rabinovich, 1998). This genetic variation shows that "Insave" rye was polymorphic for genes located on 1RS, and both translocations occurred independently of each other. The 1AL.1RS translocation is found in U.S. wheat cultivars only (Graybosch, 2001).

1BL.1RS translocations have the most impact on wheat breeding programs. 1R (1B) substitution and 1BL.1RS translocations are present in several hundred wheat cultivars worldwide (Rabinovich, 1998). Schlegel and Korzun (1997) reduced the origin of the alien chromosome to four sources – two in Germany, one in the USA, and one in Japan. Only the German translocation lines have a major impact in wheat improvement programs. Both were derived from 1R (1B) substitution lines – "Zobra" produced in Weihenstephan and "Salzmünder Bartweizen" developed in Salzmünde. "Zobra" got its rye chromatin via a

triticale line and was released in the early 1960s (Rabinovich, 1998). "Salzmünder Bartweizen" originates from the rye variety "Petkus" and was the first commercial cultivar carrying a rye translocation to be released in Germany in 1957 (Rabinovich, 1998).
Schlegel and Korzun (1997) gave evidence though, that the two German 1BL.1RS translocations might have a common progenitor which could have been the starting point for all 1BL.1RS translocations in the world. Rabinovich (1998) also acknowledges that both lines are nearly identical and presumes that "Salzmünder" could have been the initial translocation line that was eventually distributed all over Germany. In the early 1970s the Russian cultivars "Aurora" and "Kavkaz" were developed by the famous Russian wheat breeder Lukyanenko (Krassnodar, UdSSR) using the East German variety "Neuzucht", which was most probably a sister line of "Salzmünder Bartweizen". "Aurora" and "Kavkaz" were soon incorporated into the wheat breeding program of CIMMYT and became donor of the 1BL.1RS translocation of CIMMYT wheat varieties distributed worldwide.
1BL.1RS and 1AL.1RS translocations are the most successful wheat-alien translocations (Friebe et al., 1996).

In Australia a 1DL.1RS translocation was derived from "Imperial" rye, resulting in the presence of a resistance gene for stem rust (Graybosch, 2001).

1.2.3. Impact of 1RS translocations in wheat breeding programs

1RS introgressions in wheat may result in higher yields, more stress tolerance, better environmental acclimatisation and certain resistance genes along with reduced in end-use quality. Quality defects are linked to alterations in the protein composition, which are traced back to two major sources. First, the removal of the short arm of chromosome 1 in the wheat genome, either 1A, 1B or 1D, leads to the loss of loci encoding gliadins and low molecular weight glutenins, which have a direct influence on end-use quality. The second source is the presence of 1RS which results in the production of rye secalins (Kumlay et al., 2003). The locus *Sec-1* encoding for the tightly linked seed storage proteins ω-secalins and γ-secalins is part of 1RS (Baum and Apples, 1991).

Agronomic performance
Surveys on the agronomic performance as well as the end-use quality of 1AL.1RS genotypes compared to 1A genotypes showed little beneficial results for 1AL.1RS lines. Lines carrying the translocation showed slight yield improvements in non favourable environments. In end-use performance 1A lines produced higher flour yields whereas 1AL.1RS lines had higher flour protein content. Several mixing tests acknowledged that 1AL.1RS lines showed an exceptional stability in all environments. (Espitia-Rangel et al., 1999a, 1999b; Graybosch et al., 1999)

The agronomic performance of 1BL.1RS translocations is very conflicting and inconsistent in different genetic backgrounds. In a broad study about the genotype by environment interaction of 1BL.1RS no general positive or severely negative effects of the translocation were detected. Increased yield, test weight or better quality traits were found in some 1BL.1RS lines only (Lelley et al., 2004). The comparison of three draught tolerance studies is an example of contradicting conclusions. An over all improved agronomic performance of the 1BL.1RS in contrast to the 1B lines in both optimum and reduced moisture conditions, was clearly shown in the result of Villareal et al. (1998). In Hoffmann's study (2008) 1BL.1RS carrying lines developed a larger root biomass, which led to a higher harvest index and water use efficiency and resulted in less yield decrease in drought conditions compared to non-translocation lines. In contrast, Singh et al. (1998) experienced 1BL.1RS translocation lines

that were significantly lower yielding in both moisture and non-moisture conditions than genotypes without the translocations.

Kumlay et al. (2003) did various trials and research on the three possible translocation lines. In terms of agronomic performance the translocations can be ranked in the descending order - 1BL.1RS > 1AL.1RS > 1DL.1RS. The negative contribution to end-use quality is the lowest in 1AL.1RS, middle range in 1BL.1RS, and highest in 1DL.1RS.

Disease resistance
Depending on the origin of the translocation different resistance genes are present on 1RS. Race specific resistances to leaf rust (*Puccinia recondite f. sp. tritici*, *Lr26*), stem rust (*Puccinia graminis f. sp. tritici, Sr31*) and stripe or yellow rust (*Puccinia striiformis f. sp. tritici, Yr9*) were reported in progenies of "Petkus" rye (Singh et al., 1990; Mago et al., 2002) whereas the stem rust resistance gene *SrR* is of "Imperial" rye origin (Mago et al., 2002). Roux et al. (2004) mapped another leaf rust resistance gene (*Pr3*) to 1RS. Resistances to powdery mildew (*Blumeria graminis f. sp. tritici*, *Pm8*; *Erysiphe graminis* DC, *Pm17*) were confirmed by Rabinovich (1998). The expression of *Pm8* in a 1BL.1RS translocation is controlled by a dominant suppressor gene located on chromosome 1AS of wheat (Ren et al., 1997). The cultivar "Amigo", derived from "Insave" rye, carries the resistance gene *Gb2* against green bug (*Schizaphis graminum*) (Friebe et al., 1996). A further resistance gene (*Dn7*) for the Russian wheat aphid (*Diuraphis noxia*) is present on 1RS (Lapitan et al., 2007).

However, to-date many of these resistances are already overcome by the pathogens. In 2001 Graybosch acknowledged that stem rust and powdery mildew resistance genes on 1RS are still effective.

The impact of 1RS as shown above is very dependent on the source of the rye chromatin. The importance of the genetic wheat background is emphasised in several studies (Baum and Appels, 1991; Ren et al., 1997; Graybosch, 2001; Kumlay et al., 2003, Lelley et al. 2004).

1.3. Molecular markers

According to the FAO Biotechnology Forum molecular markers are defined as "identifiable DNA sequences, found at specific locations of the genome" (FAO 2000). They are classified according to various parameters. Most commonly, markers are distinguished as morphological, biochemical (protein) and DNA marker. DNA markers are distinguished according to method of detection; detected by hybridisation (RFLPs) or by PCR (e.g. RFLP, AFLP, SSR).

Molecular markers are applied in genetic and physical mapping. Markers support the assembling of contigs and the studies of quantitative trait loci (QTL). From the phylogenetic level they explore associations between populations of related species, paternity connections and go as far as the genetic identification of individuals (DNA fingerprinting). They are further applied in evolution studies of specific DNA sequences, e.g. transposable elements (TE). Depending on the application, a range of various marker types and techniques are available.

In the present study simple sequence repeat (SSR) and insertion site-based polymorphism (ISBP) markers are the dominating marker types. Their main features are explained in the following paragraphs.

1.3.1. Simple sequence repeat (SSR)

Simple sequence repeats markers also referred to as microsatellites are genomic sequences that are composed of short tandemly repeated motifs, e.g. $(AG)_n$. On average these motifs are 1-6 nucleotides long. They are found to have ubiquitous but probably not random distributions in both protein-coding and non-coding regions. These repetitive sequences are flanked by unique, highly conservative DNA stretches, which are used to design primers. Therefore, it is possible to amplify a single sequence locus. The use of SSR sequences to identify individuals and their application in genetic mapping and linkage studies, respectively, was first introduced by Diethard Tautz (1989).

Fig. 2 gives an overview on possible microsatellite structure according to Chambers and MacAvoy (2000) and Buschiazzo and Gemmell (2006).

Classification of microsatellites

(according to Chambers and MacAvoy (2000) and Buschiazzo and Gemmell (2006))

Class	**Number of repeat motifs**	**Sequence***
Pure	1	$(AC)_{14}$
Interrupted pure	1	$(CA)_8$-CT-$(CA)_3$
Compound	2	$(CA)_9$-$(GAA)_5$
Interrupted compound	2	$(CA)_9$-CAA-$(GAA)_4$
Complex	>2	$(CA)_4$-$(T)_7$-CAAT-$(CTT)_3$
Interrupted complex	>2	some complex alleles have interruption(s) within the repeat unit(s)

*Sequences are hypothetical and serve as examples only

Fig. 2 Classification of microsatellites

So far no threshold on either the minimum number of iteration or the minimum number of base pairs has been defined for a repetitive sequence to be called a microsatellite. Kofler et al. (2008) suggest a minimum length of 14bp. Furthermore, no consensus is reached on how much degeneration is still acceptable for imperfect repeats to be referred to as microsatellite (Ellegren, 2004).

Evolution

Buschiazzo and Gemmell (2006) gave a review on the life cycle concept of microsatellite evolution. This hypotheses suggests that SSRs are born, mature and grow until a "midlife crisis" is experienced, which leads to shortening, death and eventually, revival of the SSR.
Several theories on microsatellite birth exist. One hypothesis suggests that microsatellites arise spontaneously from unique sequences within the genome. These "de novo" microsatellites evolve from proto-microsatellites, which are sequences of three to four scrambled repeat units still lacking a definite tandem repeat arrangement. Proto-microsatellites are likely to emerge through base substitution. An alternative model ("adopted" microsatellites) suggests that some TEs posses sequence regions which are predisposed to form microsatellites (Buschiazzo and Gemmell, 2006). However, the fact that in plants microsatellite frequency is negatively related to genome size but stays relatively stable in transcribed regions leads to the assumption that most SSRs already existed before the large expansion of many plant genomes (Morgante et al., 2002).

In eukaryotes mutation rate of microsatellites is on average 10^{-7} to 10^{-2} per locus per generation. The most mutations length variations evolve from replication slippage (Tautz, 1989). During replication slippage the nascent DNA strands get dissociated and realign out-of-register. If a loop is formed on the template strand the resulting SSR is shortened whereas a loop on the nascent strand leads to an elongation. Most of these primary mutations are corrected by the mismatch repair system and only a small amount survives until the next replication cycle resulting in length variation. Any malfunction of the mismatch repair system will lead to a much higher mutation rate (Ellengren, 2004, Li et al. 2002, Schlötterer 2000). Generally these slippage events are single step events, the gain or loss of one repeat unit. However, multi-step events, the addition or removal of more repeat units, have also been recorded. Additionally, base substitutions and short insertions and deletions shape the repeat array and increase polymorphism of SSRs (Buschiazzo and Gemmell 2006).

The structure of the microsatellite itself also determines the mutation rate. Long perfect repeats are more likely to mutate than short ones; slippage events seem to occur more often in long iterated sequences. Therefore, interrupted microsatellites are more stable. Microsatellite length, motif length as well as nucleotide composition affect the mutation rate. The internal architecture, e.g if a SSR is pure, compound, complex or interrupted, is another bias in the mutation rate. Furthermore, mutation rate also depends on the genomic context. The positions of microsatellites in the genome, e.g. coding or non-coding regions and the flanking sequences, all may have an impact.
Environmental factors such as climate and radiation, as well as the biological condition of an individual, e.g age and sex, can influence mutation rates (Li. et el., 2002; Buschiazzo and Gemmell, 2006).

After growing in repeat length for some time microsatellites seem to reach a size limit. Although a threshold is difficult to define SSRs rarely exceed 50 repeats. Expansion by slippage is disturbed by introduction of interruptions in the perfect pattern . While expansions and contractions keep SSRs at an average length, interruptions may sometimes occur, leading to breaks in the repeat assay. The result of several such events leads to short DNA sequences in which the remnants of the original repeat pattern can be recognized. These segments can now resurrect and become new microsatellites; thus the life cycle starts again. (Buschiazzo and Gemmell, 2006)

Distribution
Microsatellites are abundant in noncoding DNA regions but occur relatively rare in protein-coding sequences. Changes in mono-, di-, and tetranucleotid SSRs lead to a frameshift. As a result, negative selection against such frameshift mutations in coding sequences explains differences in SSR distribution. The observation, that trinucleotid SSRs appear more frequently in coding regions, strengthens this argument (Li et al., 2002 and Morgante et al., 2002).

Functions
Even though SSRs were considered to be neutral DNA, a review by Li et al. (2002) showed that tandem repeats may have significant influence. SSR sequences can form special loop structures, such as hairpins, which might have an affect on gene regulation. Furthermore, repeated elements function well as promoters of recombination events. Speculations on their influence on fast genetic adaptation in organisms exist. Additionally, SSRs located in promoter regions may affect transcription and gene activity (Li et al., 2002; Ellegren, 2004).

Design
Development of microsatellites is very costly and laborious. Sequence information is needed to first of all find repetitive regions and furthermore design primers according to the flanking regions of the SSRs. Additionally, annotation and masking of the vast amount of transposable elements (TEs), which are not suitable as microsatellites, turn out to be challenging tasks.

In the past several approaches were used to develop SSRs in rye. Saal and Wricke (1999) constructed genomic libraries with more than a 160 000 clones in total and focused on two dinucleotide repeat motifs ($(CT/GA)_n$ and $(GT/CA)_n$). After screening procedures, where clones containing the desired motifs were identified, sequencing was carried out and primer pairs were designed accordingly. Hackauf and Wehling (2003) demonstrated the use of sequence information deposited in public databases. Expressed-sequence tags (EST) served as source for microsatellite development.
Another approach is to take advantage of the large number of already mapped wheat and barley microsatellites and test their amplification in the rye genome. Khlestkina et al. (2004) published a map of all seven rye chromosomes containing wheat SSRs of the Gatersleben collection. Kofler et al. (2008) constructed DNA libraries enriched for particular microsatellite motifs of which certain clones were sequenced. BAC-end-sequence information and ESTs were also used to design primer pairs. All microsatellites were searched against the Triticeae repeat database (TREP) to remove sequences that were similar to TEs.

Advantages of SSRs
Microsatellites are highly polymorphic markers due to their mutation mechanism and can be employed even in advanced breeding stocks. They are abundant and cover the whole genome.
SSRs are often monolocus and show a Mendelian co-dominant inheritance, which makes them very informative markers. SSRs are easy-to-use markers as they are PCR suitable and require only a low amount of sample DNA (Saal und Wricke, 1999; Powell et al. 1996; Korzun 2003; Ellegren 2004).

Disadvantages of SSRs:
The principal disadvantage of microsatellites is the laborious and costly development, which includes sequence knowledge for primer design. Technical problems such as stutter bands which arise from replication slippage during PCR amplification complicate scoring and data analysis (Tautz, 1989). According to Schlötterer (2004) the complex and rapid mutation pattern of SSRs limit their phylogenetic information content.

Application
Microsatellite markers can be employed in genetic and physical mapping. Furthermore, SSRs are applied in DNA fingerprinting, paternity and relatedness tests, and forensic analysis. Microsatellites are also employed in population genetic studies, such as hitchhiking mapping.
SSRs are also used to linkage-disequilibrium mapping where associations between markers and trait loci are explored. Due to their high mutation rate SSRs found their way to genetic toxicology studies, where the influence of environmental factors on genomic mutations is investigated (Ellegren 2004; Schlötterer 2004).

1.3.2. Insertion site-based polymorphism (ISBP)

Transposable elements (TE) exist in high copy numbers, are ubiquitous and evenly distributed in the genome. ISBP markers consist of two primers; one is designed on a sequence flanking a TE, the other primer lies within the TE. The amplification products result either in size polymorphism or nil alleles (Paux et al., 2006 and 2008).

1.4. Genetic and physical mapping

1.4.1. Genetic mapping

A genetic map of a chromosome describes the positions of genes or markers (loci) relative to each other along the length of the chromosome. The distance between two loci is measured in CentiMorgan (cM), a unit based on the frequency of recombination between these two loci; 1 cM equivals 1 percent recombination frequency. The technique of recombinational mapping was first introduced by A. Sturtevant in 1913. During meiosis each chromosome segregates independently. If two loci segregate together at a higher frequency than expected by random assortment of the chromosomes these loci are considered to be linked; they are located on the same chromosome. Furthermore, before the first meiotic division the homologous chromosome pairs align with each other and crossing-over, the exchange of maternally and paternally DNA sequences, takes place. The critical step during this process is the introduction of breaks in the DNA, which occur randomly along the length of the DNA. Consequently, the farther apart on a chromosome two loci are the more likely a break will happen between them and recombination takes place (Lodish, 2000, p266-278).

Recombination frequencies and correlated CentiMorgan distances are calculated by mapping softwares. Some of the most popular mapping programs are MAPMAKER (Lander et al., 1987) and JoinMap (Ooijen and Voorips, 2001) though various other programs are also available. Depending on the population types, marker types and aims, different programs and individual settings are employed.

The logarithm of odds (LOD) is an important parameter to determine linkage between traits and to build linkage groups accordingly. In general a LOD score of 3 is considered to be evidence for linkage. This implies that the likelihood of observing the same two loci inherited together if the two loci are not linked is less than 1 in 10^3. To estimate map distances from recombination frequencies two mathematical approaches can be applied. The Haldane function assumes that there is no interference, which would increase or reduce the proportion of double crossing-overs. The Kosambi function is based on empirical data and considers the varying probability of double crossing-overs at changing physical distances. Map distances are adjusted accordingly (Helms, 2009; Gisbon and Muse, 2002, p5)

1.4.2. Physical mapping

A physical map gives the actual position of genes or markers in the genome. In the best case that would be the complete nucleotide sequences of all chromosomes. More common are partial physical maps, such as ordered DNA clones like yeast artificial clones (YAC) or bacterial artificial clones (BAC) or maps of restriction sites with known nucleotide distances between the sites (Lodish, 2000, p266-278). Furthermore, deletion or addition lines, where parts of DNA are either missing or added additionally to another genome, are also employed for physical mapping.

In all cases of partial maps, markers or genes are mapped to chromosomal regions of various sizes, that have either a know position in the genome or can be put in order accordingly.

The most accurate way to determine a locus is by comparing a genetic and a corresponding physical map. Ambiguities about the order of closely linked markers of a genetic map can be resolved by physical mapping of the same markers. However, the relationship between genetic distances (cM) and physical distances varies between different regions of the genome within the same organism and between organisms. In most species the centromere regions are less recombinogenic. A rough estimation in cereals suggests that in the distal part of a

chromosome 1cM equals 440-1530kb whereby close to the centromere 1cM is between 172,000 and 234,000kb (Ma et al., 2001).

1.4.3. Mapping progress in rye

Over the past years several genetic and physical maps of rye in general and 1RS, respectively, were published. Simultaneously, with the development of new techniques, maps were composed of various different marker types. In 2001 Korzun et al. summarized available mapping data of the rye genome and published a consensus map composed of 139 RFLPs, 19 isozyme and protein markers, 13 SSRs, ten known functional sequences and two morphological genes. In the same year Ma et al. produced a linkage map in rye containing clones from rye, wheat, barley, oat and rice genomic and cDNA libraries, known-function genes and SSRs. The map contains 184 markers of which 60 have been integrated from previous rye maps. Markers cover all seven chromosomes though there was a noticeable lack of markers in the short arms of chromosomes 1 and 4. With the help of anchor markers, Hackauf and Wehling (2003), succeeded in mapping 41 EST-derived microsatellite markers into seven linkage groups and created a "functional map"; 15 of their mapped rye ESTs showed significant similarities (BlastX) to known genes. Khelstkina et al. (2004) further saturated maps of all seven rye chromosomes by introducing new microsatellite-derived markers obtained from rye EST databases and the Gatersleben collection of wheat microsatellite markers (GWM). The majority of the tested GWM loci in rye were found to be homoeologous to those mapped in *Triticum aestivum* L.

Milczarski et al. (2007) published a new genetic map of rye consisting of 148 loci; 99 RAPDs, 18 SSRs, 14 STSs, 9 SCARs (sequence characterized amplification regions) and 7 ISSRs (inter-simple sequence repeats; Camacho et al 2005). The results were eight linkage groups of which two were assigned to the first chromosome; one is located at the distal part of 1RS, the other covers 1RL. In the course of a comparative mapping study of DNA sequences in rye in relation to the rice genome, Hackauf et al. (2009) constructed another map of the rye genome, where 92 EST-SSRs, 25 genomic SSR markers, 131 AFLP and four STS markers were included. Varshney et al. (2007) derived twelve SNP loci in rye from barley ESTs and integrated them to the map of Khlestkina et al. (2004).

Mapping information focusing on 1RS in more detail was first published by Nagy et al. (2003), who developed a genetic map of 1RS based on six previously mapped STS marker and 30 SSAP markers, which were anchored in a dispersed repeat element in rye.

The above mentioned 1RS specific SSAP markers were also physically localized using three 1RS deletion lines. These deletion lines, which were produced by Friebe et al. (2000), divide the chromosome arm into a terminal, an intercalary and a proximal part (bin). Kofler et al. (2008) employed the same three deletion lines to physically map 129 1RS specific SSR markers into these three bins. Ma et al. (2001) detected a non-linear relationship between genetic linkage maps and physical distances of 1RS. Genetic distances distal to the NOR were expanded while those proximal were reduced.

Segregation distortion in rye

Many of the existing rye mapping populations suffer from significant segregation distortion, i.e. many of the loci fail to show the expected Mendelian ratio of 1:2:1 for co-dominant and 3:1 for dominant alleles. Segregation distortion in the rye genome in general has been described by Korzun et al. (2001) and Ma et al. (2001). Other reports limit this phenomenon to the 7R chromosome only (Hackauf und Wehling, 2003; Hackauf et al., 2009), while Milczarski et al. (2007) could not find any distortion. The reason for segregation distortion is

probably associated with the out-crossing nature of rye and its suffering from inbreeding depression. A competition among gametes as well as a selecting force operating at any stage of development might lead to a bias in the progeny (Ma et al., 2001; Chikmawati et al., 2006).

1.4.4. Application of maps

Maps that contain a high density of all kind of morphological, biochemical or molecular markers are a powerful tool in breeding programs and provide a good framework for scientific research. Genetic maps are further a basis for mapping of quantitative trait loci (QTL). Quantitative traits, such as grain yield, are controlled by many genes. QTLs are, well defined polymorphic DNA sequences closely linked to genes involved in the expression of a quantitative trait. Markers that again are associated with QTLs help to identify the positions of these regions in the genome (McClean, 2009a). Currently, Masojć and Milczarski (2009) are analysing the relationship between QTLs for preharvest sprouting and alphy-amylase activity in rye grain.

Marker assisted selection (MAS) is a breeding method, where markers are used for indirect selection of a trait of interest, such as disease resistance or stress tolerance. This technique is advantageous for traits that are controlled by recessive alleles, are only expressed in a late development state or are generally difficult to measure. An up-to-date MAS example is the work of Lapitan et al. (2007) where PCR-based markers linked to the Russian wheat aphid resistance gene *Dn7* were developed. *Dn7* is a rye gene which is part of the 1BL/1RS translocation in wheat.

The process of map-based cloning or positional cloning starts of with the identification of a DNA marker that is close to a gene of interest. Once such a marker is found this region is saturated with additional markers (fine mapping), and large insert genomic libraries, like BAC clones, are screened. After a clone carrying the gene in question is identified, markers that flank and co-segregate with the gene are selected. The region in-between these markers can be cloned and introduced to new plants by genetic engineering for further analysis (McClean, 2009b).
Mago et al. (2004) identified the *SrR* gene on the 1RS translocation from “Imperial” rye and now are in the process of cloning it using the method described above.

2. Material and Methods

2.1. Plant material

Genetic mapping was carried out on two F_2 populations, each consisting of 96 individuals. These populations were derived from two crosses including three rye inbred lines: Sp3 x Hy2 and Sp4 x Hy2. The parents were selected based on a relationship study (Fig. 3). The common parent (Hy2) made the joining of the two maps possible. DNA of the parents and the F_2 populations was kindly provided by Dr. B. Hackauf (Groß Lüsewitz, Germany).

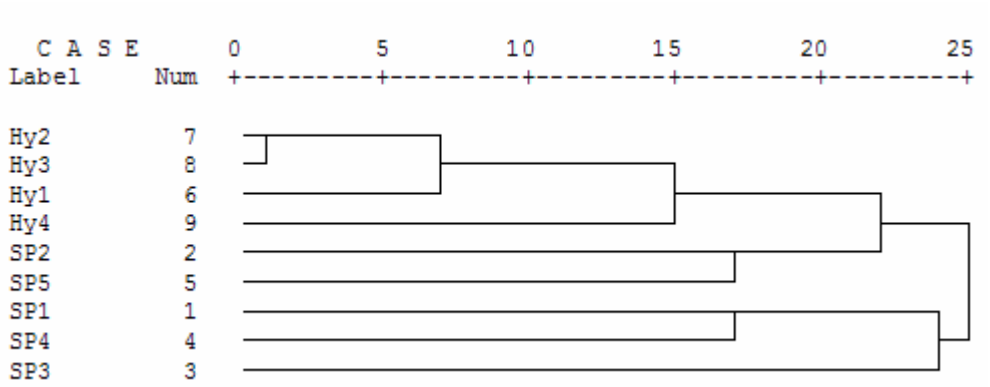

Fig. 3 Dendrogram showing relatedness of parental lines Sp3, Sp4 and Hy2 (Dr. B. Hackauf, Groß Lüsewitz, Germany).

For physical mapping twelve 1RS deletion lines (5, 14, 24, 28, 33, 34, 44, 45, 47, 59, 65, 70) of Tsuchida et al. (2008) were used (Fig. 4). Each line carries the whole wheat genome of the variety "Chinese Spring" (CS) plus a 1R chromosome of the variety "Imperial" with different lengths of the short arm deleted. Genomic DNA of the wheat genotype Chinese Spring (CS) and of the wheat-rye addition line CS+1RS'', were employed as controls. Plants were grown at the institute's green house.

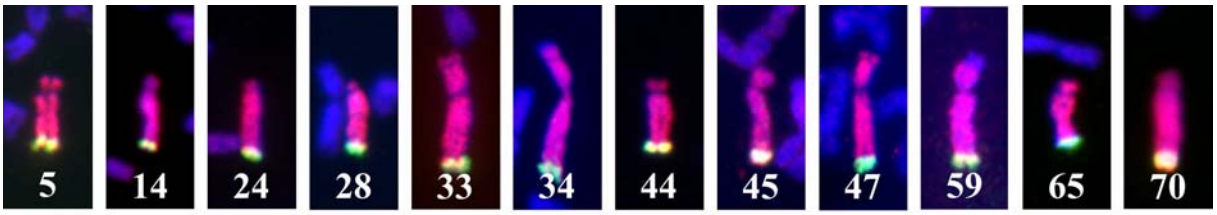

Fig. 4 Twelve 1RS deletion lines of Tsuchida et al. (2008) (1R chromosome only; lines ordered by ascending number)

2.2. Molecular markers

The aim of this project was to map 119 1RS specific markers developed by Kofler et al. (2008). These primer pairs are referred to as Tulln Secale Microsatellite (TSM) markers. Additionally, several already published and mapped DNA markers, which could be used as reference markers, were aimed to be integrated into both maps. These markers include the SSR markers Rems1135, Rems1303 (Khlestkina et al., 2004), Scm1, Scm9, Scm127 (Hackauf & Wehling, 2003) and Bmac213 (Ramsay et al., 2000), as well as the sequence tagged site (STS) marker 5S (Koebner, 1995) and the ISBP markers Ora1 – Ora12 (Bartoš et al., 2008) (Tab. 1). For detecting amplification products the method described by Schülke

(2000) was employed. All forward primers received an M13-sequence at their 5´-end and corresponding fluorescence-labeled (FAM or HEX) M13-primers were added to the PCR.

marker name	marker type	reference
all TSM	SSR	Kofler et al. (2008)
Ora1 – Ora12	ISBP	Bartoš et al. (2008)
Rems1135, Rems1303	SSR	Khlestkina et al. (2004)
Scm1, Scm9, Scm127	SSR	Hackauf and Wehling (2003)
Bmac213	SSR	Ramsay et al. (2000)
5S	STS	Koebner (1995)

Tab. 1 Applied markers and their references

2.3. Chemicals and equipment

2.3.1. DNA extraction

Plant material was grinded in 1,5ml, glass beads containing microcentrifuge tubes in a shaking device (Skandex). DNA was extracted with the Wizard® Genomic DNA Purification Kit (Promega) providing Nuclei Lysis Solution and Protein Precipitation Solution. RNase, isopropanol, 70% ethanol and TE (tris/EDTA) buffer were further ingredients.

2.3.2. PCR

The standard PCR components were primer pairs, Taq-polymerase (BioTherm^{TM}), template DNA, desoxy-nucleotide-triphosphates (dNTPs), fluorescence-labeled M13 oligo primers (FAM, HEX), buffer/$MgCl_2$ (BioTherm^{TM}) and aqua dest. If the standard PCR did not give satisfactory results and a repetition was required, hot-start PCR (HotStarTaq® by Qiagen) was applied. For physical mapping GoTaq® Green Master Mix (Promega), already containing Taq-polymerase, dNTPs, $MgCl_2$ and reaction buffer, was employed. Aqua dest. as well as all pipette tips were autoclaved before use.
Testing for polymorphism between the parents was performed in 96-well microtitre plates in a thermocycler by MWG2. General marker amplification was done in 386-well plates in a thermocycler by Biometra® (TProfessional Thermocylcer).

2.3.3. Electrophoresis

The main components of the polyacryalmid gel were TBE (Tris/Borate/EDTA) buffer, acrylamid, ammonium persulfat (APS), and tetramethylethylenamid (TEMED). Stock solutions containing acrylamid and TBE were prepared and kept in a fridge. APS and TEMED were added before use.

The loading buffer for standard and hot-start PCR products consisted of 0,25% bromphenol and 30% glycerol. PCR products performed by GoTaq® Green did not require any further loading buffer. Electrophoresis was done in a device from C.B.S Scientific Co (Model: MGV-216-33) at 400V for two hours. Electrophoreses buffer consisted of 1xTBE.

2.3.4. Detection amplification products

The polyacrylamid gels were scanned with the Typhoon Trio (GE Healthcare) variable mode imager. The photomultiplier tubes (PMT) were powered with 600-700V for scanning FAM-labeled and 680-780V for HEX-labeled amplification products.

2.4. DNA extraction

DNA extraction was only required for the 1RS deletion lines. Of each plant 8cm of fresh leave tissue was harvested, put into 1,5ml tubes and dried on silica gel for two days. The dried leaves were grinded into fine powder using glass beads and a shaking device. To dissolve cell walls and membranes Nuclei Lysis Solution was added, the tubes vortexed and incubated at 65°C (water bath) for 15 minutes. Treatment with RNase and incubation at 37°C (incubator) degraded present RNA. Next, each sample was mixed with Protein Precipitation Solution and centrifuged at high speed resulting in a protein containing pellet and a clear DNA containing supernatant. The supernatant was transferred to a new tube to which isopropanol was added. Gentle mixing allowed thread-like strands of DNA to form a visible mass. After centrifuging the precipitated DNA formed a pellet and the liquid phase was decanted. In a finale step the DNA pellet was washed in 70% ethanol and air-dried overnight. On the next day DNA was dissolved in TE buffer and stored at 4°C for further use.
The detailed DNA extraction protocol is listed in the appendix.

2.5. Template DNA

For PCR 30ng of template DNA per reaction were required. For genetic mapping all 96 individual DNA probes of one population fitted four times on a 386 well plate. Therefore, four primers that required similar PCR conditions were amplified in one PCR run.
For physical mapping the first step was to test all available plants per line with a marker known to be located close to the centromere. In this process the isolated DNA was checked for its quality and the presence of the 1RS chromosome arm was verified. Only two plants per line which showed a clear amplification product were chosen for further marker testing and mapping. To ensure that all markers are 1RS specific but do not occur in wheat each primer pair was also tested on the wheat cultivar Chinese Spring and a Chinese Spring 1RS addition line (CS+1RS'').

2.6. PCR preparation

The primers were provided in either 100µM or 50µM stock solutions. In genetic mapping an already available primer mix for each primer pair was used. The primer mix consisted of a 5:1 reverse to forward primer ratio. For physical mapping fresh primer dilutions were necessary. The proportion of reverse to forward primer was changed to 10:1.
Because of the fluorescence labelling method described by Schülke (2000) a smaller amount of forward than reverse primer was needed. In the first cycles of the PCR the primer pair amplified the template DNA sequence and incorporated the M13 tail, which was attached to the forward primer. Once the amount of forward primer was used up, the fluorescence labeled M13-primer took over, and annealed to the already existing M13 carrying PCR products and integrated its fluorescence dye.

2.7. PCR

In this work several protocols to optimize PCR results were applied. Standard PCR, hot-start and GoTaq® Green PCR all differ in utilization and price.
All PCR preparation was done on ice.

2.7.1. Standard PCR

A standard protocol was used to test the parents for polymorphism and as for most of the genetic mapping.

PCR components	per reaction [µL]	concentration of stock solution
aqua dest.	1.12	
Buffer (+$MgCl_2$)	1	10x (15mM $MgCl_2$)
BSA	1	2.5x
labeled M13 primer	0,8	10µM
dNTP	1	200µM
Taq-polymerase	0.08	5 units/µL
Primer mix (rev. and fwd.)	2	1.8µM and 0.36µM
template DNA	3	10ng/µL
total volume	10	

Tab. 2 Standard PCR protocol

To speed up the working process and to reduce the risk of measurement errors a master mix which contained all the reaction components but the Taq-polymerase and the template DNA was produced. First, a master master mix (MMM) with a volume for up to 8 reactions containing aqua dest., buffer, BSA, fluorescence dye (either FAM or HEX), and dNTPs was made. Next, aliquots of the volume needed for one reaction were pipetted into separate tubes, referred to as master mix (MM), and stored at -20°C for further use. For a new PCR the MM was defrosted and the primer mix added. As Taq-polymerase is sensitive to defrosting and refreezing it had to be added immediately before the PCR reaction.

2.7.2. Hot-start PCR

The standard protocol was adapted for hot-start PCR kit from Qiagen containing buffer, Q-solution and HotStarTaq-polymerase. Hot-start PCR was applied for those markers which gave poor results in the standard PCR and were thought worth repeating.

PCR components	per reaction [µL]	concentration of stock solution
aqua dest.	1.12	
Qiagen Buffer	1	10x (15mM $MgCl_2$)
Q-Solution	1	5x
labeled M13 primer	0,8	10µM
dNTP	1	200µM
HotStarTaq®	0.08	5 units/µL
Primer mix (rev. and fwd.)	2	1.8µM and 0.36µM
template DNA	3	10ng/µL
total volume	10	

Tab. 3 Hot-start PCR protocol

2.7.3. GoTaq® Green PCR

The master mix of GoTaq® Green (Promega) already contains all PCR chemicals. Only the three primers had to be added just before use. Green GoTaq® was applied on all markers for physical mapping.

PCR components	per reaction [µL]	concentration of stock solution
aqua dest.	1.2	
GoTaq® Green MM	5	
labeled M13 primer	0.23	10µM
forward primer	0.025	10µM
reverse primer	0.25	10µM
template DNA	3.3	8ng/µL
total volume	10	

Tab. 4 GoTaq® Green protocol

2.7.4. Thermal cycling profile

Generally a PCR cycle is split into three steps, denaturation, annealing, and extension, and is repeated a specified number of time. During denaturation (95°C) the double-stranded template DNA is separated into single strands. Next the temperature is cooled down to allow primers to attach and anneal to the template strands. The annealing temperature is primer specific. It depends on the number of bases and the C/G content of the primer sequences and varies between 35-65°C. After primer annealing, the extension step follows at the optimum working temperature of Taq-polymerase. Taq-polymerase adds bases onto the 3´-ends of the primers. The result is a newly replicated double-stranded DNA.
To achieve best amplification products, Kofler et al. (2008) had grouped their markers into three annealing temperatures, 50°, 55°, and 60°. In accordance the thermal cyclers were run with three different 'Touchdown' programs, one for each annealing temperature. For hot-start PCR the initial denaturation step was extended to 15 minutes at 95°C.
For detailed thermal cycling profiles (Touchdown) see Fig. 5.

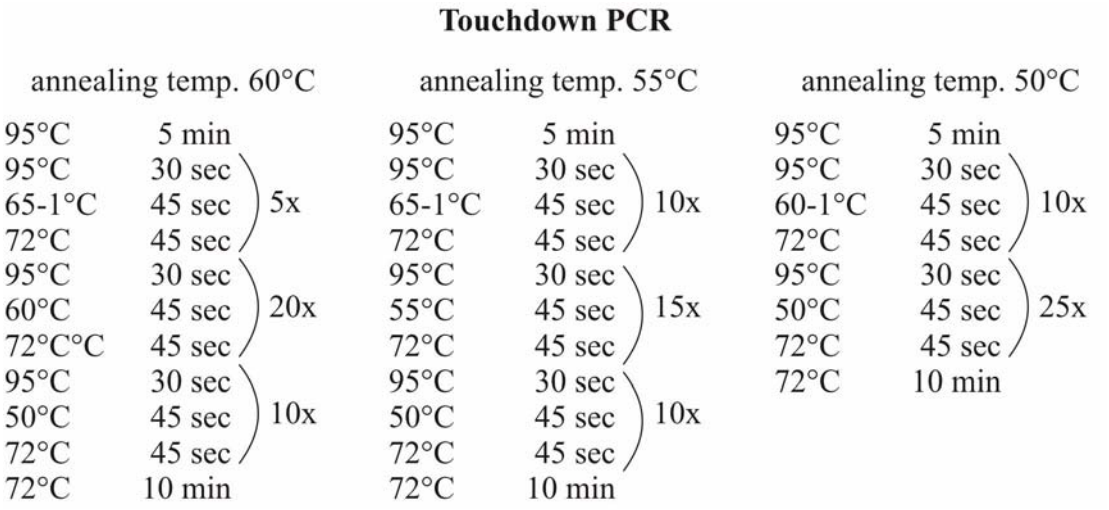

Fig. 5 Thermal cycling profile (Touchdown)

From the three PCR protocols GoTaq® Green (Promega) turns out to be the simplest in use and the most economic and can be recommended for SSR applications.

2.8. Electrophoresis

All tasks regarding preparing and pouring of the polyacrylamid gels were done under a fume hood wearing nitril disposable gloves. The gel was formed within two glass plates with rubber insulation; two spacers regulated the thickness of the gel. First, the resolving gel, which took 30 minutes to polymerise, was poured. Next, a slot forming gel comb was inserted on top of the resolving gel, and the stacking gel was poured. The solidified gel was put into an electrophoresis apparatus, top and bottom submerged in 1xTBE buffer. After the comb was removed the gel was ready for loading. If required, PCR amplification products were mixed with a loading buffer and subsequently pipetted into the gel slots. The first lane of the gel was reserved for a size marker. Electrophoresis ran for two hours at 400V. Work with polyacrylamid gels was speeded up by loading each gel with two markers of different fluorescence labelling. Additionally, gels were reused after letting them run unloaded for two hours to wash out old fragments.

12% polyacrylamid gel for SSR marker			
resolving gel			1x CBS gel
29:1 Acrylamide 30% + A.dest + TBE			40 ml
	29:1 acrylamid 30%	240 ml	
	aqua dest.	472 ml	
	TBE	80 ml	
APS 10 %			421 µl
	APS	1g	
	aqua dest.	10 ml	
TEMED			20,8 µl
stacking gel			1x CBS gel
19:1 Acrylamide 30% + A.dest + TBE			5 ml
	19:1 acrylamid 30%	12 ml	
	aqua dest.	77 ml	
	TBE	80 ml	
APS 10 %			105 µl
	APS	1g	
	aqua dest.	10 ml	
TEMED			5,4 µl

Tab. 5 12% polyacrylamid gel for electrophoreses and detecting of SSR markers

2.9. Scanning

After electrophoresis the gels were scanned with the Typhoon Trio (GE Healthcare) variable mode imager. All data were stored electronically. Each gel was scanned twice; once with FAM and once with HEX settings.

2.10. Scoring of PCR products for genetic mapping

Data analysis and calculation of the genetic map were carried out with the software CarthaGène 1.0 (Givry et al., 2005). The Kosambi function was used to calculate genetic distances (cM). The minimum LOD score was set to 3 and the maximum recombination frequency to 30%. Separate maps for both mapping populations were created (Fig. 13). Electrophoreses results were scored according to the mapping software manual. Dominant as well as co-dominant markers were integrated.

	scoring code	expected percentage [%]	expected segregation
co-dominant markers			1:2:1
homozygous for Sp3/Sp4	A	25	
homozygous for Hy2	B	25	
heterozygous	H	50	
dominant markers			1:3
homozygous for Sp3/Sp4	A	25	
homozygous for Hy2 + heterozygous	C	75	
or			
homozygous for Hy2	B	25	
homozygous for Sp3/Sp4 + heterozygous	D	75	

Tab. 6 Scoring scheme for an F_2-population

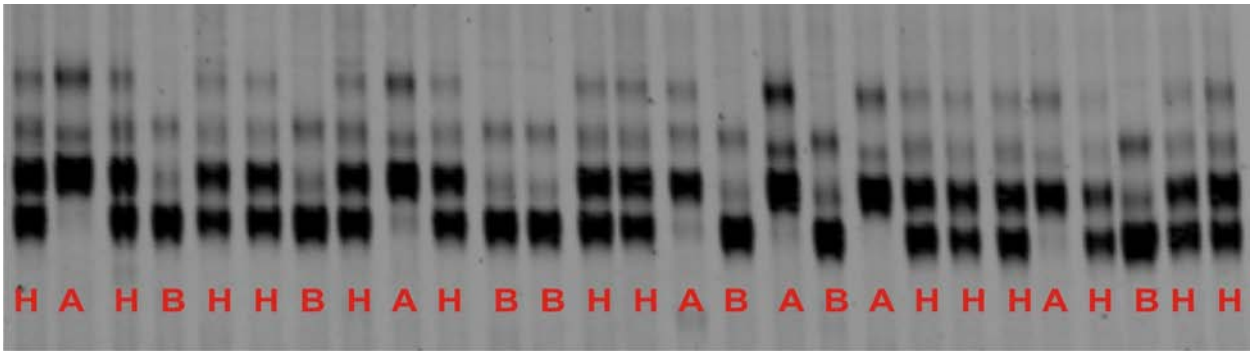

Fig. 6 Example of scoring a co-dominant marker (Tsm81 in population Sp4 x Hy2)

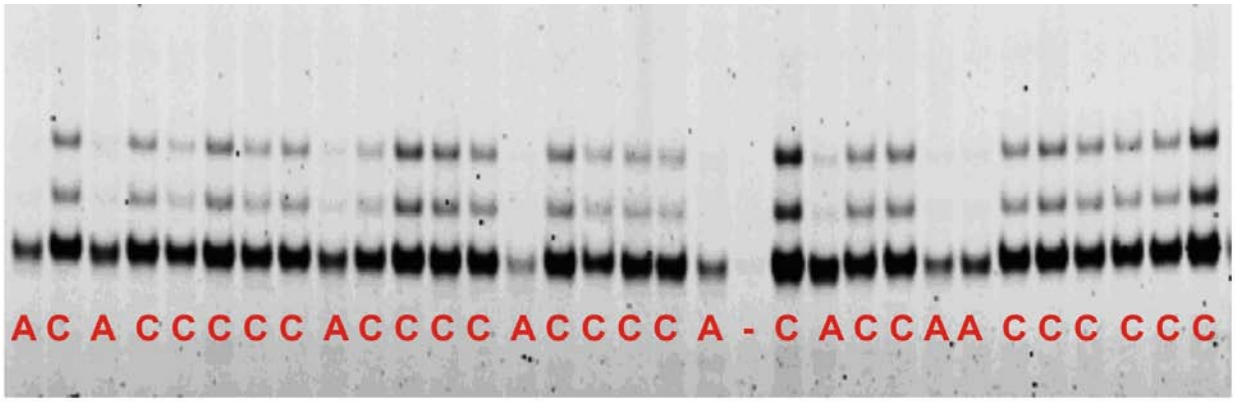

Fig. 7 Example of scoring a dominant marker (Tsm12 in population Sp3 x Hy2)

2.11. Scoring of PCR products for physical mapping

The gametocidal effect of certain *Aegilops* chromosomes was detected by Endo (1988). Their use to create deletion lines was developed by the same author (Endo, 2007). 1R deletion lines were published by Tsuchida et al. (2008).

The interspaces between each deletion line breakpoints create bins. Markers that cause amplification in all lines are located in the bin closest to the centromere whereas markers that show a band in the CS+1RS line only are placed in the most distal bin. Markers were scored with present (1) or absent (0) in all available deletion lines. Fig. 8 shows the scoring principal. After scoring all markers were put in an appropriate order and bin, respectively. The bins were given names from A to O; A being closest to the centromere.

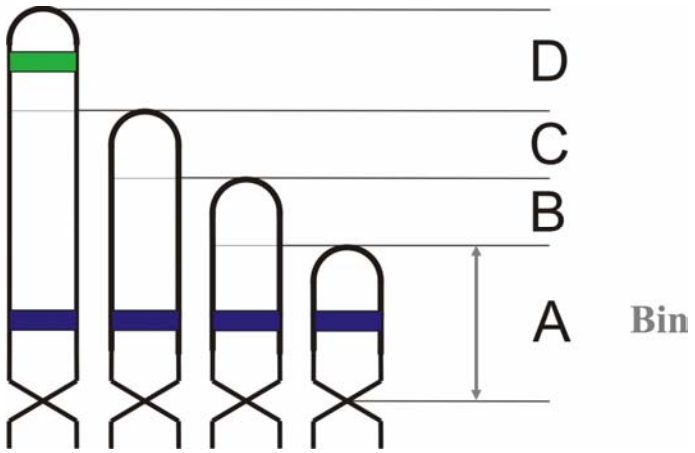

Fig. 8 Illustration of scoring principal by means of four deletion lines and two markers. The deletion lines create bins (A-D). A marker (blue) that amplifies a band in all four line is located in the bin nearest to the centromere. A marker (green) that lies in the most distal bin will only amplify in lines still carrying the region.

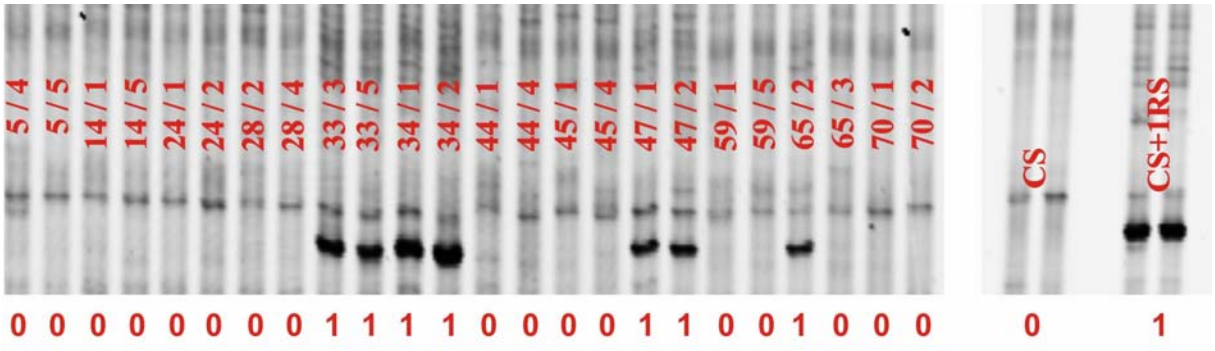

Fig. 9 Physical mapping of marker Tsm520 (12 deletion lines [2 plants/line], CS and CS+1RS)

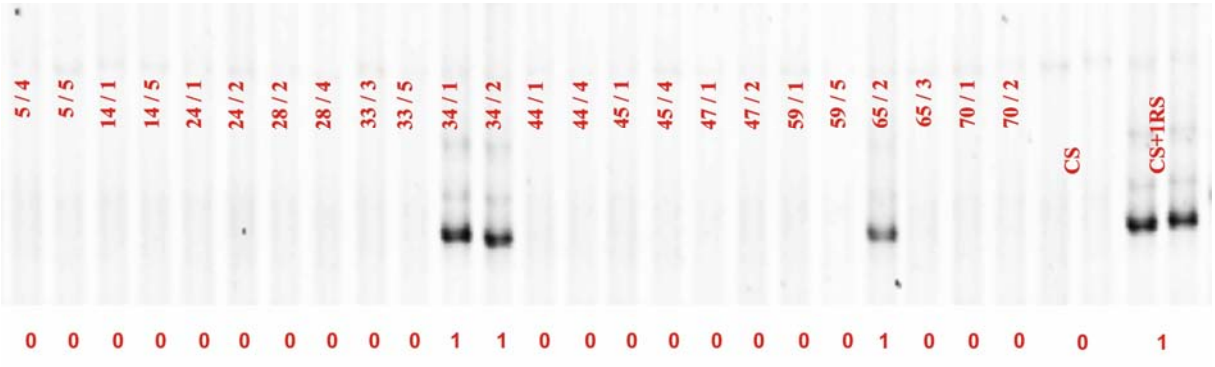

Fig. 10 Physical mapping of marker Tsm468 (12 deletion lines [2 plants/line], CS and CS+1RS)

3. Results

3.1. Genetic mapping

3.1.1. Data analysis

In total 139 markers, 119 TSM and 20 others, were available for genetic mapping. 86 markers were polymorph between the parents Sp3 and Hy2 and were consequently tested on the corresponding F_2-population. Between the parents Sp4 x Hy2 64 markers showed polymorphism and could be further employed.
After PCR and electrophoreses 28 markers could be scored in Sp3 x Hy2. Only eight of the 24 TSM markers showed a co-dominant segregation pattern whereas the rest amplified a nil-allele. In population Sp4 x Hy2 22 markers were legible; nine of the 19 TSM markers acted in a co-dominant way.
In total 53 markers could not be evaluated as either their amplification was so poorly that no banding pattern was visible or their alleles looked so similar that no proper distinction could be made between them.
The information of all scored markers was summed up in a raw data file ready for map calculating.
Fig. 11 gives an overview of the analysed data.

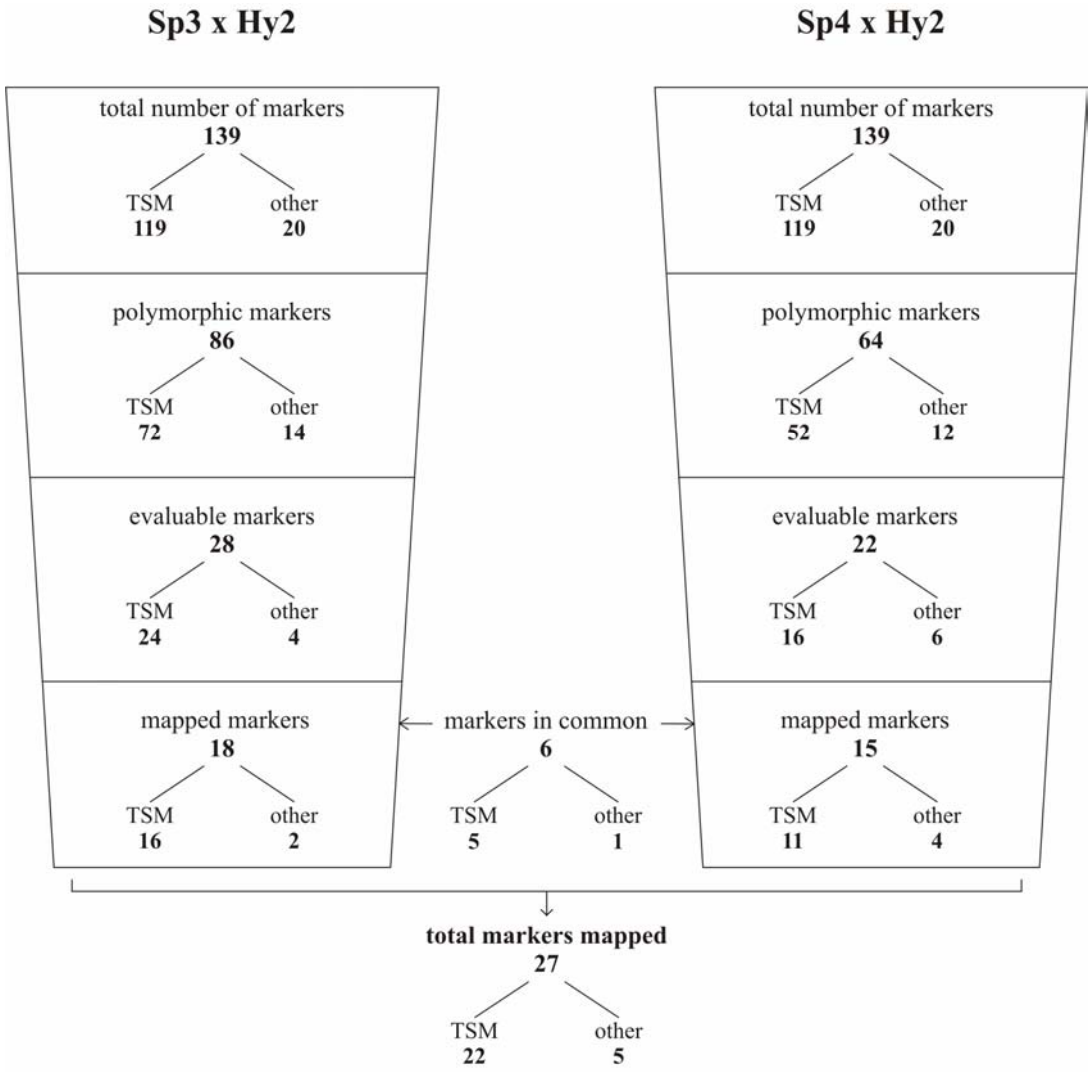

Fig. 11 Marker analysis in genetic mapping

3.1.2. Genetic distance and mapping software

Genetic mapping was carried out with the software CarthaGène 1.0 (Givry et al., 2005). The Kosambi function was used to convert recombination frequency into genetic distances (cM). As recommended by the software manual linkage groups were separated using a minimum LOD of 3 whereas the maximum recombination frequency was set to 30%.
For each population a separate map was calculated. In both populations the software could clearly distinguish one group of markers closely linked together. In population Sp3 x Hy2 28 markers were split in one large group of closely linked markers (nr. 7) and one grouped with two linked markers. All other markers appear unlinked and represent different groups. In population Sp4 x Hy2 one group contains 16 closely linked markers. The remaining six markers appear to be unlinked. Fig. 12 shows the distribution of markers in the two populations.

marker distribution

Sp3 x Hy2

group	markers
1	Xtsm471-1R
2	Xtsm387-1R
3	Xscm9
4	Xtsm12-1R
5	Xtsm61-1R
6	Xtsm221-1R
7	Xtsm16-1R.2, Xtsm81-1R, Xtsm118-1R, Xtsm197-1R, Xtsm213-1R, Xtsm279-1R, Xtsm294-1R, Xtsm315-1R, Xtsm350-1R, Xtsm468-1R, Xtsm472-1R, Xtsm587-1R, Xtsm592-1R.2, Xtsm608-1R, Xtsm634.1R, Xtsm690-1R.1, Xrems1135, Xscm1, 5S
8	Xtsm274-1R, Xtsm192-1R
9	Xtsm492-1R

Sp4 x Hy2

group	markers
1	Xtsm350-1R
2	Xrems1303
3	Xtsm461-1R
4	Xtsm468-1R
5	Xtsm221-1R
6	Xtsm24-1R
7	Xtsm81-1R, Xtsm118-1R, Xtsm275-1R, Xtsm315-1R, Xtsm326-1R, Xtsm332-1R, Xtsm472-1R, Xtsm492-1R, Xtsm552-1R, Xtsm592-1R.2, Xtsm690-1R.1, Bmac0213, Xscm1, Xscm9, Xscm127, Xora2

Fig. 12 Linkage calculated by CarthaGène 1.0 (LOD threshold = 3.00, distance threshold = 30.00)

After choosing the largest group of linked marker and further map improving settings independent maps for the two populations were calculated.

3.1.3. Genetic map

The map of population Sp3 x Hy2 covers 222cM and contains 18 markers in total, 16 TSM and two other markers. The Sp4 x Hy2 map consists of 15 markers, including eleven TSM markers, and extends 176cM. Five markers were mapped in each population and can be used to link both maps together (Fig. 13).
In summery, 27 markers, 22 TSM and 5 already published ones, could be integrated into a genetic map of 1RS.
Fig. 13 shows the new consensus map. The LOD matrix and the 2-points distance matrix are found in the appendix.

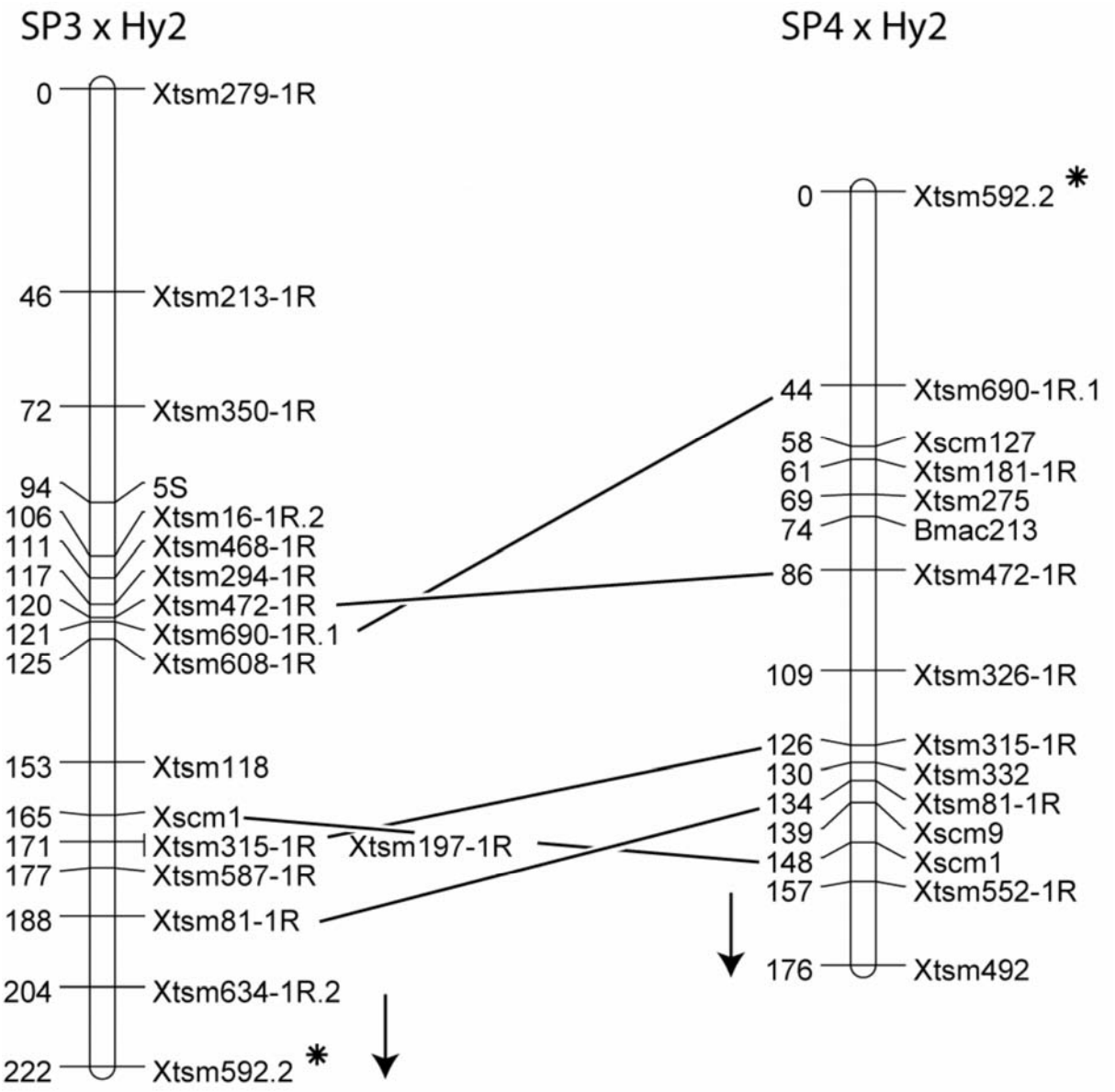

Fig. 13 Genetic maps of 1RS (lines link markers mapped in both populations; arrows indicate direction of centromere)

3.2. Physical mapping

A total of 139 markers, 119 TSM and 20 others, were tested on 12 deletion lines of Tsuchida et al. (2008). 103 TSM markers and 15 others could be scored successfully creating a physical map of 118 markers in total.

Fig. 14 shows the 1RS chromosome arm dissected into 15 regions (bins). Mapping was carried out on 24 plants, two per deletion line. In two cases, deletion lines number 44 and 65, a distinction could be made between the two plants, leading to two additional bins and resulting in 15 bins in total. We found that the breakpoint of deletion line ’44/1’ is closest to the centromere, followed by ’24’, ‘28’, ‘44/4’, ’ 70’, ‘14’, ‘5’, ‘65/3’, ’45’, ‘59’, ‘47’, ‘33’, ‘34’, and finally that the breakpoint of deletion line ‘65/2’ lies most distal.

Letters from A to O, starting at the centromere, were assigned temporarily to the bins.

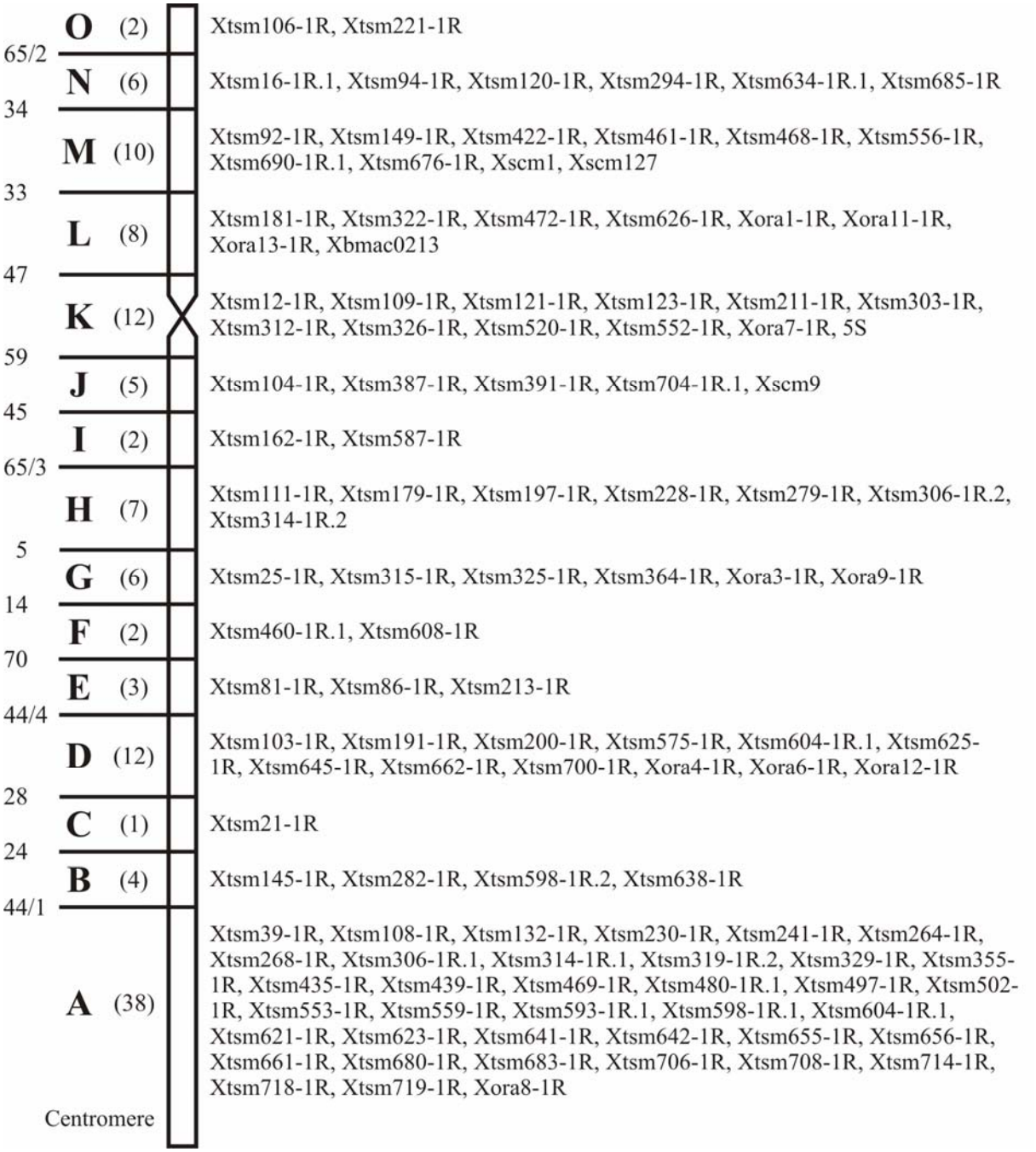

Fig. 14 Physical map of 1RS. The numbers in brackets indicate the amount of markers per bin, the numbers on the very left give the name of the deletion line breakpoint. The constriction in the bar represents the NOR.

3.3. Comparison of genetic and physical map

The results obtained by the physical and the genetic map were compared and combined (Fig. 16). In total 20 markers, 15 TSM and 5 others could be mapped physically and genetically. Markers clustered on the genetic map are generally found in neighbouring bins in the physical map. For single markers that are separated by large genetic distances the physical and genetic mapping results are conflicting.

4. Discussion

4.1. Technical aspects

4.1.1. PCR optimization

In this study three different PCR techniques, standard, hot-start and GoTaq® Green, were applied. Markers which gave no sufficient amplification products in standard conditions were repeated with the more costly hot-start PCR. In most cases significantly better results could be achieved which led to more evaluable markers.
Half way during this study a protocol for GoTaq® Green mastermix, that could keep up with standard PCR quality-wise, was developed. The advantages were faster PCR preparation and a reduced risk of pipetting errors.

4.1.2. Marker amplification

Especially in genetic mapping faint or missing amplification on the one side and nonspecific bands and stutter bands on the other side were the main reasons why markers could not be evaluated. In total 33% of the polymorphic markers in the Sp3 x Hy2 population and 25% of the Sp4 x Hy2 population could be scored.
One reason for weak amplification could lie within DNA quality. DNA of the two populations had been stored in the freezer for more than a year and was defrosted and refrozen several times, which can lead to breaks in the DNA resulting in poor DNA quality and weak, unclear PCR products. This argument is strengthened by the fact that the amount of mapped markers was much higher in physical mapping (87%), where DNA was isolated from young plants at the start of this project.
Furthermore, the annealing temperature has a great influence on the amplification products. If the actual primer annealing temperature is lower than the applied annealing temperature, poor amplification will be the result. If the latter is set too low, though, nonspecific primer annealing will be the output. However, in this work nonspecific primer annealing temperatures seem unlikely. Kofler et al. (2008) defined optimal annealing temperatures for their TSM markers based on calculation and subsequent empirical testing. All TSM primers were assigned to 50°C, 55°C or 60°C and run with ‘touchdown’ cycling programs.
Self-annealing of the primers (building of primerdimers) leads to very small fragments and reduces the amount of primers present in the PCR. Especially during PCR preparation unspecific binding of different components produces artefacts and additional, unwanted bands. To prevent pre-PCR Taq-polymerase activity PCRs were prepared on ice. In extremely sensitive cases hot-start PCR was applied which successfully reduced any background amplification.

4.2. Physical mapping

103 of 119 TSM markers were successfully mapped to the 12 deletion lines of Tsuchida et al. (2008). This high yield of 87% was possible due to good amplification results and the fact that no polymorphism was needed. Even though two plants per deletion line were employed each plant was treated separately. The high number of available markers and the clear amplification products allowed distinguishing even between plants of the same lines in two cases (44 and 65) and increased the number of created bins to 15 in total. The newly mapped order of the deletion lines is similar to the proposed order of Tschuida et al. (2008). Only lines 14 and 70 are found in a reverse order and lines 65/3 and 65/2 are located far more distal.

Lines 24 and 28 could be differentiated in this study. The satellite region is located distal from line 59 (bin K).

The region between the centromere and the deletion line 28 (which includes bins A, B, and C) contains 35% of the TSM markers whereas according to Tsuchida et al. (2008) this part represents less than 5% of the 1RS arm. In contrast bins J and K combined (breakpoints of lines 45 to 47) take up approximately 50% of the 1RS arm and hold only 13% of the mapped markers. These results indicate that microsatellites with the most frequent motifs AG and AAG are clustered close to the centromere whereas the middle part of the 1RS chromosome is poorly saturated with such SSRs.

Kofler et al. (2008) have already mapped the majority of the TSM markers to two deletion lines produced by Friebe et al. (2000). The results of this study are in good accordance with the published data of Kofler et al. (2008) who created three bins, *P*, *I* and *D*. Fig. 15 compares both physical maps. Bins A to C are equivalent to Kofler's bin *P*, bins E to I meet bin *I*, and bins K to O correspond to bin *D.* Bin D and bin J contain markers mapped to *P* and *I,* and *I* and *D*, respectively. The same distribution of microsatellites along the chromosome arm could be confirmed. Bin *P* represents approximately a fifth of the 1RS arm (Friebe et al., 2000) and contains around 38% of the TSM markers.

The physical position of the markers Bmac0213 (bin L), 5S (bin K) and Scm9 (bin J) have been mapped before in the same order by Nagy et al. (2003). In this work marker Scm1 and Scm127 are both found in the distal part (bin M) of the 1RS arm. The position of Scm127 is confirmed by Hackauf and Wehling (2003), whereas Scm1 is supposed to be located closer to the centromere. The ISBP markers Ora1, 3, 4, 6, 7, 8, 9, 11, 12, 13 were successfully mapped for the first time.

Even though markers Tsm266, Tsm366, Tsm536, Tsm596 and Tsm634-1R.2 gave good amplification results they could not be integrated into the physical map. For example, marker Tsm266 amplified a band in every deletion line except 65/3 and one line of 70; these amplification results did not correlate with any bin and so that the marker had to be excluded from the map. Mutations in the primer binding sites and consequently loss of amplification in specific plants might be the reason for this behaviour.

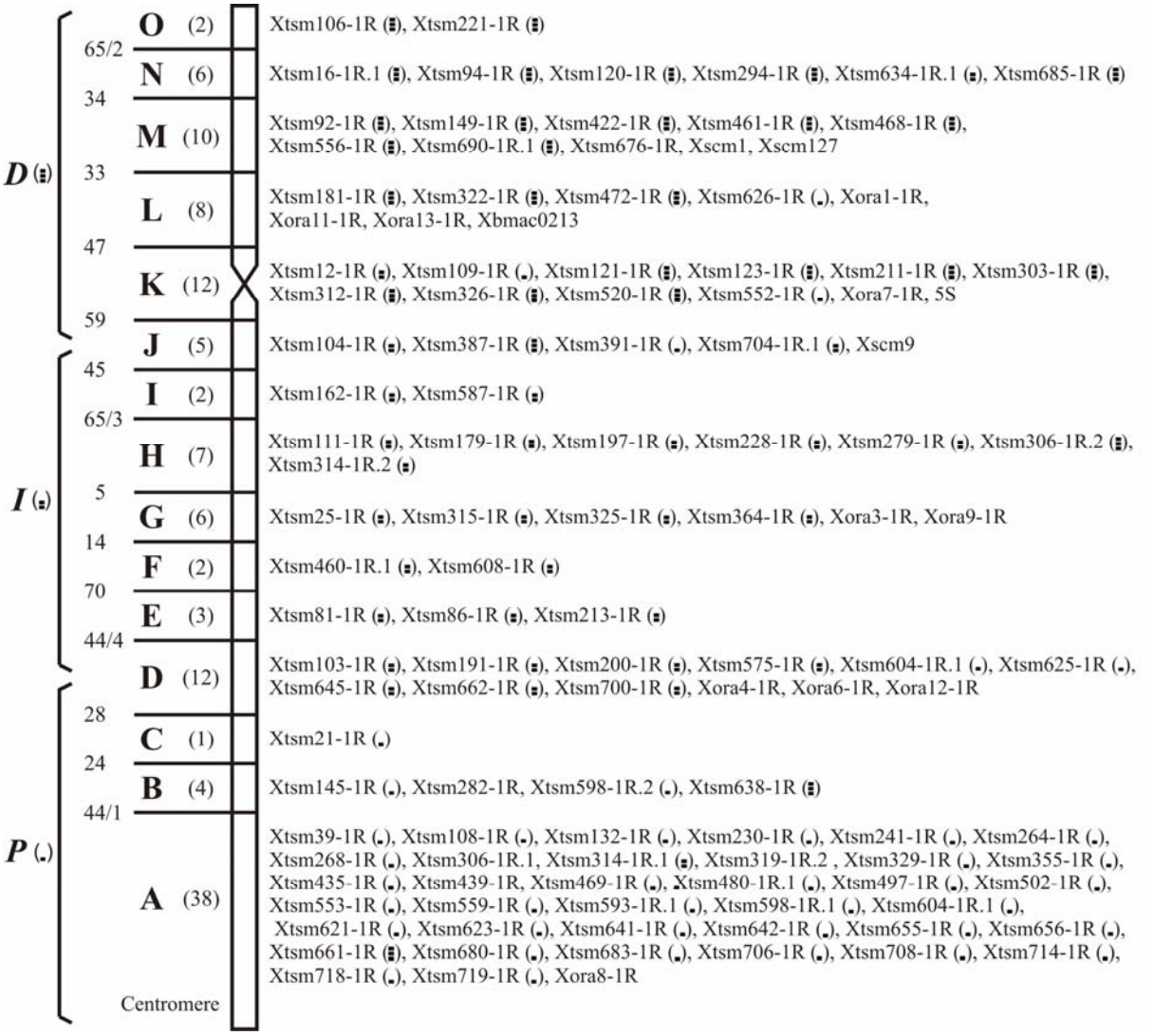

Fig. 15 Schematic representation of the 1RS chromosome. Bins A-O were created by the deletion lines given on the left side (Tsuchida et al., 2008). The symbols in brackets after each marker name correspond to the three bins proximal, intergalary and distal, *P*, *I*, and *D* on the very left side (Friebe et al., 2000). Loci within bins are ordered by ascending marker name number, starting with the TSM markers. The constriction in the bar in bin K represents the NOR.
In this figure the sizes of the bins are not correlating with their physical sizes or with the number of enclosed markers.

4.3. Genetic mapping

4.3.1. Polymorphism of markers

For mapping 119 TSM primer pairs were available of which 72 (60%) were polymorphic in the Sp3 x Hy2 population and 52 (44%) in the Sp4 x Hy2 population (Tab. 7). This degree of polymorphism is in general accordance with SSR specific rye mapping data published by Saal and Wricke (1999) [47%], Korzun et al. (2001) [61%], and Hackauf and Wehling (2003) [43%]. Khlestkina et al. (2004) found only 36 of 207 (17%) rye EST-derived microsatellite markers polymorph at least in one of the four employed mapping populations and Milczarski et al. (2007) describes a mapping efficiency of 14.4%.

In these studies the level of polymorphism between rye parents seems to be between 14% and 61%. To obtain a sufficient amount of markers that can be used for linkage mapping, it is necessary to start with a large enough number of primer pairs.

	Sp3 x Hy2		Sp4 x Hy2	
TSM makers in total	119		119	
polymorphic TSM markers	72	~ 60%	52	~ 44%

Tab. 7 Percentages of polymorphic markers

Even though microsatellite markers are praised because of their co-dominant inheritance, in this study, the majority of the evaluated and mapped SSRs showed dominant behaviour (Tab. 8). The reason for this was the fact that many of the co-dominant markers differ only slightly in base pair number and therefore their visual evaluation in the F_2 population was not reliable.

	Sp3 x Hy2		Sp4 x Hy2	
scored TSM marker	24		22	
- co-dominant	8	33%	9	41%
mapped TSM marker	16		11	
- co-dominant	5	31%	7	64%

Tab. 8 Percentages of co-dominantly inherited markers

In total 22 TSM markers were genetically mapped for the first time. All markers are physically located between bins E to O.
In the Sp3 x Hy2 population 24 TSM markers were scored of which 16 showed linkage of a LOD score of 3. In population Sp4 x Hy2 11 of the 15 evaluated markers were found to be linked. No obvious differences were found between these markers and the markers excluded from the groups by the program. No segregation distortion or differences in the evaluation of the gels could be found as a possible reason for not appearing in the map. The group of these markers consisted of dominant as well as co-dominant markers and were often found in similar bins.

Segregation distortion was analyzed in a χ^2-test employing the software program JoinMap v.3.0 (Ooijen and Voorrips, 2001). In population Sp4 x Hy2 nine markers (41%) segregated significantly different from the expected Mendelian ratios, whereas the Sp3 x Hy2 population distortion was observed in 11% of the markers only. Various degrees of segregation distortion in rye have been observed in several other mapping populations (Saal and Wricke, 1999; Korzun et al., 2001; Ma et al., 2001).

4.3.2. Distribution of markers along the 1RS chromosome

All markers which were genetically mapped are found in the intercalary to telomeric region of the chromosome arm. Fig. 16 shows the physical mapping results integrated into the genetic maps. In both populations the position of the markers in the genetic map fits well to the three bins *P*, *I* and *D* defined by Friebe et al. (2000). Only markers with large genetic distances show contradicting positions. However, the correspondence of the Sp3 x Hy2 genetic map with the more detailed physical map based on the deletion lines of Tsuchida et al. (2008) is deviating strongly from expectations, while in the population Sp4 x Hy2 the genetically

mapped order of the marker is in good correspondence with the physical map created in this study. Only the location of markers Xtsm552, Scm1 and Scm9 are not satisfactory. However, the physical position of Scm1 (bin M) has already been doubted in the previous chapter. In population Sp3 x Hy2 only a rough tendency from bin E to N is noticeable. This could be due to large gaps of markers on the one hand and regions of tightly clustered markers on the other hand. Besides, nearly two thirds of the mapped markers showed dominant inheritance which contains less accurate information. In comparison, in the Sp4 x Hy2 population 64% of the markers are co-dominant.

Both genetic maps cover mostly the terminal one third part of the chromosome arm. The noticeable absence of mapped markers located in the intercalary to proximal region has already been observed in several other studies (Korzun et al., 2001). Ma et al. (2001) could not place any marker between the centromere and the cytological marker C1-1RS. Milcazrski et al. (2007) got two linkage groups for the 1R chromosome; one referred to 1RL and one to the terminal part of 1RS. It seems that there is a general low level of polymorphism in this region. No noticeable C band variation has been recorded (Ma et al., 2001).
Polymorphism of Microsatellite markers arises through replication slippage (Tautz, 1989; Schlötterer, 2000) causing variation in repeat number which is independent of the physical location of the locus in the chromosome. However, such mutations occur rather rarely. Should such an event take place the possibility for this allelic variation to be passed on and spread through recombination is far more likely if this marker is located at the telomeric end of the chromosome. Recombination leads to a general larger allelic variation in the gene pool. Therefore the possibility of finding two different alleles in only three parent lines increases with distance from the centromere.
In this study, not solely the lack of polymorphic marker but the relatively low number of available markers for the intercalary region limited the mapping success.

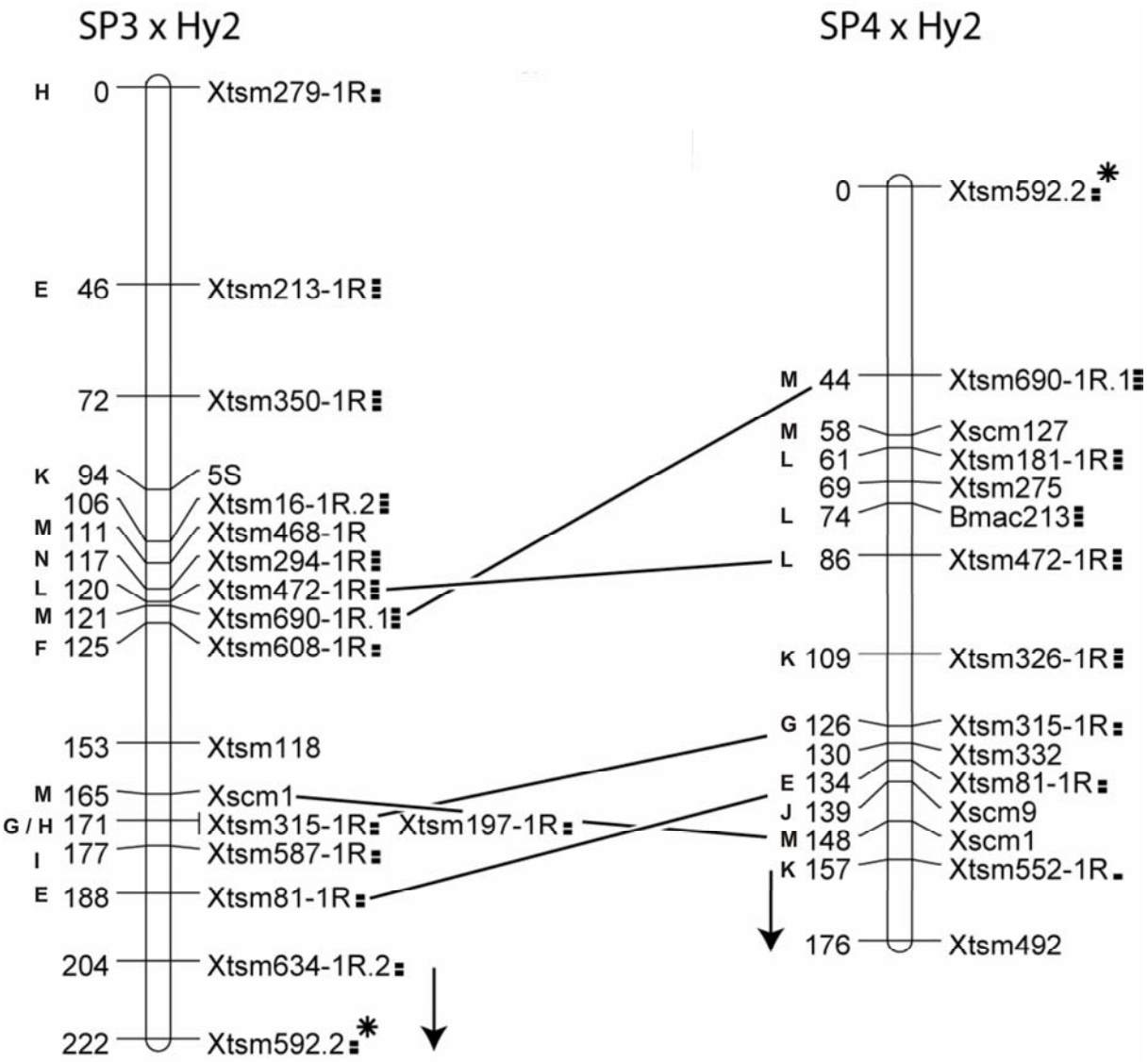

Fig. 16 Integration of the physical maps (Fig. 15) into the genetic maps. The capital letters on the left side refer to the bins created by the deletion lines of Tsuchida et al. (2008). The symbols on the right give the position of the markers according to Kofler et al. (2008) and Friebe et al. (2000).

4.3.3. Consensus map

Only five of the 27 markers mapped in total are shared between the two populations. Milczarski et al. (2007) and Hackauf and Wehling (2003) observed similar results in their rye genome studies; Of 61 mapped microsatellites only 12 common polymorphic loci were found for both maps. Khlestkina et al. (2004) found 99 microsatellite-derived loci though hardly any of those SSRs mapped to more than one of the four employed populations. This low transferability of SSRs among rye mapping populations might be due to the less than expected level of allelic variation.

In the newly created consensus map marker Scm1 is located clearly proximal to Scm127, as has been previously described by Hackauf and Wehling (2003) and Bmac0213 lies distal of Scm9 as observed earlier by Nagy et al. (2003). However, the position of the marker 5S, which is known to reside in the NOR, is not in agreement with any other study.

Even if the same primer pairs are employed comparing maps is complicated by the application of different mapping software and mapping populations. Furthermore, a positive

correlation between recombination and distance from the centromere exists (Ma et al., 2001; Nagy et al., 2003) Therefore, genetic distances can vary along the chromosome arm and between the same loci on different maps, respectively. Also slight changes in order of loci can be the result of different mapping calculations (Börner and Korzun, 1998; Ma et al., 2001).

5. Summary and conclusions

Rye (*Secale cereale* L.) is an important, out-crossing cereal crop in Northern Europe and parts of Asia. Its relatively low yield is compensated by a strong tolerance to extreme climate and poor soil conditions. Additionally, rye is part of the hybrid triticale and serves as gene pool for wheat improvement programs.
Chromosome arm 1RS, which takes about 5.6% of the rye genome, attracts special attention as it carries several resistance genes (Singh et al., 1990, Friebe et al., 1996; Ren et al., 1997; Rabinovich 1998; Mago et al., 2002; Roux et al., 2004; Lapitan et al., 2007) and the self-incompatibility locus *S* (Hackauf, 2005). Furthermore, 1RS has a huge impact on wheat varieties via the 1AL.1RS and 1BL.1RS translocations in wheat.

Compared to other cereals genomic studies in rye are still limited. Several linkage maps have been produced though the number of mapped SSR markers is still relatively low. The objective of this study was to develop a well saturated genetic and physical map of the 1RS chromosome based on 119 SSR markers (referred to as TSM) developed by Kofler et al. (2008). Additionally, 20 other 1RS specific markers were applied (Koebner, 1995; Ramsay et al., 2000; Hackauf and Wehling, 2003; Khlestkina et al., 2004; Bartoš et al., 2008).

For physical mapping all available markers were employed on twelve deletion lines, each carrying the 1RS arm in a different length (Tsuchida et al., 2008). This deletion lines are now available for mapping e.g. gene sequences, BACs, contigs, etc. In total 118 markers separate 15 physical bins. A noticeable large number of markers (35 %) are located in the three bins nearest to the centromere, which represent less than five percent of the chromosome arm. This is in a strong contradiction to the relatively scarce appearance of SSR in the middle region of 1RS. The mapped order of markers is in general good agreement with previously published data.

For genetic mapping the F_2 progeny of three rye inbreed lines, populations Sp3 x Hy2 and Sp4 x Hy2, were provided by Dr. B. Hackauf (Groß Lüsewitz). The results are two genetic maps; one for the population Sp3 x Hy2 consisting of 18 markers and one for population Sp4 x Hy2 composed of 15 markers, respectively. Both genetic maps are linked by five common markers and cover mainly the telomere region of the chromosome arm. The reasons for the low number of genetically mapped markers are manifold. 35% of the markers were localised in the proximal bin (Friebe et al., 2000) close to the centromere, lacking any polymorphism. The low level of polymorphism in rye in general further reduces the number of markers suitable for mapping. Many of the detected polymorphisms appeared as a slight difference in fragment size which made evaluation of segregation most difficult.

This study shows that microsatellite markers are not as polymorphic as often claimed in the literature which limits the success of linkage mapping; especially in chromosomal regions of little recombination. Furthermore, the low amount of common markers in different populations hinders comparison of mapping results and the construction of consensus maps.
To create a well saturated map, which shares common markers with reference maps, a large number of primer pairs is necessary.
Also, the conflicting order of markers in the genetic and physical map has yet to be explained.
Further research is needed to increase the number of mappable markers on 1RS and to expand the map towards the centromere. Employing a third mapping population, isolating new microsatellite markers from 1RS specific DNA, or using SSR motifs less frequently found in the rye genome are possible ways and are in progress.

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9. Abbreviations

A	adenin
AFLP	amplified fragment length polymorphsim
BSA	bovin serum albumin
bp	base pair
C	cytosin
CS	wheat cultivar ‘Chinese spring’
DNA	desoxyribonucleic acid
dNTP	desoxy-nucleotid-triphosphate
EDTA	ethylenediaminetetraacetic acid
F_2	second filial generation
FAM	6-carboxy-fluorescent
G	guanin
HEX	hexachloro-6-carboxy-fluorescent
NOR	nucleolus organizer region
PCR	polymerase chain reaction
RAPD	random aplified polymorphic DNA
RFLP	restriction fragment length polymorphsim
RNA	ribonucleic acid
SSR	simple sequence repeat
T	thymin
TBE	tris/borate/EDTA
TE	transposable element
TE buffer	tris/EDTA buffer

10. Appendix

App1: DNA extraction protocol (Wizard® Genomic DNA Purification Kit (Promega))

App2: Sp3 x Hy2: 2-point LOD
2-point distance (Kosambi)

App3: Sp4 x Hy2: 2-point LOD
2-point distance (Kosambi)

App1: DNA extraction protocol (Wizard® Genomic DNA Purification Kit (Promega))

1. Use 8cm of dried and grinded leaf in 1,5ml microcentrifuge tube

2. Add 600µl Nuclei Lysis Solution and vortex for 3 seconds

3. Incubate for 15 minutes at 65°C and cool down to room temperature for 5 minutes afterwards

4. Add 20µl RNase (0.4µg/µl) and mix gently by overturning the tube 2-5x

5. Incubate for 15 minutes at 37°C and cool down to room temperature for 5 minutes afterwards

6. Add 200µl Protein Precipitation Solution and vortex for 20 seconds

7. Centrifuge 2x2 minute with maximum speed in Hettich Centrifuge at 15°C

8. Transport clear supernatant (700µl) to a new tube

9. Add 700µl of Isopropanol to the tube and mix gently by overturning the tube for at least 5x

10. Centrifuge for 1 minute with maximum speed Hettich Centrifuge at room temperature

11. Decant supernatant by overturning the tube carefully

12. Add 500µl 70% ethanol and wash DNA pellet carefully

13. Centrifuge for 1 minute with maximum speed Hettich Centrifuge at room temperature

14. Decant ethanol by overturning the tube

15. Put the tube upside down on filter paper to remove ethanol drops

16. Dry over night

17. Add 100µl TE (ph 8.0) and incubate for one hour at 65°C or over night at room temperature or 4°C

18. Store at 2-8°C

19. Dilute 1:50 for PCR

App2 : Population Sp3 x Hy2

2-point LOD

	Xtsm 279	Xtsm 118	Xtsm 634.2	Xtsm 592.2	Xtsm 197	Xtsm 294	Xtsm 16.2	Xtsm 690.1	Xtsm 608	Xtsm 587	Xsm 81	Xtsm 315	Xscm1	Xtsm 213	5S	Xtsm 468	Xtsm 472	Xtsm 350	Xrems 1135
Xtsm279	----	5.2	3.5	6.6	9.0	0.1	0.1	0.2	1.3	0.2	2.9	4.2	2.8	2.2	3.5	1.5	1.4	1.4	0.0
Xtsm118	5.2	----	9.3	7.6	12.0	1.8	1.4	2.9	4.5	1.5	7.2	13.9	11.8	3.6	6.6	2.2	6.5	1.8	1.5
Xtsm634.2	3.5	9.3	----	7.2	14.2	1.9	1.5	1.9	2.3	2.5	9.7	14.7	11.7	2.5	3.2	0.6	3.3	0.8	2.4
Xtsm592.2	6.6	7.6	7.2	----	10.1	1.6	0.4	1.5	3.1	2.3	7.2	12.0	8.7	1.7	3.1	2.0	3.8	1.9	2.3
Xtsm197	9.0	12.9	14.2	10.1	----	3.4	2.0	3.6	3.8	1.7	10.8	14.5	4.9	4.9	5.9	2.2	6.0	2.6	1.7
Xtsm294	0.1	1.8	1.9	1.6	3.4	----	9.9	14.6	12.2	2.5	1.3	5.3	3.9	1.3	2.2	27	16.4	3.3	0.4
Xtsm16.2	0.1	1.4	1.5	0.4	2.0	9.9	----	10.3	10.7	2.4	1.9	3.9	2.3	1.0	2.6	3.1	13.6	5.2	0.5
Xtsm690.1	0.2	2.9	1.9	1.5	3.6	14.6	10.3	----	13.0	2.1	1.8	5.0	3.9	1.3	1.4	1.5	17.9	3.3	0.2
Xtsm608	1.3	4.5	2.3	3.1	3.8	12.2	10.7	13.0	----	3.6	4.5	7.3	5.9	1.0	8.9	10.2	28.3	5.1	0.4
Xtsm587	0.2	1.5	2.5	2.3	1.7	2.5	2.4	2.1	3.6	----	6.3	11.2	9.6	0.5	0.3	0.2	2.7	3.2	1.5
Xsm81	2.9	7.2	9.7	7.2	10.8	1.3	1.9	1.8	4.5	6.3	----	19.4	13.4	3.2	3.0	1.7	4.7	1.4	3.1
Xtsm315	4.2	13.9	14.7	12.0	23.8	5.3	3.9	5.0	7.3	11.2	19.4	----	26.2	5.5	5.2	2.2	9.7	4.2	4.3
Xscm1	2.8	11.8	11.7	8.7	14.5	3.9	2.3	3.9	5.9	9.6	13.4	26.2	----	4.0	2.9	1.0	6.1	3.8	3.0
Xtsm213	2.2	3.6	2.5	1.7	4.9	1.3	1.0	1.3	1.0	0.5	3.2	5.5	4.0	----	1.9	0.0	1.8	2.3	0.4
5S	3.5	6.6	3.2	3.1	5.9	2.2	2.6	1.4	8.9	0.3	3.0	5.2	2.9	1.9	----	8.3	12.5	2.2	0.2
Xtsm468	1.5	2.2	0.6	2.0	2.2	2.7	3.1	1.5	10.2	0.2	1.7	2.2	1.0	0.0	8.3	----	12.5	0.8	0.1
Xtsm472	1.4	6.5	3.3	3.8	6.0	16.4	13.6	17.9	28.3	2.7	4.7	9.7	6.1	1.8	12.5	12.5	----	5.7	0.5
Xtsm350	1.4	1.8	0.8	1.9	2.6	3.3	5.2	3.3	5.1	3.2	1.4	4.2	3.8	2.3	2.2	0.8	5.7	----	0.8
Xrems1135	0.0	1.5	2.4	2.3	1.7	0.4	0.5	0.2	0.4	1.5	3.1	4.3	3.0	0.4	0.2	0.1	0.5	0.8	----

2-point distance (Kosambi)

	Xtsm 279	Xtsm 118	Xtsm 634.2	Xtsm 592.2	Xtsm 197	Xtsm 294	Xtsm 16.2	Xtsm 690.1	Xtsm 608	Xtsm 587	Xsm 81	Xtsm 315	Xscm1	Xtsm 213	5S	Xtsm 468	Xtsm 472	Xtsm 350	Xrems 1135
Xtsm279	------	22.3	30.0	18.0	12.9	80.7	86.0	67.8	46.8	67.7	33.9	31.2	36.1	32.6	27.7	39.8	47.9	42.9	96.6
Xtsm118	22.3	------	16.8	16.5	9.9	27.5	33.8	17.4	25.2	29.5	17.9	8.7	11.8	27.3	19.9	33.8	20.4	30.9	29.1
Xtsm634.2	30.0	16.8	------	18.3	8.3	30.9	35.8	30.3	38.0	24.3	15.2	9.9	13.9	34.3	32.0	53.4	33.5	47.1	24.7
Xtsm592.2	18.0	16.5	18.3	------	11.0	28.4	49.4	28.6	29.5	19.1	16.1	9.7	14.7	36.6	29.4	34.2	27.5	26.6	18.8
Xtsm197	12.9	9.9	8.3	11.0	------	4.9	20.1	4.9	26.1	21.5	9.7	0.0	6.4	21.6	20.4	33.0	19.9	18.0	21.5
Xtsm294	80.7	27.5	30.9	28.4	4.9	------	10.5	3.7	6.6	28.3	39.1	20.6	25.2	24.2	19.3	4.9	3.2	26.5	50.8
Xtsm16.2	86.0	33.8	35.8	49.4	20.1	10.5	------	8.7	8.9	29.7	35.9	26.1	33.4	33.1	18.2	4.9	6.4	21.0	48.7
Xtsm690.1	67.8	17.4	30.3	28.6	4.9	3.7	8.7	------	4.8	29.1	34.5	20.8	24.2	24.4	29.5	22.8	1.2	25.5	54.8
Xtsm608	46.8	25.2	38.0	29.5	26.1	6.6	8.9	4.8	------	21.7	28.0	22.2	25.3	43.7	13.8	9.9	3.8	20.4	51.6
Xtsm587	67.7	29.5	24.3	19.1	21.5	28.3	29.7	29.1	21.7	------	15.6	7.4	10.0	41.4	55.8	56.7	27.4	26.3	32.6
Xsm81	33.9	17.9	15.2	16.1	9.7	39.1	35.9	34.5	28.0	15.6	------	8.7	14.0	27.2	28.7	34.6	28.0	41.3	24.9
Xtsm315	31.2	8.7	9.9	9.7	0.0	20.6	26.1	20.8	22.2	7.4	8.7	------	5.6	20.1	21.7	32.8	19.1	26.4	26.5
Xscm1	36.1	11.8	13.9	14.7	6.4	25.2	33.4	24.2	25.3	10.0	14.0	5.6	------	25.2	31.1	45.1	26.0	27.9	21.0
Xtsm213	32.6	27.3	34.3	36.6	21.6	24.2	33.1	24.4	43.7	41.4	27.2	20.1	25.2	------	35.8	85.6	36.7	19.2	42.9
5S	27.7	19.9	32.0	29.4	20.4	19.3	18.2	29.5	13.8	55.8	28.7	21.7	31.1	35.8	-----	14.7	9.2	25.3	58.3
Xtsm468	39.8	33.8	53.4	34.2	33.0	4.9	4.92	2.8	9.9	56.7	34.6	32.8	45.1	85.6	14.7	------	7.6	39.4	59.4
Xtsm472	47.9	20.4	33.5	27.5	19.9	3.2	6.4	1.2	3.8	27.4	28.0	19.1	26.0	36.7	9.2	7.6	------	20.7	48.9
Xtsm350	42.9	30.9	47.1	26.6	18.0	26.5	21.0	25.5	20.4	26.3	41.3	26.4	27.9	19.2	25.3	39.4	20.7	------	43.2
Xrems1135	96.6	29.1	24.7	18.8	21.5	50.8	48.7	54.8	51.6	32.6	24.9	21.0	26.5	42.9	58.3	59.4	48.9	43.2	------

App3: Population Sp4 x Hy2

2-point LOD

	Xtsm 275	Xtsm 326	Xscm 127	Xtsm 181	Xtsm 332	Xtsm 492	Xtsm 552	Xtsm 472	Xtsm 690.1	Bmac213	Xscm1	Xscm9	Xtsm 81	Xtsm 592.2	Xtsm 315	Xora2
Xtsm275	----	6.4	12.3	13.6	8.2	5.3	7.7	16.9	14.0	29.9	10.6	11.8	14.4	6.3	11.9	2.7
Xtsm326	6.4	----	4.6	5.9	8.2	5.4	6.4	7.6	2.5	5.8	7.9	8.6	9.2	0.8	10.7	2.1
Xscm127	12.3	4.6	----	18.8	6.3	3.3	6.0	13.3	12.2	13.8	5.3	4.3	6.8	0.9	5.1	0.4
Xtsm181	13.6	5.9	18.8	----	5.8	3.5	5.5	14.7	10.1	15.2	5.4	4.4	6.4	1.9	5.8	0.7
Xtsm332	8.2	8.2	6.3	5.8	----	7.1	11.4	7.8	4.8	6.1	11.3	11.3	13.4	1.4	14.1	4.6
Xtsm492	5.3	5.4	3.3	3.5	7.1	----	9.8	5.3	4.0	4.8	11.4	9.2	12.1	2.2	11.0	2.2
Xtsm552	7.7	6.4	6.0	5.5	11.4	9.8	----	7.2	5.6	6.1	19.4	14.5	17.8	2.8	14.7	2.0
Xtsm472	16.9	7.6	13.3	14.7	7.8	5.3	7.2	----	10.5	19.6	5.6	5.9	7.7	2.2	8.4	0.5
Xtsm690.1	14.0	2.5	12.2	10.1	4.8	4.0	5.6	10.5	----	17.0	7.0	5.3	7.7	4.0	7.3	0.7
Bmac213	29.9	5.8	13.8	15.2	6.1	4.8	6.1	19.6	17.0	----	8.8	9.1	11.3	5.6	9.1	1.9
Xscm1	10.6	7.9	5.3	5.4	11.3	11.4	19.4	5.6	7.0	8.8	----	20.3	25.9	5.9	19.4	4.8
Xscm9	11.8	8.6	4.3	4.4	11.3	9.2	14.5	5.9	5.3	9.1	20.3	----	24.8	5.1	19.9	5.2
Xtsm81	14.4	9.2	6.8	6.4	13.4	12.1	17.8	7.7	7.7	11.3	25.9	24.8	----	9.1	27.9	4.8
Xtsm592.2	6.3	0.8	0.9	1.9	1.4	2.2	2.8	2.2	4.0	5.6	5.9	5.1	9.1	----	8.5	2.1
Xtsm315	11.9	10.7	5.1	5.8	14.1	11.0	14.7	8.4	7.3	9.1	19.4	19.9	27.9	8.5	----	3.7
Xora2	2.7	2.1	0.4	0.7	4.6	2.2	2.0	0.5	0.7	1.9	4.8	5.2	4.8	2.1	3.7	----

2-point distance (Kosambi)

	Xtsm 275	Xtsm 326	Xscm 127	Xtsm 181	Xtsm 332	Xtsm 492	Xtsm 552	Xtsm 472	Xtsm 690.1	Bmac213	Xscm1	Xscm9	Xtsm 81	Xtsm 592.2	Xtsm 315	Xora2
Xtsm275	------	22.8	9.9	8.4	15.7	28.7	24.0	13.3	17.4	5.1	21.0	18.2	16.7	26.6	20.4	36.8
Xtsm326	22.8	------	22.8	20.1	14.1	20.1	18.1	18.4	36.5	23.6	17.5	15.3	15.6	49.4	14.3	32.2
Xscm127	9.9	22.8	------	2.2	17.4	25.7	17.6	7.4	10.3	7.4	22.5	25.7	19.0	46.9	25.2	57.8
Xtsm181	8.4	20.1	2.2	------	18.7	25.0	18.7	6.1	13.5	6.0	22.0	25.1	20.7	35.8	23.3	50.7
Xtsm332	15.7	14.1	17.4	18.7	------	12.7	6.2	14.9	24.0	20.0	8.5	7.9	6.4	39.3	7.0	14.2
Xtsm492	28.7	20.1	25.7	25.0	12.7	------	16.5	25.7	33.3	30.2	16.1	18.8	16.1	39.1	18.5	38.9
Xtsm552	24.0	18.1	17.6	18.7	6.2	16.5	------	22.2	27.1	26.0	8.6	11.4	10.0	35.4	13.8	40.7
Xtsm472	13.3	18.4	7.4	6.1	14.9	25.7	22.2	------	20.8	10.9	28.8	27.1	25.0	41.8	24.2	61.3
Xtsm690.1	17.4	36.5	10.3	13.5	24.0	33.3	27.1	20.8	------	14.2	26.2	30.1	26.0	33.7	28.2	55.4
Bmac213	5.1	23.6	7.4	6.0	20.0	30.2	26.0	10.9	14.2	------	23.0	21.8	19.9	28.4	23.9	42.5
Xscm1	21.0	17.5	22.5	22.0	8.5	16.1	8.6	28.8	26.2	23.0	------	8.6	6.7	26.1	11.8	27.9
Xscm9	18.2	15.3	25.7	25.1	7.9	18.8	11.4	27.1	30.1	21.8	8.6	------	6.1	27.0	10.3	26.2
Xtsm81	16.7	15.6	19.0	20.7	6.4	16.1	10.0	25.0	26.0	19.9	6.7	6.1	------	19.8	6.6	28.1
Xtsm592.2	26.6	49.4	46.9	35.8	39.3	39.1	35.4	41.8	33.7	28.4	26.1	27.0	19.8	------	21.9	37.6
Xtsm315	20.4	14.3	25.2	23.3	7.0	18.5	13.8	24.2	28.2	23.9	11.8	10.3	6.6	21.9	------	32.9
Xora2	36.8	32.2	57.8	50.7	14.2	38.9	40.7	61.3	55.4	42.5	27.9	26.2	28.1	37.6	32.9	------