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Identification of SSR Markers Linked to Resistance Against the Spotted Stem Borer in Sorghum

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Abstract: Sorghum (*Sorghum bicolor* (L.) Moench) is an important cereal crop grown worldwide. However, spotted stem borer (*Chilo partellus* Swinhoe) is a big threat to the sorghum production in Asia and Southern and Eastern Africa. Genetic improvement of sorghum for resistance against the spotted stem borer has been a very important component in integrated pest management. Conventional resistance breeding has been difficult due to the quantitative nature of the resistance and the complex interactions of resistance factors with environmental factors. Molecular markers linked to resistance genes or QTLs conferring resistance have been useful in cultivar development. This research project aimed at the construction of a genetic linkage map of sorghum with a mapping population developed from a cross between ICSV745 (susceptible) × PB15881-3 (resistant) and subsequent mapping of stem borer resistance QTL in the RIL population (94 RILs) segregating for resistance to spotted stem borer. Two hundred and ten SSR markers were tested for polymorphism among the parents and a few selected offspring. One hundred and four SSR markers exhibited polymorphic among individuals of the mapping population and thus enabled us to assess the segregation of the target traits in a sub set (94 RILs) of the RIL population on polyacrylamide gels. A linkage map was constructed from the data analysis with Join Map 3.0. This map consisted of twenty-two linkage groups, spanning 782 cM of the sorghum genome.

Key words: Sorghum · Simple Sequence Repeat · Linkage mapping · Spotted stem borer

INTRODUCTION

Sorghum, *Sorghum bicolor* (L.) Moench is the fifth important cereal crop, after rice, maize, wheat and barley, grown worldwide. Sorghum is still widely grown in most tropical and sub-tropical regions and will very likely play an important role in poor tropical and subtropical countries in the future as climate conditions and poor soil fertility increasingly hamper the growth of other crops those are less tolerant to water deficit [1].

Several constraints potentially affect grain yield of sorghum including drought, pests and diseases. The spotted stem borer (*Chilo partellus* Swinhoe) is one of the most important pests of sorghum. The insect attacks the aerial parts of the crop after 4 weeks of germination at all growth stages. In the early stage of stem borer, the larvae feed on leaves and cause dead hearts. The larvae feed on leaf, sheath and collar tissues and pollen and tunnel into stalk and ear shank [2]. The stalk tunneling reduces grain yield [3] by interfering with physiological processes, physically weakening the stalk and ear shoot [4] and providing points of entry for pathogens associated with stalk rot.

Development and utilization of sorghum cultivars with improved resistance to stem borer has been recognized as the most effective way for controlling the pest. In general, simply inherited traits, such as qualitative (major gene) resistance, can be introgressed relatively easily from a resistant donor parent to a susceptible breeding line. However, most traits of agricultural

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importance, such as quantitative disease or pest resistance, grain quality, or drought tolerance are more difficult targets for improvement because these traits are often controlled by several genes localized on different chromosomes. In addition, these traits often show a strong genotype environment interaction, further hampering straight forward transfer of the trait from a donor to an acceptor [5].

Conventional breeding for quantitative traits is extremely slow and laborious and the outcomes are often location specific. Application of DNA markers and mapping technology would facilitate crop breeding for complex traits. DNA markers are unlimited in number, discrete, non-deleterious and are free of environment and epistatic interactions. Many markers are even co-dominant (meaning we can differentiate a homozygous pure line from a heterozygote which is still segregating). Most importantly, when these DNA markers for a trait of interest are found, the selection can be carried out in a central location very rapidly, thus saving cost and labor for selection at each test locations as done traditionally [6].

When transferring QTL from a resistant donor to a susceptible parent, DNA markers are identified closely linked to the resistance QTL. These markers can subsequently be employed for foreground selection in a backcross breeding program, where the segment containing markers linked to the resistance QTL is transferred from a donor parent to a susceptible parent. In addition, markers unlinked to the resistance QTL are used to monitor the extent of linkage drag during the backcross breeding process [7].

There are many types of DNA markers currently available. The use of molecular markers has become widely accepted as a valuable tool for plant breeding programs as well as for evolutionary and genetic diversity studies. One of the markers widely applied in breeding programs is the Simple Sequence Repeat (SSR) marker, also called microsatellite. SSRs offer a potentially attractive combination of features that make them useful as molecular markers in crop breeding programs. First, SSRs have been reported to be highly polymorphic in plants and hence highly informative. Second, SSRs can be analyzed by a rapid, technically simple and inexpensive PCR-based assay that requires only small quantities of DNA. Thirdly, SSRs display a co-dominant and simple Mendelian inheritance. Finally, SSRs are both abundant and uniformly dispersed in both human and plant genomes [1, 5, 8]. Their high information content and other favorable characteristics make them excellent genetic markers for many types of investigations including marker assisted selection (MAS) and fingerprinting of germplasm collections. A large number of SSR loci have been genetically mapped in several agronomically important plant species, including wheat [7, 9], rice [10, 11] and sorghum [12-16]. However, using SSR markers to analyze sorghum stem borer has not been reported yet.

The objectives of this study were to screen polymorphic SSR primer pairs among 94 F_5 individuals and to establish the segregation of polymorphic markers among the progenies and to construct a linkage map based on the resultant marker-segregation data. The ultimate goal of this study was to identify QTLs associated with genetic resistance to the spotted stem borer in sorghum.

MATERIALS AND METHODS

Plant Materials: The RIL mapping population, consisting of 354 RIL lines (F_5), was derived from a cross between the two sorghum lines, ICSV745 and PB15881-3. ICSV745 is susceptible and PB15881-3 resistant to stem borer.

DNA Extraction: Mini DNA extraction was performed in 96-well plates using the CTAB method [17]. Crude DNA from 94 RILs and both parents was extracted from one week old seedlings and the DNA was further purified by an RNase digestion followed by extraction with phenol/choroform/iso-amylalcohol (25:24:1) and ethanol precipitation. Completely dried pellets were re-suspended in 100 to 150 μl of TE buffer and kept at room temperature to dissolve completely. The resulted DNA was kept at 4 °C. Agarose gel (0.8%) was used to check the quality and concentration of the DNA samples.

Selection Primers of SSR: Two hundred and ten SSR markers [(95 xtxp markers [14, 15], 33 xcup markers and 82 Celera markers (IS10)] were screened for polymorphism between both parents. One hundred and four SSR markers showed polymorphic between both parents of at least 5 bp (minimal difference in size scorable on polyacrylamide). Segregation of these markers was assessed in a subset (94 F_5 RILs out of 354 F_5 RILs) of the RIL population. Sequences of primer pairs are listed in Appendix 1).

Amplification of SSR: PCR reactions were conducted in a 384-well plate using the PE9700 Perkin Elmer DNA Thermal Cycler (Norwalk, Conn.). The SSR PCR mixtures (5 µl in total) consisted of 2 µl H_2O, 0.5 µl 10x reaction

buffer, 0.75 µl 10 mM $MgCl_2$, 0.5 µl 0.1 mM dNTP, 0.3 µl 0.25 U Taq enzyme, 0.5 µl 0.1 pM/l primer, 0.5 µl 2.5 ng DNA. The PCR program consisted of an initial denaturation for 15 min at 94 °C and then 10 cycles of denaturation for 10 s at 94°C, annealing for 20 s at 61-52°C, (the annealing temperature was reduced by 1°C with each cycle) and extension for 30 s at 72°C and 35 cycles of denaturation for 10 s at 94 °C, annealing for 20 s at 54°C and extension for 30 s at 72 °C]. The last PCR cycle was followed by a 20 min extension at 72°C. PCR products were separated in 6% non-denaturing polyacrylamide gels and silver stained using the procedure of Fritz *et al.* [18].

Data Collection and Analysis: For gel band scoring, bands in the gel were scored as A, B, H, OFF and "-" based on their patterns in comparison with those of the parents. "A" was defined as the presence of the allele from P1 (ICSV 745); "B" was defined as the presence of the allele from P2 (PB15881-3); "H" was defined as heterozygous (both P1 and P2 allele present); "OFF" was defined as an allele from neither P1 nor P2 and "-" was a missing sample.

The SSR linkage map was constructed using the Join Map 3.0, a software package available to develop primary linkage maps from molecular data obtained from experimental crosses. This is a geneticist-friendly computer environment to build linkage maps by simultaneous estimation of all recombination fractions for dominant, recessive, codominant markers. A map composed of markers ordered at a LOD score ¡Ý3.0 was constructed. The LOD score, defined as log 10 of the odds ratio, indicates the "strength" of the linkage between markers, a high LOD score between two markers indicates close linkage between these markers [19].

Recombination frequencies were converted to centiMorgans (cM) using the Haldane mapping function. LOD Groupings thresholds: Lower 1.00, Upper 13.5, step 0.5

The following mapping parameters were used: use of linkage with REC smaller than 0.49, LOD larger than 0.10, performing a ripple each time after adding 1 loci, thresholds for removal of loci with respect to jump in goodness-of-fit 5.00 and showing genotype probabilities with lg10 (p) value larger than 2.00.

RESULTS

Selection of Primer Pairs: In this study, 210 SSR markers were evaluated for their polymorphism in this mapping population. Differential responses of the sorghum lines to the SSR genotyping were documented in PAGE gel (Fig. 1). Among those markers, one hundred and four markers revealed sufficient polymorphism between ICSV745 and PB15881-3 (both alleles differed at least 5 bp in size). Those polymorphic markers included 56 xtxp SSR markers, 14 xcup markers and 34 IS10 markers.

Construction of Linkage Map of Sorghum Stem Borer: The segregation of the 104 polymorphic SSR markers was evaluated among 94 F_5 RIL individuals, representing a subset of the 354 RIL populations. The segregation of the 104 SSR markers was evaluated on polyacrylamide gels, where two markers were pooled per gel. The patterns of amplification products with the SSR primer pairs were shown in Fig. 2. By scoring the specific distribution pattern of polymorphic markers, the markers can be mapped. One hundred and four polymorphic SSR primer pairs were screened on F_5 individuals.

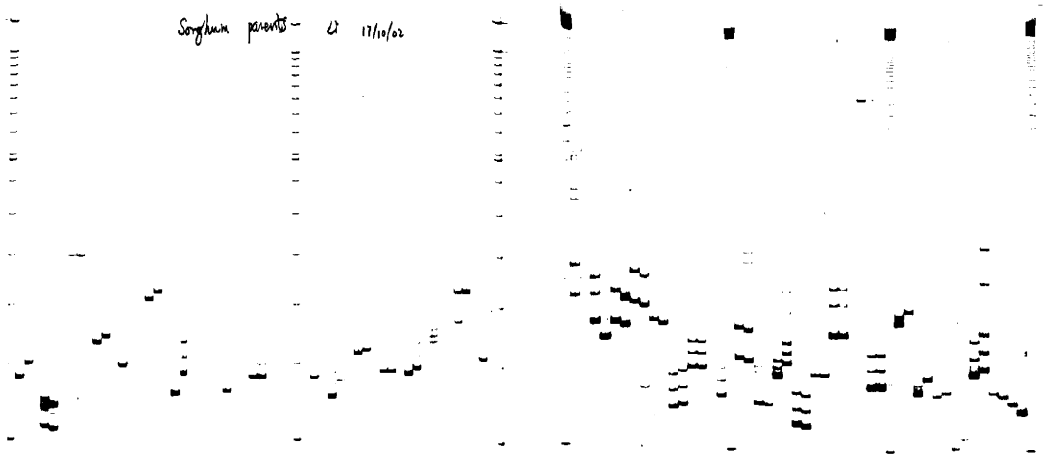

Fig. 1: Images of two PAGE gels for screening parental polymorphism

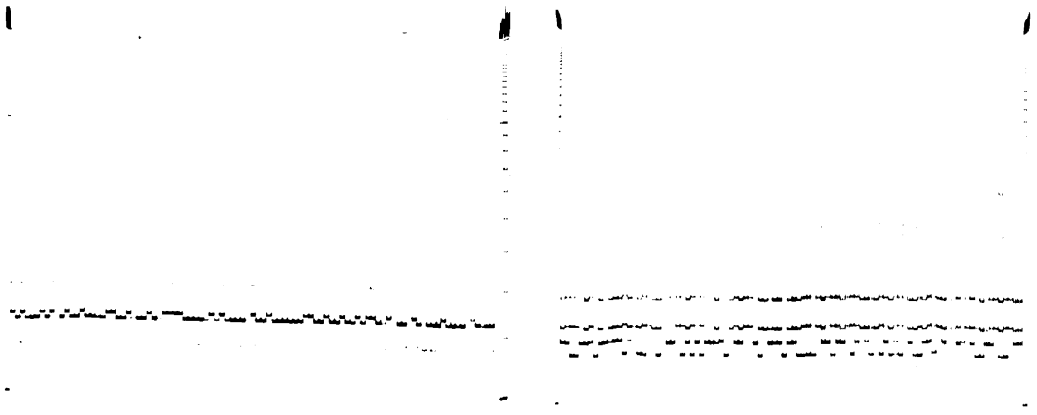

Fig. 2: Images of two PAGE gels revealing the segregation of 8 SSR markers among 94 RILs

Table 1: The length and number of Linkage Group (LG)

Linkage group		The number of xtxp primer pairs	The number of xcup primer pairs	The number of IS10primer pairs	The length of LG(cM)	The number of loci
A	1	4	1	3	84	8
A	2	4	0	1	54	5
A	3	1	1	0	11	2
B	1	2	0	0	25	2
B	2	2	1	1	15	4
B	3	2	1	1	28	4
B	4	5	0	5	69	10
C	1	3	2	1	39	6
C	2	4	0	1	43	5
C	3	2	2	3	43	7
D	1	3	1	1	48	5
E	1	2	0	0	6	2
E	2	1	0	0	23	3
F	1	2	1	0	32	3
G	1	1	0	2	18	3
G	2	1	0	1	12	2
H	1	5	1	3	86	9
I	1	4	0	4	77	8
J	1	1	0	1	18	2
J	2	2	0	0	10	2
J	3	2	0	1	33	3
	1	0	1	1	12	2

Table 2: Comparison of certain marker data with reported research results

	Identity markers			LG length (cM)	
LG	Same order	Different order	New markers	This study	Bhatramakki's
A	xtxp46, xtxp248, xtxp279, xtxp32, xtxp43, xtxp88, xtxp357, xtxp302		IS10322, IS10330, IS10237, IS10359, xcup53, xcup06	149 149/15£½9.9	209
B	xtxp96, xtxp63, xtxp25, xtxp50, xtxp55, xtxp298, xtxp1, xtxp100, xtxp207, xtxp7, xtxp296		IS10245, IS10282, IS10200, IS10334, IS10074, IS10259, IS10228, xcup64, xcup07	137 137/20£½6.9	196.1

Table 2: Continued

C	xtxp69, xtxp34, xtxp38, xtxp31, xtxp205, xtxp 183, xtxp228	xtxp31, xtxp 205, xtxp183	IS10323, IS10307, IS10277	125 125/18£½6.9	165
D	xtxp12, xtxp24, xtxp177	xtxp24, xtxp177	xcup20, IS10343	48 48/5£½ 9.6	140.9
E	xtxp159, xtxp312, xtxp168		IS10365, IS10344	29 29/5£½ 5.8	141
F	xtxp10, xtxp258		IS10365, IS10344	32 32/3 £½10.7	121.9
G	xtxp331 xtxp141		IS10340, IS10263, IS10272,	30 30/5 £½6	125.5
H	xtxp294, xtxp292, xtxp354, xtxp250, xtxp105		IS10198, IS10279, IS10333	86 86/9£½ 9.6	124.5
I	xtxp145, xtxp274, xtxp265, xtxp57		IS10328, IS10264, IS10347, IS10225	77 77/8£½ 9.6	95.7
J	xtxp65, xtxp15, xtxp225, xtxp23, xtxp262		IS10258, IS10350	61 61/7 £½8.7	86.5

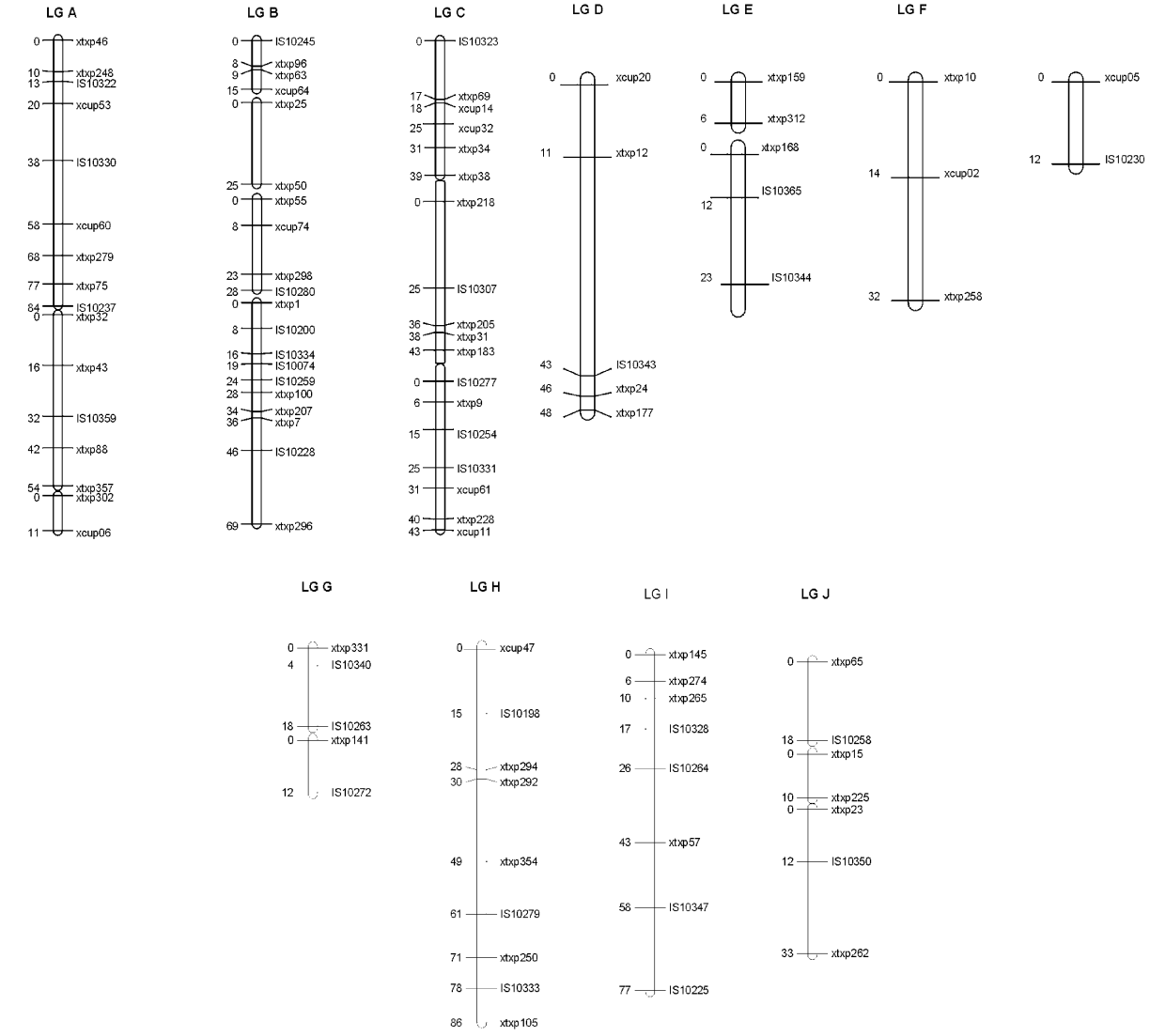

Fig. 3: Genetic linkage map of sorghum

An SSR linkage map was constructed based on the genotyping data using the computer program JoinMap. This linkage map consists of 22 groups, 97 SSR loci, including 54 xtxp loci, 13 xcup loci and 32 IS10 loci (Fig. 9, Fig. 10 and Fig. 11). These loci are in LGA, LGB, LGC, LGD, LGE, LGF, LGG, LGH,LGI. There are 3 groups with 16 loci (9 xtxp primer pairs, 3 xcup primer pairs and 4 IS10 primer pairs) in the LGA. There are 4 groups with 20 loci (11 xtxp primer pairs, 2 xcup primer pairs and 7 IS10 primer pairs) in the LGB. There are 3 groups with 18 loci (9 xtxp primer pairs, 4 xcup primer pairs and 5 IS10 primer pairs) in the LGC. There is 1 group with 5 loci (3 xtxp primer pairs, 1 xcup primer pairs and 1 IS10 primer pairs) in the LGD. There are 2 groups with 5 loci (3 xtxp primer pairs and 2 IS10 primer pairs) in the LGE. There is 1 group with 3 loci (2 xtxp primer pairs, 1 xcup primer pairs) in the LGF. There are 2 groups with 5 loci (2 xtxp primer pairs and 3 IS10 primer pairs) in the LGG. There is 1 group with 9 loci (5 xtxp primer pairs, 1 xcup primer pairs and 3 IS10 primer pairs) in the LGH. There is 1 group with 8 loci (4 xtxp primer pairs and 4 IS10 primer pairs) in the LGI. There is 1 group with 7 loci (5 xtxp primer pairs and 2 IS10 primer pairs) in the LGJ. The length of each linkage group (LG) was showed in Table 5. Linkage analyses revealed twenty-two linkage groups, spanning 782 cM of the sorghum genome.

DISCUSSION

According to the above experimental results, the SSR marker technique has been successfully applied for the construction of sorghum genetic linkage map using a population segregating for resistance to the spotted stem borer. Of the 210 SSR markers screened, 104 markers revealed polymorphism between both parents and hence contributed to successful construction of the genetic linkage map.

The data of the 104 markers were analyzed with JoinMap 3.0 software and 97 markers were mapped into 22 linkage groups (clusters), of which 21 could be assigned to the linkage groups as described by Peng *et al.* [6] and Bhatramakki *et al.* [14]. The 21 linkage clusters consisting of 97 markers were further located in A, B, C, D, E, F, G, H, I and J linkage groups (LG). The map spanned approx.782 cM of the sorghum genome.

The mapping data of the study were compared with those reported by Bhatramakki *et al.* [14] in view of marker similarity, difference and length of linked chromosomal segment of each LG. In linkage group A (LGA), the markers including xtxp46, xtxp248, xtxp279, xtxp32, xtxp43, xtxp88, xtxp357 and xtxp302 are in the same order as reported by Bhatramakki *et al.* [14] and Bowers *et al.* [20], but the distances between the markers were different from that of their reports. Moreover, the length of the LGA in the current map is 149 cM while that in Bhatramakki's report was 209 cM. In this linkage group, several new markers including IS10322, IS10330, IS10237, IS10359, xcup53 and xcup06 were added. In LGB, the markers including xtxp96, xtxp63, xtxp25, xtxp50, xtxp55, xtxp298, xtxp1, xtxp100, xtxp207, xtxp7 and xtxp296 are the same as that reported by Bhatramakki *et al.* [14]. The length of our report of LGB was 137 cM while Bhatramakki *et al.* reported 196.1 cM. The markers identified in this study including IS10245, IS10282, IS10200, IS10334, IS10074, IS10259, IS10228, xcup64 and xcup74 were not included in Bhatramakki's linkage group B.

In LGC, the markers xtxp69, xtxp34, xtxp38, xtxp31, xtxp205, xtxp 183 and xtxp228 are same as that reported by Bhatramakki e atl. [14] while the order of xtxp31, xtxp 205 and xtxp183 was different. In addition, three new markers, IS10323, IS10307 and IS10277, were added to this linkage group. The length of the LGC is 125 cM in this study but 165.0 cM in the previous report.

The comparison for LGD to LGJ are presented in Table 3. In each LG, there were similar markers and some new markers when compared to the previously reported data. However, only a few markers in LGC and D were found to have different order from the reported work. All the lengths of the LGs were shorter than the reported ones, indicating that the number of markers identified in this study seemed still limited. Therefore, future research effort needs to be directed toward the development of more SSR marker for this mapping population to generate a fine map and a better understanding relationship between the molecular markers and the target gene(s) conditioning the genetic resistance to the spotted stem borer.

The closest linkage between markers in this study is 1.0 cM while the longest distance was 85.0 cM. Technically, there might be a way to increase the marker number by using an ABI Prism 3100 genetic analyzer that could distinguish the difference of segments up to one bp, which is necessary for fine mapping. In summary, this study represents the first report on the identification of molecular markers linked to sorghum resistance to the spotted stem borer. These DNA markers developed in this study will be useful in marker-assisted selection for insect resistance breeding against this pest insect in sorghum.

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