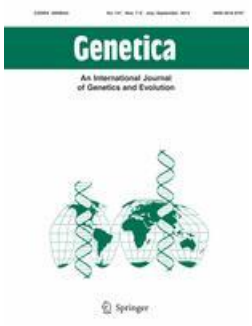

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Genetic analysis and molecular mapping of a new fertility restorer gene *Rf8* for *Triticum timopheevi* cytoplasm in wheat (*Triticum aestivum* L.) using SSR markers

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Abstract

A study on mode of inheritance and mapping of fertility restorer (*Rf*) gene(s) using simple sequence repeat (SSR) markers was conducted in a cross of male sterile line 2041A having *Triticum timopheevi* cytoplasm and a restorer line PWR4099 of common wheat (*Triticum aestivum* L.). The F_1 hybrid was completely fertile indicating that fertility restoration is a dominant trait. Based on the pollen fertility and seed set of bagged spikes in F_2 generation, the individual plants were classified into fertile and sterile groups. Out of 120 F_2 plants, 97 were fertile and 23 sterile (based on pollen fertility) while 98 plants set ≥5seeds/spike and 22 produced ≤4 or no seed. The observed frequency fits well into Mendelian ratio of 3 fertile: 1 sterile with χ^2 value of 2.84 for pollen fertility and 2.17 for seed setting indicating that the fertility restoration is governed by a single dominant gene in PWR4099. The three linked SSR markers, Xwmc503, Xgwm296 and Xwmc112 located on the chromosome 2DS were placed at a distance of 3.3, 5.8 and 6.7cM, respectively, from the *Rf* gene. Since, no known *Rf* gene is located on the chromosome arm 2DS, the *Rf* gene in PWR4099 is a new gene and proposed as *Rf8*. The closest SSR marker, Xwmc503, linked to the *Rf8* was validated in a set of fertility restorer, maintainer and cytoplasmic male sterile (CMS) lines. The closely linked SSR marker Xwmc503 may be used in marker assisted backcross breeding facilitating the transfer of fertility restoration gene *Rf8* into elite backgrounds with ease.

Key words: Hybrid wheat, fertility restorer gene, bulked segregant analysis (BSA) and molecular mapping

Introduction

Globally, wheat (*Triticum aestivum* L.) is the second most important crop after maize. It contributes to 21 percent of the food calories and 20 per cent of protein to more than 4.5 billion people in developing countries (Braun et al (2010) . Demand for wheat in the developing world is projected to increase 60 percent by 2050 (Anonymous 2007). Improving wheat productivity will be essential to meet the growing demand for food under shrinking cultivable land area. Hybrids, which exploit heterosis and generally exhibit higher yields than the high yielding semi-dwarf varieties are seen as one of the possible approaches for improving wheat productivity. Wheat is strictly a self-pollinated crop with chasmogamous flowers and it needs change in pollination system to facilitate hybrid breeding. Therefore, the crucial and important requirement for heterosis breeding is to promote natural out-crossing through induction of male sterility. Genic male sterility in wheat was reported long ago (Pugsley and Oram 1959) and its utilization was also reported in mid-sixties (Suneson 1962; Athwal et al. 1967). Other different types of male sterility inducing systems were also been reported for production of hybrid wheat. These include chemical hybridizing agent (CHA) (Striff et al. 1997; Asfaw 2005) and thermo-photo-sensitive genic male sterility (T/PGMS), which is controlled by nuclear recessive gene (Zhang et al. 2006; Tang et al. 2012). However, to develop commercial hybrid seeds, cytoplasmic male sterility (CMS), which is often caused by defects in mitochondrial function, has been exploited in many crops (Ma 2013). Mitochondrial genome rearrangements in CMS lines result into chimeric and abnormal (toxic) open read frames (ORFs), which leads to reduction in respiration and other mitochondrial defects ultimately leading to pollen sterility (Bentolila et al. 2002). The nuclear genes which counteract the effects of mitochondrial sterility factors, protecting normal mitochondrial function and male fertility are known as fertility restore genes (*Rf*) (Schnable and Wise 1998; Ma 2013)

The development of hybrids in wheat is a promising approach to break the yield barriers and to get the quantum jump in wheat production (Cisar and Cooper 2002). Work on hybrid wheat was started by Kihara (1951) who discovered an effective cytoplasmic male sterility (CMS) in an alloplasmic line containing nuclear genome of common wheat and cytoplasm of *Aegilops caudata* (goatgrass). Remarkably, to develop commercial hybrid wheat, dependable male sterility systems were identified in the genetic background of *Triticum timopheevi* Zhuk. cytoplasm with the substitution of the nuclear genome of wheat (*Tritcum aestivum*) by Wilson and Ross (1962). Fertility

restoration using *T. timopheevi* cytoplasm is crucial component for successful hybrid wheat breeding program because the identification of suitable fertility restorers using conventional approach is tedious and cumbersome process (Wilson 1968). To increase hybrid vigour, it is desirable to select genetically diverse male-sterile lines and their fertility restorer lines (Singh et al. 2010; 2011). This will help in developing widely adaptable hybrids across different agro-ecological areas and cropping systems. For successful exploitation of diversity in hybrid breeding programme, analysis of agronomic traits is an important criterion for identification of superior fertility restorers. Tomar et al. (2009) studied agro-morphological and molecular diversity among exotic and indigenous fertility restorers against *T. timopheevi* cytoplasm and reported that fertility restorers were genetically diverse. To utilize the diverse restorer lines in the hybrid breeding programme, it is essential to know the genetic architecture and the location of fertility restorer genes in the lines.

Earlier seven *Rf* genes have been reported to restore fertility against *T. timopheevi* cytoplasm (G-type), and their chromosomal locations have been determined, as, *Rf1* (1A) (Du et al. 1991), *Rf2* (7D) (Bahl and Maan 1973; Maan et al. 1984), *Rf3* (1B) (Tahir and Tsunewaki 1969; Zhou et al. 2005), *Rf4* (6B) (Maan et al. 1984), *Rf5* (6D) (Bahl and Maan 1973), *Rf6* (5D) (Bahl and Maan 1973) and *Rf7* (7B) (Bahl and Maan 1973). In addition, some minor QTLs involved in fertility restoration have also been reported on chromosomes 2A, 2B, 4B, 5A 6A and 7D (Ahmed et al. 2001; Zhou et al. 2005). Out of seven known fertility restorer genes (*Rf*), only one gene *Rf*3 was localized with the help of molecular markers like Restriction Fragment Length Polymorphism (RFLP) markers (Kojima et al. 1997; Ahmed et al. 2000). Subsequently, Zhou et al. (2005) identified the closely linked SSR markers, Xbarc207, Xgwm131 and Xbarc61 to the fertility restorer gene *Rf3* on chromosome 1B.

Tomar et al. (2004) developed different CMS lines using lines of Chinese Spring carrying *T. timopheevi*, *T. araraticum* Zhuk., *Ae.caudata* and *Ae. speltoides* cytoplasm through backcross breeding . Subsequently, with a view to develop highly heterotic hybrids in Indian Sub-continent, a highly diverse fertility restorer for *T. timopheevi* (PWR4099) cytoplasm was identified (Tomar et al., 2004 and 2009), which showed higher level of heterosis in comparison to the high yielding varieties (HYVs). Keeping this in view, during the present study, genetic analysis was carried out to understand the mode of inheritance of fertility restoration and to map the chromosomal location of the identified fertility restorer (*Rf*) gene in PWR4099. The validation of the SSR marker linked to the fertility restorer gene was also done using a set of CMS, maintainer and restorer lines.

Methods

Plant material

The cytoplasmic male sterile (CMS) line 2041A (Lok-1*7//Sunstar*6/C80-1) developed through repeated backcross breeding, carrying *T. timopheevi* cytoplasm and the fertility restorer line PWR4099 of wheat (*T. aestivum* L.) (Table 1) were sown in 14" size pots in net house at the Indian Agricultural Research Institute, New Delhi, India. PWR4099 an exotic line, is dwarf, have shy tillering ability and large spikes producing 90 to 100 grains per spike. The CMS line 2041A was crossed with the fertility restorer line PWR4099 during the winter (November to March of 2008-09). The F_1 seeds were grown in National Phytotron Facility, IARI, New Delhi, India (at 20 to 25°C) during the summer (June to September) of 2009. All the spikes of F_1 plants were covered with butter paper bags prior to anthesis to obtain selfed seeds. The seeds harvested from one solitary F_1 plant only were used to rise the F_2 generation. The 120 F_2 plants were planted in rows with seed to seed distance of 15 cm and row to row distance of 30 cm in net house during winter 2009-10. The data on pollen fertility and seed set per spike were recorded on individual plants in F_2 population and subjected to chi-square (χ^2) analysis to determine the mode of inheritance.

Phenotyping of F_2 segregants for pollen fertility

Pollen fertility was used as the main criterion for assessing male fertility and sterility. Anthers from three florets were randomly selected from each of the lower, middle and top portions of the main spike at the time of anthesis. The anthers were smeared in a drop of 1% Iodine-Potassium Iodide (I-KI) solution on a glass slide to examine pollen under the microscope at 10× and 40× magnifications. The pollen grains that were completely round and deeply stained were counted as fertile and those, which were unstained or stained but withered, were considered as sterile. Three microscopic fields were taken for counting the number and fertility percentage of pollen grain. F_2 generation individual plant data in respect to pollen fertility and seed set was plotted on a graph for the purpose to distinguish between fertile and sterile groups. Based on this plotting, F_2 plants were classified into four classes, namely, fully fertile (FF) (61 -100 % pollen fertility), partially fertile (PF) (31 to 60% pollen fertility), partially sterile (PS) (1-30% pollen fertility) and fully sterile (FS) (0% pollen fertility). For carrying out genetic analysis, the FF and PF groups of plants were merged together to form one category of fertile (F), and PS and CS plants were

merged into sterile (S) group considering inflicting point at 30% of pollen fertility (see later). Data on observed frequency of plants thus obtained were subjected to χ^2analysis.

Phenotyping of F_2 segregants on the basis of seed setting

In addition to the pollen fertility analysis, the data on number of seeds set on the main spike (seed set/spike) of individual F_2 plants was also recorded to further confirm the inheritance of the fertility restoration. Based on seed set/spike, the F_2 plants were classified into the following four categories, namely fully fertile (FF) (>35seeds /spike), partial fertile (PF) (5-35 seeds/spike), partially sterile (PS) (1-4 seeds /spike) and fully sterile (FS) (no seed set) following Anbalagan (2003) and Ali et al. (2011). Merging of different categories of plants, as for pollen fertility was also considered for seed set. The FF and PF group of plants were merged together to form fertile (F) category and PS and CS plants were merged into sterile (S) group for the purpose of genetic analysis. Data on observed frequency of plants thus obtained were subjected to χ^2 analysis.

SSR marker analysis

Genomic DNA was extracted from young leaf tissues (at 2-3 leaf stage) of two parental lines (2041A and PWR4099) and their derived 120 F_2 population and 15 additional lines for validation, using CTAB (Cetyl- Tetra Methyl Ammonium Bromide). For the genetic mapping of *Rf* gene, a set of 994 SSRs of Xgwm, Xwmc and Xbarc series (Röder et al. 1995; Somers et al. 2004) were used during the present study for polymorphism survey between the parental genotypes covering the entire genome. The primer sequence were obtained from Grain Genes database (http://wheat.pw.usda.gov/GG2/index.shtml) and synthesized on contract by Sigma Life Science, Bangalore, India. The PCR products were resolved on 3.5 % Metaphor® gels stained with ethidium bromide and photographed using gel documentation system.

Bulk segregant analysis and construction of linkage map

Bulk segregant analysis (Michelmore et al. 1991) was used to identify putatively linked SSR markers to the targeted fertility restorer gene. Two DNA bulks were prepared using equal amounts of genomic DNA from 10 fertile and 10 sterile plants using pollen fertility data. Markers exhibiting polymorphism between the parental genotypes, fertile and sterile bulks were used to screen the entire population. MAPMAKER v.3.0 was used for linkage analysis

(Lander et al. 1987). The marker order was established using multipoint analysis at LOD 3.0 and above. Kosambi mapping function was used to determine the distance in centimorgan (cM) between the markers (Kosambi 1944).

Marker trait association

The association of all the markers with the fertility restoration trait was analyzed in F_2 population. For this purpose, t-test was performed to test the significance of difference (at 5% level of significance) between the mean values of the pollen fertility (%) of the F_2 plants carrying A-type alleles (sterile parent allele), both A- and R-types of alleles (i.e. heterozygous=H) and R-type of alleles (fertile parent type allele).

Validation of linked molecular markers

A total of 17 lines (15 additional line and 2 parental lines of mapping population) were considered for validation of SSR marker(s) linked to the *Rf* gene (Table 1). The 15 lines which were used are consisted of 12 different restorer lines of which seven were developed using PWR4099 as one of the parent. Two maintainer line having *T. timopheevi* cytoplasm and one CMS line 2019A which had cytoplasm of *T. araraticum* were also used in validation study.

Results

Phenotyping of F_2 population

2041A was used as female parent and crossed with PWR4099 as male to generate 35 F_1 seeds. Further, 10 plants were selected for analysis of pollen fertility and seed sets per main spike in the F_1 plants, which was bagged prior to avoid any contamination. The pollen fertility of all F_1s was more than 90% and seed set per main spike ranged from 53 to 61 and indicated that fertility restoration is dominant over male sterility. The seeds from only single plant were taken to grow 120 F_2 plants to take utmost care to avoid any possible mixture of seed. To define the cut off point for merging groups on the basis of pollen sterility and seed setting data of F_2 plants, polygons were generated two different peaks with well-defined valley between them (Figure 2). Based on polygon data of pollen fertility and seed setting data, 30% and $\leq$ 4 seeds per main spike, respectively was considered as cut off point for merging groups and for inheritance and mapping studies.

Genetics of fertility restoration

The F_1 plants were fertile having >90% pollen fertility suggesting that fertility restoration is a dominant trait. Based on the pollen fertility per cent, 77 F_2 plants out of 120 were grouped into fully fertile (FF) class, 20 into partially fertile (PF) class, 6 into partially sterile (PS) and 17 plants were grouped into completely sterile (CS) class. The 77 FF and 20 PF plants were merged together into one fertile (F) class and 6 PF and 17 CS plants were grouped into the sterile (S) category. Thus, the total number of plants in fertile category was 97 and in the sterile category the number of plants was 23. The observed frequency of plants fit well to expected segregation ratio of 3(fertile): 1(sterile) with a χ^2 value of 2.84 (P value = 0.091) at 5% level of significance (Table 2).

The data on pollen fertility was further confirmed with the data on seed set/spike.As per the classification of F_2 individuals in different categories based on pollen fertility, the 120 F_2 plants were grouped into four categories: 82 FF plants(>35 seeds per spike), 16 PF plants (5-35 seeds per spike), 4 PS plants (1-4 seeds per spike) and 18 CS plants producing no seeds. The FF and PF group of plants were merged together into fertile (F) group while PS and CS plants were merged into sterile (S) group. The observed frequency of 98 fertile and 22 sterile plants in F_2 population showed a good fit to the Mendelian segregation ratio of 3 (fertile): 1 (sterile), with a χ^2value of 2.17 (P value = 0.140) at 5% level of significance (Table 2). This data of pollen fertility had good correspondence with data of seed set in individual F_2 plants. Segregation ratios in the F_2 population using data on pollen fertility percent and seed setting indicated that the fertility restoration is controlled by a single dominant gene, which is derived from the exotic spring wheat line PWR4099.

Identification of molecular markers linked to fertility restorer gene

A set of 994 SSRs covering all the 21 chromosomes of wheat was used for polymorphism survey between the two parental genotypes 2041A and PWR4099 of the F_2 mapping population (derived from 2041A × PWR4099). Out of 994 SSRs marker, 105 SSRs detected polymorphism between the two parental genotypes, namely 2041A and PWR4099. All the polymorphic markers were used to screen the two bulks (sterile bulk and fertile bulk). Out of these 105 SSRs, three SSR markers namely, Xwmc503, Xwmc112 and Xgwm296 located on chromosome arm 2DS were polymorphic in the set of two bulks. The sequence information of three putatively linked SSR markers is presented in Table 3.

Genotyping of F_2 population and segregation analysis

A total of 120 F_2 plants, derived from the cross 2041A × PWR4099, were genotyped using above three SSR markers showing polymorphism between the two parental genotypes as well as between the two DNA bulk samples. The results of genotyping are presented in Table 4. The goodness of fit of segregation ratio at each of the three SSR loci was tested using χ^2 test against expected Mendelian segregation ratio of 1:2:1. Chi -square values for the SSRs Xwmc503, Xgwm296 and Xwmc112 were 2.2, 1.46 and 0.13, respectively (Table 4.). This suggested a good fit to Mendelian segregation ratio of 1:2:1 for each of the three SSR markers. The representative gel picture of random 44 F_2plants (out of 120 plants), using closely linked SSR marker Xwmc503 is presented in Figure 2.

The mean values of pollen fertility (%) data of F_2 plants carrying A-type (CMS) of allele, R-type (restorer) of allele and H (heterozygous) plants along with the probability values are presented in Table 5. The mean pollen fertility value of A and R type and A and H type showed significant difference at 5 % level of significance. However, the mean pollen fertility values of R and H type of plants did not show significant difference suggesting dominant nature of fertility restorer gene.

Construction of linkage map

The co-segregation analysis for individual SSR marker genotype and the fertility restoration phenotype based on pollen fertility per cent of 120 individual F_2 plants using MAPMAKER ver. 3b software showed the following best order: Xwmc503, *Rf*, Xgwm296 and Xwmc112. The position of linked SSRs in relation to the fertility restorer (*Rf*) locus is shown in Figure 4. The SSR, Xwmc503 was located at a distance of 20 cM from the telomere of the short arm of chromosome 2DS in the genetic map reported by Somers et al. (2004). This SSR was located at a distance of 3.3 cM from the *Rf* gene (10.12 LOD score value). On the proximal side of the *Rf* gene, SSRs Xgwm296 and Xwmc112 were located at genetic distances of 5.8 cM and 6.7 cM with LOD score values of 8.58 and 7.17, respectively. The results suggest that the *Rf* gene mapped during the present study has not been reported earlier. To the best of our knowledge, no *Rf* gene has so far been reported on chromosome 2DS in wheat so, we propose that the newly identified gene may be designated as *Rf8*.

Validation of closely linked markers in a set of restorer and maintainer lines

The closely linked SSR marker Xwmc503 was used to validate in a set of 17 lines including two parental lines used for the development of F_2 population and a set of each of the CMS, maintainer and restorer lines. The marker Xwmc503 amplified 170 bp fragment in sterile parent (CMS line 2041A) and 200 bp fragment in fertile parent (restorer line PWR4099). However, one novel allele of 140 bp was also observed during the validation in a set of different lines (Table 6, Figure 5). Out of 12 fertility restorer lines (excluding parental line PWR4099) tested, 10 restore lines amplified restorer specific allele of 200 bp. The remaining two primary fertility restorer genotypes PWR4101 and EC368169R of exotic origin, amplified 140 bp (novel allele) and 170 bp (similar to sterile parent allele) allele, respectively. The maintainer (B line) line HW2041and its corresponding CMS line 2041A amplified 170bp allele, which is similar to sterile parent of mapping population. However, the other maintainer HW2019 (B line) and its corresponding CMS line 2019A carried the novel 140 bp allele, similar to exotic restorer line EC368169R which was not found in any of the remaining 14 genotypes. Together, the above results suggested that none of the CMS lines and the maintainer lines carried 200 bp restorer specific allele and 10 of the 12 fertility restorer lines studied carried 200bp allele, which is similar to the one associated with the proposed *Rf8* gene in PWR4099 detected during the present study. It is therefore, concluded that marker Xgwm503 is closely linked with newly identified *Rf8* gene.

Discussion

In the past, a number of studies have been conducted with a view to unravel the genetics of nuclear fertility restoration in wheat. These studies reported varying results suggesting variability in the genetic control of the fertility restoration in wheat. While studying the genetics of fertility restoration, Wilson (1968) reported one major factor and some minor factors. However, Schmidt and Johnson (1963) reported two dominant genes controlling fertility restoration. While both dominant and recessive genes were reported by Maan (1992) and two independent dominant genes (one with a major effect) exhibiting semi-epistatic interaction were reported by Tomar et al. (2004). Further, Zhou et al (2005) observed that the fertility restoration gene *Rf3* behave as partially dominant to confer fertility restoration. Nonaka et al. (1993) observed that one dose of *Rfv*1 gene was enough to restore complete fertility in *Ae. kotschyi* cytoplasm but contrary to it, Ikeguchi et al (1999) stated that a single dose of *Rfv*1 was insufficient to restore a high level of fertility. Classical studies conducted in rice involving different fertility restorer lines also indicated that fertility restoration of WA cytoplasmis controlled by a single gene as well as two dominant

genes (Chaudhury et al. 1981; Govind Raj and Virmani, 1988; Ganesan and Rangaswamy 1997). Similarly, Fu and Xue (2004) clarified that one *Rf* gene in restorer lines T984 and H921 and two *Rf* genes in the restorer lines Milyang46 and H804 in rice controlled fertility restoration for ID-type CMS lines. In *Secale cereale* also the restoration is determined by at least three major genes (*Rfg1, Rfg2* and *Rfg3*) (located on chromosome arms 1RS, 4RL and 6R) and a number of genes with smaller effects (on chromosome arms 3RL, 4RL, 5R and 1RS) identified using different mapping populations (Miedaner et al. 1997).

In wheat, seven fertility restorer genes (*Rf1* to *Rf7*) have been reported so far and out of these genes, only *Rf3* was mapped on short arm of chromosome 1B (Zhou et al. 2005). During the present study, the distribution of seed set per spike (≥5 to 82) observed in F_2 populations comprising FF and PF plants, correspond in appearance to continuous phenotypic variation governed by a single major gene as evident from the polygon generated through seed setting data (Figure 2B), which converts an otherwise qualitative character into quantitative one. It is therefore, assumed that some modifying genes that are segregating in the mapping population have conspicuous effect on the fertility/sterility phenotype in the F_2 population. These modifying genes seem to have cumulative small effect on seed set controlled by a major *Rf* gene. It is likely that these modifier genes affecting fertility restoration, may be dispersed throughout the genome and if their number is not determinable, it is not possible to cull out the effect of individual modifiers in the *T. timopheevi* cytoplasm.

The variation in pollen fertility per cent observed during the present study may also be due to the genetic background of F_2 segregants. However, the frequency distribution of F_2 plants with respect to pollen fertility showed that the actual situation is much more complex most probably due to the segregation of the modifier genes in F_2. The observed seed set in FF was very high number of seeds per plant (82) in single plans indicating that homozygous and heterozygous plants set almost equal number of seeds per spike. However 16 PF plants had seed set per spike ranging from 5 to 35. Borner et al (1998) considered the plants setting on average ≤ 5 seeds per spike as male sterile and those setting ≥20 seeds per spike as male fertile plants. However they excluded plants producing 6-19 seeds per spike from the mapping population, which is good approach to eliminate any spurious associations. Li et al (2008) considered plants setting ≤5 seeds per spike as partially sterile, but the present study considered ≤4 seeds per spike as PS. Ali et al (2011) concluded that the modifiers largely influence phenotypes of the heterozygous (*Rf rf*) plants both in negative and positive directions. However the F_1 plants that are heterozygous (*Rf rf*) are generally not

affected because of complementarity of fertility restoring genes and the modifiers thus making them highly fertile. They studied the pollen fertility in F_2 generation derived from the crosses, 2041A × EC368169R and 2019A × T2003R (fertility restorer), and the inflicting point was observed at 10% and 60% pollen fertility. In fact the number of fertile plants in F_2 generation showed continuous variation in seed set. The continuous variation may be described due to the minor genes or modifiers influencing the expression of seed set. The inflicting point at 4 seeds/ spike is chosen on the basis of our earlier report (Ali et al. 2011) to fit the hypothesis and the goodness of fit of fertile and sterile plants to a 3 (fertile): 1 (sterile) segregation ratio and concluding that fertility restoration is controlled by a single dominant gene. The seed set in respective B (maintainer line) varied from 35 to 64. Similarly, the insisting point at 30% of pollen fertility was considered for the purpose of classification of fertile and sterile groups, as mentioned above in materials and method section. The F_2 segregants based on pollen fertility and seed set were plotted on a graph (Figures 2 A and 2B), which formed clear-cut fertile and sterile groups rather than the normal distribution indicating that fertility restoration is not a polygenic trait. In present study both pollen fertility (%) and seed set per spike were considered to classify F_2 population to the purpose of genetic analysis.

So far, seven genes (designated from *Rf1* to *Rf7*) have been reported to control the fertility restoration against *T. timopheevi* cytoplasm (Zhou et al. 2005) and except for one gene (*Rf3*), chromosomal locations of the six remaining genes have been determined using monosomic analysis. The, gene *Rf3*, has been mapped using SSR markers (Xbarc207, Xgwm131, and Xbarc61). The present study reports a new and distinct fertility restorer (*Rf*) gene that is located on the short arm of 2D chromosome, which we have designated as *Rf8*, because no other *Rf* gene(s) has been reported on 2D chromosome of wheat in the past.

The validation of marker linked with the novel *Rf* gene may indirectly help in identification of the potential donor genotypes for introgression of *Rf8* gene into new genetic backgrounds using MAS. Therefore, validation of the SSR marker Xwmc503 linked to the new *Rf8* gene at a distance of 3.3 cM on 2DS reported during the present study was carried out using a set of 13 restorer lines (including PWR4099, the parental genotype of the mapping population), two maintainer lines and two CMS lines, which are in the pipeline for development of three line hybrid wheat breeding system at IARI, New Delhi. The SSR marker Xwmc503, closely linked to *Rf8* gene was found to be highly useful in discriminating between the restorer lines and non-restorers i.e. maintainer and male sterile lines of wheat particularly the lines derived from the cross involving PWR4099 as one of the parents. It may be noted that 10 of the

12 restorer lines had similar allele of 200 bp at the SSR locus Xwmc503 linked with *Rf8* gene. Out of these 10 restorer lines, seven restorer lines (T892R, T917R, T918R, T921R, T926R, T963R and T965R) derived from PWR4099 amplified 200 bp allele specific to PWR4099. This suggests that during the course of introgression of *Rf8* gene into different genetic backgrounds, no crossover had occurred between the marker locus Xwmc503 and the gene. Another indigenous primary fertility restorer line PWR2003 also carried the 200bp allele at Xwmc503 locus, suggesting that this may also be carrying *Rf8* gene for fertility restoration. Furthermore, the two fertility restorers, namely PWR4101 and EC368169R, which carried the alleles of 140 bp and 170 bp size, respectively, at the Xwmc503 locus possibly did not possess the *Rf8* gene. Overall, the marker Xwmc503 linked to *Rf8* gene showed high selection accuracy when related materials were used for testing the presence of *Rf8*. Therefore, we are tempted to conclude that the marker Xwmc503 could be used effectively in marker assisted selection (MAS) aimed at introgression the *Rf8* gene from PWR4099 into different genetic backgrounds. In future, fine-mapping of the genomic region carrying *Rf8* gene may be carried out to identify the candidate gene(s) responsible for fertility restoration in wheat.

In conclusion, the SSR marker Xwmc503 linked to new fertility restorer gene *Rf8* may play a crucial role in MAS to accelerate breeding of elite fertility restorer lines with enhanced efficiency. In addition, the marker may also be used for evaluation of seed purity of hybrid seed at the seedling stage and can become an alternative to the time consuming and laborious grow out test (GOT).

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Conflict of interest statement

None declared.

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Legend to Figures:

Figure 1. Pollen fertility analysis of parental lines

A view of stained pollen grains of cytoplasmic male sterile lines (A) and restorer line (fertile) (B) of wheat under 10 × and 40 × magnifications

Figure 2. Frequency distribution in F_2 population of the cross 2041A × PWR4099

A. Distribution based on per cent pollen fertility of single plant bagged before anthesis

B. Distribution based on seed set per main spike

Figure 3. Genotyping of linked markers in mapping population

Representative gel picture showing results of genotyping of the two parental genotypes (PWR4099 and 2041A) and representative 44 plants of the F_2 mapping population of wheat derived from the cross 2041A × PWR4099 using SSR Xwmc503.

Figure 4. Genetic position of *Rf8* gene in chromosome

A. Reference wheat consensus SSR map (source: gramene.org). B. Genetic map of the region of the wheat chromosome arm 2DS containing fertility restoration (*Rf8*) locus. Markers are indicated on the right side and map distances (in cM) are given on the left side.

Figure 5. Validation of closely linked marker in a set of known lines

Amplification profile of SSR Xwmc503 linked with fertility restorer (*Rf8*) gene in a parental lines and additionally set of 12 fertility restorer (R) lines, two maintainer (B) lines and one cytoplasmic male sterile (A) lines of wheat with a view to validate the marker.

Table 1. Pedigree and sources of genotypes used in the study

S. No.	Genotypes	Pedigree	Source
1	PWR4099	CBHW-R CHN QI RR925 OCHN S-4 BV97 = EC414149	Mexico
2	2041A	Lok1*7//Sunstar*6/C80-1	India
3	PWR4101	CBHW-R CHN 89R 4294 OCHN S-2 BV97 = EC414148	Mexico
4	T 892R	ACMS2099/(PWR4099/ PWR4101)	India
5	T 917R	HW2045/PWR4099	India
6	T 918R	HW2045/PWR4099	India
7	T 921R	HW2045/PWR4099	India
8	T 926R	ACMS2022/PWR4099	India
9	T 939R	2042A/EC368169	India
10	T 955R	2041A/EC368169	India
11	T.963R	PBW226/Lr37/PWR4099	India
12	T 965R	PBW226/Lr37/PWR4099	India
13	PWR2003	HD69/NP839//S310//NP830	India
14	EC368169R	Not known, Exotic Collection	France
15	2019A[a]	WH542*6/TR380-14*7/3Ag #14	India
16	HW2019 (B)	WH542*6/TR380-14*7/3Ag#14	India
17	HW2041(B)	Lok1*7//Sunstar*6/C80-1	India

PWR4099: perfect restorer line and male parent of mapping population; 2041A: cytoplasmic male sterile line having *T. timopheevi* cytoplasm and female parent of mapping population; A: cytoplasmic male sterile line; B: maintainer line; R: restorer line; [a] cms line having *Triticum araraticum* cytoplasm.

Table 2. Segregation for pollen fertility and seed set in F_2 mapping population derived from the cross 2041A × PWR4099

Genotype	Generation	Pollens/seed set in F_2 plants			Expected segregation ratio	χ^2 value	P-value (5%)
		Total number of plants	Number of fertile plants	Number of sterile plants			
Pollen fertility							
2041A	P_1	6	0	6	-	-	-
PWR4099	P_2	10	10	0	-	-	-
2041A × PWR4099	F_1	10	10	0	-	-	-
2041A × PWR4099	F_2	120	97	23	3:1	2.84	0.091
Seed setting							
2041A	P_1	6	0	6			
PWR4099	P_2	10	10	0			
2041A × PWR4099	F_1	10	10	0			
2041A × PWR4099	F_2	120	98	22	3:1	2.17	0.140

Table 3. Details of polymorphic markers linked to the fertility restorer (*Rf)* gene

SSR marker	Primer sequence (5'-3')	Tm	Product size (bp)	
			Sterile parent allele	Restore parent allele
Xwmc503	F: GCAATAGTTCCCGCAAGAAAAG R: ATCAACTACCTCCAGATCCCGT	61	170	200
Xgwm296	F: AATTCAACCTACCAATCTCTG R: GCCTAATAAACTGAAAACGAG	55	150	132
Xwmc112	F: TGAGTTGTGGGGTCTTGTTTGG R: TGAAGGAGGGCACATATCGTG	61	230	220

Tm: annealing temperature of primers; F: forward primer sequence; R: reverse primer sequence

Table 4. Segregation pattern of three SSR markers in the F_2 population derived from the cross 2041A × PWR4099 of wheat

SSR marker	Total number of plants	Segregation pattern of SSR alleles			χ^2 value	P-value (5%)
		AA	AR	RR		
Xwmc503	120	23	64	33	2.20	0.33
Xgwm296	120	25	66	29	1.46	0.48
Xwmc112	120	27	62	31	0.13	0.93

AA: defines presence of sterile parent allele in homozygous conditions; AR: defines the presence of both sterile and fertile alleles in heterozygous conditions and RR: defines the presence of fertile parent alleles in homozygous conditions

Table 5. Mean values of pollen fertility (%) in F_2 plants belonging to different allele classes and significance of difference between their mean pollen fertility (%) values

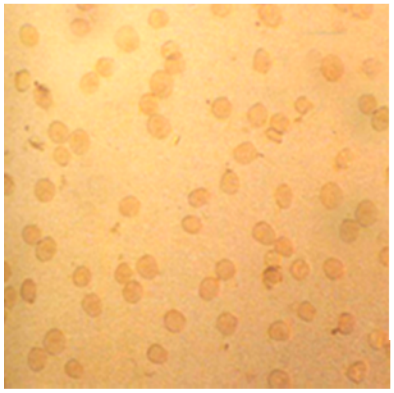
SSR Marker	Mean values of pollen fertility (%)			Significance of difference of mean pollen fertility (%) values		
	A ± SD	H ± SD	R ± SD	A-R	A-H	R-H
Xwmc503	7.13±9.10	84.88±16.45	81.83±16.90	*	*	NS
Xgwm296	8.43±7.67	81.98±14.58	80.57±15.54	*	*	NS
Xwmc112	7.29±7.92	86.07±12.15	82.22±17.66	*	*	NS

A: plants carrying sterile parent type allele; H: heterozygous plants carrying both the fertile and sterile parent types of alleles; R: plants carrying fertile parent type allele; SD: standard deviation; *difference of means significant at 5% level of significance; NS: difference of means not significant at 5% level of significance.

Table 6. Validation of molecular marker Xwmc503 linked with *Rf*-gene on a set of known restorer, maintainer and CMS lines

S. No.	Genotypes	Details	Xwmc503		
			Sterile parent allele (170bp)	Fertile parent allele (200bp)	Other allele (140bp)
1	PWR4099[a]	Restorer	-	+	-
2	2041A[b]	CMS	+	-	-
3	PWR4101	Restorer	-	-	+
4	T 892R	Restorer	-	+	-
5	T 917R	Restorer	-	+	-
6	T 918R	Restorer	-	+	-
7	T 921R	Restorer	-	+	-
8	T 926R	Restorer	-	+	-
9	T 939R	Restorer	-	+	-
10	T 955R	Restorer	-	+	-
11	T.963R	Restorer	-	+	-
12	T 965R	Restorer	-	+	-
13	PWR2003	Restorer	-	+	-
14	EC368169R	Restorer	+	-	-
15	2019A[c]	CMS	-	-	+
16	HW2019B	Maintainer	-	-	+
17	HW2041B	Maintainer	+	-	-

[a]Perfect restorer line for *T. timopheevi* cytoplasm and male parent of mapping population; [b]Cytoplasmic male sterile line having *T. timopheevi* cytoplasm and female parent of the mapping population; [c]CMS line having *Triticum araraticum* cytoplasm. The details of pedigree and origin of each line are mentioned in Table 1



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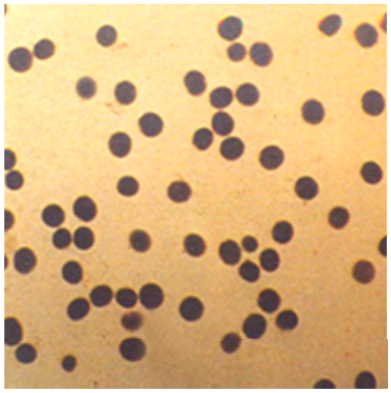

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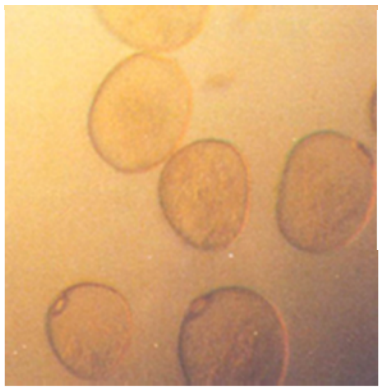

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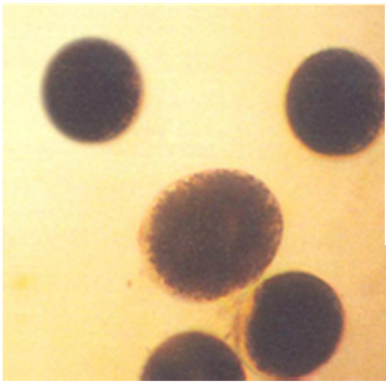

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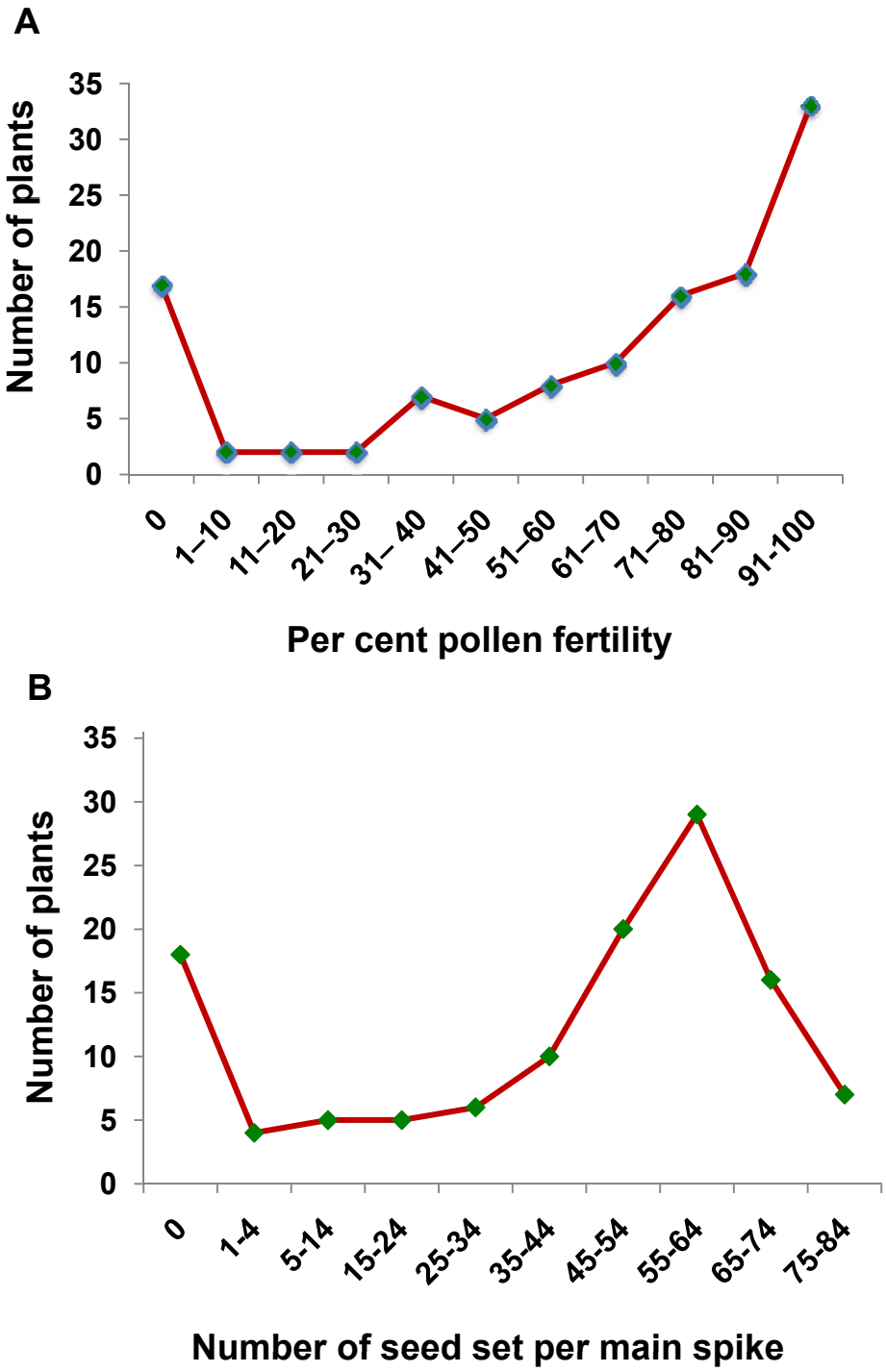
Figure 1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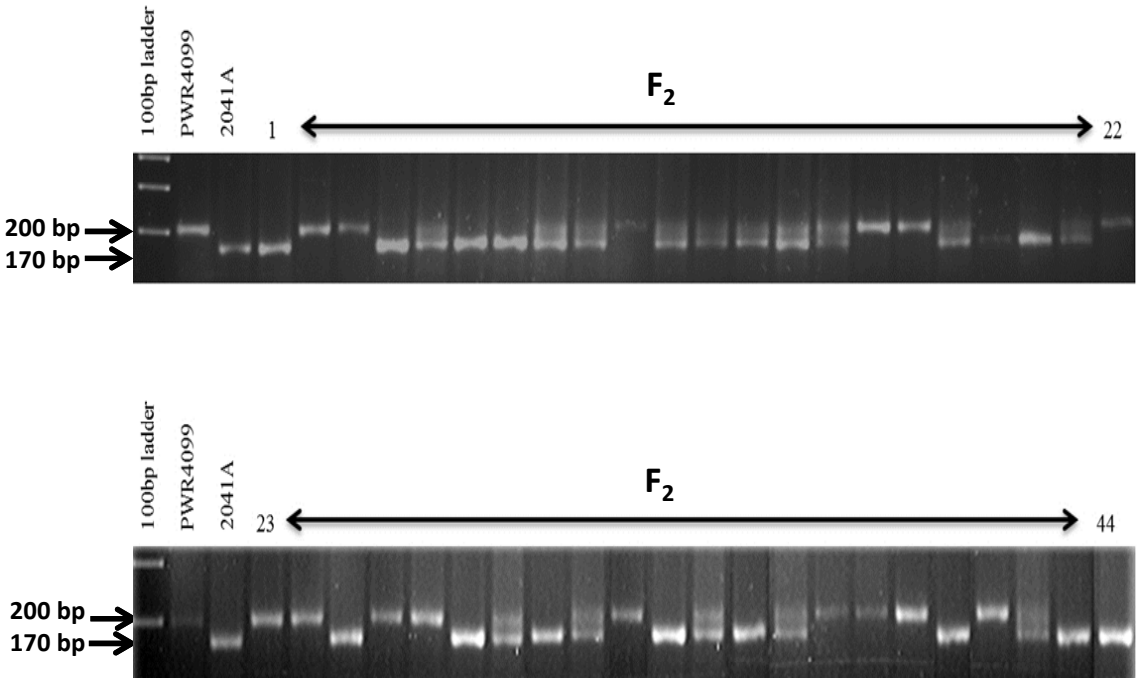
Number of plants
35
30
25
20
15
10
5
0
0
1–10
11–20
21–30
31– 40
41–50
51–60
61–70
71–80
81–90
91-100
Per cent pollen fertility
B
Number of plants
35
30
25
20
15
10
5
0
0
1-4
5-14
15-24
25-34
35-44
45-54
55-64
65-74
75-84
Number of seed set per main spike

Figure 2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PWR4099
2041A
1
F2
22
200 bp
170 bp
100bp ladder
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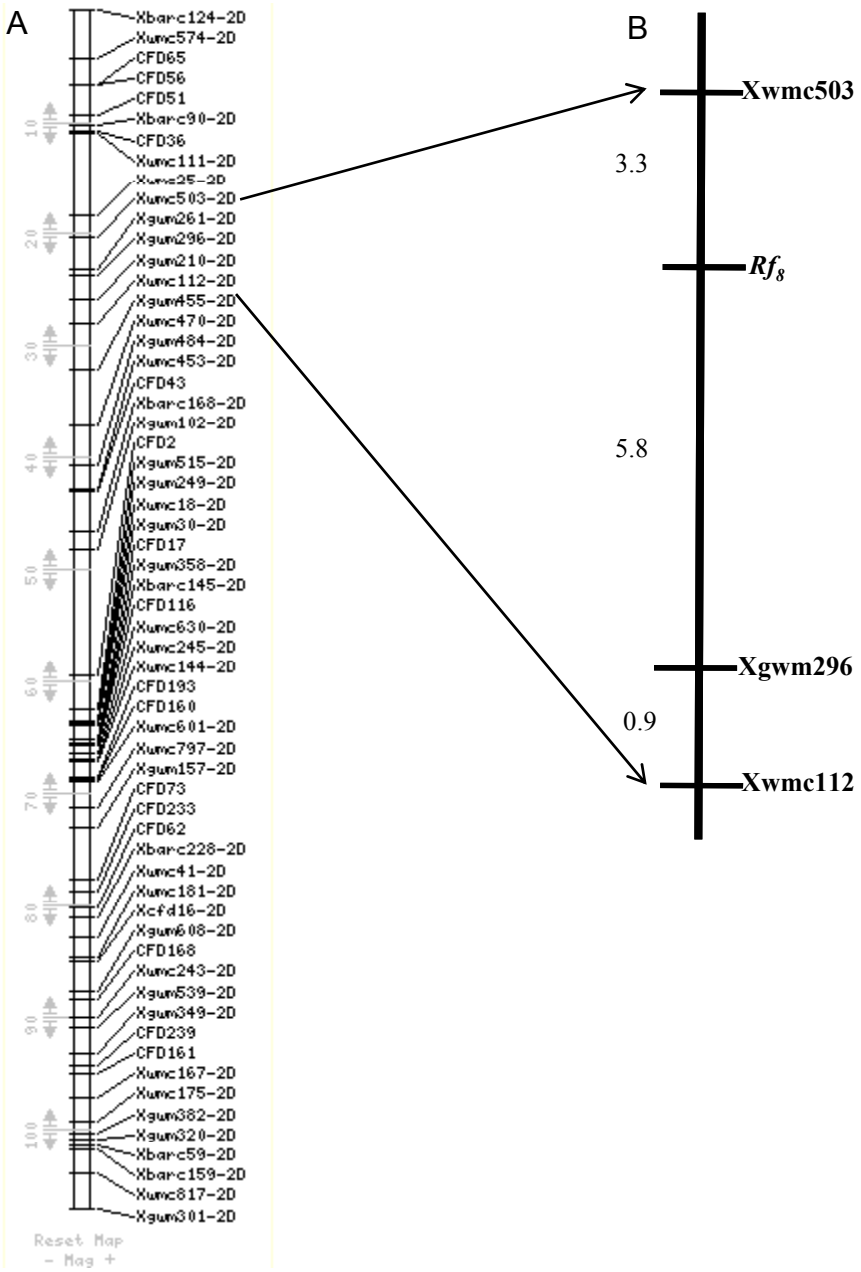
2041A
23
F2
44
200 bp
170 bp

Figure 3.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CFD36
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Xgum261-2D
Xgum296-2D
Xgum210-2D
Xumc112-2D
Xgum455-2D
Xumc470-2D
Xgum484-2D
Xumc453-2D
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CFD2
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CFD193
CFD160
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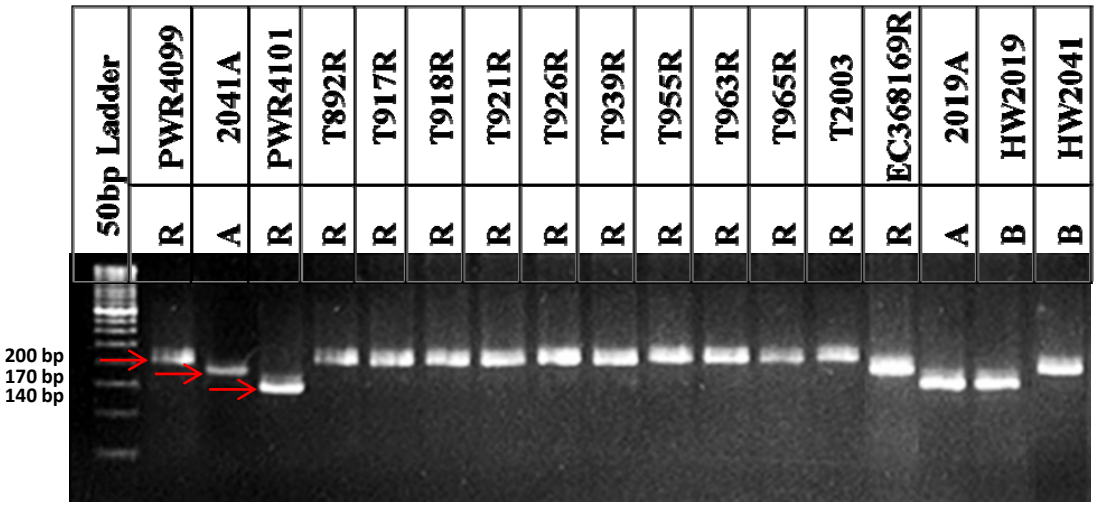
Xumc797-2D
Xgum157-2D
CFD73
CFD233
CFD62
Xbarc228-2D
Xumc41-2D
Xumc181-2D
Xcfd16-2D
Xgum608-2D
CFD168
Xumc243-2D
Xgum539-2D
Xgum349-2D
CFD239
CFD161
Xumc167-2D
Xumc175-2D
Xgum382-2D
Xgum320-2D
Xbarc59-2D
Xbarc159-2D
Xumc817-2D
Xgum301-2D
Reset Map
- Mag +
B
Xwmc503
3.3
Rf8
5.8
Xgwm296
0.9
Xwmc112

Figure 4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T965R
T2003
EC368169R
2019A
HW2019
HW2041
R
A
R
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R
R
R
R
R
R
R
R
R
R
A
B
B
200 bp
170 bp
140 bp

Figure 5.