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Genetic studies and a search for molecular markers that are linked to *Striga asiatica* resistance in sorghum

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Sorghum [*Sorghum bicolor* (L.) Moench] is important both as a food and feed crop in Zimbabwe. Its yield losses can be up to 100% when the crop is heavily infested by witchweeds [*Striga asiatica* (L.) Kuntze]. Witchweed resistant cultivars offer the most practical control option under smallholder (SH) farmer conditions and could become part of a sustainable integrated control strategy. Development of *S. asiatica* resistant cultivars by conventional breeding is slow and has been hampered by the lack of efficient and reliable screening techniques in breeding programs. Molecular markers that are linked to witchweed resistance can expedite the development of resistant cultivars through adoption of appropriate marker-assisted selection (MAS) strategies. The objectives of this investigation were to study the inheritance of low germination stimulant (*Igs*) production in cultivar SAR 29 and to identify molecular markers that are linked to this trait. Low germination stimulant production is one of the recognised mechanisms of witchweed resistance. A segregating F₂ population derived from crosses between cultivars SV-1 (high germination stimulant producer, *Striga*-susceptible) and SAR 29 (low germination stimulant producer, *Striga*-resistant) was used for this purpose. Parental and F₂ genotypes were screened for *Igs* production using the agar gel technique (AGT). Maximum germination distance (MGD) was used as the index of resistance. Deoxyribonucleic acid was extracted from agar gel-screened F₂s, and DNA bulks were created from 16 resistant (MGD<10 mm) and 25 extremely susceptible (MGD>25 mm) progeny. Bulked segregant analysis (BSA) was done using random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers. Ninety-nine of the primers that were polymorphic between parent genotypes (10 SSR and 89 RAPD) were then used to screen a total of 77 segregating F₂ progeny. Linkage analysis was performed using the computer software MAPMAKER 3.0b. Segregation ratios of high to low F₂ stimulant producers did not differ significantly ($P \geq 0.05$) from the expected ratio of 3:1. It was therefore deduced that a single recessive gene controlled *Igs* production in cultivar SAR 29. No molecular marker was found to be linked to the *Igs* locus. Instead, linkage analysis resulted in the construction of a molecular marker linkage map consisting of 45 markers that were distributed over 13 linkage groups (LGs). The other fifty-four loci, including the locus for *Igs* production, were completely unlinked and could not be assigned to any linkage group. The LGs consisted of 2-8 markers, identified at a LOD grouping threshold of 4.0. The map spanned a total distance of 494.5 cM, Haldane.

Key Words: Witchweed, *Striga asiatica*, bulked segregant analysis (BSA), Linkage map, marker assisted selection (MAS).

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Abbreviations: *Striga asiatica* resistant = SAR; low germination stimulant = *Igs*; agar gel technique = AGT; Maximum germination distance = MGD; Smallholder = SH; Linkage group = LG; likelihood of odds ratio = LOD.

INTRODUCTION

Sorghum is a drought tolerant cereal grain crop that is grown by smallholder (SH) farmers in marginal rainfall areas of Zimbabwe. It is ranked as the third most important cereal crop in Zimbabwe, after maize and wheat (FAO, 1996). White sorghum is ground into flour and used mostly for making both "Sadza" (thick

porridge) and thin porridge. Its grain is used for making a rice-like product which is cooked in a mixture with groundnuts, cowpeas or bambara nuts to improve the flavour and nutritional value (Mushonga et al., 1992). Both SH farmers and breweries use red sorghum malt to make opaque beer and non-alcoholic beverages. Sorghum is also used in livestock and poultry feeds. This small grain crop is cultivated in areas considered to be too dry and hot for maize production because it is tolerant to drought and heat stress.

The major biological constraint to increased sorghum production in the SH sector in Africa is attack by *Striga* (DeVries and Toenniessen, 2001) or witchweeds. Witchweeds are very devastating obligate root parasites of cereal crops of the family *Gramineae* that includes sorghum, millets [*Pennisetum americanum* (L.) Leek and *Eleusine coracana* (L.) Gaertn], maize (*Zea mays* L.), rice (*Oryza sativa* L.) and sugar cane (*Saccharum* species) (Stroud, 1993). *Striga asiatica* is the most common and devastating parasitic weed in Zimbabwe (Mabasa, 1996). Crop yield losses may be up to 100% when a susceptible cultivar is grown under high levels of infestation (Obilana and Ramaiah, 1992; Haussmann et al., 2000b). Currently recommended control measures require costly additional inputs, which are beyond the means of SH farmers. Resistant cultivars may provide the most economically promising *Striga* control measure since such cultivars can be grown without any additional costly inputs by the SH farmer. *Striga* could therefore be effectively managed using an integrated control approach that utilizes affordable cultural, agronomic and chemical options, in addition to *Striga* resistant cultivars.

Conventional breeding for *Striga* resistance is slow and greatly constrained by lack of reliable screening methods, as well as a lack of knowledge of the genetics of witchweed resistance (Haussmann et al., 2000a). Hess and Ejeta (1992) attributed the lack of progress on inheritance studies and breeding for resistance to rarity of genotypes that exhibit stable resistance across geographical regions, problems of failing to produce uniform field infestations, and the difficulty encountered in evaluating individual segregating progenies for resistance. In addition, there is also a lack of alternative and reliable laboratory screening assays that can adequately predict the field performance of a given genotype (Omanya et al., 2001). Screening for witchweed resistance without regard for the underlying resistance mechanism is not effective. In sorghum, resistance to *S. asiatica* is a manifestation of one or more potential mechanisms that inhibit the development of parasitism (Ejeta et al., 1993; Mohamed et al., 2001). In this investigation, focus was placed on the mechanism of low germination stimulant (*lgs*) production. The advantage of this mechanism is that it is able to impart field resistance to a genotype, even in the absence of other mechanisms (Hess et al., 1992). However, the positive correlation between stimulant production and *Striga* emergence

under field conditions depends on the genetic materials under investigation and the environments in which they are tested (Rattunde et al., 2000).

Molecular (DNA) markers can provide a powerful arsenal for use by the plant breeder. Detecting witchweed resistance genes by their linkage to DNA markers makes it possible to screen for many different resistance mechanisms simultaneously, without a need for inoculation with the *Striga* parasite. Pyramiding of resistance mechanisms to provide durable witchweed resistance is therefore facilitated. Integration of marker-assisted selection (MAS) into *S. asiatica* resistance breeding could permit more rapid movement of desirable genes among varieties (Haussmann et al., 2000b). Marker-assisted backcross breeding would be particularly handy because the procedure can considerably shorten the time required to introgress this recessive trait into an elite cultivar. The objectives of this study were therefore to determine the inheritance mechanism, and identify molecular markers that are linked to *lgs* production.

MATERIALS AND METHODS

Agar gel assay

Surface sterilization of sorghum and *S. asiatica* seeds, conditioning of *S. asiatica* seeds and the agar gel assay were done following procedures described by Hess et al. (1992) with minor modifications. When setting-up the agar assays, 100 l of preconditioned *S. asiatica* seeds were pipetted into petri dishes. Water agar (0.7 %) was then poured over the *S. asiatica* seeds. Roots of germinating sorghum seeds were placed in the solidifying agar with the root tip pointing across the plate. The plates were incubated in the dark at 28°C for five days. Maximum germination distance (MGD), that is, the distance between a host root and the most distant germinated *S. asiatica* seed, was used as the index of resistance (Hess et al., 1992). After classifying parental and F2 genotypes as high or low *lgs* producers in agar, the same seedlings were transplanted into kaolite crates filled with river sand. After seven days, the seedlings were transplanted again into 16 cm diameter by 16 cm depth clay pots that were filled with red clay soil. The seedlings were allowed to grow in the greenhouse for 14 days prior to DNA extraction.

One hundred and thirty- four F2 progenies were assessed for *lgs* production. Analysis of variance was performed for MGD of parent genotypes. Chi-square values and their probabilities were calculated for a single gene hypothesis of 3:1 for individuals of the segregating F2 population. Frequency distributions of MGD were plotted and standard errors calculated for each class with a frequency of more than two genotypes. Phenotypic variances were also calculated for each parent and F2 population. Genetic variance was calculated for MGD following the partitioning of variance components for measurements made in different generations following formulae by Strickberger (1968) as follows:

$$\begin{aligned} V_{P1} &= E \\ V_{P2} &= E \\ V_{F2} &= V_G + E \\ \text{Therefore, } V_G &= V_{F2} - (V_{P1} + V_{P2})/2 \end{aligned}$$

Where, V_{P1} , V_{P2} , and V_{F2} are the phenotypic variances of parent one, parent two and F2 generation, respectively, and V_G is the genetic

Table 1. Characterisation of parent cultivars and their F2-derived progeny for *S. asiatica* seed germination stimulant production.

Genotype	No. of seedlings	Mean [†]	H ² _i	Expected ^o Ratio	Observed ratio ^φ		χ ²	P
					High	Low		
SV-1 x SAR 29	134	2.08 ab	0.70	3:1	106	28	1.20 ^{ns}	0.54
SV-1	5	2.66 a						
SAR 29	5	0.70 c						

[†] Means followed by the same letter are not significantly different ($P \leq 0.05$).

ⁱ Broad sense heritability = $[V_{F2} - (V_{P1} + V_{P2})/2] / V_{F2}$.

^o Expected segregation ratio for a single dominant gene for high stimulant production.

^φ High: maximum distance for germination of *S. asiatica* seeds was ≥ 10 mm. Low: maximum distance for germination of *S. asiatica* seeds was < 10 mm. ^{ns}-Non-significant, $P \leq 0.05$.

variance. Environmental variance is depicted by E . Broad sense heritability (H^2) was estimated according to Klug and Cummings (1994) as follows;

$$H^2 = V_G / V_P$$

where, V_P is the total phenotypic variation for each set of parents and their F2 derived progeny.

DNA extraction and construction of DNA bulks

Extraction of DNA was done using 0.5 g of fresh leaf tissue and a buffer containing sodium dodecyl sulfate (SDS) following a method that was modified from Picknett et al. (1997). Deoxyribonucleic acid concentration for the extracted samples was measured using a Biospec-1601 uv-visible spectrophotometer (Shimadzu Corporation). On the basis of the spectrophotometer readings, 10 ng/ul solutions were prepared and were used in the construction of DNA pools. Resistant and susceptible bulks were prepared by pooling DNA from extremely resistant (MGD <10 mm) and susceptible (MGD >25 mm) F2 genotypes, respectively. The resistant bulk had 16 genotypes while the susceptible bulk had 25 F2 genotypes.

Primer screening and segregation analysis of polymorphic markers

Parent genotypes were screened for polymorphisms using RAPD and SSR primers. Primers that were polymorphic between the parents were then used to screen DNA bulks for differences in DNA sequence at the *Igs* locus as outlined by Michelmore et al. (1991). After failing to detect polymorphisms between bulked DNA samples, the strategy was then to analyse the segregation of individual molecular markers on a total of 77 F2 progeny. Second filial generation progeny that were genotyped consisted mostly of the extremely resistant and susceptible F2 genotypes, with a few samples having intermediate values for MGD. Ninety-nine primers (10 SSR and 89 RAPD) were used to amplify DNA of these F2 progeny.

RAPD and SSR procedures

A total of 440 RAPD primers were screened in this study. They consisted 12 primer kits from Operon technologies (Kits A, B, C, G, H, I, J, K, L, M, Q and R) and two from the University of British Columbia, Canada (UBC100 and UBC400 series). There were 29 *Xtxp* sorghum microsatellite, or SSR, primers that were originally developed at Texas A & M University. A total of seven maize

primers from the *phi* and *nc* series were sourced from CIMMYT (International Maize and Wheat Improvement Centre) Mexico and also used in this study. Polymerase chain reaction conditions and cycling profile for maize primers were based on a protocol published by Senior (<http://www.agron.missouri.edu>). A GeneAmp PCR System 9700 (Perkin-Elmer Applied Biosystems) thermocycler was used for all RAPD and SSR thermal cycling reactions in this investigation. After amplification, RAPD products were separated by agarose gel electrophoresis while SSRs were electrophoresed using 6 % non-denaturing polyacrylamide gels. All gels were stained with ethidium bromide to a concentration of 5 g/l and viewed under UV light using a Gene Genius Bioimaging system (Syngene, Synoptics Ltd). Gel pictures were subsequently scored for polymorphisms prior to data analysis.

Segregation and linkage analyses

Segregation of individual markers was analysed by a chi-square test for goodness-of-fit to a 1:2:1 (ABH) and 3:1 (present:absent) ratios for the codominant SSRs and dominant RAPDs, respectively. Linkage analysis of the entire set of markers was performed using the computer software MAPMAKER/EXP 3.0b (Lander et al., 1987). Mapmaker uses the Lander and Green algorithm to calculate the maximum likelihood genetic linkage map for any given order of loci. Mapping analysis was conducted using a likelihood of odds ratio (LOD) threshold score equal to or above 4.0 and a 0.50 maximum recombination frequency. A molecular linkage map of the resulting linkage groups was subsequently drawn using the software Mapplot.

RESULTS

Parent characterisation

The two sorghum cultivars differed significantly ($P < 0.01$) for MGD. Cultivar SV-1 had a MGD of 26.6 mm and SAR 29 had a MGD of 7 mm (Table 1). Cultivar SAR 29 was therefore resistant while SV-1 was susceptible, following the classification by Hess et al. (1992). Figure 1 shows a picture from the agar gel assay.

Inheritance of low *S. asiatica* seed germination stimulant production in cultivar SAR 29

Maximum germination distances ranged from 0 to 44 mm. Segregation ratios of high to low F2 stimulant



Figure 1. Picture showing the root of cultivar SV-1 that produced germination stimulants causing *S. asiatica* seeds to germinate.

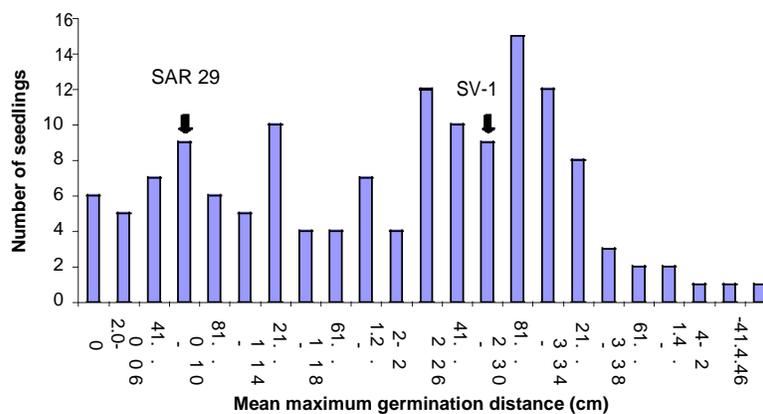


Figure 2. Frequency distributions of segregating F2 progeny from crosses between SV-1 (high germination stimulant producer) and SAR 29 (low germination stimulant producer).

producers did not differ significantly ($P \geq 0.05$) from a ratio of 3:1 (susceptible: resistant) (Table 1). It was therefore apparent that a single recessive gene controlled *Igs* production in cultivar SAR 29. Broad sense heritability (H^2) estimates for MGD were 0.70 (Table 1). This high heritability value indicated that the observed variation for MGD was mostly genetically determined.

The frequency distribution of MGD for the segregating F2 progeny is shown in Figure 2. The class interval for the frequency distributions of MGD among the F2 progenies was 0.2 cm. Block arrows in the graph show mean parental values obtained during the time when their F2 progeny were screened. The F2 progeny were generally put into two groups, low and high *S. asiatica* seed germination stimulant groups. There was, however, considerable variation for MGD within each group as seen in Figure 2. Standard error of means ranged from 0.01 to 0.04 for all different classes of the progeny.

Molecular marker analysis

A total of 2532 bands were amplified using 440 RAPD

primers and thus 7.3 loci were amplified and examined per primer. The number of RAPD fragments that were amplified ranged from 1- 14, and the sizes ranged from 200 to about 2500 bp. One hundred and eighty-seven RAPD primers (7.3%) gave polymorphic bands between the parent genotypes. This low rate of polymorphisms for RAPDs may be attributed to the fact that the variation is intraspecific, in addition to being between genotypes that are in the cultivated gene pool of sorghum.

Out of 29 sorghum Xtxp primers, five failed to amplify while 10 (34.48%) of them amplified bands that were polymorphic between the parents. The remaining 14 Xtxp sorghum SSR primers amplified bands that were monomorphic between SAR 29 and SV-1. Out of seven maize SSR primers, one failed to amplify. Two of them generated polymorphisms while four primers were monomorphic between the parent genotypes.

Segregation analysis of individual marker loci using the chi-square goodness-of-fit test revealed that 63 (63.6%) of the loci segregated normally while 36 (36.4%; 1 SSR and 35 RAPD) exhibited distorted segregation ($P < 0.05$). At a LOD grouping threshold of 4.0, 13 linkage groups (LGs) with 2 to 8 markers were identified. Five LGs

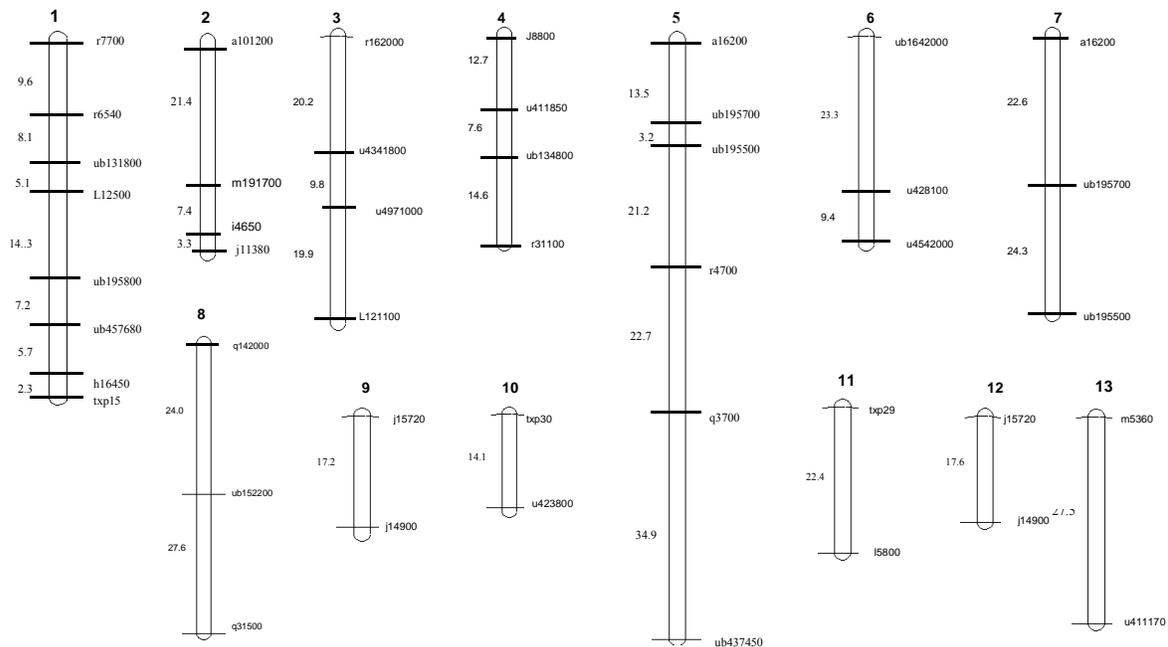


Figure 3. Linkage map constructed from F2 progeny from crosses between SV- 1 and SAR 29. Names on the right of each linkage group are the marker names. Numbers on the left of each linkage group indicate distance between markers in cM (Haldane).

(groups 9, 10, 11, 12 and 13) consisted of two markers each. The shortest was LG 10, with 14.1 cM. The longest LG (group 1) had 6 loci and covered a distance of 95.5 cM (Figure 3). The final map consisted of 45 (2 SSR and 43 RAPD) markers spanning a total of 494.5 cM (Haldane) (Figure 3). Fifty-four loci (54.5 %) were completely unlinked and could not be included in the map. Unlinked loci included the locus for *Igs* production. The average, minimal and maximal distances between markers were 10.99 cM, 2.3 cM and 34.9 cM, respectively.

DISCUSSION

A single recessive gene largely controlled *Igs* production in cultivar SAR 29. This was in agreement with results from similar investigations with other sorghum genotypes (Vogler et al., 1996; Haussmann et al., 2000a; Greiner, et al., 2001). The fact that different sorghum genotypes differ by as much as a billion fold in the amount of germination stimulants that they produce (Ejeta et al., 1993) necessitates that each genotype be considered as a unique case and hence it was important to investigate the inheritance of this trait in cultivar SAR 29. However, considerable variation for MGD was observed among F2 progeny within the low and high germination stimulant groups. This suggested that there were additional minor genes that modified MGD in the F2 populations. It has been reported that witchweed host plants produce several compounds that stimulate *Striga* seed germination. In sorghum, four active compounds that are found in root exudates have been shown to act as germination

stimulants. These sorghum derived germination stimulants are sorgoleone, sorgolactone, strigol and a water-soluble compound with a quantitative biosynthetic pathway that has not yet been identified (Siame et al., 1993; Vogler et al., 1996). The major stimulant from sorghum is sorgolactone and the minor stimulants are structurally related to strigol (Siame et al., 1993). It is therefore possible that all these stimulants were produced by this cultivar causing *Striga* seeds to germinate.

Stuber et al (1999) reported that traits that are considered to be simply inherited might be “semi-quantitative”. Many genes, such as a major gene plus several modifiers, govern trait expression in this case. The fact that *S. asiatica* resistant genotypes support fewer emerged *Striga* under field conditions, or have short germination distances in agar gel assays (<1 cm) may point out to some continuity in variation for resistance as depicted in Figure 2, and hence the existence of modifier genes. The observed variation for MGD was predominantly genetically determined as highlighted by the fairly high heritability estimate (0.70) for MGD. This heritability estimate is comparable with results from other investigations (Haussmann et al 2000a; Haussmann et al., 2001; Vogler et al 1996). These results show that genetic gains can be made if selection for the *Igs* trait is imposed upon populations that are segregating for this trait.

Bulked segregant analysis and segregation analysis of individual molecular markers were both unable to detect a marker that was linked to witchweed resistance. It has been reported that a high level of genetic uniformity exists in *S. bicolor* (Tao et al., 1993). This implies that a much

larger number of primers might have had to be screened in order to identify the ones that would have generated polymorphisms associated with *S. asiatica* resistance in this population. In addition, increasing the sample size of segregating F2 progeny is an alternative way that could have increased chances of identifying markers linked to the *Igs* locus in this investigation. However, the *Igs* locus is now known to be located on linkage group "J" (Bennetzen et al., 2000), or chromosome "1" (Hausmann et al., 2001) of sorghum. It is therefore recommended to start by testing markers that have been found to be linked to this locus in other segregating populations. In addition, markers that are located close to the *Igs* locus, preferably about 20-30 cM on either side of the locus, should be tested for linkage in this population.

RAPD markers are inherited as dominant genetic markers. This limits the application of this marker type, particularly in cases where one would like to distinguish homozygous from heterozygous genotypes. The PCR amplification that generates RAPD fragments of interest is very sensitive to specific reaction conditions (Karp and Edwards, 1997). RAPD 2-8 markers are therefore not reproducible outside mapping populations in which they have been detected. A different population will produce different amplification profiles using the same primers. This is what necessitates the conversion of a RAPD marker that is linked to a gene of interest into a more breeder friendly and reproducible marker such as a sequence characterised amplified region (SCAR). The basis of our approach was to develop a SCAR once a RAPD marker linked to the *Igs* locus would have been identified. The enormous attraction of RAPDs is that the technique is quick, simple, uses small amounts of DNA, sample throughput can be high and the procedure is automatable (Karp et al., 1997). There is also no requirement for DNA probes or sequence information for primer design when one uses RAPDs.

The chromosomal locations of sorghum SSR markers were used as a basis for identifying the possible chromosomes to which some LGs might belong. Marker Xtxp 15 and Xtxp 30 are both located on chromosome 'J' of sorghum (Bhatramakki et al., 2000). In this study, however, they were located on LGs 1 and 10, respectively. Linkage groups 1 and 10 are therefore part of the same linkage group. If additional markers are mapped, then the gaps between these two LGs can be filled. The chromosomal location of sorghum primer Xtxp29 (on LG 11) in the sorghum genome is not known (Bhatramakki et al., 2000). Of all the mapped markers, 19 showed distorted segregation. With the exception of LGs 1, 10 and 12, all the other LGs contained at least one marker with distorted segregation. All the six markers on LG 5 exhibited distorted segregation. Winter et al (2000) reported that the extent of segregation distortion for a given marker is depended upon the overall segregation distortion of the region where they reside. Some genomic regions are more prone to distorted

segregation than others. Clustering of markers with distorted segregation has been observed in sorghum (Hausmann et al., 2002) and several other crops (Menendez et al., 1997; Lu et al., 1998; Sosinski, Reighard, Baird and Abbott, 1998). It is therefore logical to assume that LG5 consists of a cluster of markers with distorted segregation. Segregation distortion has been reported to be around 20 % on average for most segregating populations (Winter et al., 2000). The higher level of distortion observed in this study, 36 %, may be attributed to a small sample size of F2 progeny assayed per primer.

The currently available molecular map was derived from an intraspecific cross, the parent materials having been chosen from the cultivated gene pool of sorghum. Such a map is very useful for practical breeding applications. This is because the segregating SSR markers that were identified were polymorphic within the cultivated gene pool, and they are likely to be present in other crosses between cultivated sorghum genotypes that are of interest to breeders (Menendez, et al., 1997). As already seen, however, informative RAPDs have to be converted to SCARs for them to be reproducible and useful in other segregating populations. It is recognized that most sorghum genetic maps have been constructed using wide crosses. The disadvantage with such maps is that loci that are identified may be polymorphic only between divergent genotypes. Molecular maps based on crosses involving wild progenitors also have little direct application in breeding programmes that usually exploit intraspecific variation within cultivated forms (Menendez et al., 1997).

CONCLUSION

The low *S. asiatica* seed germination stimulant trait in SAR 29 was inherited as a single recessive gene. However, minor genes modified *Igs* production, judging by the variation for MGD that was observed among the

low and high germination stimulant groups of F2 progeny. Broad sense heritability (H^2) values obtained for the population that was studied revealed that genetic factors largely explained the observed phenotypic variation for MGD in this investigation. No molecular marker was linked to the loci for *Igs* production. Linkage analysis resulted in the subsequent construction of a molecular marker linkage map consisting of 45 markers that were distributed over 13 linkage groups and spanning a total distance of 494.5 cM. Fifty-four loci were completely unlinked and could not be included in the map. Unlinked loci included the locus for low *S. asiatica* seed germination stimulant production.

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