GENETICS AND MECHANISMS OF BRUCHID RESISTANCE IN SELECTED COMMON BEAN (*Phaseolus vulgaris* L.) LANDRACES FROM TANZANIA AND MALAWI

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GENERAL ABSTRACT

Bean bruchids are the devastating pests of common bean which causes huge losses during storage. Two landraces Kalubungula and KK25 were identified to be resistant to bruchid infestation and damage but the mechanism and genetics of resistance were unknown. The aim of this study was to (i) characterize the storage protein related to resistance in these landraces (ii) determine genomic region and map QTL's related to resistance (iii) phenotype progenies response to bean bruchid infestation.Two mapping populations KSy and KSw and Nagaga x KK25 recombinant inbred lines was created in this study. We reported no association between resistance to the storage proteins in KSw and KSy. Resistance in Nagaga x KK25 recombinant inbred lines reported to be due to storage proteins Arcelin-5 and Phytohaemagglutinin found at 25KDa. On determination of genomic region and QTL's related to bruchid resistance, a total of 328 novel single nucleotide polymorphisms (SNP's) were reported from KSy and 435 SNP's from KSw assembled in 11 linkage groups of *P. vulgaris*. QTL's associated with bruchid resistance were mapped on linkage group five (PV05) and linkage group nine (PV09) in KSy and KSw mapping populations except for seed size and 50% adult bruchid emergence. Seed size QTL's were distributed on different linkage groups indicating little or no association between the resistance and seed size. To evaluate the response of progenies to bruchid infestation, 15 lines out of 53 (28.3%) were resistant in KSy population and 5 lines (10.4%) out of 48 were resistant in KSw population. Resistant lines identified may be important sources of bruchid resistance in breeding programs and the QTL's may be of importance in marker development for marker assisted selection in common bean.

DECLARATION

I, CAROLINE MARO, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor concurrently submitted in any other Institution.

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DEDICATION

This dissertation is dedicated to God the Almighty, my nieces Neema Faustine, Genevieve Godfrey and Genesee Godfrey to encourage them to be the future scientists.

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ABBREVIATIONS:

APA	-	Arcelin-Phytohaemmaglutinin-Alfa amylase inhibitor
SNP's	-	Single Nucleotide Polymorphisms
RIL's	-	Recombinant Inbred Lines
QTL's	-	Quantitative Trait Loci
GBS	-	Genotyping By Sequencing
PCR	-	Polymerase Chain Reaction
DAE	-	Days to Adult bruchid Emergence
SI	-	Susceptibility Index
LOD	-	Logarithm of the Odds
CAPS	-	Cleaved Amplified Polymorphic Sequences
dCAPS	-	derived Cleaved Amplified Polymorphic Sequences
CIAT	-	Centro International de Agriculture Tropical
SUA	-	Sokoine University of Agriculture
OSU	-	Oregon State University
FAOSTAT	-	Food and Agriculture Organization Statistics
PICS	-	Purdue Improved Crop Storage
SDS-PAGE	-	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
S.E.D	-	Standard Error of Difference

CHAPTER ONE

1.0 INTRODUCTION

1.1 Importance of Common Bean

Common bean (*Phaseolus vulgaris* L.) is the grain legume that is grown worldwide and adapted to broad environmental conditions. It is the food that is consumed as a substitute for meat as fresh, dry and green and can be kept for 3-4 years if stored in a cool dry place although as time goes its nutritive value and taste decrease (Rusike, 2012). Bean provide important source of protein (~ 22%), vitamins (folate) and minerals (Ca, Cu, Fe, Mg, Mn and Zn) for human diets especially in developing countries (Broughton *et al.,* 2003). Beans have been found to be the second important source of dietary protein and third important source of calories after maize and cassava (Sarikamis *et al.,* 2009). The crop is also used as a source of income for farmers households hence contribute in improving their livelihood (Muimui, 2010). Apart from dietary and income generation, common bean also has medicinal properties linked with reduced cholesterol and mitigation of chronic heart conditions, diabetes as well as certain types of cancers especially prostate, breast and colon cancer (Hangen and Bennink, 2003).

1.2 Bean Production Trend

The common bean is produced worldwide with North and South America being the major producers (50% of the total world production) mainly in Brazil, Mexico and United States followed by Africa which produce 25% of the total world production (Beebe *et al.,* 2013). In East Africa 4.7 million tons was produced in 2014 with

Tanzania being the leading country in production (1.0 million tons) exceeding Uganda (0.88 million tons) and Kenya (0.62 million tons). Total production in Tanzania ranges from 1.2 million tons in 2012 to 1.0 million tons in 2014 (FAOSTAT 2012/2014). More than 90% of the production is done by small scale farmers under the farm size ranging 0.5-2.0ha (Ndakidemi *et al.,* 2006). While Tanzania may be the top producer in East Africa, there was a decrease in production from 2012 to 2014. Both biotic and abiotic factors may contribute to reduced yields which includes genetically low yielding varieties, diseases, poor soil fertility, drought and insect pests that attack beans both in field and in storage (Hillocks *et al*., 2006).

1.3 Effects of bean bruchids to common bean

Bean bruchids also called bean weevils are among the pests that cause decrease in bean production due to high postharvest losses they cause during storage. Two species of bruchids namely *Zabrotes subfasciatus* (Boheman) and *Acanthoscelides obtectus* (Say) are the main bruchids known to infest common bean (Blair *et al.,* 2010). The occurrence of these pests is influenced by the environment whereby *Z. subfasciatus* is confined to warmer areas and infest beans only in storage while *A. obtectus* is found in cooler climates (high altitude or latitude) infesting beans both in field and during storage (Teshale, 2010). In Tanzania both species co-exist and a considerable yield loss of up to 48% has been reported due to bruchid infestation. Total crop loss also can occur upon longer storage periods (Kusolwa *et al.,* 2009). Due to these losses, farmers grow small quantities of beans and sell most of their beans soon after harvest to avoid large storage losses (Nchimbi and Misangu, 2002).

1.4 Bruchid management strategies and limitations

Farmers use both cultural and chemical management strategies to prevent heavy losses due to bruchid attack. The strategies commonly used are short storage period, reduction in quantity of production in any one season (both strategies allow the farmer to sell the beans in the market quickly), mixing beans with wood ashes, the use of dust, storing beans with threshing residues, frequently sun drying, tumbling of the storage containers, the use of botanicals as well as use of chemicals (Mbongo *et al*., 2013; Kusolwa *et al.,* 2013). The use of chemicals is expensive to small scale farmers and may pose toxicity problems to environment and consumers and are therefore less frequently used by small scale farmers. The uses of non-chemical methods are only effective when small seed lots are stored. Hermetic technologies like the use of Purdue Improved Crop Storage (PICS) bags was reported to be effective control for bruchids but sustainable introduction requires the technology to be profitable to producers as PICS bags found to provide substantial returns to storage for marketing producers in most marketing regions but do not provide the highest returns to storage (Jones *et al*., 2011). Nevertheless the methods are less effective to *A. obtectus* as the pest starts infestation from the field hence farmers may begin to incur losses before harvest and application of control measures (Beaver *et al*., 2003).

1.5 Genetic resistance

Genetic resistance in common bean shows promising results against bean bruchids. High levels of resistance were identified in wild bean accessions from central highlands of Mexico (Singh and Schwartz, 2011). The resistance is associated with the plant derived insect defense seed storage protein arcelin which is found in a

complex locus that includes α-amylase inhibitor and phytohaemagglutinin (Kusolwa and Myers, 2012). Screening of these wild accessions was performed at CIAT whereby 210 accessions were tested for resistance to *A. obtectus* and *Z. subfasciatus* and high levels of resistance to each species of seed weevils were found (Schoonhoven *et al.*, 1983). Resistance transfer from the wild accessions to cultivated genotypes has been done and satisfactory levels of resistance have been reported but only one arcelin derived bruchid resistant germplasm AO-1012-29-3-3A have been officially released (Kusolwa 2007; Kusolwa *et al.*, 2009; Kusolwa *et al.*, 2012; Kusolwa *et al.*, 2016).

Screening for bruchid resistance of cultivated genotypes has been performed at Sokoine University of Agriculture to identify if there are resistant cultivated genotypes apart from the wild resistant genotypes reported previously. The cultivated common bean landraces among other varieties were collected from farmers in major bean growing areas in Tanzania and Chitedze Agriculture Research Institution in Malawi (Kananji, 2007). Screening of these landraces was performed using both *A. obtectus* and *Z. subfasciatus* in no choice controlled insect feeding experiment. Two landraces known Kalubungula and Nagaga X KK25 were found to be resistant based on number of damaged seeds, number of bruchid emerged, delay in days to 50% adult bruchid emergence and susceptibility index but the genetics of resistance and the mechanisms of resistance in these landraces are not known.

1.6 General Objective

The general objective of this study is to establish parameters for genetic resistance found in landraces from Tanzania and Malawi against bean bruchids for reduced postharvest losses in common bean.

1.7 Specific Objectives

1) To characterize the seed storage protein related to bruchid resistance in the developed progenies and parents.
2) To determine the genomic region related to resistance and map QTL in the resistant parents and their progenies.
3) To evaluate the response of progenies in bean populations developed from crosses between resistant and susceptible parents to bean bruchid attack.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History of Common Bean and its Distribution

Common bean evolved from wild plants growing as vines distributed in the highlands of Middle American and Andes with domestication occurring around 2500 years for Mesoamerican and 4400 years for Andean beans. This led to two major gene pools namely Andean and Mesoamerican (Gepts and Debouk, 1991). More than 30 species exist but five of them *P. vulgaris, P. lunatus, P. coccineus, P. acutifolius and P. polyanthus* were domesticated with *P. vulgaris* being mostly grown (Debouck, 2000). Following domestication, the crop was disseminated and introduced by Spanish and Portuguese into other parts of the world. Prior to the Columbian exchange, Mesoamerican cultivars became pre-dominant in Brazil and Southwest United States whereas Andean cultivars were introduced by European traders and became pre-dominant in Africa, Europe and Northeast United States (Gepts and Bliss, 1988). The crop is now widely spread and cultivated as a major food crop in many tropical, subtropical and temperate areas of America, Europe, Africa and Asia (Wortmann *et al.,* 2006).

Two market classes of *P. vulgaris* exist known as snap beans and dry beans with the later having large production and consumption (Blair *et al*., 2006). The difference between these two market classes is that dry bean have thin mesocarp and their pods have fibers while snap beans have thick succulent mesocarp and their pods as well as sutures have little or no fiber at all (Myers, 2000). Dry beans are normally harvested

and consumed as fresh or dried before consumption but snap bean are harvested and consumed as fresh, frozen or canned (Singh, 2001).

Generally it has been proved that America particularly Andean region of South America, Argentina and Mexico are the centers of common bean origin and primary center of domestication based on morphological and molecular levels (Mensack *et al*., 2010). Now the crop is distributed throughout the world and consumed as essential part of human diet.

2.2 Bean Economic and Nutritional Importance

Common bean is a crop with high profit margins compared to other crops such as cereals. In Brazil, bean was reported to give quick investment return to large scale farmers. In Central America small scale farmers indicate that the best income generation comes from producing beans (Broughton *et al*., 2003). In addition to its value bean as an important commercial crop contributing significant income to rural smallholders, it provides food security to many rural peasant farmers especially women in Sub- Saharan Africa (Wortmann *et al*., 2004).

In East Africa per capita consumption is 50-60 kg/year in Rwanda, Kenya and Uganda, which is higher than in Latin America where per capita consumption is 4-17 kg/year in Columbia and Brazil respectively (Beebe *et al*., 2013). Generally high per capita consumption of 13-40 kg/year was observed in developing countries especially in low-income families in rural and urban areas (Singh and Munoz, 1999).

Nutritionally bean provides 60-65% of calories which are from carbohydrate in form of digestible starch, resistant starch and small amount of non-starch polysaccharides. The grains have lower glycemic index relative to glucose and white bread which

helps to reduce blood glucose level (Anderson *et al.*, 2004). Beans also contain soluble and insoluble fibers, the soluble fibers helps to lower blood levels of choresterol and insoluble fibers enhance transit time of the waste through the colon thus helps to combat constipation and colon cancer (Guillon and Champ, 2002). The vitamins and proteins contained in common bean are very essential for human and minimize cost relative to production of animal protein sources.

2.3 Bean Production Constraints

Apart from bean importance in small-scale household's food security and income generation, there is still low yield of the crop due to both biotic and abiotic constraints. The use of unimproved varieties, diseases, low soil fertility (particularly phosphorus) and insect pests are among the vital factors for decrease in bean production (Hillocks *et al.*, 2006). Diseases such as common bacterial bright (CBB), angular leaf spot (ALS), bean common mosaic virus (BCMV) and bean common mosaic necrotic virus (BCMNV) have been a constraint in bean production whereby tremendous decrease in yield has been reported due to these disease attacks. This is exemplified by angular leaf spot which has been reported to cause a yield loss of up to 50-80% (Tryphone *et al.,* 2015). It has been reported also by Bucheyeki and Mmbaga (2013) that most of farmers especially in Tanzania use unimproved varieties, they use locally available varieties which have low yield potential as a result low yield per area and reduced bean production. Nutrient availability in the soil is another factor that constraint bean production in which compounds such as phosphorus has been reported to cause high yield loss when unavailable in the soil. Maurice and Muhamba (2012) reported a decrease in bean yield due to decrease in

phosphorus content in the soil. Insect pests that infest bean both in field and during storage also cause decrease in bean production with the storage pests been the most serious problem wherever beans are produced as their effects are irreversible (Blair *et al.,* 2010).

2.4 Bean Bruchids Classification

Bean bruchids belong to the order Coleoptera in the family Bruchidae. Bruchidae has got 56 genera which are divided into 5 subfamilies namely bruchinae, cubaptinae, kytorhininae, amblycerinae and pachynaemerinae (Howe and Curie, 1964). The family consists of about 1300 species of seed weevils out of which 20 species are recognized as being pests in stored legume seeds especially in developing countries (Southgate, 1979). Four species are of cosmopolitan importance namely *Callosobruchus malacus, C. chinensis, A. obtectus* and *Z. subfasciatus* (Southgate *et al.,* 1978). *Z. subfasciatus* and *A. obtectus* are the main bean bruchid species distributed in nearly all bean producing areas. *Z. subfasciatus* is confined to warmer areas and infests bean only in storage while *A. obtectus* is confined to cooler areas (high altitude or latitude) infesting bean both in field and during storage (Blair *et al*., 2010).

2.4.1 Bruchid developmental biology

Both *Z. subfasciatus* and *A. obtectus* have similar developmental biology except *Z. subfasciatus* glues its eggs to the seed testa of the stored beans while *A. obtectus* scatters its eggs among stored seeds (Dendy and Credland, 1991). After hatching from the egg, the larvae penetrate the seed coat. In the seed the larvae of both species molt four times before pupating. During the last larvae instar, the feeding and

pupation cells become visible externally as circular windows on the seed. After pupation the adults push their way out of the window and soon thereafter mate (Schoonhoven and Cardona, 1986). *Z. subfasciatus* female lay an average of 36 to 56 eggs and the eggs lasts for 5-6 days. Different larvae instar takes 14 days, pupa stage 6-7 days and adults lives 10-13 days. *A. obtectus* female lay an average of 45-60 eggs and their eggs lasts 6-7 days. Larvae and pupa state takes 23 days and adult lives 14 days (Cardona, 1989).

2.4.2 Losses due to bean bruchid infestation

The two-bruchid species are of economic importance worldwide. Higher losses occur when the pests have multiple generations in a season than when only produce a single generation and controlled (Dobie *et al.*, 1988). The eggs hatch into larvae that burrow through the seeds and feed on nutritious cotyledons. They develop within the seed and when they emerge reproduction cycles continues while beans are still in storage causing both quantitative and qualitative losses. The levels of damage due to bruchid infestation of 1-20% has been reported which is relatively low, but a loss of up to 100% can be reached depending abundance of bruchid species, harvesting time, storage methods, hygiene in the storage and management techniques employed by farmers (Kusolwa *et al*., 2013). In Rwanda and Burundi grain losses of about 30% have been reported while in Mexico the loss was about 35% (CIAT, 1986; Jones 1999). Damage is related to the number of larvae that hatch and burrow into the seed as the adult emerges leaves typical damage in form of empty feeding chambers (Parsons and Credland, 2003). Up to a total of 26 larvae can develop in a grain making the grain useless for consumption and sowing as seed. Other indirect

damages include contamination by insect frass, dead insects, lower germination rate of the damaged seeds as well as susceptibility to diseases of plants resulted from bruchid damaged seeds **(**Chipungahelo *et al.*, 2001). Therefore effective control measures are needed to reduce heavy postharvest losses. One promising strategy is to develop varieties that are resistant to infestation by bean bruchids.

2.5 Bruchid Resistance in Common Bean

Almost all cultivated common bean genotypes are susceptible to bean weevils. It is the wild genotypes of bean that were obtained from central highlands of Mexico that were originally found to be resistant to bean bruchid infestation (Singh and Schwartz, 2011). CIAT screened and used these wild accessions to develop cultivated bean lines known as RAZ lines that are resistant to bruchid infestation (CIAT, 1986). Different researchers used these accessions from CIAT and promising levels of resistance has been obtained. This was verified by the work done by Myers et al. (2001) who obtained a wild tepary (*P. acutifolius*) accession from CIAT and tested it at SUA whereby resistance to *A. obtectus* was observed. Additional research conducted by Teshale, (2010) as well as Kusolwa and Myers (2011) revealed the presence of high levels of resistance to bruchids of the progenies resulted from crosses from the tepary, RAZ ph- and Arc2.

2.6 Mechanism of Resistance to Bruchids

Insect pest resistance consists of four mechanisms namely antixenosis, antibiosis, tolerance and escape. Tolerance and escape are effective for field infestation but not for storage pests and the other two mechanisms can be effective for storage pests (Keneni *et al.,* 2011). Antibiosis is an effective mechanism for storage pests by

which a colonized host is resistant because it has got an adverse effect on insect development, reproduction and survival. Antibiosis is associated by plants producing secondary metabolites that offers defense against pests as repellents, feeding inhibitors and anti-nutritional factors (Panda and Kush, 1995; Kusolwa 2007).

Common bean contains defensive compounds with insecticidal effects that protect their seeds against widely different herbivores. Among these are tannins, non-protein amino acids and proteins such as protease, α-amylase inhibitors, lectins, chitinases, β-1,3-glucanases and arcelins (Sales *et al*., 2000). Lectins in which phytohaemagglutinin is a major lectin of the common bean and lectin- like proteins which comprise α-amylase inhibitors and arcelins are bean storage proteins that protect beans against bean bruchids infestation (Lioi *et al*., 2003). Phytohaemagglutinin has lectin-like properties as it exhibits agglutination properties whereas α-amylase inhibitors has no sugar binding sites and arcelins shows only weak agglutinin properties- hence they are called lectin-like proteins (Hamelryck *et al*., 1996). These three proteins are called APA (α-amylase- phytohaemagglutinin-arcelin) proteins as they are tightly linked at one locus in linkage group Pv04 and apparently arose through duplication of a single gene of an ancestral lectin (Kusolwa and Myers, 2012). The APA proteins vary in their biochemical and physiological properties but have similar expression patterns with all being synthesized only in the embryonic axis and cotyledons during seed formation (Moreno *et al.*, 1990).

2.6.1 Phytohaemagglutinin (PHA)

Phytohaemagglutinin is a major lectin of common bean made up from two polypeptides sub-units that occurs in many accessions of beans. The two polypeptide sub- units are called leucoagglutinin (PHA-L) and erythroagglutinin (PHA-E) and are closely related proteins formed by tandemly linked gene. They are responsible for agglutination of leucocytes (white blood cells) and erythrocytes (red blood cells) respectively (Mirkov *et al*., 1994). The mature de-glycosylated polypeptides of phytohaemagglutinin have the molecular weight of 27000 (Cardona, 1989). Phytohaemagglutinin binds to glycoprotein in the intestinal mucosa of mammals and insects whereby it inhibits nutrient absorption across the walls and its toxicity results from its initial binding properties (Jones, 1999). By enhancing the activities of lectin-like proteins, phytohaemagglutinin contributes to resistance of common bean to bruchids (Goosens *et al*., 2000).

2.6.2 α-amylase inhibitors (α-AI)

α-amylase inhibitor is a lectin-like protein that inhibits bruchids and other insect species infestation in common bean due to its porcine pancreatic α-amylase inhibitory activities that deter feeding, prevent starch digestion, retard instar development and even cause larvae mortality (Ishimoto *et al*., 1995). α-amylase inhibitors are found in both cultivated and wild accessions of common bean. Electrophoretic mobility and pancreatic α-amylase inhibitory activities led to identification of eight α-amylase inhibitors. Among the eight α-amylase inhibitors identified, α-amylase inhibitor-1 (αAI- 1) and α-amylase inhibitor-2 (αAI- 2) which are allelic, are found to have adverse effects on bruchid development. αAI-1 is

closely linked to phytohaemagglutinin and exists in both wild and cultivated common bean accessions whereas αAI-2 is found only in wild accessions with various arcelin variant (Suzuki *et al*., 1995). These two variants of α-amylase inhibitors provide different levels of resistance to bruchids. . αAI- 1 found in genotypes that lacks arcelins and has inhibitory effects to *C. maculatus* and *C. chinensis* but offers weak resistance to some bruchid species and do not inhibit α-amylase of *Z. subfasciatus*. αAI- 2 is contained in genotypes with arcelins and offers high resistance to bruchids as well as inhibit α-amylase of *Z. subfasciatus* (Kusolwa, 2007).

2.6.3 Arcelins (Arc)

Arcelins are abundant lectin-like seed storage proteins that are present in wild *P. vulgaris* accessions. The protein occurs in the globulin -2 fraction and its presence is highly correlated with reduced phaseolin content (Romero, 1984). Arcelin like other related proteins are heat labile and its anti-nutritional as well as harmful effects are lost during prolonged cooking (Singh, 2011). Genetic analysis shows that the presence of arcelin protein is inherited as a monogenic trait and the trait is controlled in a simple Mendelian fashion. Expression of alleles for presence of different arcelin variants is co-dominant with respect to each other and dominant with respect to alleles for absence of arcelins (Osborn *et al*., 1986; Kusolwa, 2007). The polypeptides for arcelins are closely related phytohaemagglutinin and α-amylase inhibitors. Arcelins have different intrinsic specificity for complex sugars that makes it toxic to bruchids (Minney *et al*., 1990).

Different scientists have conducted research on arcelins. The earliest research was performed by Osborn et al. (1986) whereby they discovered that arcelins associated with inhibition of development of some bruchid species. Many other works has been done after that, including the work done by Goosens et al. (2000) who was pessimistic about the function of arcelin 5. He reported no correlation between the presence of arcelin 5 and the insecticidal effects observed in wild genotype G02771 seeds. Goosens claimed there was inadequate resistance against *Z. sufasciatus* even in presence of elevated levels of arcelin 5. Another work was performed by Kusolwa (2007) who described the new arcelin-like proteins, which are novel variants of arcelin in wild tepary bean genotype G40199 inherited as a dominant allelic block. He discovered ARL-3pa which is homologous to ARL-2pa is a functional gene in G40199 and its interspecific lines as well as the gene was not expressed in brown tepary beans. He also described ARL-4pa, which falls into separate branch from ARLpa earlier described by Mirkov et al. (1994). The two arcelins expressed in a homozygous condition and were not been reported previously.

Based on electrophoretic patterns and genetic analysis, eight arcelins variants are known and designed as *Arl-1* to *Arl-8* allelic forms (Zaugg *et al*., 2013). Five allelic variants have been characterized in which *Arl-2* and *Arl-5* contain dimeric arcelin protein , *Arl-3* and *Arl-4* contain tetrameric arcelin protein and *Arl-1* have both dimeric and tetrameric proteins (Hartweck *et al*., 1991; Goosens *et al*., 1994). Based on complimentary DNA sequence homology, six variants group into three clusters. The first cluster consists of *Arl-1, Arl-2* and *Arl-6*, the second cluster consists of *Arl-3* and *Arl-4* and the last on consists of *Arl-5*. These variants have different levels of resistance with *Arl-1* and *Arl-5* having high resistance against *Z. subfasciatus* while

Arl-4 contain high resistance to *A. obtectus* (Hartweck *et al.*, 1997). High levels of resistance to bruchids of Arcelins was studied by Cardona et al.,(1990) who observed a delay in adult emergence and larvae mortality especially in first and second instar. Antibiosis properties of arcelins are proposed to be due to the lysis of epithelial cells of the intestines by binding to the carbohydrate moieties of these proteins as well as poorly digestible by gut proteases of the insects (Janarthanan *et al.*, 2002).

The use of host plant resistance as a major bruchid control may be the most effective control measure and less costly to farmers, especially small holder farmers who have less income to be able to afford the use of pesticides, and many of whom have little knowledge of pesticides control tactics (Kornegay and Cardona, 1991; Mulungu *et al.*, 2007; Paul *et al.*, 2009). In previous breeding efforts for bruchid resistance, resistance was transferred from wild accessions of *P. vulgaris* to cultivated genotypes through intraspecific hybridization. The discovery of bruchids resistance in landraces from Malawi and Tanzania will give a new insight in the breeding for resistance to bruchid programs especially in these two countries. Knowing the resistance source and mechanisms in these landraces will be an effective way towards simplicity to incorporate this resistance into large seeded cultivated Andean bean genotypes with fewer backcrossing to regain seed size among farmers preferred bean varieties or can be promoted to farmers directly if they meet local market specificities and other adaptation requirements. This will give advantages of reduced postharvest losses to farmers, prolonged storage time, increased bean production, maintain quality and quantity of bean during storage, sustain market value of the crop and serve as source of breeding materials to other breeders.

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CHAPTER THREE

3.0 CHARACTERIZATION OF SEED STORAGE PROTEIN RELATED TO BRUCHID RESISTANCE IN COMMON BEAN LANDRACES FROM TANZANIA AND MALAWI

3.1 ABSTRACT

Acanthoscelides obtectus and *Zabrotes subfasciatus* are the major storage pests of common bean. In the effort to control these pests cultivated common bean genotypes were screened at Sokoine University of Agriculture (SUA) to search for genetic resistance and two landraces known Kalubungula and KK25 were identified as resistant. In order to get in-depth understanding of the mechanism and genetics of resistance two recombinant inbred populations KSy and KSw were created from crosses between Soya X Kalubungula and Soworo X Kalubungula respectively and genotypes from Nagaga X KK25 were used. Protein characterization and sequencing was conducted to evaluate the progenies and determine if the mechanisms of the resistance is associated with antibiosis effects of defensive storage protein reported to be approximately at arcelin like seed storage protein in 33KDa known as APA protein. Results revealed no polymorphic banding pattern observed in KSy, KSw and their parents suggesting a different mechanism of resistance other than the storage protein. Unique banding pattern at 25KDa was visualized by SDS-PAGE in Nagaga X KK25 and their progenies corresponding to Arcelin-5, leucohaemagglutinin (PHA-L) and erythrohaemagglutinin (PHA-E) suggesting antibiosis effect of these storage proteins. Also one hypothetical protein sequence (uncharacterized) was reported that may add new knowledge shared in the protein database. Refining of the proteins identified, characterization of the hypothetical protein and exploring the mechanism

of resistance in KSy and KSw may be potential in breeding for resistance to bean bruchids in common bean.

3.2 INTRODUCTION

Common bean is the principle legume grown and consumed as major source of protein as well as source of income by most of farmers in developing countries (Broughton *et al.*, 2003). Common bean is mostly cultivated by small scale farmers in which most of them cannot afford the improved storage equipment and technology hence they store the grains in farm where they incur a wide range of postharvest losses including insect pest infestation (Cardona, 2005). The most serious storage pest of beans are the bean weevils. Two species namely *A. obtectus* and *Z. subfasciatus* are the major species infesting beans whereby their distribution is temperature dependent with Z. *subfasciatus* being confined to warmer areas and *A. obtectus* being confined to cooler areas (Blair *et al.*, 2010). Most of small scale farmers use botanicals as their indigenous bruchid control (Mbongo *et al*., 2013, Kusolwa *et al*., 2013) and few of them afford pesticides uses but these methods are less efficient in most cases due to shortage supply of botanicals and environmental hazards related to the use of pesticides. The use of improved storage structures can be effective way of controlling these bruchid species but this methods is unaffordable to small scale farmers as a results less practically used, therefore one of the best ways of controlling them is by the use of host plant resistance.

Legume seeds contains different compounds that are essential for embryo and seed development as well as defense against insect pests which most of them are stored as proteins. Among these compounds are tannins, cynogenic glucosides, non-protein

amino acids and proteins such as protease, α-amylase inhibitor, lectins, chitinases, β-1, 3-glucanases, phaseolin and arcelins among which some are nutritional and some are anti-nutritional with antibiosis effects against different herbivores (Sales *et al*., 2000). In common bean, the important seed storage proteins that were identified and characterized are phaseolin, lectins, trypsin inhibitor and lectin-like proteins (Gepts, 1988; Lioi 2003).

Phaseolin is the major storage protein in common bean that account for 50% of the total seed storage protein. It is an important nutritional protein to animals as it provide essential amino acids for animal's nutrition (Bollini and Chrispeels, 1978). Apart from nutritional role, phaseolin has provided the evidence for protein diversity studies particularly for subdividing *Phaseolus vulgaris* in two major gene pools: the Andean and Mesoamerican (Gepts, 1990).

Lectins and lectin-like proteins are the anti-nutritional seed storage proteins that defend bean seeds against insect pests. These proteins accumulate in the bean cotyledons as a reserve for amino acids required during germination and seed development. The lectin and lectin-like proteins includes phytohaemagglutinin, arcelins and α-amylase inhibitors. The three proteins are tightly linked together in one locus called APA locus arose through duplication of a single gene located in linkage group four (PV04) of *P. vulgaris* chromosomes (Kusolwa and Myers, 2012). Phytohaemagglutinin is the major lectin protein of common bean responsible for agglutination of erythrocytes and leucocytes. It interferes with starch digestion in the insect pest intestine which results into starvation by binding to carbohydrates, thus provide defense against insect pests. In addition to agglutination properties,

phytohaemagglutinin being part of the APA locus contribute to resistance of bean against pest infestation by enhancing the activity of lectin like proteins (Goosens *et al*., 2000).

Lectin like proteins include α-amylase inhibitors, phytohaemagglutinins and arcelins. α-amylase inhibitor is a lectin like protein that inhibit pest infestation on bean due to its porcine pancreatic α-amylase inhibitory activity as well as chitinolytic activity by having the ability to hydrolyse chitin possessed by external exoskeleton and internal perithropic gut membrane of insect pests (Dayler *et al*., 2005).

Arcelins are also lectin like proteins with polypeptides that are closely related to phytohaemagglutinin and α-amylase inhibitor but different intrinsic specificity for complex sugars that make it toxic to insect pests. Its toxicity is due to interaction with glycoproteins and other constituents of digestive track membranes as well as direct binding to intestinal cells of the insect (Minney *et al*., 1990).

Generally phytohaemagglutinin and α-amylase inhibitor are present in wild and cultivated genotypes of common bean while arcelins are found only in wild genotypes of common bean. Arcelins and α-amylase inhibitor are also present in some accessions of tepary and lima bean described as arcelin-like and α-amylase inhibitor-like proteins (Sparvoli, 2001; Kusolwa and Myers, 2010).

Accession G40199 of tepary bean is among the wild accessions found to confer high level of resistance to bruchid infestation (Cardona, 2005). Transfer of this resistance to common bean genotypes was performed by Kusolwa (2007) by interspecific hybridization and the progenies were observed to be resistance with the mechanisms

of resistance being associated the presence of storage protein of approximately 33KDa corresponding to the Arcelin – Phytohaemmaglutinin – α-amylase inhibitor protein collectively known as APA protein. On the other hand common bean landraces collected from major growing areas in Tanzania and Malawi were screened for bruchid resistance and some of them were found to be resistance to bruchid infestation and damage. Two landrace namely Kalubungula from Tanzania and KK25 from Malawi were found to be resistant. The mechanisms of resistance and storage protein related to resistance in these landraces are unknown. Therefore this study focused of characterizing and investigating the seed storage protein related to bruchid resistance in KK25, Kalubungula and derived progenies from Kalubungula crosses with susceptible parents.

3.3 MATERIALS AND METHODS

3.2.1 Study area

The study was conducted at Sokoine University of Agriculture (SUA) and Oregon State University (OSU). Seed multiplication was done at Sokoine University of Agriculture whereby F_2 seeds were advanced to F_3 generation. After harvesting and drying the grains were stored in a deep freezer (-20ºC) for two days in order to eliminate any field acquired bruchid infestations. The F_3 seeds were then taken to OSU for laboratory analysis of storage protein.

3.2.2 Plant materials

The plant materials used in this study included bean landraces Kalubungula and Nagaga x KK25 from Tanzania and Malawi respectively collected by Bean Bruchid Resistance Project supported by McKnight Foundation at SUA. These landraces

were part of the major bean collection from farmers saved seed in major bean growing regions in Tanzania and Chitedze Research Institution in Malawi. Two populations namely KSy resulted from the cross between variety "Soya" X Kalubungula and KSw resulted from "Soworo" X Kalubungula was created from Tanzanian landraces by crossing susceptible x resistant genotypes that results into 101 $F_{2:3}$ families with 53 genotypes from Kalubungula x Soya and 48 from Kalubungula x Soworo. Kalubungula is bruchid resistant red seeded bean landrace, Soya and Soworo are two farmers preferred bean varieties that are susceptible to bruchids in Tanzania. Nagaga X KK25 is the resistant bean genotype in Malawi generated from crosses with susceptible variety "Nagaga" (Kananji, 2007). These bean genotypes together with the Tanzanian lines were used in protein profiling and sequencing described in Appendix 1.

3.3.3 Protein extraction

The samples were prepared as described by Osborn et al., (1986) with some modification. Cotyledons of individual seeds was scraped on sand paper to obtain fine powder. Ten milligram (0.01 g) of the cotyledon flour of each seed was placed in microfuge tube and suspended in 200 µl of extraction solution (0.5 M NaCl, pH 2.4) by vigorous shaking and vortexing. The mixture was left to settle at room temperature for 30 minutes and centrifuged at 10000 rpm for 2 minutes. 3 µl of the supernatant was mixed in a microfuge tube with 3µl of 0.5 M NaCl pH 2.4 and 6 µl of 2x protein based sample buffer from BIORAD (65.8 mM tris HCl pH 6.8, 26.3% (w/v) glycerol, 2.1% SDS, 0.5% 2-mercarpto-ethanol, 0.01% Bromophenol blue).The mixture were transferred to PCR plates and heated for 5 minutes in a thermos cycler at 94^0C to denature the tertiary protein structure into primary

structure. 10 µl of each sample were immediately loaded onto a 10% pre-cast Tris-glycine SDS-PAGE running gels (BIORAD) at 200 V constant for 50 minutes in 1x Laemmli SDS-PAGE running buffer (25 mM Tris base, 192 mM glycine, 0.1% w/v SDS, pH 8.3). The gels were stained by placing them in a sealable plastic container with 100 ml staining solution (40% methanol, 10% acetic acid, 0.1% Coomasie Brilliant Blue R-250®) for 1-2 hours on a platform shaker at low speed followed by destaining (40% methanol, 10% acetic acid) for overnight. The gels were washed three times with gentle shaking in deionized water for 15 minutes then placed between pre-wetted cellophane (BIORAD) to dry. The gels were scored with reference to 33 kDa protein subunit from electrophoretic mobility of size standard proteins and documented.

3.3.4 Protein isolation from SDS-PAGE gels and sequencing

Protein isolation and sequencing was performed as described by Kusolwa, (2007) with little modification. Unique bands from the gels were excised with a sterile scalpel and cut into 1mm pieces then placed in microfuge tubes. The gel plugs washed 2x by adding 200 µl of deionized water, soaked for 15 minutes with occasional vortexing followed by centrifuging for 5 minutes and the liquid was removed by aspirating with a pipette after each spin. The gel plugs was washed 2x to remove Coomasie Brilliant Blue stain by adding 200 µl of a 50%50% acetonitrile/ 50 mM NH_4HCO_3 solution, soaked for 30 minutes with occasional vortexing and centrifuged for 5 minutes, the liquid was removed by aspirating with the pipette. The gel plugs was dehydrated by adding 500 µl acetonitrile and left to stand with occasional vortexing until they turn opaque, centrifuged for 5 minutes and the liquid was removed. Drying of the plugs for 30 minutes in a vacuum centrifuge was done

then the plugs was rehydrated by adding 25 mM NH_4HCO_3 containing 20 ng/µl trypsin pH 8.0, chilled on ice for 45 minutes. More buffer was added to ensure well rehydration of the plugs followed by trypsin digestion for six hours in the dark at 37^0C. The supernatant was extracted to new microfuge tubes, the gel plugs were then extracted 3x by adding 50 µl of 50% acetonitrile, vortexed briefly, centrifuged for 5 minutes and the supernatant was combined in a new centrifuge tube. The samples were submitted to Mass Spectrophotometry Laboratory (MS-MS Lab.) for sequencing at OSU.

3.4 RESULTS

3.4.1 Protein profiles

The evaluation of total seed storage protein profiles from cotyledons of the crosses between Soya x Kalubungula, Soworo x Kalubungula, Nagaga x KK25 and their parents was visualized by one dimension SDS-PAGE gels. There were no polymorphic bands of seed storage proteins observed in the progenies of Soya x Kalubungula, Soworo x Kalubungula and their parents, instead a monomorphic band pattern at approximately 33KDa was observed which exists also in susceptible checks (Fig. 3.1). A polymorphic band at approximately 25KDa was observed in one of the Nagaga x KK25 and its progenies which was absent in crosses of Soya x Kalubungula and Soworo x Kalubungula (Figure.3. 2). The bean lines with arcelin backgrounds were used as bruchid resistant checks to compare with the unique band observed in Nagaga x KK25 and similarity of the banding pattern at 25KDa between them was observed (Fig. 3.3). Both Kalubungula, Nagaga X KK25 and their progenies were of Andean type based on banding pattern for phaseolin protein (Fig. 3.1 and Fig. 3. 2) between 37-50KDa with triple bands.

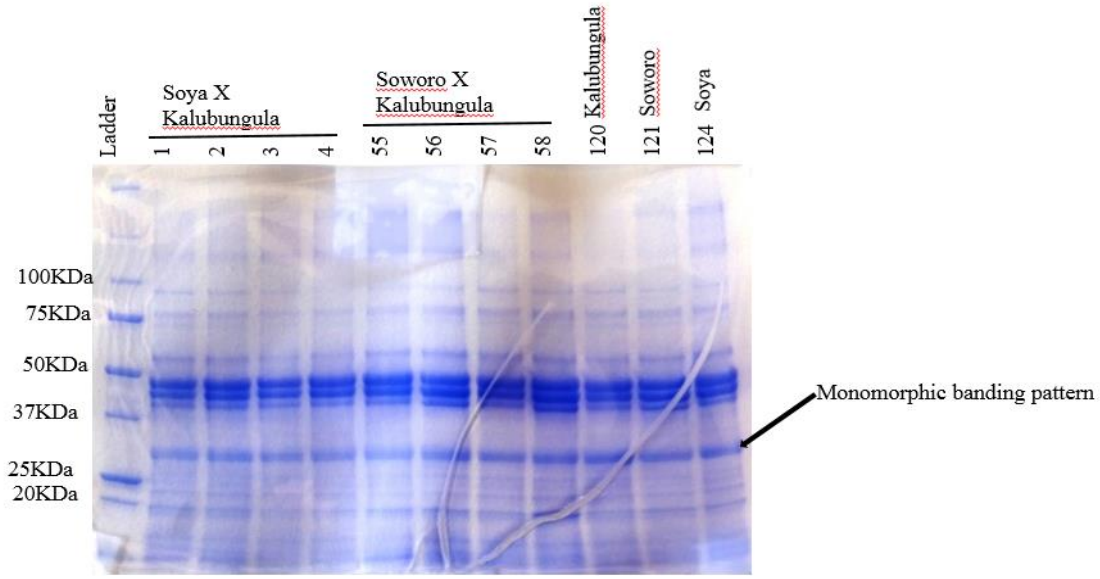

Figure 3. 1. Bean seed storage profile separated on 15% SDS-PAGE gel with an arrow indicating monomorphic banding pattern between parents and progenies. Protein ladder molecular weight on left, 1-6 are Kalubungula X Soya RIL's, 55-58 are Kalubungula X Soworo RIL's, 120 is Kalubungula, 121 is Soworo and 120 is Soya.

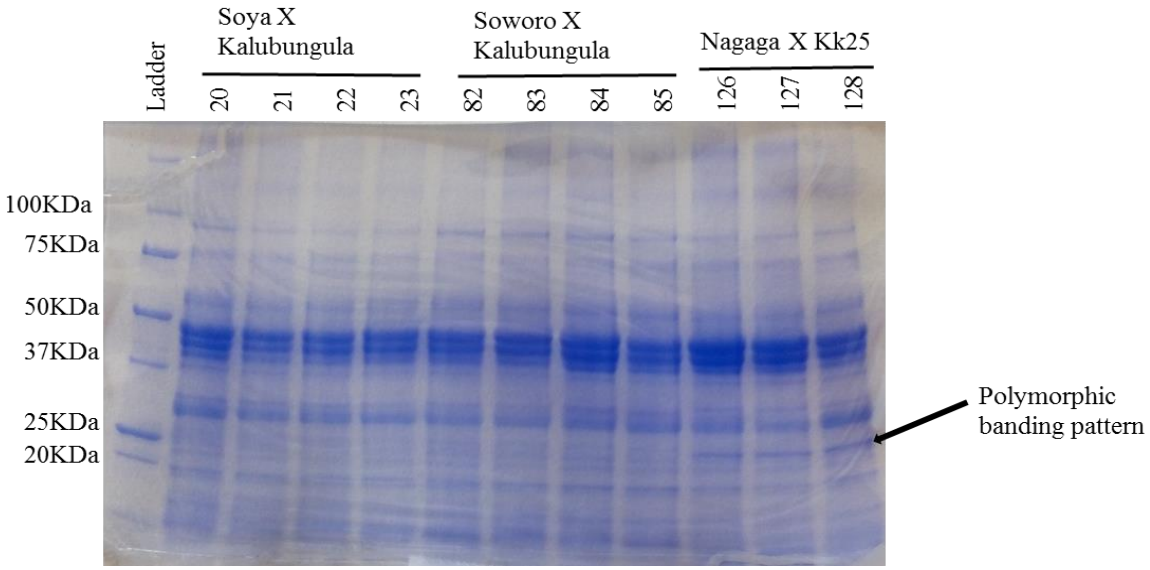

Figure 3. 2. Bean seed storage profile separated on 15% SDS-PAGE gel. Protein ladder molecular weight on left, 20-23 are Soya X Kalubungula RIL's, 82-85 are Kalubungula X Soworo RIL's, 126-127 are Nagaga X KK25 RIL's. An arrow indicates the unique band observed.

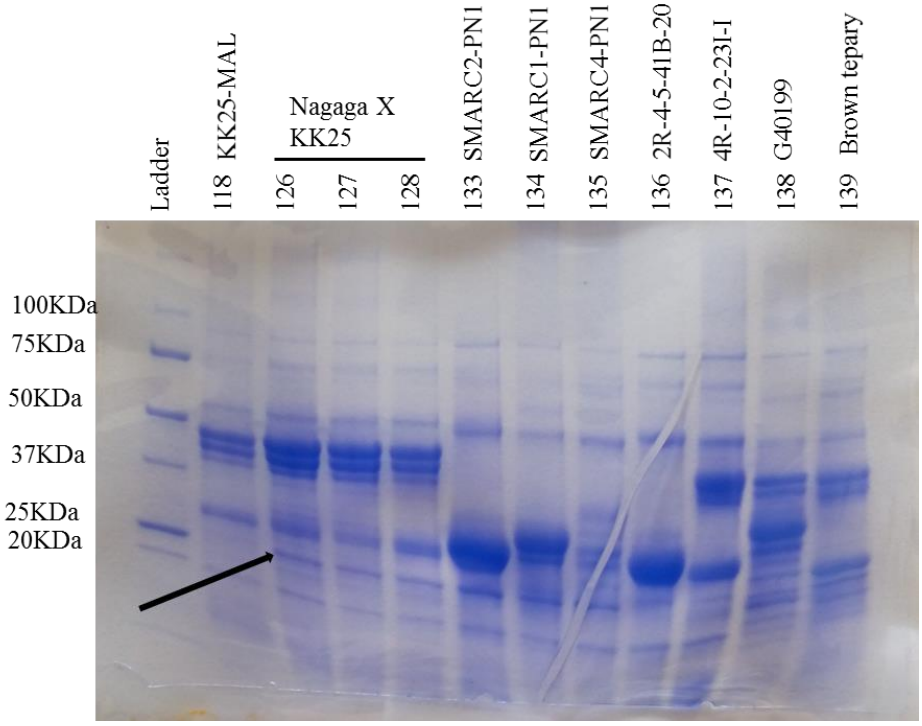

Figure 3. 3. Bean seed storage profile separated on 15% SDS-PAGE gel. Protein ladder molecular weight on left, 118 is KK25- MALI, 126-127 are Nagaga/KK25 RIL's, 133-139 are the arcelin-2, arcelin-1 and arcelin-4 containing lines. An **arrow** pointing unique band in Nagaga/KK25 RIL's resembling to arcelin containing lines.

3.4.2 Amino acid sequencing

Sequencing of the excised protein bands at 25KDa from Nagaga X KK25 recombinant inbred lines identified the presence of two major proteins, the arcelin and phytohaemagglutinin. Unfortunately one of the amino acid sequences from ML10 (indicated by lane 127 in Fig. 3.2 and Fig.3.3) were not corresponding to any previously reported protein in genus *Phaseolus* but rather being uncharacterized or hypothetical *Phaseolus vulgaris* protein. The observed amino acid sequences and their corresponding protein match from NCBI are shown in table 3.

Table 1. Amino acid sequences produced from 25KDa protein fragments of Nagaga X KK25 RIL's with their matching proteins from NCBI database.

Protein fragment size	Observed	Peptide sequence	Match sequence	Matched protein reference
		…….....ML3……		
25KDa	25.58KDa	YTDDMELDDAVHTAILTLKEGFEGQISGK	1	ARC-5- Hamelryck et al. 1996
	26.35KDa	HSLLGASGEISDFQEILRYLDELILYDNMWDDGNSLGPK	6	PHA-e- Nagae et al.2016
	26.35KDa	FNPLWNALVLGGVK	3	PHA-l- Chrispeels et al. 1996
		……ML10 (Nagaga x KK25)……		
25KDa	96.77KDa	ATFLGEIITSLPTLGAGQSAFK	1	ARC-5-Hamelryck et al.1996
25KDa	96.77KDa	IYDYDVYDNLGDPDK	1	PHA-l-Chrispeels et al.1991
25KDa	96.77KDa	LDSQVYGDHTSQITK	-	Hypothetical *Phaseolus vulgaris* protein

BLAST search of the observed amino acid sequences revealed that the sequences were identical to Arcelin-5 of the *P. vulgaris* accession 101A but the similarity was only two protein sequence match (Fig. 4). It was also observed that two sequences from these lines matched to leucoagglutinin with matching ranged from one to three match and one sequence resembled erythroagglutinin type of phytohaemagglutinin with six sequence match (Fig.5 and Fig.6). Having low protein matching (peptide length of 1-6) between the amino acid sequences and the matched protein sequences shows that there is no significant association of the observed amino acid sequences to the reference protein in the databank.

```
  1 sndiyfnfqr fnetnlilqr dasvsssgql rltnlngnge prvgslgraf ysapiqiwdn
 61 ttgtvasfat sftfniqvpn nagpadglaf alvpvgsqpk dkggflglfd gsnsnfhtva
121 vefdtlynkd wdpterhigi dvnsirsikt trwdfvngen aevlitydss tnllvaslvy
181 psqktsfivs dtvdlksvlp ewvsvgfsat tginkgnvet ndvlswsfas klsdettseg
241 lnlanlvlnk il
```

Figure 3. 4. Amino acid sequences of the 25KDa protein band from ML3 and ML10, the Nagaga X KK25 RIL's matched to leucoagglutinin (PHA-l) of *Phaseolus vulgaris.* Matched sequences are shown in red colour and bold.

```
  1  massnllsla lflvllthan sasqtsfsfq rfnetnlilq rdatvsskgq lrltnvndng
 61  eptlsslgra fysapiqiwd nttgavasfa tsftfnidvp nnsgpadgla fvllpvgsqp
121 kdkggllglf nnykydsnah tvavefdtly nvhwdpkprh igidvnsiks iktttwdfvk
181 genaevlity dsstkllvas lvypslktsf ivsdtvdlks vlpewvivgf tattgitkgn
241 vetndilsws fasklsdgtt sealnlanfa lnqil
```

Figure 3. 5. Amino acid sequences of the 25KDa protein band from ML3 and ML10, the Nagaga X KK25 RIL's matched to erythroagglutinin (PHA-e) of *Phaseolus vulgaris.* Matched sequences are shown in red colour and bold

```
  1     atetsfnfpn fhtddklilq gnatisskgq lqltgvgsne lprvdslgra fysdpiqikd
 61     snnvasfntn ftfiiraknq sisayglafa lvpvnsppqk kqeflgifnt nnpepnartv
121     avvfntfknr idfdknfikp yvnencdfhk yngektdvqi tydssnndlr vflhftvsqv
181     kcsvsatvhl ekevdewvsv gfsptsglte dttethdvls wsfsskfrnk lsnillnnil
```

Figure 3. 6. Amino acid sequences of the 25KDa protein band from ML3 and ML10, the Nagaga X KK25 RIL's matched to arcelin-5 of *Phaseolus vulgaris.* Matched sequences are shown in red colour and bold

3.5 Discussion

Protein characterization from KSy and KSw populations shows the presence of a monomorphic band at approximately 33KDa that would have been suspected to correspond to profiles of the APA protein banding pattern. However this band was different though being observed in a relatively similar molecular size as those of arcelins-like proteins but this was also observed in susceptible bean genotypes presented as checks. This indicates that the near-33KDa storage proteins in these bean lines is not related to resistance to bruchids. This result agrees with that obtained by Kananji (2007) who found out that this storage protein arcelin was not involved in any significant role in conferring resistance to bruchids in some bean lines KK35, KK73 and KK90.

Presence of storage protein at 25KDa in Nagaga X KK25 and its recombinant inbred lines suggests the resistance to be conferred by another storage protein in these lines since the protein was not observed in KSy and KSw populations therefore this may be associated with bruchid resistance in Nagaga X KK25 derived lines. Amino acid sequencing from the trypsin digested protein fragments from the 25KDa banding pattern revealed the presence of trace amount of protein peptides corresponding to arcelin-5 similar to that observed by Hamelryck *et al*. (1996), phytohaemagglutinin-l observed by Chrispeels (1991) and phytohaemagglutinin-e of *P. vulgaris* similarly observed by Nagae *et al*. (2016) that have a special property of binding glycan in a complex structure of a back-fold conformation which affects activities of glycosyltransferases enzymes and localization of carrier glycoproteins in an insect. Since these seed storage proteins are known to be involved in defending common

bean against bruchids by interacting with the glycoprotein, carbohydrate digestion and binding to the intestinal cells of insect. It is possible that the mechanisms of resistance in these Nagaga x KK25 crosses is due to antibiosis effects conferred by presence of this traceable expression of arcelin-5 and phytohaemagglutinins though other factors might be involved. Kusolwa and Myers, (2012) observed the presence of multiple variants of the antibiosis seed storage proteins of the complex APA locus arcelin- phytohaemagglutinin-α-amylase inhibitor in some progenies of the crosses between wild tepary bean (*P. acutifolius*) accession G40199 highly resistant to bean bruchids and a susceptible cultivar ICA Pijao**.** Having our protein peptide sequencing demonstrated low or weak amino acid sequence match (1-6 match) with reference proteins in the protein databank implies little association between the resistance observed and the storage protein at 25KDa and therefore it is not confirmed whether the presence of the storage protein is the only source of resistance in these line or whether there may be other factors that contribute to the observed resistance. Presence of uncharacterized sequence in one of the KK25 progeny may be of breeding importance and may add a new knowledge to the database since we do not know yet what protein correspond to this sequence and play which role, it might be a factor that contribute to the resistance observed in this line.

On the other hand Kananji, (2007) recorded emergence of *A. obtectus* on bruchid resistant SMARC 2 lines after the test period and that the seed coat played a significant role in conferring resistance to bruchids in these lines due an increased number of *A. obtectus* adult bruchid emergence when the seed coat was removed. This was also confirmed by the resistant genotype KK35 being susceptible to *A.*

obtectus after removal of the seed coat therefore seed coat physical and/or chemical barrier to seed attack by bruchids was the source of resistance other than arcelins. It was reported by Sales et al. (2000) that presence of vicillins and legumins in the seed coat of broad bean *Vicia faba* deter development of the first instar larvae of cowpea weevils *C. maculatus*. Silva et al. (2004) also supported the evidence that vicillins or phaseolin present in the seed coat of *P. vulgaris* are detrimental to the development of *C. maculatus*. He found out that the thickness of the seed coat was not a factor important in the resistance but rather high concentration of vicillins in the seed coat. Another scientist Lattanzio *et al*. (2005) reported that high concentration of tannins (13 times) in undamaged seeds than damaged seeds in the seed coat of cowpea seeds confers a biochemical defense mechanism which can deter, poison or starve bruchid larvae that feed on cowpea. The same mechanism may be applying to bean bruchids *Z. subfasciatus* and *A. obtectus* but it is not confirmed as there is no literature reported and therefore more studies has to be done on investigating the effects of the seed coat contents on conferring resistance to bean weevils.

In general, all progenies of crosses between Kalubungula x Soya and Kalubungula x Soworo and their parents demonstrated the absence of storage protein which confer resistance to bruchids suggesting that there is a different mechanism of resistance involved other than the storage proteins. Presence of some variants of arcelin-like seed storage proteins observed in KK25 and the progenies suggests for antibiosis to be the resistance mechanism in these lines. Breeders can use these resistant lines for more evaluation and improve of bruchid resistance in commercial cultivars.

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CHAPTER FOUR

4.0 LINKAGE MAPPING AND QUANTITATIVE TRAIT LOCI FOR BRUCHID RESISTANCE IN COMMON BEAN LANDRACES FROM TANZANIA

4.1 ABSTRACT

Bean bruchids are storage pests of common bean (*Phaseolus vulgaris* L.) that constrain bean production. Recombinant inbred lines were developed from crosses of resistant Kalubungula and susceptible Soya and Soworo which resulted into 53 Kalubungula X Soya namely KSy population and 48 Kalubungula X Soworo namely KSw population. The populations were evaluated for resistance to *Acanthoscelides obtectus*, one of the bruchid species and sequenced using genotyping by sequencing (GBS). We found nine lines from KSy and two lines from KSw that were resistant to bruchids. Linkage map assembled with 328 novel single nucleotide polymorphisms (SNP's) from KSy and 435 SNP's from KSw spanning around 596.4cM and 711.9cM in 11 chromosomes with average map distance of 0.5cM and 0.6cM, respectively. Two Quantitative Trait Loci (QTL's) found in chromosome five (PV05) and one candidate QTL from PV09 were associated with bruchid resistance in KSy and three QTL's located in PV08 in KSw were associated with resistance. QTL's for seed size in both populations mapped on different chromosome with QTL's for bruchid resistance indicating that there is no association between seed size and the resistance to bruchid in these lines as the two traits are under different genetic control. The resistant lines identified in this study will be an important source

of bruchid resistance in breeding programmes and the QTL's can be used to develop markers for marker assisted selection (MAS) in common bean.

4.2 INTRODUCTION

Common bean is the legume grown and consumed as staple food for the developing countries. The crop is used as source of income for farmers household hence improve the livelihood (Muimui, 2010). Common bean production is constrained by both biotic and abiotic factors such as disease, drought, low soil fertility and insect pest including *A. obtectus* and *Z. subfasciatus* which are the major storage pests of bean grain (Blair *et al*., 2010). In Tanzania both species exist and have been reported to cause a yield loss of up to 48% or total crop loss upon longer storage (Mbogo *et al*., 2009). On the effort to combat losses due to bruchid infestation, genetic source of resistance from wild relative of *Phaseolus vulgaris* have been found (Miklas *et al*., 2006). The resistance is associated with the presence of storage protein with insecticidal effects to bruchid. The storage proteins are namely arcelins associated with bruchid resistance which is tightly linked with phytohaemagglutinin and α-amylase inhibitor together produced by the APA gene family located in one locus in the common bean genome (Blair *et al*., 2010).

Different methods have been used to characterize the APA seed storage protein. The methods include electrophoretic techniques that have been mostly used as one or two dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the techniques revealed that the APA protein is found at approximately 33KDa (Kusolwa, 2007). Genomic DNA sequencing is another technique used to compare the homology of the sequence of the APA protein containing parent and the

progenies, the study revealed that there are quantitative trait loci (QTL's) for the APA protein found at linkage group four of the *Phaseolus vulgaris* chromosome (PVO4) (Blair *et al*, 2010). Similarly bean landraces were collected from farmers in a major growing region in Tanzania and screened for bruchid resistance. One landrace known as Kalubungula was found to be resistant to bruchid infestation but the mechanism of its resistance is not known. Therefore the aim of this study is to characterize resistance and to determine the QTLs responsible for the resistance observed in Kalubungula.

4.3 MATERIALS AND METHODS

4.3.1 Study area

The study was conducted at Sokoine University of Agriculture (SUA) and Oregon State University (OSU). Seed multiplication was done at SUA whereby F_2 seeds were advanced to F_3 generation. After harvesting and drying the grains were stored in a deep freezer (-20ºC) in order to eliminate any field acquired infestations. The F_3 seeds were then taken to OSU for laboratory analysis.

4.3.2 Plant materials

The plant materials used in this study were landraces from Tanzania and Malawi collected from Bean Bruchid Resistance Project supported by McKnight Foundation at SUA. The landraces were collected by the project team from farmers in major bean growing regions in Tanzania. Two mapping populations namely KSy and KSw was created from susceptible x resistant genotypes consisting of 101 $F_{2:3}$ families with 53 genotypes from Kalubungula x Soya and 48 from Kalubungula x Soworo. Kalubungula is the resistant landrace, Soya and Soworo are susceptible lines both

originating from Tanzania. The bean populations were advanced at SUA by selfing F_2 plants and harvesting F_3 seeds. The F_3 seeds were taken to OSU for genotyping. The F_3 seeds were also advanced by selfing to F_4 population, which was used for progenies phenotyping for bruchid resistance back at SUA.

4.3.3 Genotyping by sequencing (GBS)

Genotyping by sequencing was done as described by Elshire et al, 2011 with modifications. The leaf samples were collected from bean plants at trifoliate stage. Genomic DNA were isolated from leaf samples using Omega Biotek's Mag Bind Plant DNA DS 96 Kit (#M1130-01). Extraction process was on a King Fisher Flex Extraction Robot and yielded 100µl of purified genomic DNA at various concentrations. The DNA's were quantified using Quant-iT high sensitivity dsDNA assay Kit from Thermo Fisher using a Fluorophore on a synergy HTplate reader from Biotek. The DNA plate was normalized using the "epMotion" 5-75 liquid handling robot to 100 ng in 10 µl. restriction digestion was then performed in a 20 µl reaction using ApeKI at 75^0C for 2 hours. Barcode adaptors were then ligated to the cut ends using T4 ligase for 2 hours at 22^0C. Pooling of the libraries (5 µl of each sample) into a single tube was done followed by polymerase chain reaction clean up (PCR-clean up) using QIA quick PCR purification Kit from Qiagen and eluted in 30 µl of elution buffer. Polymerase chain reaction (PCR) was then performed for library amplification using 25 µl of 2x Phusion Taq Master Mix containing 3µl of each illumina primers designed by Gargiulo et al. (2014) at 10 µl each (Forward primers: 5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTC TTCCGATCT3' and Reverse primers: 5'CAAGCAGAAGACG

GCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT3'), 3 µl of the clean pooled library, 16 µl of PCR water that adds up 50µl reaction. The thermal parameters for the PCR were (1) 98^0C 30 sec, (2) 98^0C 10 sec, (3) 68^0C 30 sec, (4) 72^0C 30 sec (2-4 for 15 cycles), (5) 72^0C 5 minutes, (6) 4^0C hold. PCR product clean up was done using a QIA quick PCR purification kit (Qiagen) and eluted in 30 µl elution buffer. Quantification of the library using a Qubit leader (Thermo Fisher) and run on the Agilent 2200 Bioanalyser for quality control was done. The library was run on a HiSeq 3000 from illumina on a 150 bp paired-end flow cell which connected to the Tassel analysis pipeline where by the data was packaged and run for sequence generation. Manual data cleaning was performed in excel to remove bad SNP's (insignificant SNP's) and further in Joinmap®4.0 where SNP's with no calls and those which were monomorphic between parents as well as those having the heterozygosity value of more than 20% were all removed. The data were then run into Joinmap®4.0 using Kosambi mapping function for linkage groups detection then the map graphics was done using MapChart 2.0.

4.3.4 Progenies Phenotyping for bruchid resistance

Bruchid feeding trials was performed as described by Kusolwa, 2007. A colony of A. obtectus was obtained from bean stock in SUA bruchid management project. The colony was multiplied and maintained in susceptible Soya and Soworo to get large number of bruchids enough for the experiment. The mapping populations were tested for bruchid resistance by infesting the seeds with one colony of bean bruchid species *Acanthoscelides obtectus*. The experiment was performed at SUA in bruchid rearing chamber at room temperature (25^0C). The F_4 seeds of each bean line were weighed

and placed in transparent plastic vials with perforated covers. Twenty adult bruchids were introduced into the vials, left for 15 days for oviposition then the bruchids were removed. Starting from the thirty days since the experiment was set, data were collected over a period of 60 days. The data collected included initial seed weight before infestation, final seed weight after infestation, seed size, number of clean seeds, number of damaged seeds, and total number of emerged adult bruchids, number of days to first bruchid emergence and days to 50% adult bruchid emergence (50% DAE). The susceptibility index (SI) and percentage weight loss (%Wt loss) was calculated.

$$\text{SI} = \frac{\log(number\ of\ adult\ emerged)}{50\%\ DAE} \text{X100}$$

$$\%\text{Weight loss} = \frac{initial\ seed\ weight - final\ seed\ weight}{initial\ seed\ weight} \text{X100}$$

Data collected were analyzed using GENSTAT 17.0 for statistical test using t-test for the comparison of means of the two populations. Correlation analysis between susceptibility index and days to 50% adult bruchid emergence, susceptibility index and number of adult bruchid emerged, weight loss and number of bruchids emerged, and weight loss and susceptibility index were done as well.

4.3.5 QTL analysis

The genotyping data obtained in this study together with the phenotyping data for bruchid resistance related traits including days to 50% adult bruchid emergence, seed size, seed weight, susceptibility index (SI) and damaged seeds were used for quantitative trait loci (QTL) analysis using MapQTL® 6. Quantitative trait loci were then aligned into the linkage map using MapChart 2.0.

4.4 RESULTS

4.4.1 Genotyping by sequencing

A total of 7736 and 6273 marker loci were generated from illumina sequencing for mapping population KSy and mapping population KSw respectively. Following data cleaning where by the SNP's with distorted segregation ratios and those having >20% missing values were removed, 328 Novel SNP's for genetic mapping were identified from mapping population KSy and 435 novel SNP's for the mapping population KSw distributed around the 11 linkage groups of *Phaseolus vulgaris* (PV1-PV11). The total map distance was 596.4cM and 711.9cM for the mapping population KSy and mapping population KSw with the average map distance per SNP's of 0.5cM and 0.6cM respectively (Fig.4.1 and Fig.4.2). The length of individual linkage groups varied from 2.9cM in PV6 to 200.1cM in PV4 for mapping population KSy and 43.4cM in PV10 to 123.4cM in PV8 for mapping population KSw. The linkage groups had clusters of markers due to low recombination of markers in which large genomic region were lacking polymorphic markers in chromosome prevented the consolidation of the markers into a single linkage group as a result a single linkage group was having clusters of markers such as PV01 had 1a, 1b and 1c. Linkage group PV4, PV7 and PV9 had three SNP's clusters between four groups (highest number of clusters) while PV1 and PV 10 had the lowest number of SNP's clusters (one cluster between two groups) in the KSy population. In the mapping population KSw, PV1 had the highest number of clusters (clusters of breaks of splits from the mother linkage group) while the lowest number of clusters (one cluster of markers between two groups) was in PV6 and PV7. In both mapping population KSy and KSw, some SNP's were co-segregating with other SNP's

mapping on the same position in the linkage maps (for example S3_40089496 mapped on the same position with S3_39113410 in linkage group PV3A).

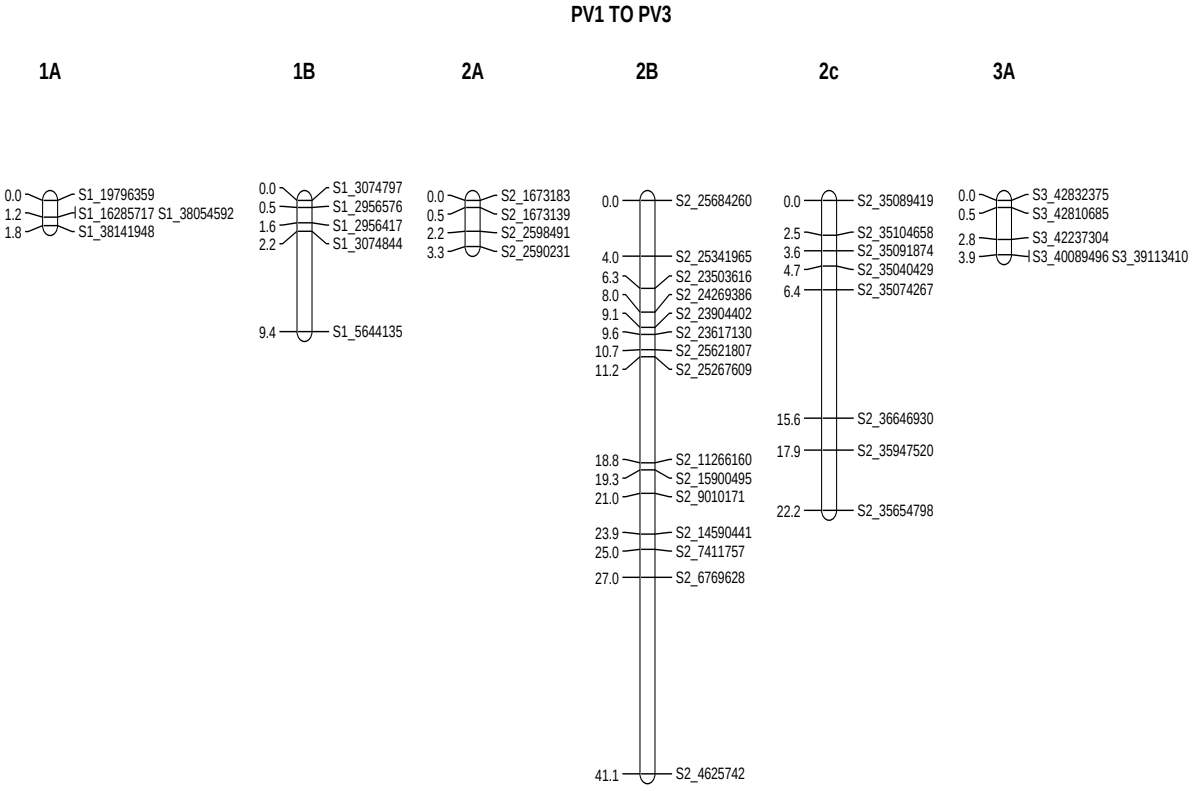

Figure 4. 1. Recombinant inbred lines in a mapping population KSy with 11 linkage groups generated from 328 SNP's

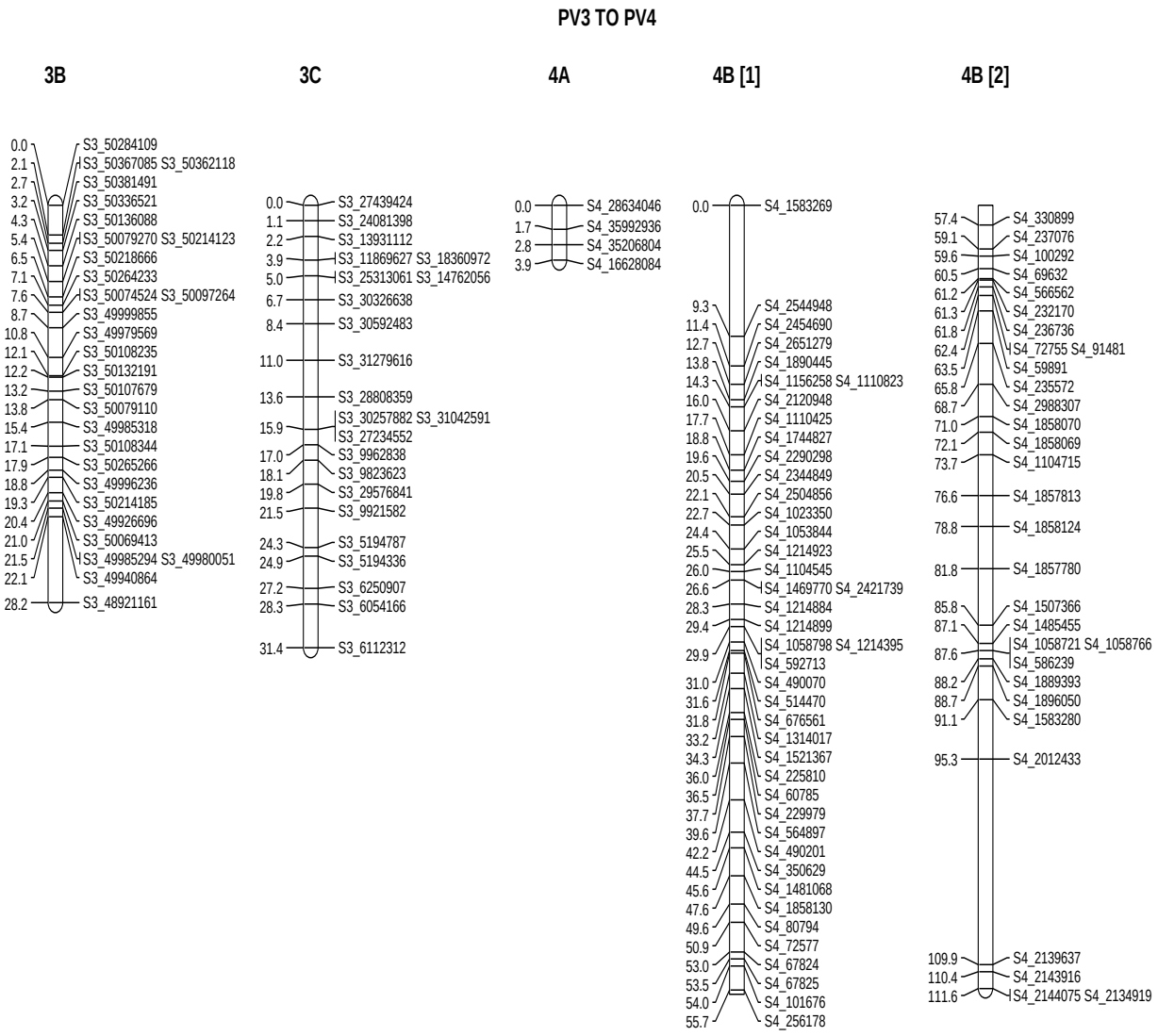

Figure 4.1 (cont.)

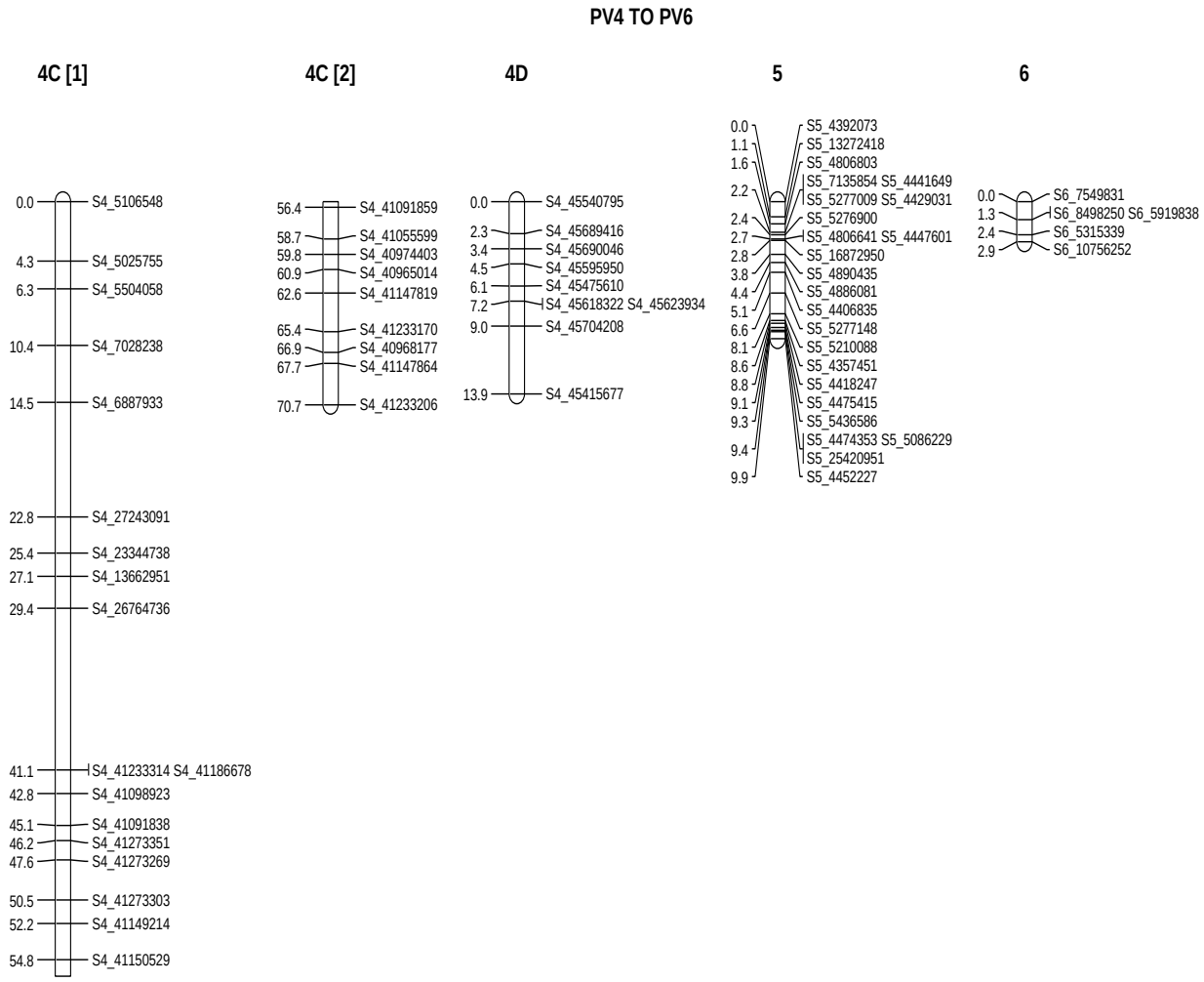

Figure 4.1 (cont.)

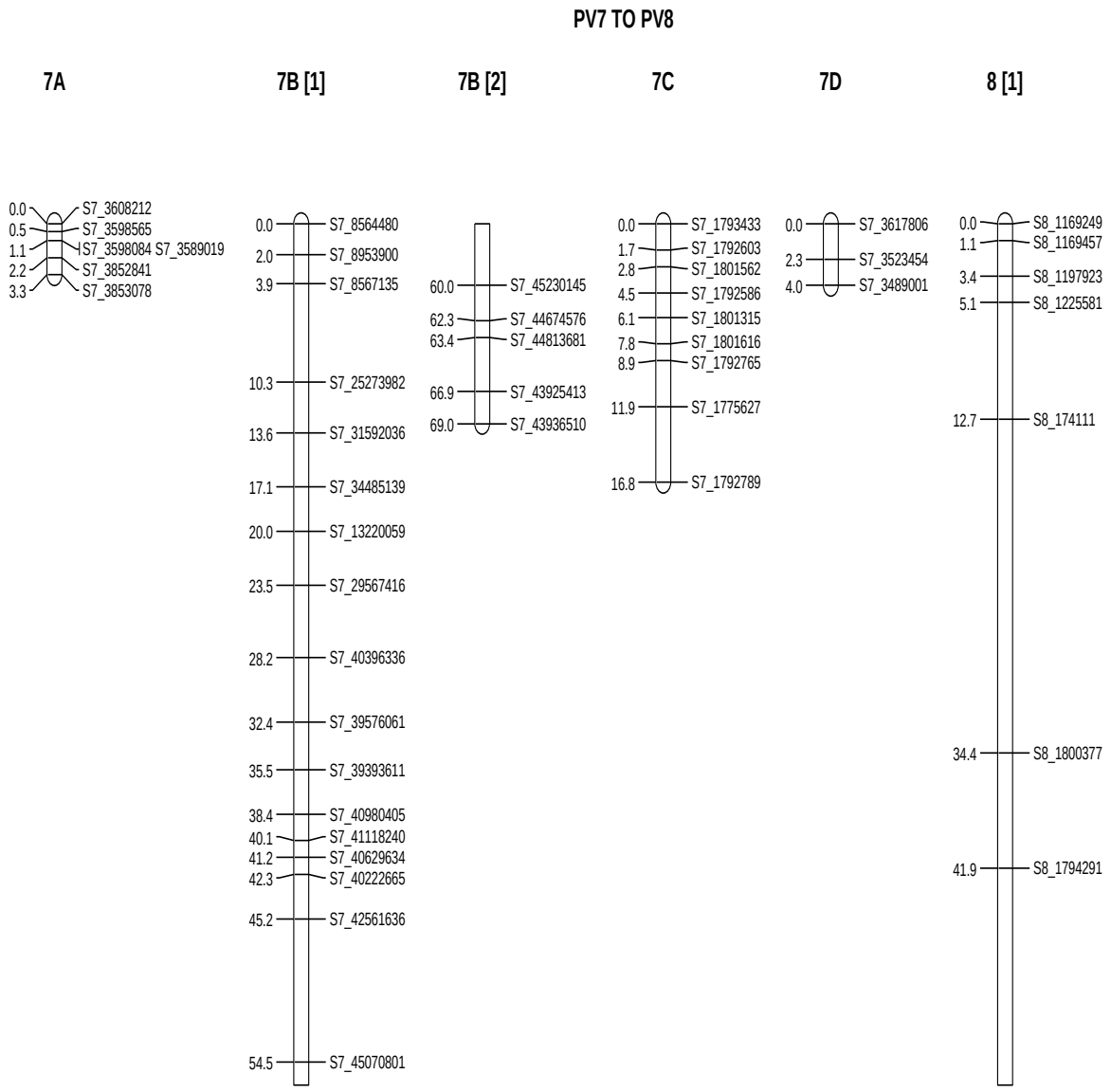

Figure 4.1 (cont.)

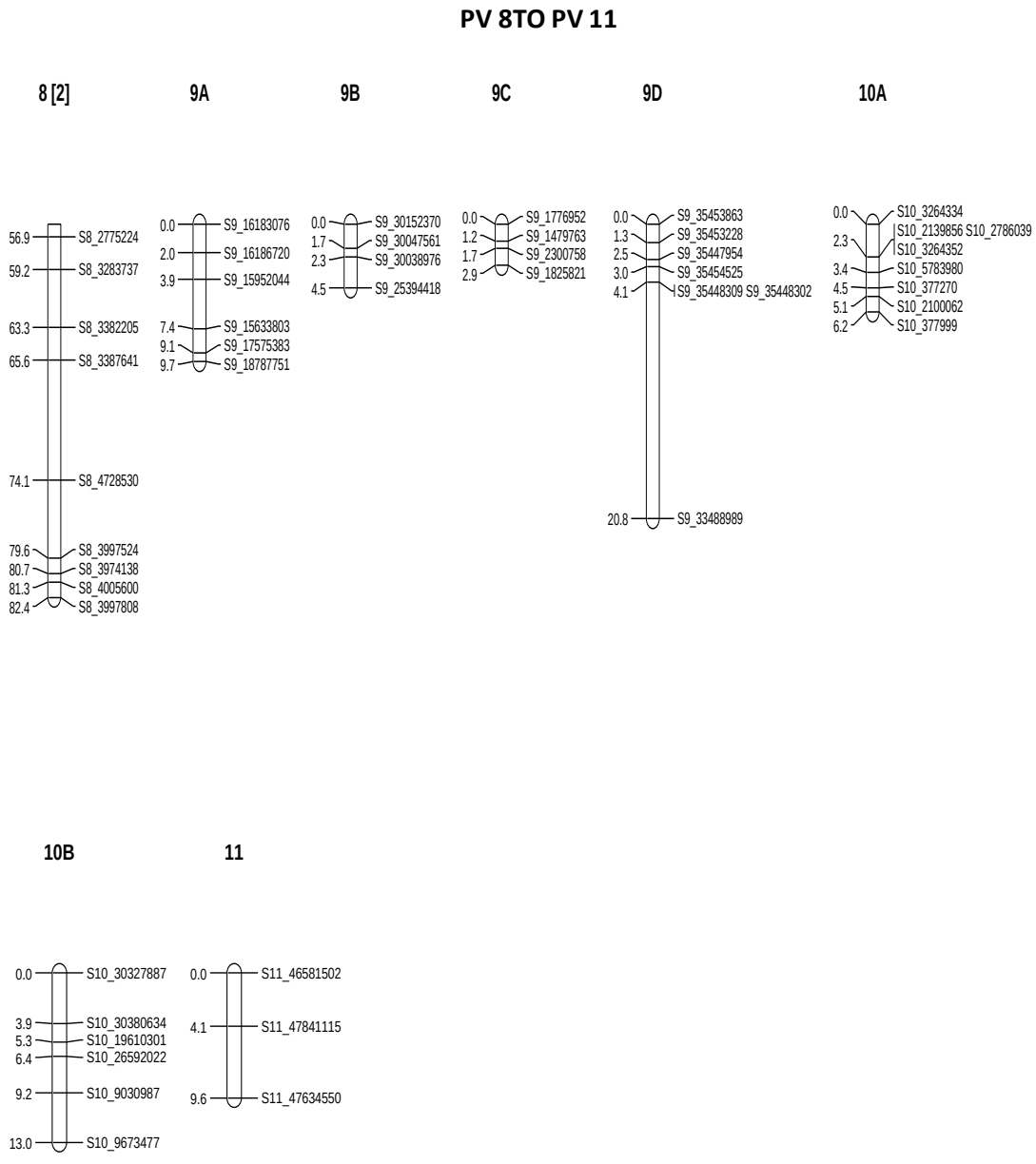

Figure 4.1 (cont.)

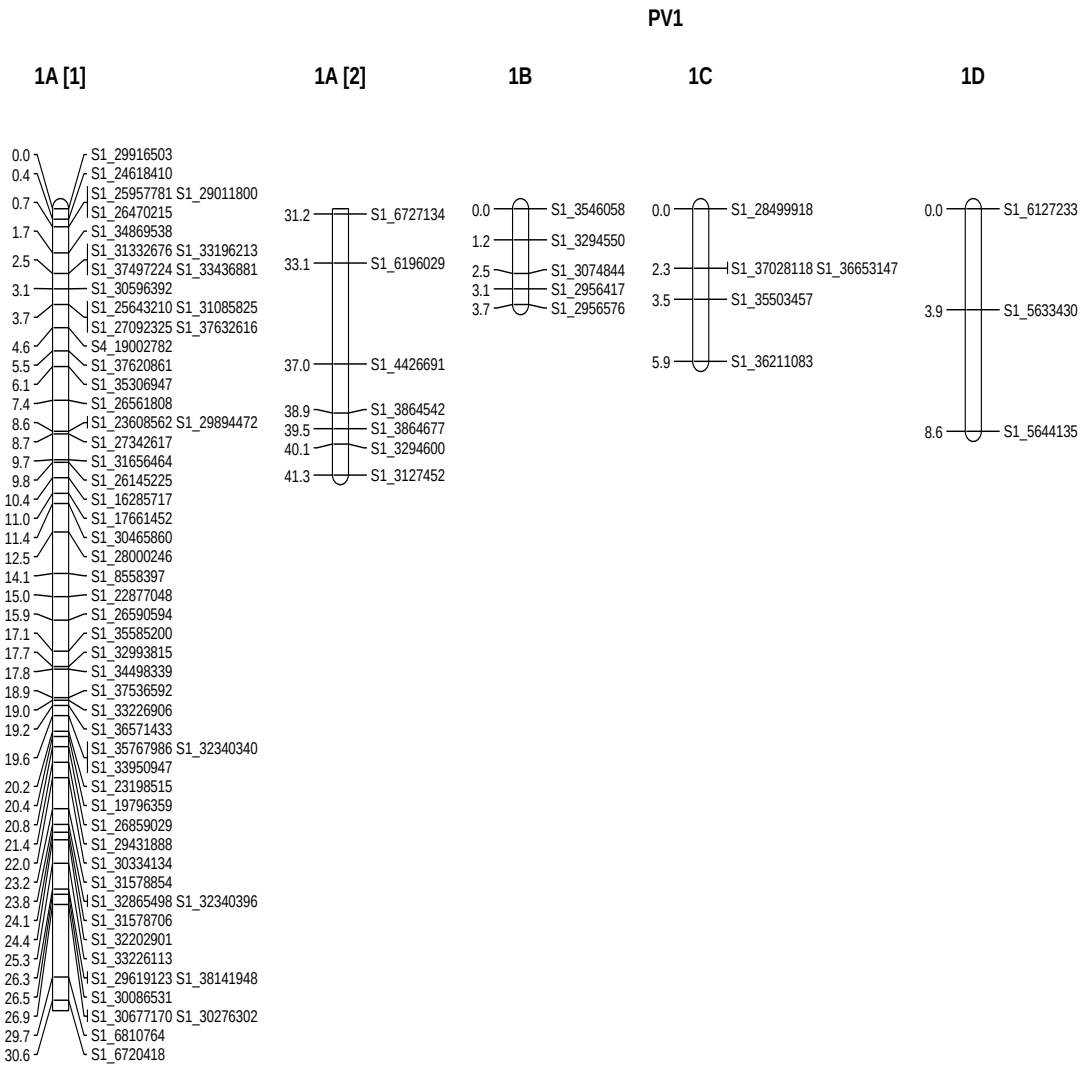

Figure 4. 2. Recombinant inbred lines in a mapping population KSw with 11 linkage groups generated from 435 SNP's

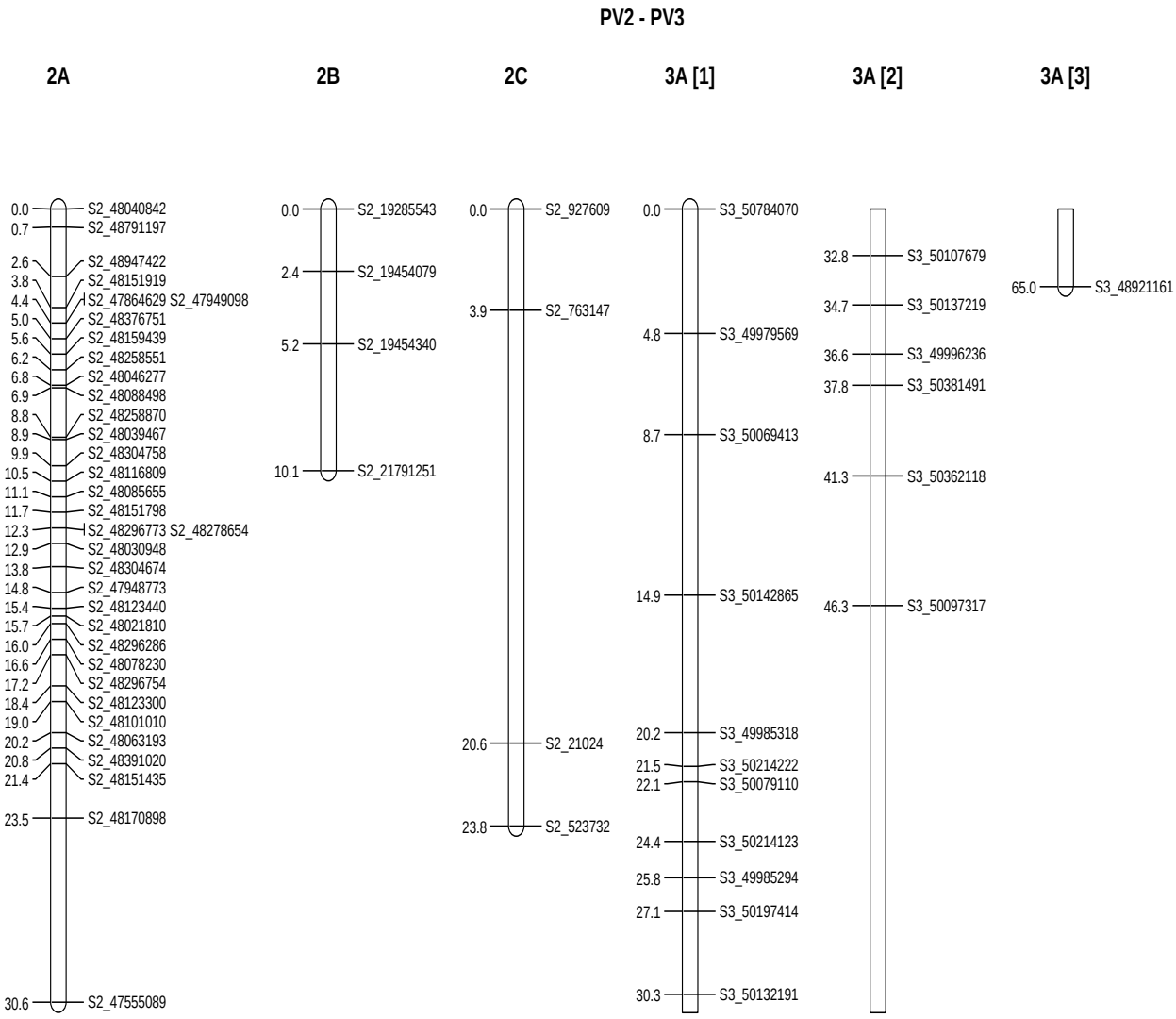

Figure. 4.2 (cont.)

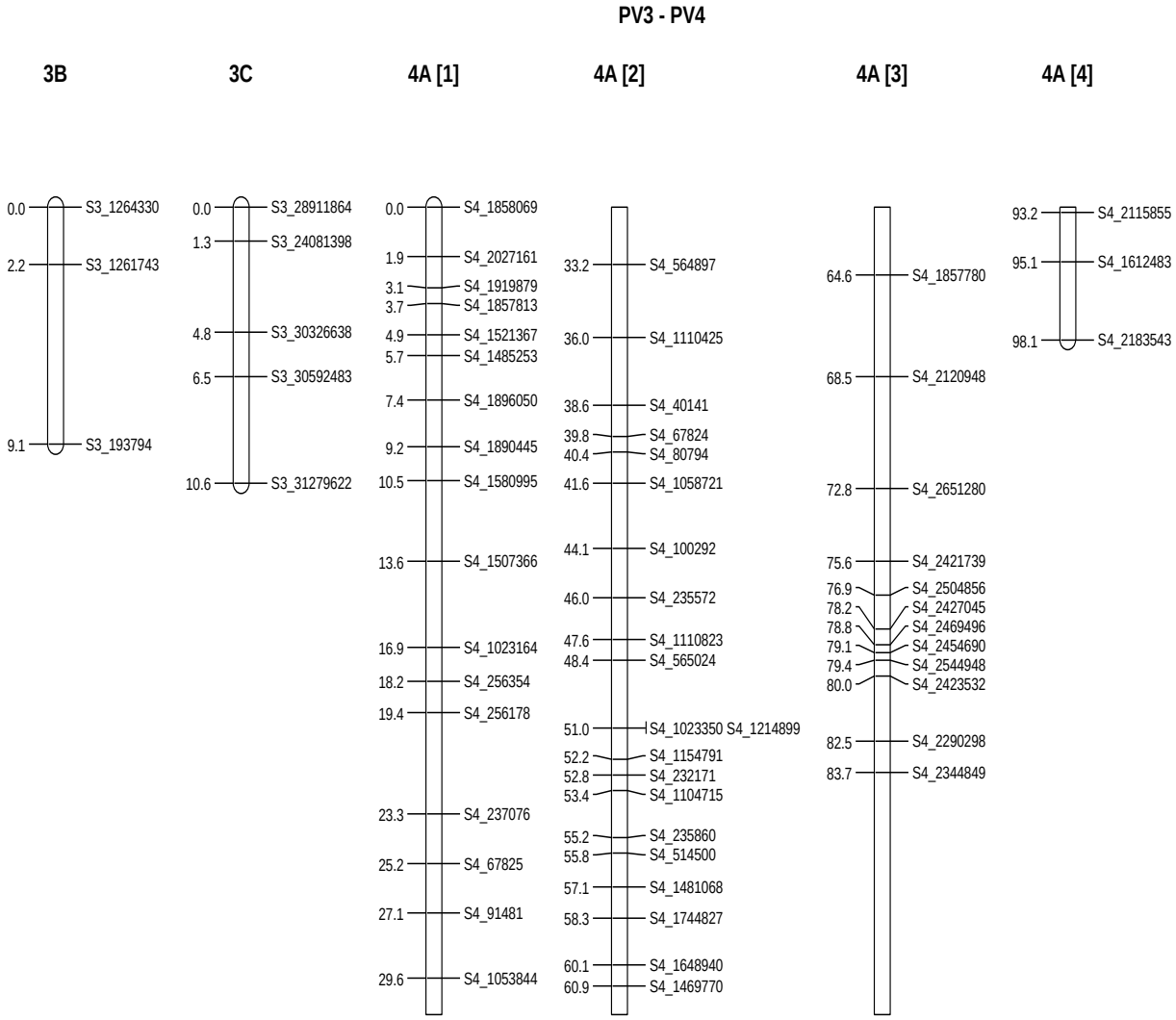

Figure. 4.2 (cont.)

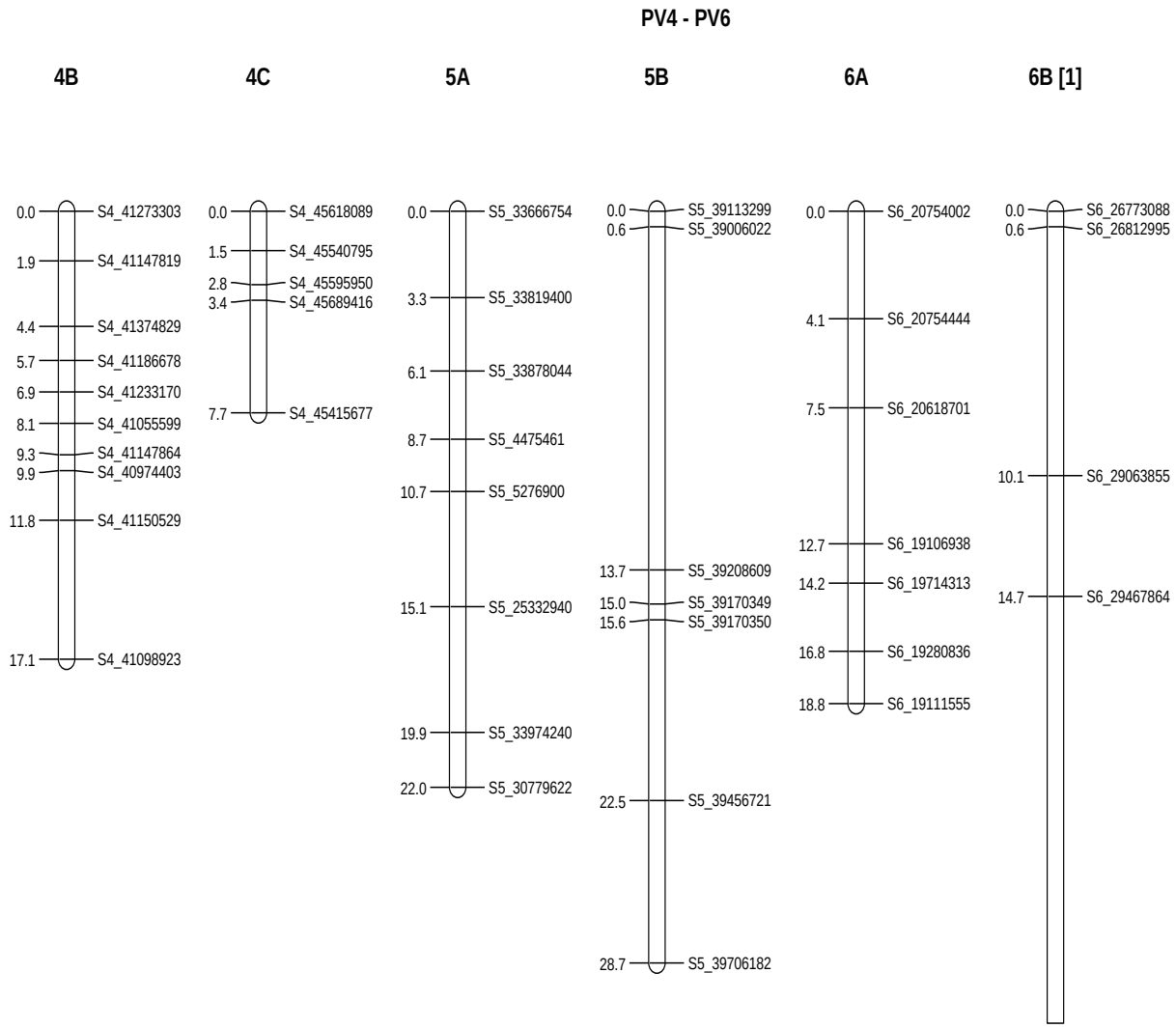

Figure. 4.2 (cont.)

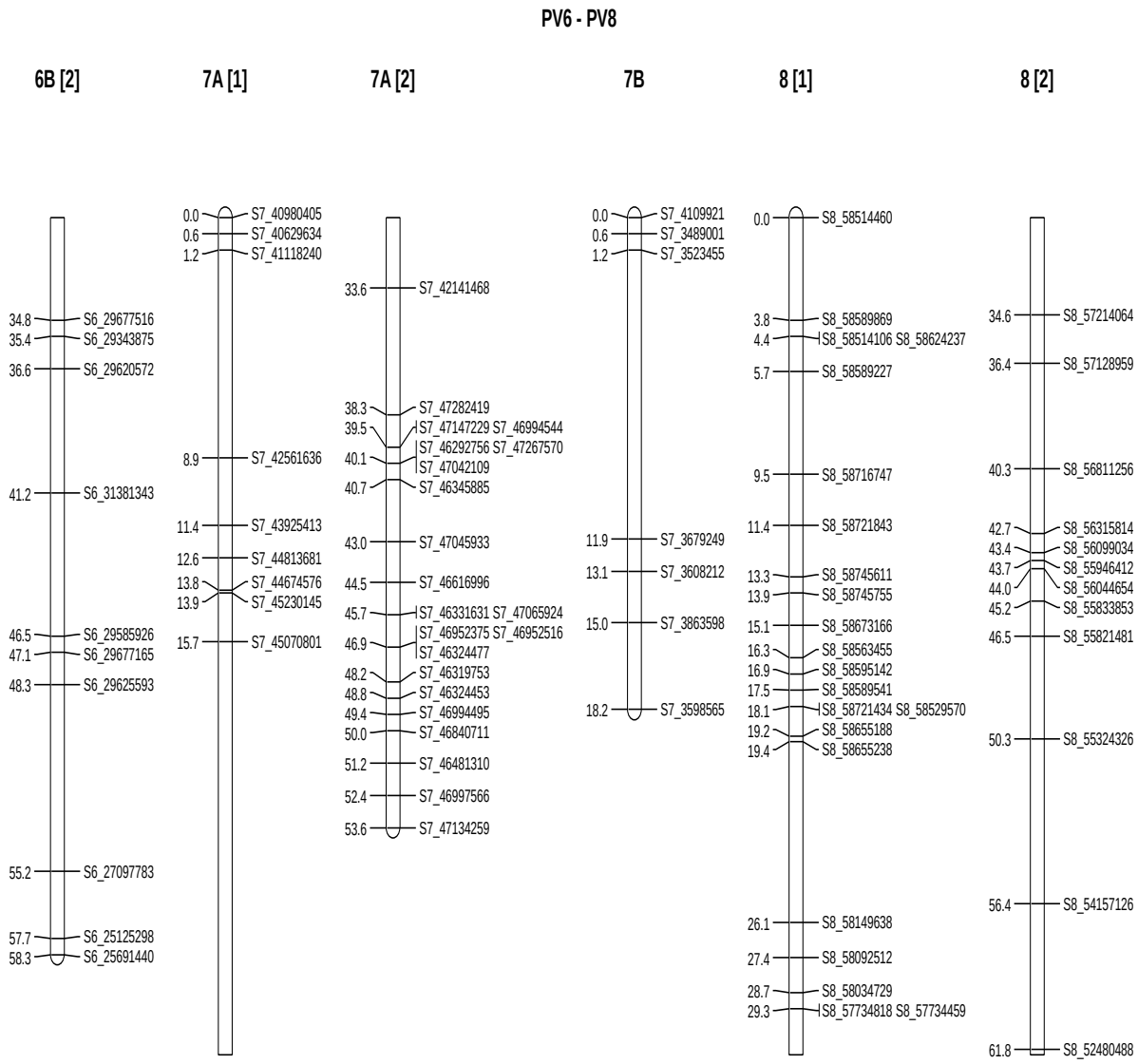

Figure. 4.2 (cont.)

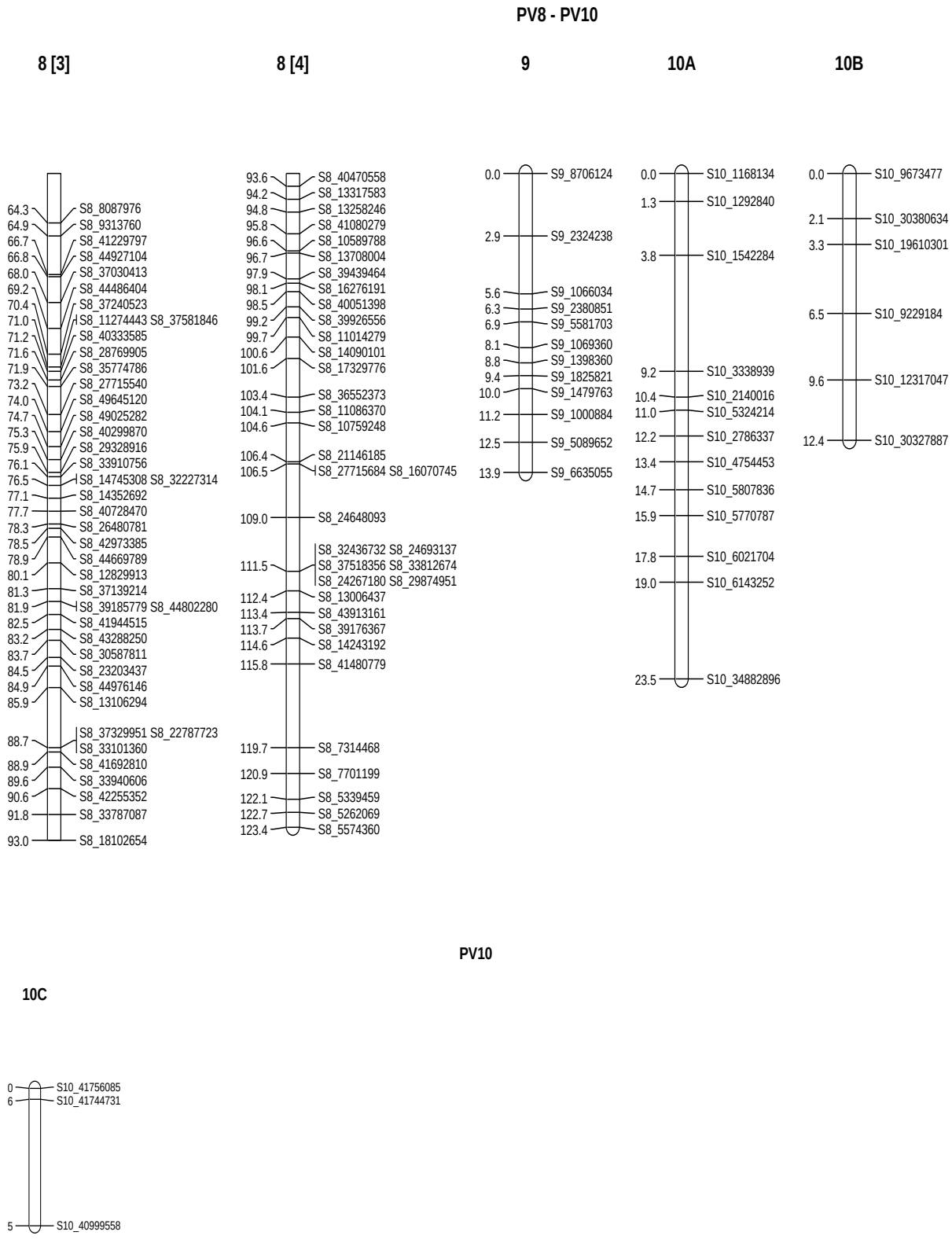

Figure. 4.2 (cont.)

4.4.2 Progenies phenotyping for bruchid resistance

The results obtained from the two populations evaluated for bruchid resistance showed that there were variations in response to bruchid infestation between population KSy and population KSw based on number of bruchids emerged, damaged seeds, susceptibility index (SI) and percentage weight loss (%wt loss).

In KSy population, six lines which are KSy2, KSy5, KSy9, KSy32, KSy35 and KSy38 had the lowest number of bruchid emergence of zero indicating that the lines were highly resistant as there were no bruchid emerged. Nine lines KSy1, KSy3, KSy7, KSy10, KSy14, KSy34, KSy42, KSy47 and KSy48 were also significant resistant by having lower number of bruchid emergence ranging from one to seven bruchids emerged while the highest number of bruchid emerged was 85 observed in line KSy21 showing that this line is highly susceptible to bruchid attack (Fig. 4.3).

The lowest number of damaged seeds was zero observed in lines KSy2, KSy5, KSy9, KSy32, KSy35 and KSy38. Lower number of damaged seeds was also observed in lines KSy1, KSy3, KSy7, KSy10, KSy14, KSy34, KSy42, KSy47 and KSy48 ranging from one to five damaged seeds while the highest number of damaged seeds was 24 observed in susceptible parent Soya (Fig. 4.4). The lowest susceptibility index (SI) observed in lines KSy1, KSy2, KSy5, KSy9, KSy32, KSy35 and KSy38 with susceptibility index of zero till the end of the experiment, nine lines KSy1, KSy3, KSy7, KSy10, KSy14, KSy34, KSy42, KSy47 and KSy48 had lower susceptibility index ranged from 0.75 to 1.95 compared to KSy21 with highest susceptibility index of 5.41 (Fig.4.5)

The percentage weight loss (%Wt) ranged from zero with lines KSy2, KSy5, KSy9, KSy32, KSy35 and KSy38 having the lowest percentage weight loss of zero to 16.47 which was the highest observed in line KSy21 (Fig. 4.6).

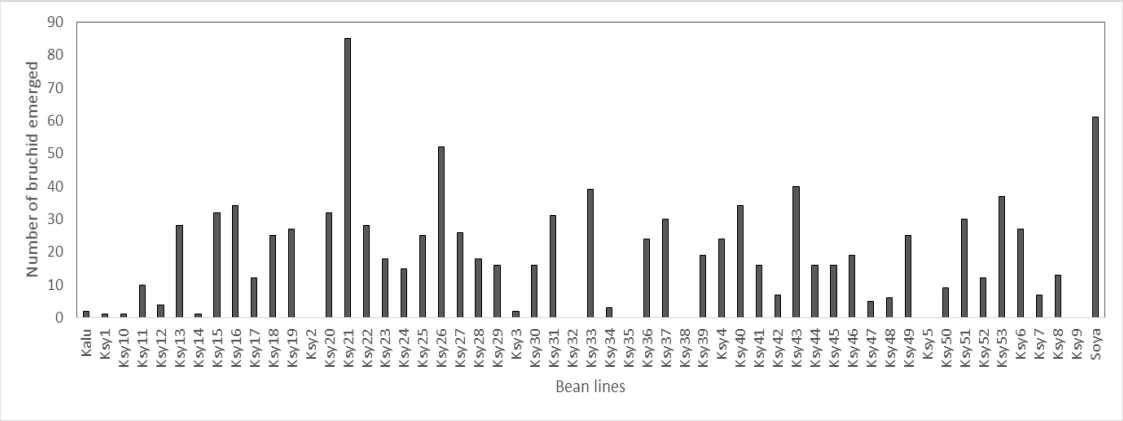

Figure 4. 3. Number of *A. obtectus* adult bruchids emerged in population KSy monitored for more than 60 days following bruchid infestation

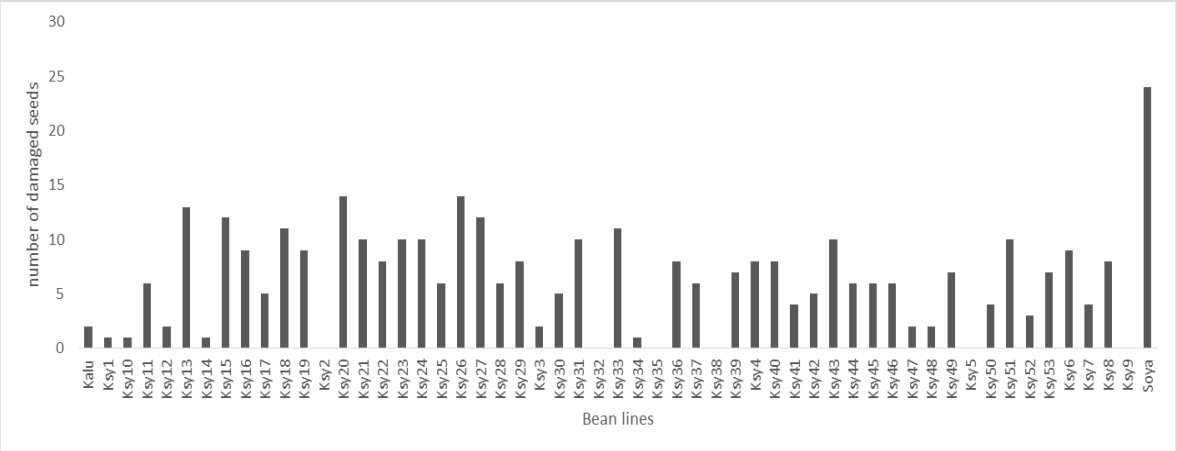

Figure 4. 4. Number of damaged seeds in population KSy infested by *A. obtectus*

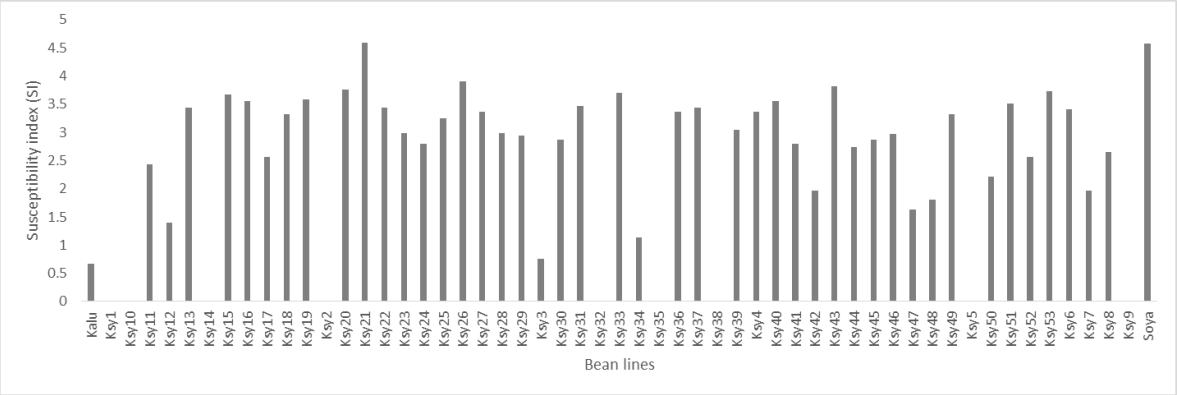

Figure 4. 5. Susceptibility index in population KSy infested by *A. obtectus*

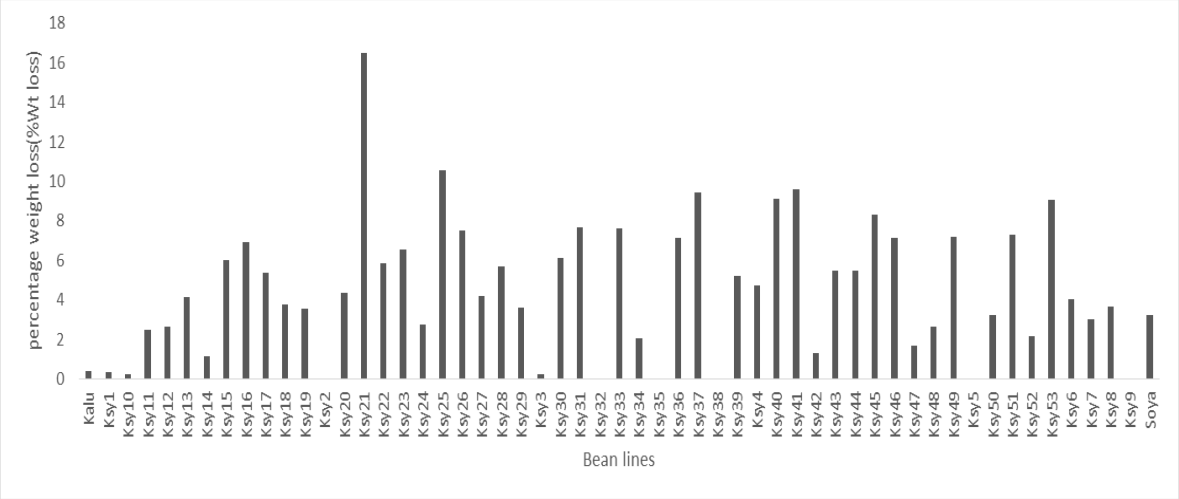

Figure 4. 6. Percentage weight loss in population KSy infested by *A. obtectus*

In contrast to KSy population, KSw population had only one line with zero number of bruchid emerged which was KSw26 while the highest number of bruchid emerged was 138 in line KSw48 exceeding that in population KSy by 53 (Fig. 4.7). Four lines KSw5, KSw6, KSw10 and KSw16 were having lower number of bruchid emerged (5-16 bruchids emerged) indicating that the lines were resistant to bruchid emergence too. No damaged seed was observed in line KSw26, four to nine damaged seeds were observed in lines KSw5, KSw6, KSw10 and KSw16 while in line KSw47 there were highest number of damaged seeds of 33 exceeding that of KSy population by 9 (Fig.

4.8). The lowest SI was zero observed in line KSw26, lower SI value ranged from 1.7-2.27 were shown by lines KSw5, KSw6, KSw10 and KSw16 and the highest was 5.09 observed in line KSw48 higher than that of Ksy population (Fig. 4.9). The results also showed that the lowest percentage weight loss was zero in line KSw26 while the highest was found in line KSW42 with 20.42 percentage weight loss (Fig. 4.10).

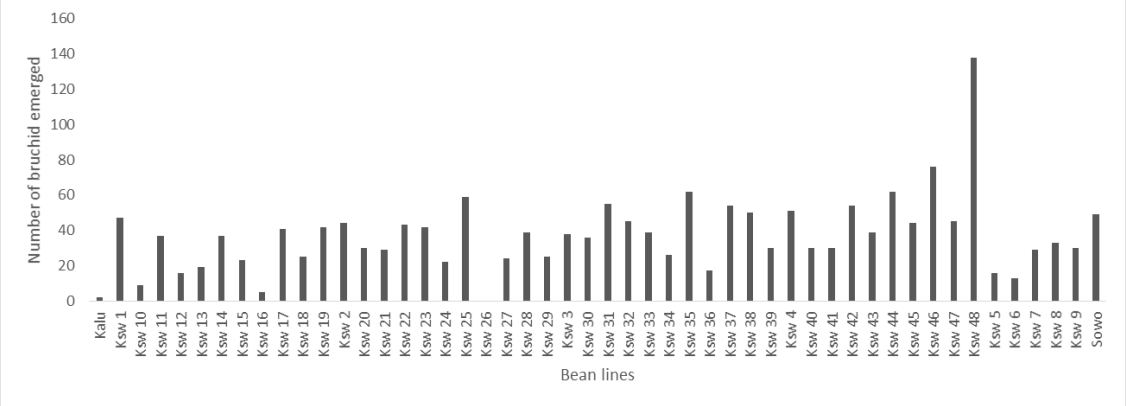

Figure 4. 7. Number of *A. obtectus* adult bruchids emerged in population KSw monitored for more than 60 days following bruchid infestation

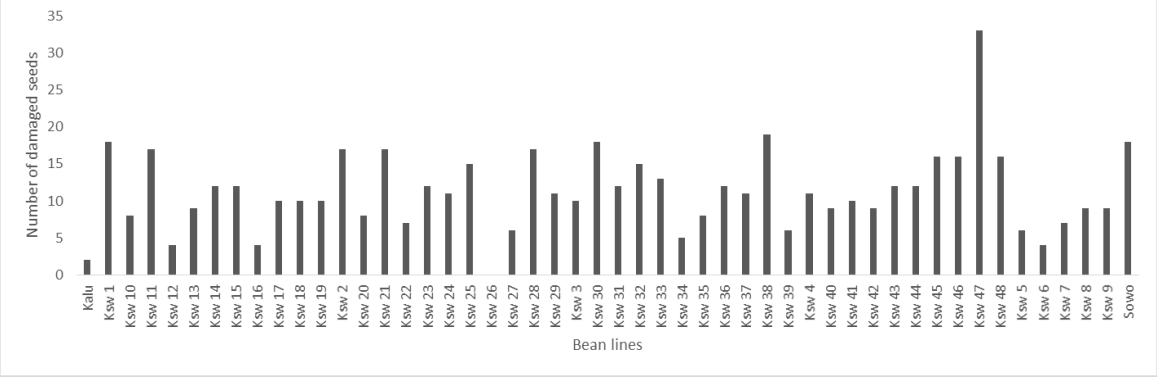

Figure 4. 8. Number of damaged seeds in population KSw infested by *A. obtectus*

Figure 4. 9. Susceptibility index in population KSw infested by *A. obtectus*

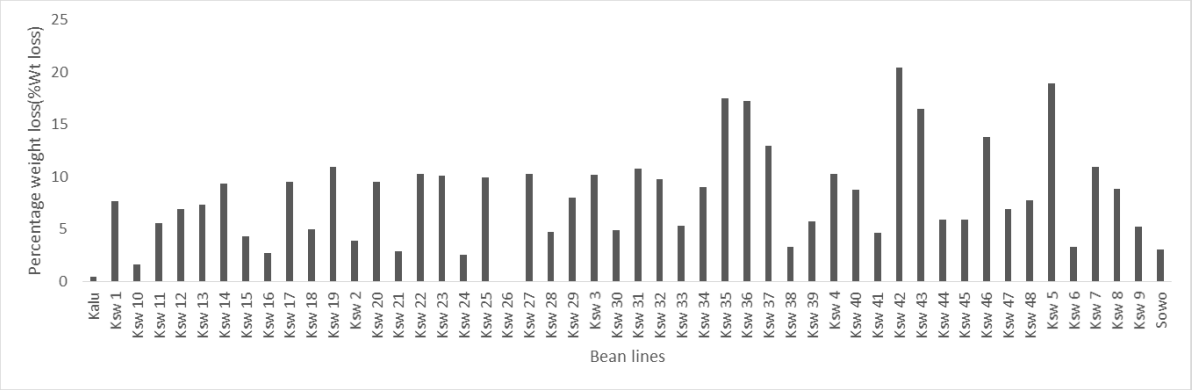

Figure 4. 10. Percentage weight loss in population KSw infested by *A. obtectus*

Generally eight lines were identified to be highly resistant from this experiment based on number of adult bruchid emergence, number of damaged seeds, susceptibility index (SI) and percentage weight loss (% Wt loss) with six lines KSy2, KSy5, KSy9, KSy32, KSy35 and KSy38 from KSy population and two lines KSw16 and KSw26 from population KSw. Twelve lines were identified to be resistant including KSy1, KSy3, KSy7, KSy10, KSy14, KSy34, KSy42, KSy47 and KSy48 from KSy population and KSw5, KSw6 and KSw10 from KSw population (Table 4.1).

Table 4. 1. The best performing lines in KSy and KSw populations based on damaged seeds, susceptibility index (SI), number of adult bruchid emerge, days to 50% adult emergence and percentage weight loss (% Wt loss)

Bean line	Damaged seeds	Susceptibility Index	# of bruchid emerged	50%DAE	%Wt loss
KSy1	1	0	1	36	0.359
KSy2	0	0	0	60	0
KSy3	2	0.75	2	40	0.22
KSy5	0	0	0	60	0
KSy9	0	0	0	60	0
KSy10	1	0	1	44	0.235
KSy14	1	0	1	42	1.146
KSy32	0	0	0	60	0
KSy34	1	1.13	3	42	2.06
KSy35	0	0	0	60	0
KSy38	0	0	0	60	0
KSy42	5	1.96	7	43	1.32
KSy47	2	1.62	5	43	1.70
KSy48	2	1.8	6	43	2.6
KSw5	6	2.8	16	43	18.9
KSw6	4	2.5	13	43	3.33
KSw10	8	2.27	9	42	1.65
KSw16	4	1.705	5	41	2.756
KSw26	0	0	0	60	0

Statistical analysis was performed for the comparison between the two populations using t-test (Table 4.2). The results indicated that there were highly statistical significance ($P \leq 0.05$) among the two populations based on number of damaged seeds, susceptibility index (SI), number of adult bruchid emerged and percentage weight loss (% Wt loss) with population KSy having lower means in all the parameters than population KSw. The results also showed a statistical significance in days to first adult bruchid emergence and there were no statistical significance in number of days to 50% DAE among the two populations (Table 4.2).

Table 4. 2. t-test means of days to 50% DAE, number of damaged seeds, days to first bruchid emergence, Susceptibility index (SI), number of bruchid emerged and percentage weight loss (%Wt) of two mapping populations infested by *A. obtectus*

	50%DAE	Damaged seeds	1st bruchid emergence	Susceptibility index (SI)	# of bruchid emerged	%Wt loss
Population KSy	44.02 ± 0.7822	6.418 ± 0.6312	41.51 ± 0.9323	2.472 ± 0.1841	19.27 ± 2.267	4.521 ± 0.4577
Population KSw	42.88 ± 0.3996	11.260 ± 0.7818	38.48 ± 0.7212	3.468 ± 0.1226	37.02 ± 3.083	8.047 ± 0.6607
S.E.D (±)	0.878	0.997	1.179	0.221	3.780	0.804
P-Value	0.199	< 0.001	0.012	< 0.001	< 0.001	< 0.001

Correlation analysis ($P \leq 0.01$) of the two populations was performed between susceptibility index and 50% DAE, susceptibility index and number of bruchid emerged, susceptibility index and damaged seeds, susceptibility index and days to 50% bruchid emergence, damaged seeds and number of bruchid emerged and percentage weight loss and number of bruchid emerged.

Susceptibility index indicated a strong correlation with number of bruchid emerged (80.5%) indicating that susceptible seeds with high susceptibility index have large number of adult emerged. Susceptibility index demonstrated a strong correlation (75.9%) with damaged seeds showing that susceptible seeds with high susceptibility index are more damaged by bruchids. Susceptibility index was negatively correlated with days to 50% adult bruchid emergence (-28.1%) indicating that resistant seeds with lower susceptibility index have the ability to take many days without bruchid emergence. The results also revealed a positive correlation between damaged seeds and number of bruchid emerged (67.9%) showing that seeds that were severely perforated due to larvae feeding had high number of bruchid emergence. There was also positive correlation between percentage weight loss and number of bruchid emerged (59.6%) indicating that as more bruchids emerge from the susceptible seeds the weight was lost more (Table 4.3)

Table 4. 3.Correlation between susceptibility index and 50% DAE, susceptibility index and number of bruchid emerged, percentage weight loss and number of bruchids emerged, number of bruchid emerged and number of damaged seeds and percentage weight loss and susceptibility index

	# of Bruchid emergence	Days 50% emergence	Damaged seed	SI	%Weight loss
# of Bruchid emergence	1				
Days 50% emergence	-0.147	1			
Damaged seed	0.679**	-0.185	1		
SI	0.805**	-0.281**	0.759**	1	
%Weight loss	0.596**	-0.114	0.358**	0.650**	1

**. Correlation is significant at the 0.01 level (2-tailed).

4.4.3 Quantitative Trait Loci Mapping

Bruchid resistance in the mapping populations measured by 50%DAE, adult emergence, damaged seeds, weight loss and susceptibility index (SI) was significant associated with the markers in the linkage map.

In KSy population, quantitative trait loci (QTL's) for days to 50% adult bruchid emergence (50%DAE) mapped on PV03 explained 14.4% of the total genetic variation with single nucleotide polymorphism (SNP's) S3_48921161 being associated with this trait. Three QTL's were associated with adult emergence, one mapped on PV05 and two mapped on PV09 which explained 16.2%, 13.9% and 11.7% of the total genetic variation with S5_4886081, S9_30152370 and S9_15952044 being the SNP's for this trait respectively. Percentage Damaged seeds had two QTL's mapped on PV05 and PV09 with S5_4886081 and S9_15952044 being the SNP's for this trait which explained 15.1% and 12.7% of the total genetic variation respectively. QTL's for susceptibility index mapped on PV05 with S5_4886081 being the single nucleotide polymorphism QTL's explained 15.7% of the total genetic variation. Percentage weight loss QTL's mapped on the same linkage group with susceptibility index and with the same SNP's S5_4886081 but explained 10.4% of the total genetic variation. Four QTL's were associated with percentage adult emergence mapped on PV05, PV09 and PV05 with S5_4886081, S9_15952044, S4_35992936 and S9_25394418 being the SNP's for this trait explaining 16.3%, 14.1%, 11.5% and 11.3% of the total genetic variation respectively (Table. 4.4 and Fig.4.11).

Table 4. 4. **Bruchid resistance QTL's for mapping population KSy**

LINKAGE GROUP	Flanking SNP's for bruchid resistance QTL's	QTL TRAITS	POSITION	LOD	%EXPL
PV 3	S3_48921161	50% DAE	28.25	1.78	14.4
PV4	S4_35992936	Percentage Adult Emergence (PAE)	1.731	1.4	11.5
PV5	S5_4886081	Number of Adult emergence	4.384	2.03	16.2
	S5_4886081	% Damaged seeds	4.384	1.89	15.1
	S5_4886081	SI	4.384	1.96	15.7
	S5_4886081	%wt loss	4.384	1.26	10.4
	S5_4886081	Percentage Adult Emergence	4.384	2.05	16.3
PV9	S9_15952044	Number of Adult emergence	3.946	1.44	11.7
	S9_30152370	Number of Adult emergence	0	1.73	13.9
	S9_15952044	%Damaged seed	3.946	1.57	12.7
	S9_15952044	Percentage Adult Emergence	3.946	1.75	11.3
	S9_25394418	Percentage Adult Emergence	4.512	1.37	11.3

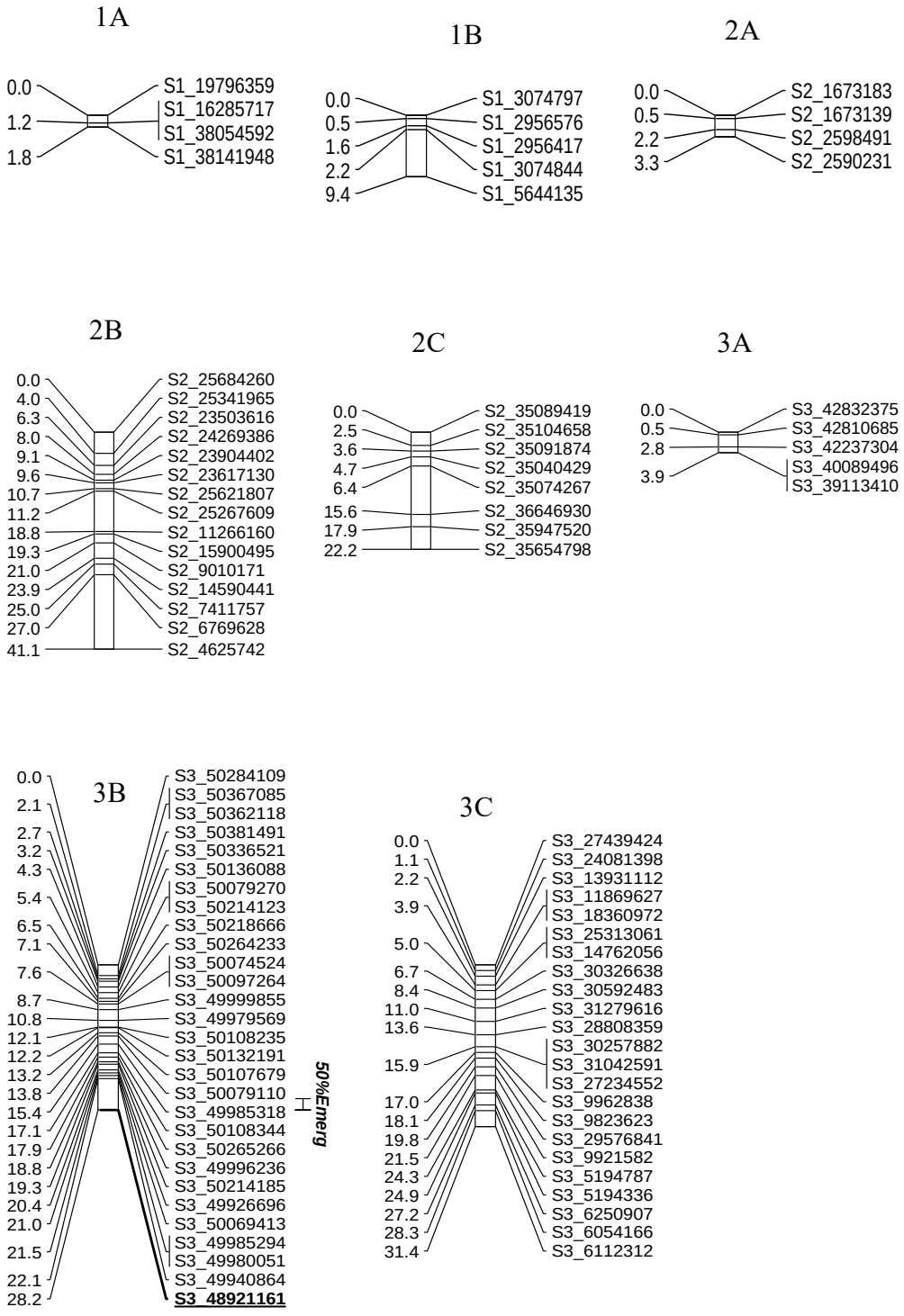

Figure 4. 11. QTL's for days to 50% adult emergence (50% DAE) in KSy population with underlined markers related to 50% DAE.

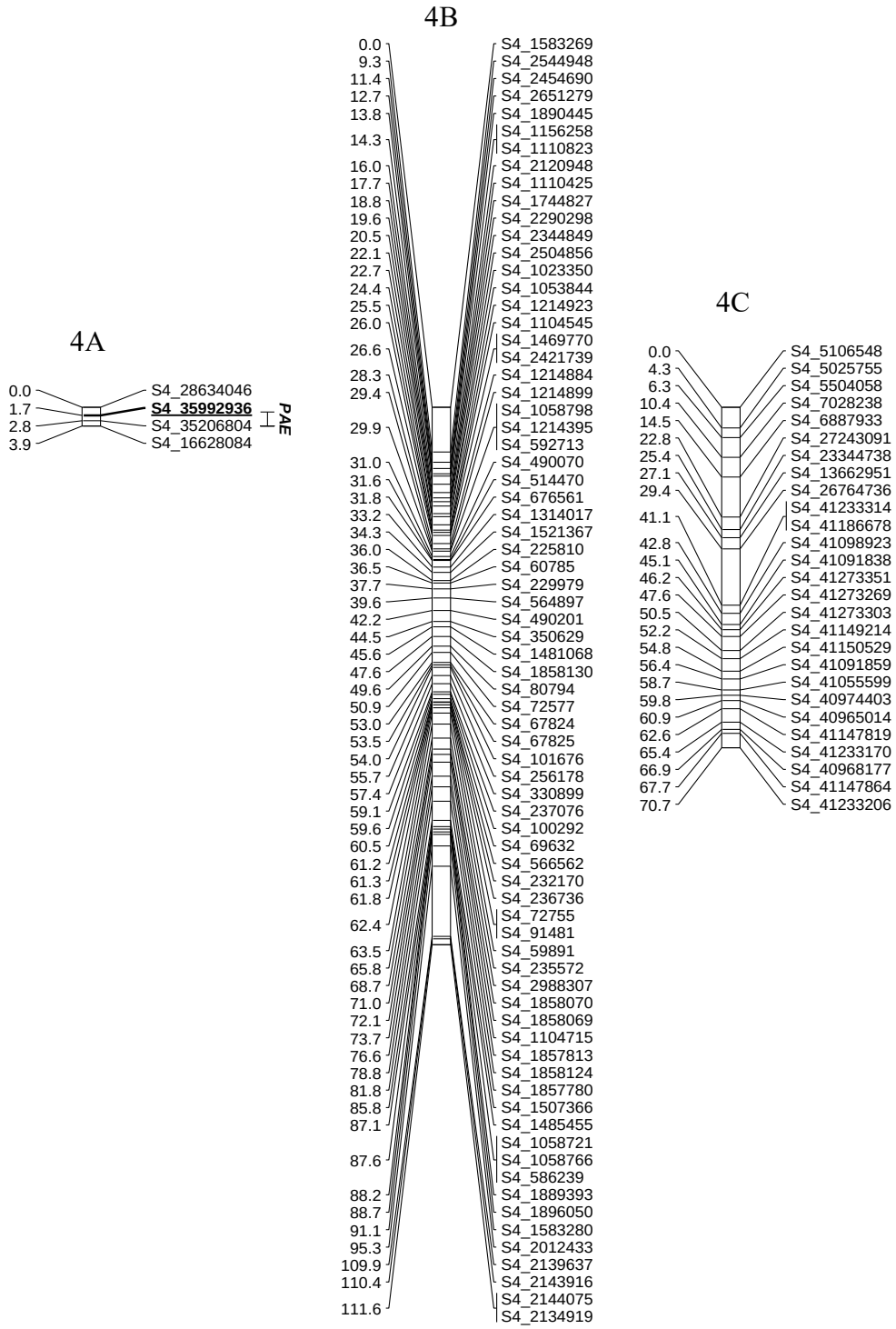

Figure 4. 12. QTL's for Percentage adult emergence (PAE) in KSy population with markers related to PAE being underlined.

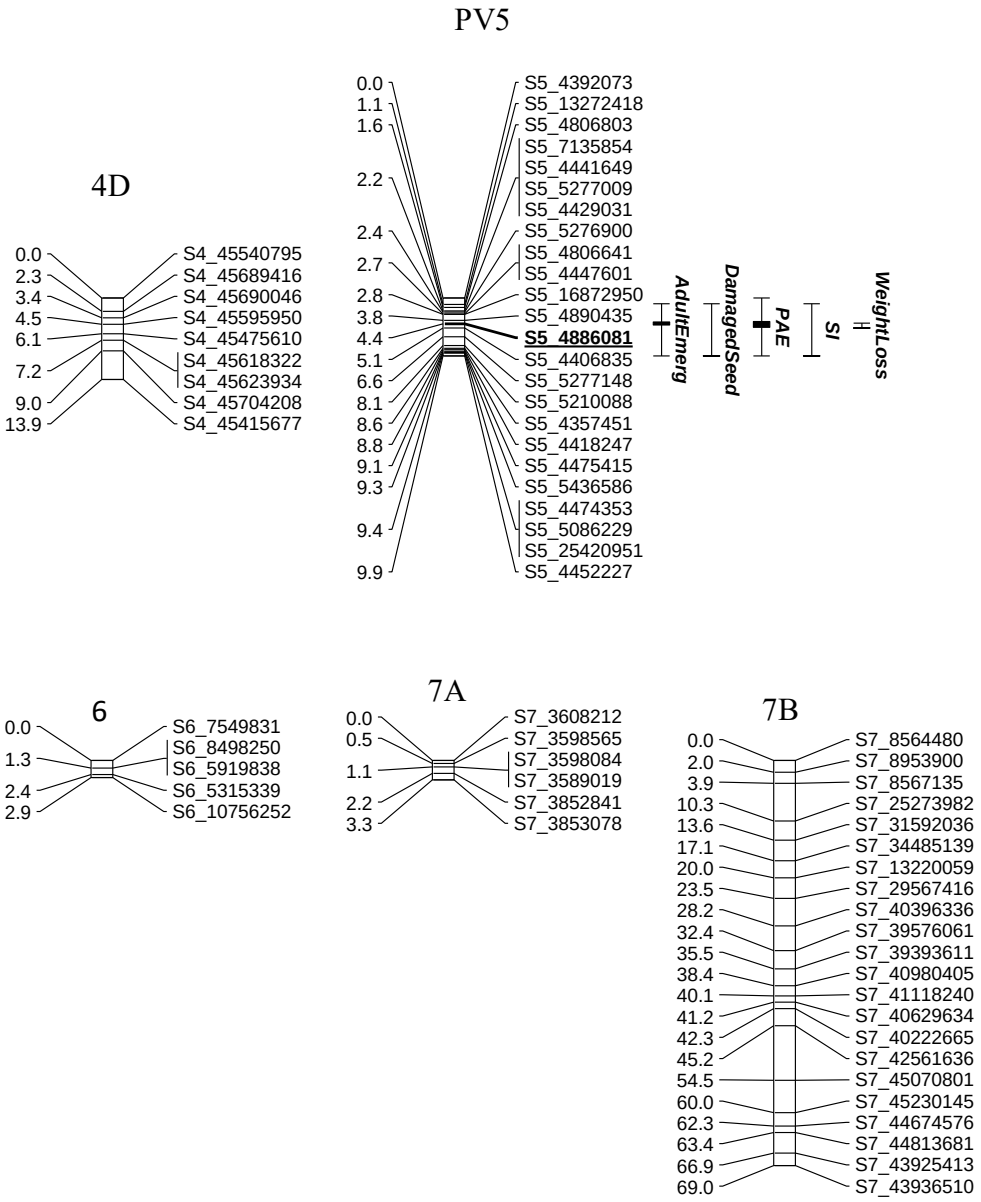

Figure 4. 13. Percentage weight loss (%Wt loss), susceptibility index (SI), percentage adult emergence (PAE), percentage damaged seeds and number of adult bruchid emergence QTL's in KSy population with markers related to these traits being underlined.

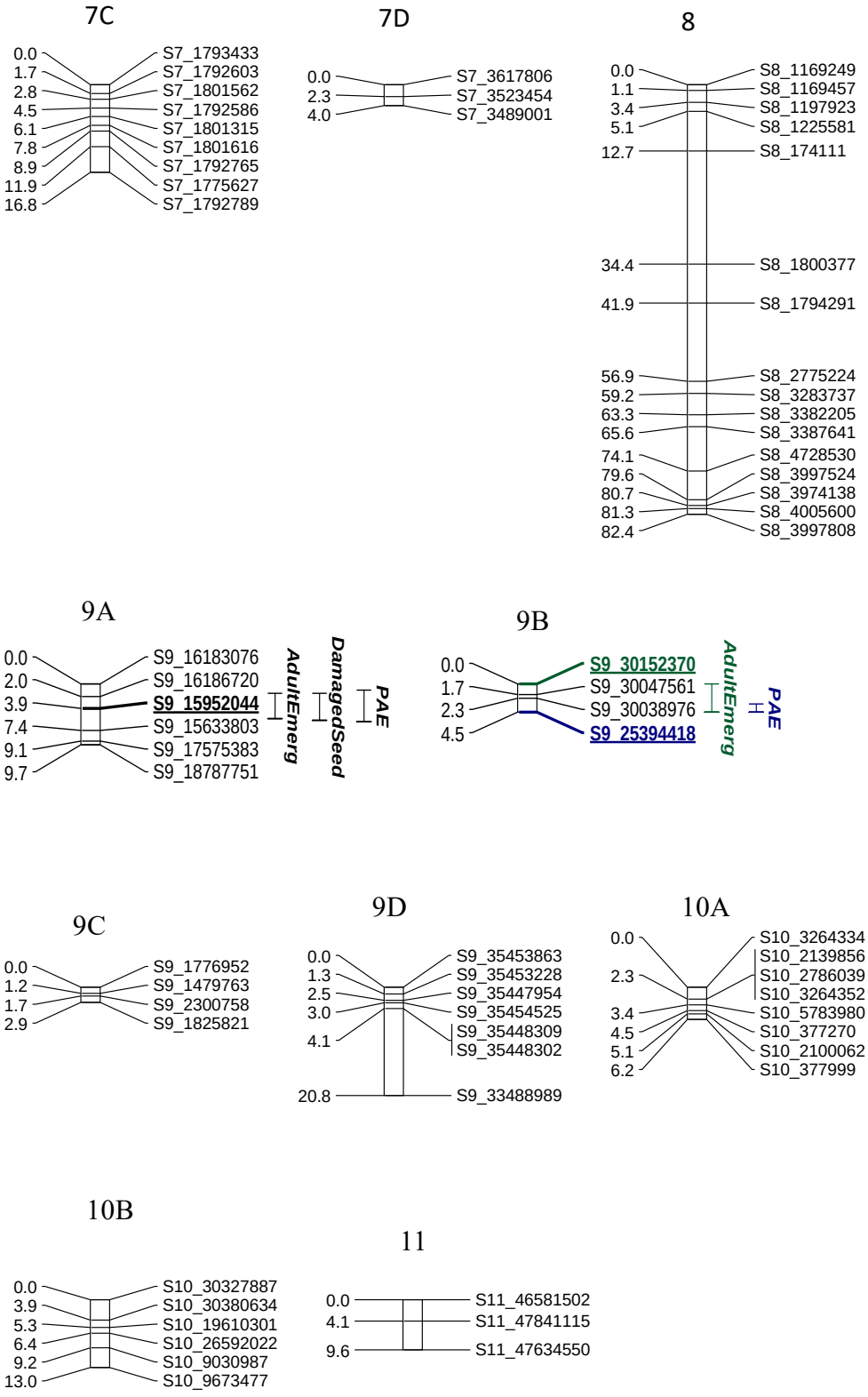
7C
0.0 S7_1793433
1.7 S7_1792603
2.8 S7_1801562
4.5 S7_1792586
6.1 S7_1801315
7.8 S7_1801616
8.9 S7_1792765
11.9 S7_1775627
16.8 S7_1792789
7D
0.0 S7_3617806
2.3 S7_3523454
4.0 S7_3489001
8
0.0 S8_1169249
1.1 S8_1169457
3.4 S8_1197923
5.1 S8_1225581
12.7 S8_174111
34.4 S8_1800377
41.9 S8_1794291
56.9 S8_2775224
59.2 S8_3283737
63.3 S8_3382205
65.6 S8_3387641
74.1 S8_4728530
79.6 S8_3997524
80.7 S8_3974138
81.3 S8_4005600
82.4 S8_3997808
9A
0.0 S9_16183076
2.0 S9_16186720
3.9 S9_15952044
7.4 S9_15633803
9.1 S9_17575383
9.7 S9_18787751
AdultEmerg
DamagedSeed
PAE
9B
0.0 S9_30152370
1.7 S9_30047561
2.3 S9_30038976
4.5 S9_25394418
AdultEmerg
H
PAE
9C
0.0 S9_1776952
1.2 S9_1479763
1.7 S9_2300758
2.9 S9_1825821
9D
0.0 S9_35453863
1.3 S9_35453228
2.5 S9_35447954
3.0 S9_35454525
4.1 S9_35448309
S9_35448302
20.8 S9_33488989
10A
0.0 S10_3264334
S10_2139856
2.3 S10_2786039
S10_3264352
3.4 S10_5783980
4.5 S10_377270
5.1 S10_2100062
6.2 S10_377999
10B
0.0 S10_30327887
3.9 S10_30380634
5.3 S10_19610301
6.4 S10_26592022
9.2 S10_9030987
13.0 S10_9673477
11
0.0 S11_46581502
4.1 S11_47841115
9.6 S11_47634550

Fig. 4.13 (Cont.).

Similarly on population KSw3, QTL's for days to 50% adult bruchid emergence mapped on PV08 with S8_56811256 being a SNP's associated with this trait which explained 10.9% of the total genetic variation. QTL's for adult emergence and susceptibility index mapped on PV09 with two SNP's S9_5581703 and S9_8706124 being associated with these traits respectively which accounted for 10.9% and 10.6% of the total genetic variation. QTL's for percentage adult emergence mapped on PV09 and PV05 with SNP's S9_6635055 from PV09 being the SNP's associated with this trait explained 12.8% of the total genetic variation and S5_25332940 from PV05 but explained less (8.2%) of the total genetic variation. Seed size QTL's mapped on six linkage groups PV01, PV02, PV03, PV05, PV08, PV09 and PV10. S9_1000884 is the SNP's from PV05 associated with this trait explained 26.3% of the total genetic variation, S1_26590594, S10_1542284, S2_19454079 and S8_57734818 explained 19.8%, 19.5% and the two had 19.4% of the total genetic variation. S5_33974240 from linkage group PV05 explain 16.4% of the total genetic variation and lastly S3_1261743 which explained 13.9% of the total genetic variation of this trait (Table 4.5 and fig.4.14).

Table 4. 5. Bruchid resistance QTL's for mapping population KSw

LINKAGE GROUP	Flanking SNP's for bruchid resistance QTL'S	QTL TRAITS	Position	LOD	% Exp
PV1	S1_26590594	Seed size	15.917	2.3	19.8
PV2	S2_19454079	Seed size	2.415	2.25	19.4
PV3	S3_1261743	Seed size	2.228	1.56	13.9
PV5	S5_25332940	Percentage Adult Emergence	15.134	0.9	8.2
	S5_33974240	Seed size	19.853	1.87	16.4
PV8	S8_56811256	50% DAE	40.315	1.2	10.9
	S8_57734818	Seed size	29.252	2.25	19.4
PV9	S9_5581703	Number of adult emergence	6.927	1.2	10.9
	S9_8706124	Susceptibility Index (SI)	0	1.16	10.6
	S9_6635055	Percentage Adult Emergence	13.941	1.43	12.8
	S9_1000884	Seed size	11.192	3.18	26.3
	S10_1542284	Seed size	3.849	2.26	19.5

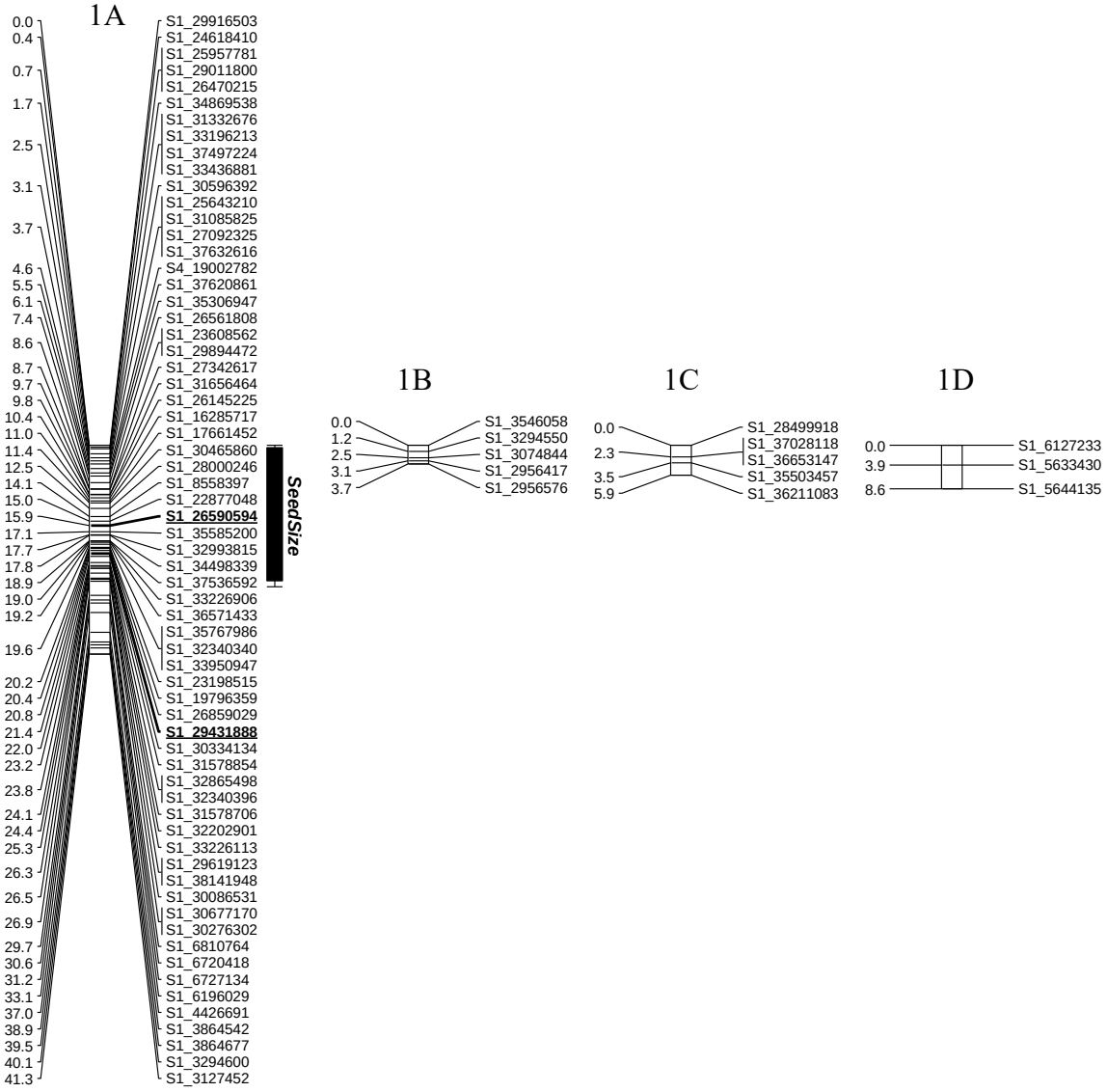

Figure 4. 14. QTL's seed size in KSw population with markers related to seed size being underlined

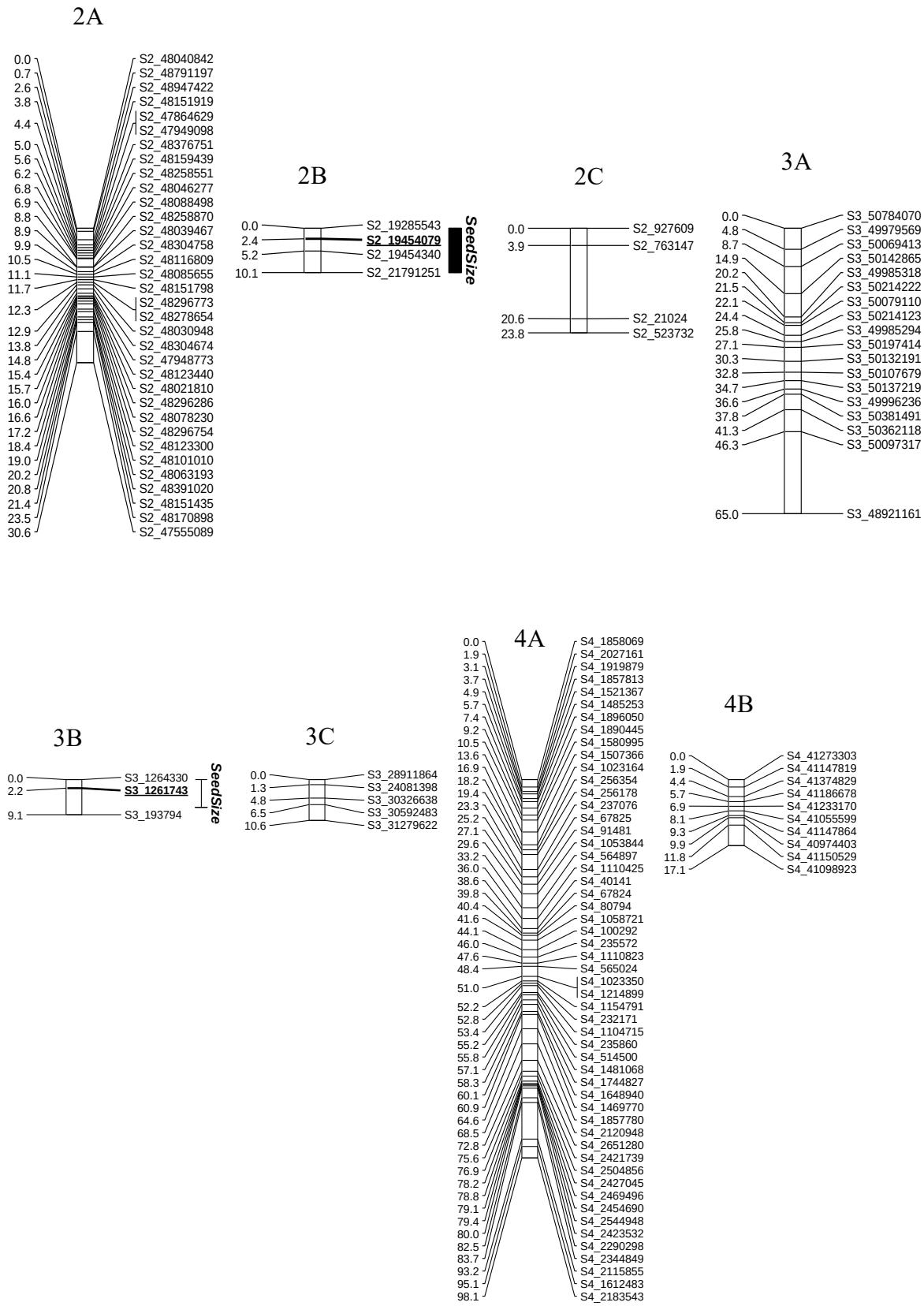

Figure 4.14 (Cont.).

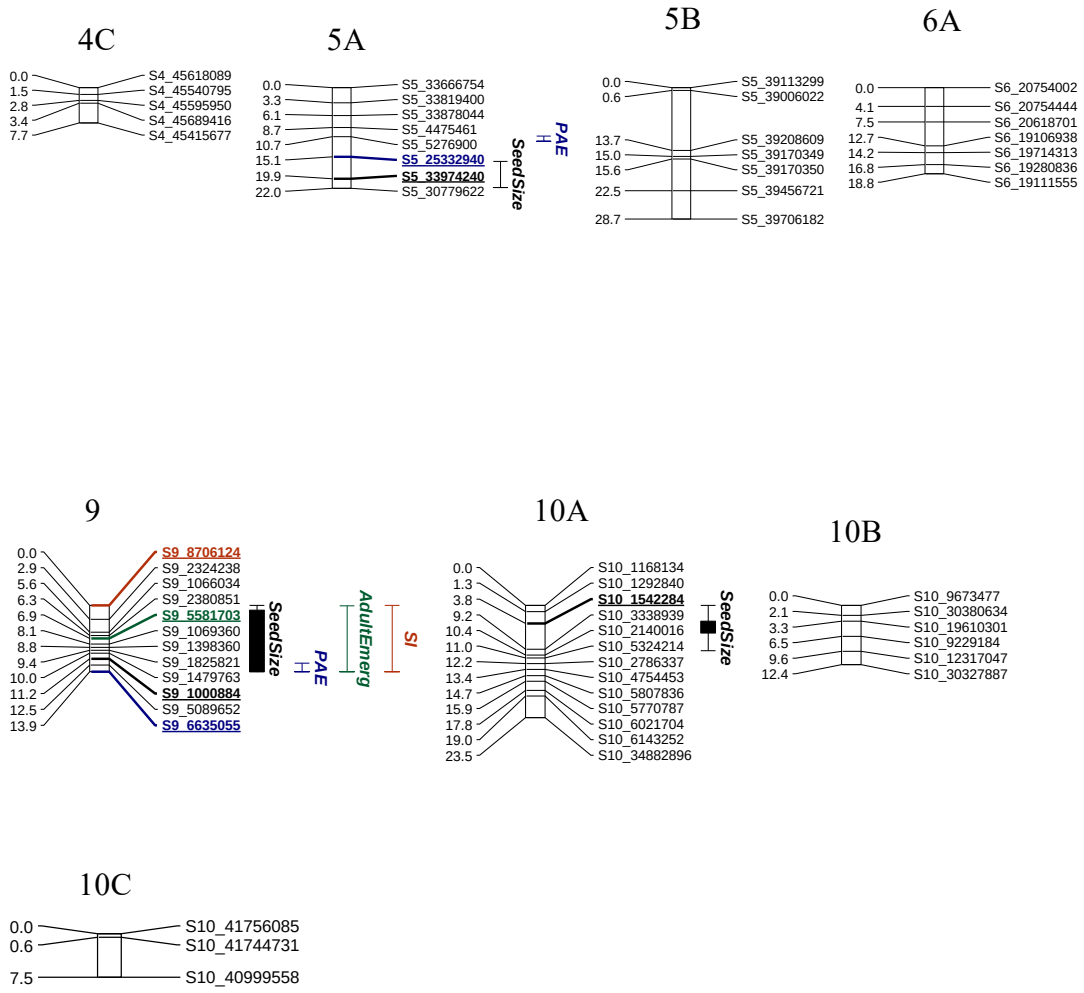

Figure 4. 15. Seed size, percentage adult emergence, number of adult emergence and susceptibility index QTL's in KSw population with underlined markers related to the mentioned traits.

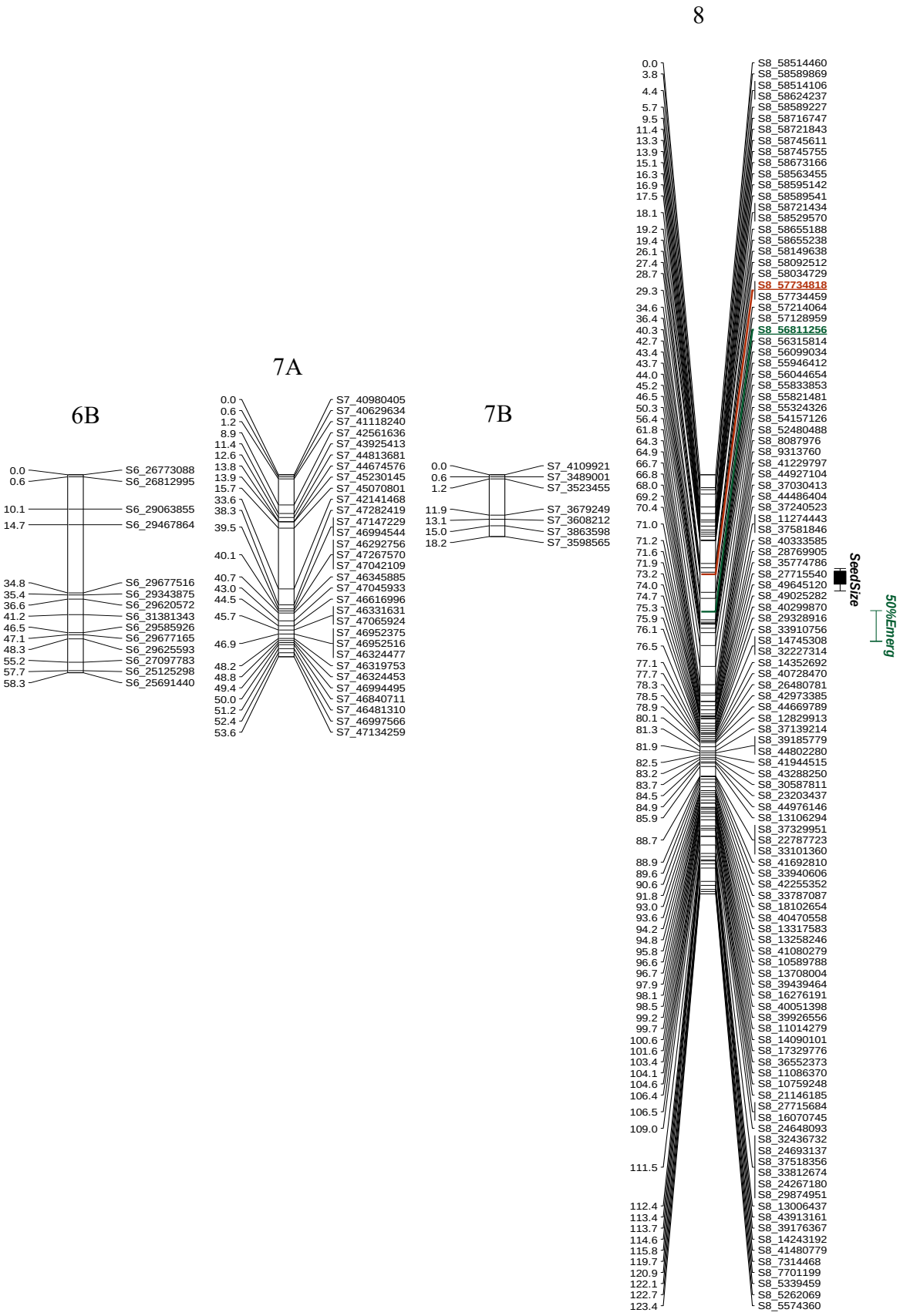
6B
0.0 S6_26773088
0.6 S6_26812995
10.1 S6_29063855
14.7 S6_29467864
34.8 S6_29677516
35.4 S6_29343875
36.6 S6_29620572
41.2 S6_31381343
46.5 S6_29585926
47.1 S6_29677165
48.3 S6_29625593
55.2 S6_27097783
57.7 S6_25125298
58.3 S6_25691440
7A
0.0 S7_40980405
0.6 S7_40629634
1.2 S7_41118240
8.9 S7_42561636
11.4 S7_43925413
12.6 S7_44813681
13.8 S7_44674576
13.9 S7_45230145
15.7 S7_45070801
33.6 S7_42141468
38.3 S7_47282419
39.5 S7_47147229
S7_46994544
S7_46292756
40.1 S7_47267570
S7_47042109
40.7 S7_46345885
43.0 S7_47045933
44.5 S7_46616996
45.7 S7_46331631
S7_47065924
S7_46952375
46.9 S7_46952516
S7_46324477
48.2 S7_46319753
48.8 S7_46324453
49.4 S7_46994495
50.0 S7_46840711
51.2 S7_46481310
52.4 S7_46997566
53.6 S7_47134259
7B
0.0 S7_4109921
0.6 S7_3489001
1.2 S7_3523455
11.9 S7_3679249
13.1 S7_3608212
15.0 S7_3863598
18.2 S7_3598565
8
0.0 S8_58514460
3.8 S8_58589869
4.4 S8_58514106
5.7 S8_58624237
9.5 S8_58589227
11.4 S8_58716747
13.3 S8_58721843
13.9 S8_58745611
15.1 S8_58745755
16.3 S8_58673166
16.9 S8_58563455
17.5 S8_58595142
18.1 S8_58589541
19.2 S8_58721434
19.4 S8_58529570
26.1 S8_58655188
27.4 S8_58655238
28.7 S8_58149638
29.3 S8_58092512
34.6 S8_58034729
36.4 S8_57734818
40.3 S8_57734459
42.7 S8_57214064
43.4 S8_57128959
43.7 S8_56811256
44.0 S8_56315814
45.2 S8_56099034
46.5 S8_55946412
50.3 S8_56044654
56.4 S8_55833853
61.8 S8_55821481
64.3 S8_55324326
64.9 S8_54157126
66.7 S8_52480488
66.8 S8_8087976
68.0 S8_9313760
69.2 S8_41229797
70.4 S8_44927104
71.0 S8_37030413
71.2 S8_44486404
71.6 S8_37240523
71.9 S8_11274443
73.2 S8_37581846
74.0 S8_40333585
74.7 S8_28769905
75.3 S8_35774786
75.9 S8_27715540
76.1 S8_49645120
76.5 S8_49025282
77.1 S8_40299870
77.7 S8_29328916
78.3 S8_33910756
78.5 S8_14745308
78.9 S8_32227314
80.1 S8_14352692
81.3 S8_40728470
81.9 S8_26480781
82.5 S8_42973385
83.2 S8_44669789
83.7 S8_12829913
84.5 S8_37139214
84.9 S8_39185779
85.9 S8_44802280
88.7 S8_41944515
88.9 S8_43288250
89.6 S8_30587811
90.6 S8_23203437
91.8 S8_44976146
93.0 S8_13106294
93.6 S8_37329951
94.2 S8_22787723
94.8 S8_33101360
95.8 S8_41692810
96.6 S8_33940606
96.7 S8_42255352
97.9 S8_33787087
98.1 S8_18102654
98.5 S8_40470558
99.2 S8_13317583
99.7 S8_13258246
100.6 S8_41080279
101.6 S8_10589788
103.4 S8_13708004
104.1 S8_39439464
104.6 S8_16276191
106.4 S8_40051398
106.5 S8_39926556
109.0 S8_11014279
111.5 S8_14090101
112.4 S8_17329776
113.4 S8_36552373
113.7 S8_11086370
114.6 S8_10759248
115.8 S8_21146185
119.7 S8_27715684
120.9 S8_16070745
122.1 S8_24648093
122.7 S8_32436732
123.4 S8_24693137
S8_37518356
S8_33812674
S8_24267180
S8_29874951
S8_13006437
S8_43913161
S8_39176367
S8_14243192
S8_41480779
S8_7314468
S8_7701199
S8_5339459
S8_5262069
S8_5574360
SeedSize
50%Emerg

Fig. 4.15 (Cont.).

4.5 Discussion

4.5.1 Progenies phenotyping against *A. obtectus*

The findings from this study showed that population KSy were more resistant to bruchid infestation than population KSw. In this experiment 6 lines out of 53 in population KSy which accounted for 11.3% were strongly resistant to *A. obtectus* and 9 lines accounted for 16.9% were resistant while only two lines out of 48 (4.6%) in population KSw were strongly resistant and three lines (6.2%) were resistant to this pest. The results agreed with those found by Kananji (2007) who screened 135 bean lines involving 77 landraces and 58 improved varieties for resistance to *A. obtectus* and obtained 12.5% of the lines were resistant to bruchid infestation with landrace KK35 showing consistently high levels of resistance.

Susceptibility index (SI) was positively correlated with all variables showing that it's a good indicator of resistance or susceptibility of the bean line. Based on the susceptibility index, lines KSy2, KSy5, KSy9, KSy32, KSy35 and KSy38 in population KSy and KSw26 in population KSw possessing high levels of resistance with susceptibility index of zero indicating that no seeds were damaged. Lines KSy1, KSy3, KSy10, KSy14, KSy34, KSy42, KSy47 and KSy48 in population KSy and KSw5, KSw6, KSw10 and KSw16 in population KSw were observed to be little damaged by bruchids indicating that they were also resistant to bruchid infestation therefore its potential to continue advancing and evaluating these lines in breeding pipeline for bruchid resistance. The results also revealed that lines KSy21 in population KSy and KSw48 in population KSw were highly susceptible to bruchid infestation based on number of adults emerged, susceptibility index, and damaged

seeds with line KSy having all seeds damaged and KSw having 16 seeds damaged out of 18.

These results agree with those found by Ebinu *et al.* (2016) who evaluated common bean germplasm from Uganda for resistance to bruchids using 45 genotypes and discovered all 45 genotypes were susceptible being severely damaged by bean bruchid by supporting bruchid development, reproduction and feeding resulting in significant reduction of seed germination. In general the results showed that the progenies from the two populations screened possess different levels of resistance to *A. obtectus* although they shared the male resistant parent Kalubungula indicating difference in combining ability with susceptible parent Soworo showing lower combining ability with Kalubungula and Soya showing higher combining ability with Kalubungula.

4.5.2 Linkage mapping and QTLs

The results obtained from this study showed the presence of QTL's relating to bruchid resistance in all of the two mapping populations distributed in different linkage groups of *P. vulgaris* with most of traits mapping on linkage group five (PV05) and linkage group nine (PV09). QTL's for percentage damaged seeds, susceptibility index, percentage weight loss (%Wt loss), percentage adult emergence and number of adult emergence mapped on PV05 and PV09 in population KSy indicating that the two linkage groups are responsible for bruchid resistance traits control. In KSw population, number of adult emergence, susceptibility index and percentage adult emergence mapped on PV09 indicating that the linkage group have QTL's relating to bruchid resistance. Looking at susceptibility index which shows

the vulnerability or resistance to damage by bruchid on bean lines, the results revealed the presence of QTL's for bruchid resistance in two different linkage groups PV05 and PV09 for KSy and KSw mapping populations with different markers controlling the traits indicating the presence of different genes donated by the same parent Kalubungula responsible for bruchid resistance. These results are different from those found by Kami *et al*. (2006) and Blair *et al*. (2010) who reported the QTL's for APA gene family controlling resistance to bruchids in wild common bean genotypes mapped on PV04.

Seed size QTL's on KSw population were distributed on different chromosomes with many QTL's controlling the trait indicating the presence of multiple genes controlling this trait. Unfortunately there were no any seed size QTL's mapped on KSy population due to lower LOD threshold that was insignificant at $P \leq 0.05$. The presence of QTL's for seed size on different chromosomes apart from PV09 where bruchid resistance traits mapped revealed that there is little or no association between bruchid resistances in this population to the seed size. The findings are against those found by Mei *et al*. (2009) who reported the QTL's for resistance to mung bean bruchid *C. chinensis* was co-located with seed size QTL's and were highly significant suggesting the incremental decrease in seed size accompanied resistance to *C. chinensis*. The small seed size being associated with bruchid resistance was also supported by Sibakwe and Donga (2015) who reported that small seeded varieties are more resistant as compared to medium seeded and large seeded varieties. They reported variety BCB2, a small seeded variety to be more resistant to bruchid damage based on number of holes, number of damaged seeds, bruchid

developmental period, Dobie susceptibility index and seed weight loss. On the other hand the results agreed with those found by Schoonhoven et al. (1983) who reported the bruchid resistance in non-cultivated common bean to be related to small seed size although other factors were probably more important. Misangu (1997) reported the preference of bruchids to large-seeded bean lines than small seeded bean lines hence large seeded bean lines are more prone to bruchid infestation and damage. Kalubungula being a large seeded resistant genotype shows that resistance in this genotype is little or not associated with resistance. By having one QTL's for seed size in PV09 it might be associated with resistance to bruchid as it maps with other bruchid resistance traits but other factors might be possibly important because of having seed size QTL's mapping on different chromosomes.

Results also showed that days to 50% adult bruchid emergence QTL's mapped on PV03 in KSy population and PV8 in KSw population apart from PV05 and PV09 were the bruchid resistance traits mapped showing that the delay in bruchid emergence is not an indicator of resistance in these lines. This is in line with what was reported by Kusolwa (2011) who reported the resistance to be exhibited as delay for 50% adult insect emergence reduced number of emerged F1 of *A. obtectus* in crosses between resistant wild and cultivated susceptible genotypes. Generally there are QTL's for resistance based on number of bruchid emergence traits in population KSy and KSw which accounts for 16.2% and 10.9% of the total genetic variation respectively.

In conclusion, 20 bruchid resistant lines were identified from this study with 15 resistant lines from KSy population and 5 resistant lines from KSw population.

QTL's associated with resistance to bruchid mapped on linkage PV05 and PV09 different from the previously known linkage group PV04. The resistant lines identified should be advanced and further selection for better line to use in breeding for bruchids resistance for prolonged storage period of dry beans and the QTL's identified should be published as well as converted into markers to add information on bruchid resistance in common bean.

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion

In conclusion, this study revealed that resistance from the two populations KSy and KSw is not controlled by storage proteins which confers resistance to bruchids. This gives an insight on investigating other mechanisms that might be involved in the resistant parent and progenies. The evidence of presence of some traces of arcelin-like proteins in KK25/Nagaga and their progenies will add information on presence of storage proteins which confers resistance to bruchid in cultivated common bean in which previously it was mostly reported to be found on wild genotypes. The results from this study also confirmed the presence of QTL's associated with bruchid resistance on PV05 and PV09 out of the known linkage group PV04. This indicates that there is more than one gene involvement in conditioning resistance hence will add genetic information on the gene bank database. Generally the 20 resistant lines obtained from this study KSy1, KSy2, KSy3, KSy5, KSy9, KSy10, KSy14, KSy32, KSy34, KSy35, KSy38, KSy42, KSy47, KSy48, KSw5, KSw6, KSw10, KSw16 and KSw26 and will be a valuable source in breeding for bruchid resistance in common bean.

5.2 Recommendations

The following are recommended for future studies:

1) More evaluation and crossing should be done to obtain better lines for effective breeding programs.

2) Further studies should be done on investigation of other mechanisms of resistance in the identified resistant lines including the role of the seed coat in conferring resistance to bruchids like seed coat hardness, shape, slippery and biochemical contents of the seed coat.
3) A study should also be done on identification of the observed hypothetical protein as it might be a factor contributing to resistance of the line ML10, a progenie of Nagaga x KK25.
4) QLT's validation and enrichment for studying the stability of the identified QTL's from one generation to another should be done as it is important step towards finding of putative SNP's markers for selection of resistant lines.
5) Converting the SNP's associated with resistance from this study into markers such as cleaved amplified polymorphic sequences (CAPS) and derived CAPs (dCAPS) markers that are cost effective will be of help in breeding for resistance to bruchid using marker assisted selection (MAS).

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APPENDICES

Appendix 1: List of bean lines used for Genotyping By Sequencing

BEAN LINE	PEDIGREE
Ksy1	Soya X Kalubungula-F3-1
Ksy2	Soya X Kalubungula-F3-2
Ksy3	Soya X Kalubungula-F3-3
Ksy4	Soya X Kalubungula-F3-4
Ksy5	Soya X Kalubungula-F3-5
Ksy6	Soya X Kalubungula-F3-6
Ksy7	Soya X Kalubungula-F3-7
Ksy8	Soya X Kalubungula-F3-8
Ksy9	Soya X Kalubungula-F3-9
Ksy10	Soya X Kalubungula-F3-10
Ksy11	Soya X Kalubungula-F3-11
Ksy12	Soya X Kalubungula-F3-12
Ksy13	Soya X Kalubungula-F3-13
Ksy14	Soya X Kalubungula-F3-14
Ksy15	Soya X Kalubungula-F3-15
Ksy16	Soya X Kalubungula-F3-16
Ksy17	Soya X Kalubungula-F3-17
Ksy18	Soya X Kalubungula-F3-18
Ksy19	Soya X Kalubungula-F3-19
Ksy20	Soya X Kalubungula-F3-20
Ksy21	Soya X Kalubungula-F3-21
Ksy22	Soya X Kalubungula-F3-22
Ksy23	Soya X Kalubungula-F3-23
Ksy24	Soya X Kalubungula-F3-24
Ksy25	Soya X Kalubungula-F3-25
Ksy26	Soya X Kalubungula-F3-26
Ksy27	Soya X Kalubungula-F3-27
Ksy28	Soya X Kalubungula-F3-28
Ksy29	Soya X Kalubungula-F3-29
Ksy30	Soya X Kalubungula-F3-30
Ksy31	Soya X Kalubungula-F3-31
Ksy32	Soya X Kalubungula-F3-32
Ksy33	Soya X Kalubungula-F3-33
Ksy34	Soya X Kalubungula-F3-34
Ksy35	Soya X Kalubungula-F3-35
Ksy36	Soya X Kalubungula-F3-36
Ksy37	Soya X Kalubungula-F3-37
Ksy38	Soya X Kalubungula-F3-38
Ksy39	Soya X Kalubungula-F3-39
Ksy40	Soya X Kalubungula-F3-40

Ksy41	Soya X Kalubungula-F3-41
Ksy42	Soya X Kalubungula-F3-42
Ksy43	Soya X Kalubungula-F3-43
Ksy44	Soya X Kalubungula-F3-44
Ksy45	Soya X Kalubungula-F3-45
Ksy46	Soya X Kalubungula-F3-46
Ksy47	Soya X Kalubungula-F3-47
Ksy48	Soya X Kalubungula-F3-48
Ksy49	Soya X Kalubungula-F3-49
Ksy50	Soya X Kalubungula-F3-50
Ksy51	Soya X Kalubungula-F3-51
Ksy52	Soya X Kalubungula-F3-52
Ksy53	Soya X Kalubungula-F3-53
Ksw 1	Soworo X Kalubungula-F3-1
Ksw 2	Soworo X Kalubungula-F3-2
Ksw 3	Soworo X Kalubungula-F3-3
Ksw 4	Soworo X Kalubungula-F3-4
Ksw 5	Soworo X Kalubungula-F3-5
Ksw 6	Soworo X Kalubungula-F3-6
Ksw 7	Soworo X Kalubungula-F3-7
Ksw 8	Soworo X Kalubungula-F3-8
Ksw 9	Soworo X Kalubungula-F3-9
Ksw 10	Soworo X Kalubungula-F3-10
Ksw 11	Soworo X Kalubungula-F3-11
Ksw 12	Soworo X Kalubungula-F3-12
Ksw 13	Soworo X Kalubungula-F3-13
Ksw 14	Soworo X Kalubungula-F3-14
Ksw 15	Soworo X Kalubungula-F3-15
Ksw 16	Soworo X Kalubungula-F3-16
Ksw 17	Soworo X Kalubungula-F3-17
Ksw 18	Soworo X Kalubungula-F3-18
Ksw 19	Soworo X Kalubungula-F3-19
Ksw 20	Soworo X Kalubungula-F3-20
Ksw 21	Soworo X Kalubungula-F3-21
Ksw 22	Soworo X Kalubungula-F3-22
Ksw 23	Soworo X Kalubungula-F3-23
Ksw 24	Soworo X Kalubungula-F3-24
Ksw 25	Soworo X Kalubungula-F3-25
Ksw 26	Soworo X Kalubungula-F3-26
Ksw 27	Soworo X Kalubungula-F3-27
Ksw 28	Soworo X Kalubungula-F3-28
Ksw 29	Soworo X Kalubungula-F3-29
Ksw 30	Soworo X Kalubungula-F3-30
Ksw 31	Soworo X Kalubungula-F3-31

Ksw 32	Soworo X Kalubungula-F3-32
Ksw 33	Soworo X Kalubungula-F3-33
Ksw 34	Soworo X Kalubungula-F3-34
Ksw 35	Soworo X Kalubungula-F3-35
Ksw 36	Soworo X Kalubungula-F3-36
Ksw 37	Soworo X Kalubungula-F3-37
Ksw 38	Soworo X Kalubungula-F3-38
Ksw 39	Soworo X Kalubungula-F3-39
Ksw 40	Soworo X Kalubungula-F3-40
Ksw 41	Soworo X Kalubungula-F3-41
Ksw 42	Soworo X Kalubungula-F3-42
Ksw 43	Soworo X Kalubungula-F3-43
Ksw 44	Soworo X Kalubungula-F3-44
Ksw 45	Soworo X Kalubungula-F3-45
Ksw 46	Soworo X Kalubungula-F3-46
Ksw 47	Soworo X Kalubungula-F3-47
Ksw 48	Soworo X Kalubungula-F3-48

PARENTS

Kalubungula
Soya
Soworo

Appendix 2: Number of bruchid emerged, Days to 50% adult amergence , number of damaged seeds, susceptibility index, percentage weight loss and percentage damaged seed of KSy population infested by A. obtectus

Bean line	# bruchid emerged	Days 50% emergence	damaged seed	SI	**%WT Loss**	%Damaged seed
Ksy1	1	36	1	0	0.359066	10
Ksy2	0	60	0	0	0	0
Ksy3	2	40	2	0.752575	0.222058	5.405405405
Ksy4	24	41	8	3.366369	4.754601	66.66666667
Ksy5	0	60	0	0	0	0
Ksy6	27	42	9	3.408009	4.022989	50
Ksy7	7	43	4	1.965344	3.039514	50
Ksy8	13	42	8	2.652246	3.661327	57.14285714
Ksy9	0	60	0	0	0	0
Ksy10	1	44	1	0	0.235018	4.347826087
Ksy11	10	41	6	2.439024	2.483444	40
Ksy12	4	43	2	1.40014	2.631579	28.57142857
Ksy13	28	42	13	3.445614	4.132231	68.42105263
Ksy14	1	42	1	0	1.146789	10
Ksy15	32	41	12	3.671098	6.006006	80
Ksy16	34	43	9	3.561579	6.944444	90
Ksy17	12	42	5	2.569479	5.362776	71.42857143
Ksy18	25	42	11	3.328429	3.793627	68.75
Ksy19	27	40	9	3.578409	3.534778	52.94117647
Ksy20	32	40	14	3.762875	4.341534	77.77777778
Ksy21	85	42	10	4.593855	16.47856	100
Ksy22	28	42	8	3.445614	5.842697	72.72727273
Ksy23	18	42	10	2.988744	6.571936	62.5
Ksy24	15	42	10	2.800217	2.737752	62.5
Ksy25	25	43	6	3.251023	10.55276	85.71428571
Ksy26	52	44	14	3.900008	7.538462	100
Ksy27	26	42	12	3.368984	4.198473	66.66666667
Ksy28	18	42	6	2.988744	5.671642	85.71428571
Ksy29	16	41	8	2.936878	3.636364	88.88888889
Ksy30	16	42	5	2.866952	6.130268	71.42857143
Ksy31	31	43	10	3.468283	7.692308	100
Ksy32	0	60	0	0	0	0
Ksy33	39	43	11	3.70015	7.635009	91.66666667
Ksy34	3	42	1	1.136003	2.068966	25
Ksy35	0	60	0	0	0	0
Ksy36	24	41	8	3.366369	7.142857	100
Ksy37	30	43	6	3.435166	9.440559	100
Ksy38	0	60	0	0	0	0
Ksy39	19	42	7	3.044651	5.191257	87.5
Ksy40	34	43	8	3.561579	9.117647	88.88888889

Ksy41	16	43	4	2.800279	9.574468	80
Ksy42	7	43	5	1.965344	1.323251	16.66666667
Ksy43	40	42	10	3.814429	5.487805	71.42857143
Ksy44	16	44	6	2.736636	5.498282	66.66666667
Ksy45	16	42	6	2.866952	8.333333	100
Ksy46	19	43	6	2.973846	7.118644	100
Ksy47	5	43	2	1.625512	1.702128	22.22222222
Ksy48	6	43	2	1.809654	2.643172	28.57142857
Ksy49	25	42	7	3.328429	7.210031	100
Ksy50	9	43	4	2.219169	3.236246	44.44444444
Ksy51	30	42	10	3.516955	7.328605	100
Ksy52	12	42	3	2.569479	2.15311	23.07692308
Ksy53	37	42	7	3.733814	9.043928	100
Kalu	2	45	2	0.668956	0.413907	7.692307692
Soya	61	39	24	4.577769	3.263708	80

Appendix 3: Number of bruchid emerged, Days to 50% adult amergence , number of damaged seeds, susceptibility index, percentage weight loss and percentage damaged seed of KSw population infested by A. obtectus

Bean line	# bruchid emerged	Days 50% emergence	damaged seed	SI	**%WT Loss**	%Damaged seed
Ksw 1	47	43	18	3.8886	7.722513	85.71428571
Ksw 2	44	42	17	3.912983	3.884235	65.38461538
Ksw 3	38	44	10	3.590417	10.20942	100
Ksw 4	51	42	11	4.065643	10.32258	91.66666667
Ksw 5	16	43	6	2.800279	18.95911	85.71428571
Ksw 6	13	43	4	2.590566	3.333333	36.36363636
Ksw 7	29	42	7	3.4819	10.99291	77.77777778
Ksw 8	33	42	9	3.615509	8.894879	75
Ksw 9	30	42	9	3.516955	5.271318	64.28571429
Ksw 10	9	42	8	2.272006	1.652893	50
Ksw 11	37	43	17	3.646981	5.543933	65.38461538
Ksw 12	16	43	4	2.800279	6.944444	66.66666667
Ksw 13	19	42	9	3.044651	7.345972	81.81818182
Ksw 14	37	43	12	3.646981	9.363296	75
Ksw 15	23	42	12	3.242209	4.33925	80
Ksw 16	5	41	4	1.704805	2.755906	57.14285714
Ksw 17	41	42	10	3.839962	9.52381	90.90909091
Ksw 18	25	44	10	3.177136	5.032823	71.42857143
Ksw 19	42	42	10	3.864879	10.94675	100
Ksw 20	30	44	8	3.357094	9.556314	80
Ksw 21	29	42	17	3.4819	2.865995	54.83870968
Ksw 22	43	44	7	3.712428	10.27778	63.63636364
Ksw 23	42	42	12	3.864879	10.12931	100
Ksw 24	22	38	11	3.532691	2.5878	42.30769231
Ksw 25	59	43	15	4.11826	9.929078	88.23529412
Ksw 26	0	60	0	0	0	0
Ksw 27	24	43	6	3.209794	10.29412	100
Ksw 28	39	42	17	3.788249	4.705882	65.38461538
Ksw 29	25	43	11	3.251023	8.042895	100
Ksw 30	36	43	18	3.619308	4.881101	90
Ksw 31	55	39	12	4.462468	10.76923	92.30769231
Ksw 32	45	40	15	4.133031	9.819967	83.33333333
Ksw 33	39	42	13	3.788249	5.357143	81.25
Ksw 34	26	43	5	3.290636	8.99654	71.42857143
Ksw 35	62	43	8	4.168353	17.53247	100
Ksw 36	17	45	12	2.734331	17.23301	100
Ksw 37	54	42	11	4.124747	12.96296	100
Ksw 38	50	42	19	4.045167	3.333333	61.29032258
Ksw 39	30	43	6	3.435166	5.761317	85.71428571
Ksw 40	30	45	9	3.282492	8.812261	45

Ksw 41	30	42	10	3.516955	4.64191	55.55555556
Ksw 42	54	44	9	3.937259	20.42553	90
Ksw 43	39	44	12	3.616056	16.54676	100
Ksw 44	62	42	12	4.267599	5.933682	80
Ksw 45	44	42	16	3.912983	5.933852	66.66666667
Ksw 46	76	44	16	4.274576	13.81323	94.11764706
Ksw 47	45	44	33	3.757301	6.896552	84.61538462
Ksw 48	138	42	16	5.09495	7.777778	88.88888889
Kalu	2	45	2	0.668956	0.413907	7.692307692
Sowo	49	40	18	4.22549	3.062201	78.26086957