TRAIT CORRELATION AND CONFIRMATION OF QTLS FOR RHIZOME GROWTH AND OVER-WINTERING IN SORGHUM

A Thesis

by

JACOB D. WASHBURN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2012

Major Subject: Plant Breeding

Trait Correlation and Confirmation of QTLs for Rhizome Growth and Over-wintering in Sorghum

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Approved by:

Co-Chairs of Committee,	Russell W. Jessup
	Seth C. Murray
Committee Member,	Byron L. Burson
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ABSTRACT

Trait Correlation and Confirmation of QTLs for Rhizome Growth and Over-wintering in Sorghum. (August 2012)

Jacob D. Washburn, B.S., Brigham Young University

Co-Chairs of Advisory Committee, Dr. Russell W. Jessup
Dr. Seth C. Murray

A growing world population drives an ever-increasing need for food and energy. These challenges, along with depletion of water and fossil fuel resources, call for improvements in crop production systems and the cultivars used within them. Perennial cropping systems present an attractive solution to many of these problems. A greater understanding of the genetic control of over-wintering ability within crop species is one way to begin the process of making perennial cropping systems a possibility.

In this study an F_3:F_4 family derived from a cross between *Sorghum bicolor* (L.) Moench and *S. propinquum* (Kunth) Hitchc. segregating for rhizome production was phenotyped in both field and greenhouse environments for traits relating to rhizomatousness and over-wintering. Several statistical models were created to correlate rhizome growth and over-wintering. A known rhizome quantitative trait locus (QTL) region was saturated with SSR markers and the QTL interval was reduced from previous estimates of about 16 Mb or 7 cM to 12 Mb or 2 cM, a 25% or 71% reduction in physical or linkage distance respectively. Two previously unidentified QTL regions

associated with over-wintering were also identified. Our results also support the hypothesis that rhizome growth is important and possibly necessary for over-wintering in *Sorghum*.

DEDICATION

To my mother and father who taught me to love learning and the pursuit of excellence. To my wife and son who have supported me and sacrificed for my education.

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I would like to thank my committee members; Dr. Jessup, Dr. Murray, and Dr. Burson for helping me successfully navigate the research process.

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NOMENCLATURE

QTL	Quantitative Trait Loci
RDS	Rhizome Derived Shoot

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CHAPTER I

INTRODUCTION

Rising world populations drive an ever increasing demand for food, shelter, and energy while at the same time causing a steady decrease in agricultural land. The availability of water, nitrogen, and other resources necessary for agriculture is also decreasing and these resources are becoming ever more expensive. Some traits that have traditionally been less desirable in agronomic crops are now more useful because of their ability to make cropping systems more sustainable. One such trait is perennialism.

In the past, perennial crops have been less desirable because they must siphon off some of their energy, and therefore harvestable yield, into belowground storage organs that can sustain them during winter months. Although this is a valid argument, it is also likely that perennial crops will need less fertilizer and leave a smaller carbon footprint (Jessup 2009) because they can store many of the nutrients they need and reuse them the following year. With living root systems in the ground year-round, perennial cropping systems are also preventative to soil erosion (Piper and Kulakow 1994; Cox et al. 2002) and able to retain more water. In the springtime, perennials also take advantage of earlier growth than annual systems which allows them to harvest solar energy that would otherwise be unutilized. For these reasons, perennial systems offer promising potential towards the increased use of marginal soils for crop production (Cox et al. 2002).

This thesis follows the style of Theoretical and Applied Genetics.

In order to take advantage of perennial cropping systems, advancement in genetic, biological, agronomic, and ecological knowledge about these systems is necessary. This knowledge can be used to provide tools for modifying and enhancing agricultural crops. One tool that has been developed and applied in this way is marker assisted breeding. In order to use this tool for a given species, new markers must be developed for each trait that one desires to enhance. The process of finding and creating genetic markers for use in marker assisted breeding is time consuming but can eventually yield a system of easy trait transfer between any cultivar within a species and sometime across species.

CHAPTER II

LITERATURE REVIEW

Rhizomes

Rhizomes are modified underground stems that act as storage organs for many perennial plants. Plants that have rhizomes include invasive weeds such as Johnsongrass (*Sorghum halepense* (L.) Pers.) as well as important forages and turf grasses (Jang et al. 2009). Johnsongrass, and probably other sorghum, rhizomes are not cold tolerant in and of themselves, however with the soil as insulation they can survive very cold winters (Hull 1970; Monaghan 1979).

The resources from a rhizome that overwinters will be completely used up during the following season. Many rhizomes disintegrate within 19-24 days after shoots emerge suggesting that they are only temporary storage organs (McWhorter 1961; Monaghan 1979). Another indication that rhizomes are primarily a winter storage organ is the fact that after flowering their production increases and fresh weights double in as little as 4 days under ideal conditions (McWhorter 1961; Monaghan 1979). Aside from storage there is also evidence that Johnsongrass rhizomes have allelopathic properties, which might help to explain their ability to outcompete plants around them (Abdul-Wahab 1967; Monaghan 1979).

It seems that at least in sorghum, rhizomes are a major factor influencing overwinter survival (Warwick et al. 1986). Rhizome depth, and therefore insulation from the cold temperatures, is the biggest factor in overwinter survival of Johnsongrass

(Warwick et al. 1986). Interestingly enough, as soil temperatures drop in northern latitudes *S. halapense* populations become more and more reliant on seed production for survival and therefore use an annual strategy rather than a perennial strategy in these climates (Warwick et al. 1986).

Genetics of Rhizomes

Rhizome genetics has been studied in several *Digitaria* hybrids. When accessions with rhizomes were crossed with accessions without rhizomes, F_2 segregation was approximately 1:2 rhizomatous to tufted (Hacker 1983). The authors of the study concluded that rhizomatousness must be controlled by incompletely recessive genes (Hacker 1983).

Rhizome genetics in sorghum has been studied several times over the years, however there are still many questions left to be answered. One suggestion is that rhizomes may be controlled by three dominant additive genes with all three absent in *S. bicolor* and one or more present in rhizomatous sorghums (Monaghan 1979). Another possibility is that that several incompletely dominant genes may control rhizomatousness (Yim and Bayer 1997). Taken together, these models suggest that rhizomatousness is most likely not controlled by recessive gene action.

When a rhizomatous sorghum such as *S. halapense* is crossed with a completely non-rhizomatous cultivar such as *S. bicolor*; the F_2 progeny of the cross segregate in a 3:1 ratio indicating that dominant gene action in one gene controls rhizome presence (Yim and Bayer 1997). Hybrids between *Sorghum sudanense* (Piper) Stapf and *S.*

halepense also show similar segregation patterns, supporting the one dominant gene hypothesis (Ramaswamy 1973; Yim and Bayer 1997). However, some results from crosses between *S. bicolor* and *S. halapense* show presence of rhizomes in all F2 progeny, but with varying degrees of expression (Sangduen 1984; Yim and Bayer 1997). One caveat to these studies however, is that all of them were performed within a tetraploid background which could have limited their ability to determine the true method of gene action.

In contrast to the former studies, Paterson et al., (1995) used crosses between two diploid sorghum varieties (*S. bicolor* and *S. propinquum*). He found at least nine distinct genome regions directly associated with quantitative rhizome traits, and several of these regions were also associated with tillers and/or plant re-growth after overwintering (Paterson et al. 1995). Jang et al. (2006) found matching QTL for many of the same loci for rhizomes in rice, indicating that the rhizome expression mechanisms may be evolutionarily conserved between these two species (Jang et al. 2006). Research in both rice and sorghum indicate that these QTL are most likely dominant for rhizomatousness (Hu et al. 2003). This data taken together leads to the conclusion that quantitative rhizome expression may be controlled by several additive genes working together. The data also support the idea that rhizomatousness and perenniality are the ancestral traits of sorghum and rice with annuals developing through the collection of recessive, perhaps lack of function, genes over time (Hu et al. 2003).

Model for Rhizome Study

Sorghum has become a model crop for the scientific study of rhizomatousness (Ramaswamy 1973; Paterson et al. 1995; Yim and Bayer 1997; Jang et al. 2006; Jang et al. 2009). One reason the sorghum genus fits so well as a model is because it has species that are highly rhizomatous (i.e., *S. halapense* and *S.propinquum*) as well as many that are completely non-rhizomatous (i.e., *S. bicolor*). These species can be either crossed artificially and then self-pollinated or backcrossed in order to create lines that segregate for rhizomatousness (Paterson et al. 1995). Another reason sorghums make a good model for the study of rhizomes is that the DNA sequence of the *S. bicolor* genome is now complete (Paterson et al. 2009). This sequence opens the doors to many genetic studies in sorghum that were previously impossible. Also, several mapping projects have been done on the genetic control of rhizomes and many genetic markers are characterized and readily available for use (Paterson et al. 1995; Jang et al. 2009).

Sorghum

According to the U.S. Grains council, sorghum is the third most important cereal crop among those grown in the U.S. (USGC 2010). Sorghum is widely used for human consumption in many parts of the world, but in the US it is manly used for animal consumption. Products that come from sorghum include animal feed (as forage, fodder, or grain) for cattle, poultry, pets, and swine (USGC 2010). Up to 12% of sorghum grown in the U.S. also goes to the production of ethanol products (USGC 2010). In 2009, 254,000 acres of sorghum were harvested for use as silage (USDA 2009).

Although this is just under 4% of the total sorghum planted that year it is still a large and important market.

The species *S. bicolor* contains most cultivated sorghums (De Wet 1978). It has a chromosome number of 2n=20 (Hoang-Tang and Liang 1988). Although it is generally referred to as a diploid with haploid number x=10, there is however evidence to suggest that it is actually an allotetraploid [x=5] (Hoang-Tang and Liang 1988). *Sorghum bicolor* has undergone many nomenclatures, and classification has changed over the years. Originally many of its subspecies and races were considered to be separate species altogether. Much of this combining of species is documented in a series of papers by J.M.J. De Wet and others (De Wet et al. 1970; De Wet and Harlan 1971; De Wet and Harlan 1975; De Wet 1978).

CHAPTER III

RHIZOMES, OVERWINTERING, AND QTL CONFIRMATION

Introduction

Desirable attributes of perennial crops include their ability to use less fertilizer and reduce humanities carbon footprint by storing nutrients and reusing them the following year (Jessup 2009). With living root systems remaining in the ground throughout multiple years, perennial cropping systems reduce soil erosion and are able to retain more water in the soil profile than annual systems (Piper and Kulakow 1994; Cox et al. 2002). Perennials also take advantage of earlier and later seasonal growth than annual systems, which allows them to harvest solar energy that would otherwise be lost during the spring and fall. For these reasons, perennial systems have potential environmental benefits as well as the ability to increase the use of marginal soils for crop production (Cox et al. 2002).

The genus *Sorghum* is a desirable model for investigating and utilizing perennial cropping systems because it consists of species that behave as both annuals and perennials in cold climates, some of which are inter-fertile. Cultivated *Sorghum bicolor* (L.) Moench. also has a sequenced genome (Paterson et al. 2009) along with considerable germplasm, genetic, and genomic resources. Because of this, it has become a model for studying rhizome inheritance, a trait that is correlated and linked to overwinter survival in sorghum (Ramaswamy 1973; Paterson et al. 1995; Yim and Bayer 1997; Jang et al. 2006; Jang et al. 2009).

Sorghum has both annual crop and perennial wild species

Grain from sorghum is widely used for human consumption in many parts of the world, but in the US it is primarily used for animal consumption (USGC 2010). Sorghum is also used for forage and other purposes. In 2009, 254,000 acres of sorghum were harvested for silage and 4,813,000 acres were harvested for grain in the U.S. (USDA 2009).

The species *S. bicolor* (2n=2x=20) has undergone numerous nomenclature and classification changes over the years and currently contains all cultivated sorghums (De Wet 1978). Originally many of its subspecies and races were considered separate species. Classification of these species as *S. bicolor* is documented in a series of papers (De Wet et al. 1970; De Wet and Harlan 1971; De Wet and Harlan 1975; De Wet 1978). *Sorghum bicolor* is an annual without rhizomes, although rhizomes have been reported in at least one accession of *S. bicolor* (L.) Moench subsp. *verticilliflorum* (Steud.) de Wet ex Wiersema & J. Dahlb. (Bhatti et al. 1960; Mouftah and Smith 1969). *Sorghum halepense* (L.) Pers. (2n=4x=40) is a weedy sorghum species commonly known as Johnsongrass. It has the ability to persist as a perennial in very cold climates (Warwick et al. 1986). Cold climate perennialism in *S. halapense* seems to be correlated with rhizomatousness (Warwick and Black 1983; Warwick et al. 1984; Warwick et al. 1986). Similar correlations have been found in other perennial sorghum species (Paterson et al. 1995).

Sorghum propinquum (Kunth) Hitchc. (2n=2x=20) is a wild rhizomatous perennial sorghum found in Asia and the pacific islands (Magoon 1961). *Sorghum*

propinquum, like *S. bicolor*, has only 20 chromosomes; however, its behavior during meiosis is dissimilar to *S. bicolor* because the nucleolus is organized on a different chromosome (Magoon 1961). *Sorghum propinquum* is also cross compatible with *S. bicolor* (De Wet 1978) making it a desirable source for the transfer of perennialism genes into cultivated sorghums. It is believed that genes or alleles for rhizomes in *S. halepense* were originally donated by an ancestor of *S. propinquum* in a cross with an ancestor of *S. bicolor* (Celarier 1958; Magoon 1961; Paterson et al. 1995).

Rhizomes are important storage structures for maintaining perenniality

Rhizomes are modified underground stems that act as carbohydrate storage organs (Whitmire 2011) for many perennial plants. Some plants with rhizomes such as Johnsongrass are invasive weeds. Johnsongrass rhizomes are not innately cold-tolerant; however, with soil as insulation, they can survive harsh winter conditions (Hull 1970; Monaghan 1979).

In sorghum, rhizomes appear to be the major factor influencing overwintering (Warwick et al. 1986). Rhizome depth provides insulation from the cold temperatures and is the most important factor in winter survival of Johnsongrass (Warwick et al. 1986). As soil temperatures drop in northern latitudes, Johnsongrass populations become more reliant on seed production for survival; therefore, an annual strategy rather than a perennial strategy is naturally selected for in these climates (Warwick et al. 1986).

The carbohydrate resources from a rhizome that overwinters are generally depleted during the following season. In fact, many rhizomes disintegrate within 19-24

days after shoots emerge, indicating that they are only temporary storage organs (McWhorter 1961; Monaghan 1979). Another indication that rhizomes are primarily a winter storage organ is the fact that their production increases after flowering with fresh weights doubling within 4 days under ideal conditions (McWhorter 1961; Monaghan 1979). Aside from storage, there is also evidence that Johnsongrass rhizomes have allelopathic properties that might help explain their ability to out-compete adjacent plants (Abdul-Wahab 1967; Monaghan 1979).

Genetics of rhizomes

Both classical and molecular genetics of rhizomes have been studied in several genera, including *Digitaria*, *Oryza*, *Zea,* and *Sorghum*. In the genus *Digitaria*, when accessions with rhizomes were crossed with accessions without rhizomes, the F_2 hybrids segregated at a ratio of about 1:2 rhizomatous to non-rhizomatous (Hacker 1983). The authors concluded that rhizomatousness was controlled by incompletely recessive genes (Hacker 1983). Rhizomatous and non-rhizomatous species in the genus *Oryza* have also been crossed with varying results (Tao et al. 2003). Tao et al (2003) indicated that rhizomatousness is most likely controlled by several QTL's with dosage effects. Analysis of rhizome production and perennialism in wide crosses within the genus *Zea* also support a quantitative inheritance pattern (Westerbergh and Doebley 2004).

The genetics of sorghum rhizomes have undergone more investigation than most other species because of weediness as well as the optimism of potential temperately-adapted perennial sorghum applications; however, there are still many questions left to

be answered. Classical genetic studies have shown that when a rhizomatous species such as *S. halepense* is crossed with a completely non-rhizomatous species such as *S. bicolor* the F_2 progeny segregate in a 3:1 ratio indicating that dominant gene action in one gene controls rhizome presence (Yim and Bayer 1997). Hybrids between *S. sudanense* (Piper) Stapf and *S. halepense* show this segregation pattern, supporting the one dominant gene hypothesis (Ramaswamy 1973; Yim and Bayer 1997). It has also been suggested that rhizomes segregate as if controlled by three dominant additive genes (Monaghan 1979). The idea being that all three alleles are recessive in *S. bicolor* while rhizomatous sorghums contain at least one, or a combination of several alleles (Monaghan 1979). Others have suggested that one of more incompletely dominant genes may control rhizomatousness (Yim and Bayer 1997). This conclusion is based on the fact that studies have shown differing results for rhizome gene action. Some results from crosses between *S. bicolor* and *S. halepense* show rhizomes presence in all F_2 progeny, but with varying in degrees of expression (Sangduen 1984; Yim and Bayer 1997) indicating the trait is controlled by many genes. One downside to the experiments listed above is that they all used a tetraploid model which may have limited their ability to determine the true inheritance pattern of rhizomatousness.

Paterson et al. (1995) used a diploid model and supported a polygenic theory when he identified at least nine chromosomal regions directly associated with quantitative rhizome traits in sorghum. Several of these regions were also associated with tillering and/or plant re-growth after over-wintering (Paterson et al. 1995). Jang et al. (2006) found matching QTLs for many of the same loci for rhizomes in rice,

indicating that the rhizome expression mechanisms may be evolutionarily conserved between these two species. Research in both rice and sorghum indicate that these QTLs are most likely dominant for rhizomatousness (Hu et al. 2003). This data taken together leads to the conclusion that quantitative rhizome expression may be controlled by several additive genes. The data also support the idea that rhizomatousness and perenniality are ancestral traits of sorghum and rice with annuals developing through the collection of recessive genes over time. (Hu et al. 2003).

The genus *Sorghum* has become a model for rhizome genetics because it has a highly rhizomatous weedy species distributed worldwide, a less aggressive wild species with rhizomes, and a major food crop that completely lacks these structures. Some of these species can be cross pollinated to produce hybrids that segregate for rhizomatousness (Paterson et al. 1995). Sorghum benefits from a wealth of genetic and genomic information such as a genome sequence of *S. bicolor* (Paterson et al. 2009), multiple high density mapping populations (Mace et al. 2009), a number of expression libraries (Jang et al. 2006; Paterson et al. 2009), and importantly a small and tractable genome (Paterson et al. 2009). Of these resources, both mapping populations and expression data have been collected on the genetic control of rhizomes and many genetic markers are characterized and readily available for use (Paterson et al. 1995; Jang et al. 2009).

In this study we sought to further investigate the relationship between rhizomatousness and perennialism, the longitivity of rhizomatous lines, as well as to

confirm and fine map previously identified rhizome and perennialism QTLs in the F_4 progeny of *S. bicolor* x *S. propinquum* hybrids.

Materials and Methods

A total of 476 plots were planted in a randomized design which included: 16 plots of F_2:F_3 plants, 410 plots of F_3:F_4 plants, and 50 plots of perennial sorghum material from the Land Institute for control purposes (Piper and Kulakow 1994; Cox et al. 2002). The land institute material was derived independently from the other material and used different parents. These were planted at College Station, Texas on May 24, 2010. From these 476 plots we chose to focus on family #13 which consisted of 130 F_4 lines derived from a cross between S. *bicolor* and S. *propinquum* (Paterson et al. 1995). Standard sorghum agronomic practices were followed throughout the season and supplemental irrigation, fertilizer, weed, and pest control were used as needed.

On November 29, 2010, near the time of the first frost, phenotypic measurements were taken to determine the rhizomatousness of each line. These measurements were a modification of those used by Paterson, 1995. They included the number of living plants per line prior to first frost, the average number of rhizome derived shoots (RDS) originating from each plant, and the average distance of these shoots from the center of each row. Plants were left in the field to overwinter and were scored phenotypically for re-growth from rhizomes the following spring (March 31, 2011). The surviving plants were allowed to grow throughout the summer of 2011 and overwinter in the field for a second season during the winter of 2011-2012 in order to obtain data on the longevity of

these lines. In the spring of 2012 the plants were again rated phenotypically for over-wintering. Special caution was taken during both spring measurements to ensure that any seedling derived plants were uprooted at an early stage so as not to be confused with rhizome derived re-growth. Leaf tissue was harvested from 10-20 plants from each line (except those with fewer surviving plants) and bulked by line for DNA marker analysis.

A separate greenhouse study was also undertaken using the same material. Seeds of family #13 F_4 lines were planted in 3 replicates in 10 centimeter pots and placed in a randomized complete block design. Temperatures in the greenhouse were moderated but fluctuated between 60° and 80° F. After three weeks of growth, seedlings were thinned to 4-6 plants per pot and allowed to continue growth. In early January of 2012, leaf tissue was harvested for DNA extraction from five plants of each line across replications. All plants that were not used for DNA extraction were disposed of so they would not be used during phenotyping. In early April of 2012, the plants were removed from their pots and rated visually for rhizome presence and abundance. Each pot was given a score from 1 to 10 based on the amount of rhizome growth. No rhizome growth was represented by a score of 1 and the maximum amount of rhizome growth was represented by a score of 10.

Several statistical models were created to test the hypothesis that over-wintering can be predicted based on rhizome growth. Three linear regressions, a logistical ANOVA, and a logistical regression model were tested. We used different combinations of RDS number per plot and RDS distance from the center of the row to create the

models. Statistical models were created and tested using JMP® Software, Version 9 (JMP® 2011).

DNA extraction was carried out using two different protocols. From the field study, plant leaf tissue was frozen at -80° C. Frozen tissue was placed in a freeze drier and dried for 3 days. A 6mm hole punch was used to remove several discs of tissue from each sample equaling about 0.17g of dry tissue and this was ground using stainless steel beads in a Talboys High Throughput Homogenizer (Troemner, Thorofare, NJ) DNA was extracted following a CTAB procedure (Doyle and Doyle 1987). DNA extraction from the greenhouse samples was performed using a modified salt extraction protocol (Aljanabi and Martinez 1997; Whitmire 2011; Dowling 2011). Whitmire (2011) describes the exact procedure used including all modifications. DNA from both greenhouse and field samples was quantified using a Nanodrop 1000 (Thermo Fisher Scientific , Wilmington, DE). Working samples of 50µg/ml were then created by dilution with double deionized water. PCR was performed on individual samples using 0.5µl of the DNA working sample with 5µl of PCR Readymix®, 3.5µl PCR grade water, and 1µl of the primer set (includes 0.5µl forward and 0.5µl reverse primer) (Whitmire 2011).

The DNA fragments were separated using gel electrophoreses and scored as co-dominant markers following a published scoring procedure (Rodríguez et al. 2001; Whitmire 2011). For electrophoresis, a MEGA-GEL (C.B.S. Scientific, Del Mar, CA) high-throughput vertical unit was used. Gels had a final concentration of 6% acrylamide, 0.5X TBE (tris-borate-EDTA) Buffer, 0.07% ammonium persulfate, and

0.08% TEMED (Tetramethylethylenediamine) (Wang et al. 2003; Whitmire 2011; Dowling 2011).

Eight *S. bicolor* genomic SSR markers within the region of interest were selected from a set of 329 (Whitmire 2011) and used for analyses. According to the published *S. bicolor* genome sequence, these markers are distributed over a 15.71 Mb region within chromosome 1 (Gramene 2011). This region was previously shown to be associated with various traits related to rhizomatousness (Paterson et al. 1995; Jang et al. 2006). The SSR markers were used to assay DNA from all 130 lines described above. Marker and trait data were entered into QTL iciMapping (ICIM) version 3.1 software (Wang et al. 2012) for linkage analysis and QTL detection.

For the linkage map, marker data was entered into ICIM as a recombinant inbred line and grouped by a LOD score of 3.00 and a distance of 37.20 cM. Ordering was performed using the SER algorithm within the software package and then rippling with the LogL algorithm. After the ordering was complete, two of the markers were manually ordered to match the published *S. bicolor* genome (Gramene 2011).

The data was analyzed with an F_3 population structure using biparental populations (BIP) mode. The ICIM-ADD (composite interval mapping) method was used with a step size of 0.5 cM and a probability in stepwise regression of 0.001. Deletion was selected as the means for dealing with missing phenotypic data and the LOD threshold was chosen through 1,000 permutations with a type I error level of 0.05. Single marker analysis (SMA) was also performed using the same number of permutations and error level.

Trait data had to be transformed prior to analysis in order to achieve normality of the residuals. All field data was transformed using by the natural log (ln) function except for RDS count for 2011 which was transformed using an ln(n+1) function (Paterson et al. 1995). Graphical representations of the linkage map and QTLs presented in this paper were created using MapChart version 2.2 software (Voorrips 2002).

Results

Perennialism

Of the 476 plots planted, seed in 446 germinated with an average of 23.10 plants per plot. Based on the data across all families, we chose to focus on family #13 which had the largest number of rhizomatous plots germinated. In family #13, seed from 131 plots germinated with an average of 14.30 plants. The winter of 2010-2011 had a low temperature of -7° C and freezing temperatures were experienced on 22 days (OCS 2012). Within family #13, only 25.2% of the plots had surviving plants after the winter and only 8.2% of the total plants within the family survived. The winter of 2011-2012 was warmer with a minimum temperature of -3° C and freezing temperatures were experience on 8 days (OCS 2012). Of the 33 plots that survived the first winter 26 survived the second winter as well (81.8% survival rate). Because of severe drought, wildlife pressure, and moderate temperatures during the second year it is difficult to say if this year's mortality rate can be attributed solely to winter kill and indeed some plants had spread much further in the second year.

After the second year phenotypic data was taken, some of the plants with very few RDS and short RDS distances were unearthed in order to observe their rhizome growth. None of these plants were found to be completely absent of rhizome growth but several had very limited growth and one had very few RDS some of which appeared to be serving no function other than as a storage organ because they were not sending out RDS growth or spreading.

Summary statistics of pre-winter and post-winter phenotyping indicate that the number of surviving plants per plot was reduced over both winters while the number of rhizome derived shoots per plot and the average distance of those shoots from the center of their rows increased (see table 1). The differences between RDS and RDS distance across years were compared using paired t-tests and all differences were statistically significant at the 0.05 level. All statistical models (see table 2) were significant at the $\alpha = 0.01$ level, but had varying degrees of predictive power in R^2 coefficients. The logistical model was the most useful model with an $R^2 = 0.33$ indicating that 33% of the variation in over-wintering can be explained by the variables measured. The implications of these results will be discussed in the conclusions section.

Table 1 Summary statistics from field trails

	November 2010			March 2011			April 2012		
	Number of plants per plot	RDS per plant	Distance of RDS from row center	Number of plants per plot	RDS per plant	Distance of RDS from plant	Number of Plants per plot	RDS per plant	Distance of RDS from plant
Mean	7.76	2.53	4.13	2.52	14.30	29.24	2.60	32.30	46.43
St.Dev	5.35	2.75	3.53	3.25	20.34	24.71	2.11	38.82	26.38
Min	1.00	0.00	0.00	1.00	1.00	4.33	1.00	1.00	9.80
Max	28.00	19.50	20.33	16.00	94.75	96.33	11.00	181.67	100.00

Table 2 Parameter estimates from over-wintering regression models

LogRDS only Model			R^2=	0.25
Term	Estimate	Std Error	t-ratio	Prob>|t|
Intercept	0.11	0.04	2.64	0.0094*
LogRDS	0.25	0.04	6.39	<0.0001*
LogAvgDist only Model			R^2=	0.14
Term	Estimate	Std Error	t-ratio	Prob>|t|
Intercept	-0.10	0.09	-1.03	0.3032
LogAvgDist	0.28	0.06	4.42	<0.0001*
Whole Model			R^2=	0.30
Term	Estimate	Std Error	t-ratio	Prob>|t|
Intercept	-0.13	0.08	-1.58	0.1168
LogRDS	0.25	0.05	5.23	<0.0001*
LogAvgDist	0.18	0.06	2.90	0.0045*
Logistic Regression Model			R^2=	0.33
Term	Estimate	Std Error	ChiSquare	Prob>|t|
Intercept	3.94	0.81	23.66	<0.0001*
LogRDS	-2.06	0.55	14.13	0.0002*
LogAvgDist	-0.81	0.53	2.37	0.1239
(LogRDS-0.70) x (LogAvgDist-1.31)	-2.06	0.87	5.60	0.0180*

Genotyping and QTL analysis

Of 16 new markers assayed only 6 were usable and informative. The linkage map for the region analyzed had a total linkage distance of 6.78 cM and a total physical distance of 15.71 Mb (see figure 1). Significant QTL regions were found for RDS distance in 2010, RDS count in 2010, RDS distance in 2011 and Over-wintering in 2011 (see table 3). To our knowledge, this is the first identification of over-wintering QTLs in this area of sorghum chromosome one. The other traits, RDS count 2011, RDS count 2012, RDS distance 2012, and Over-wintering 2012 were not significant for any QTL regions. The greenhouse study data was not significant for any QTL regions but showed small broad peaks which covered and extended beyond the same regions as the significant QTL's from the field.

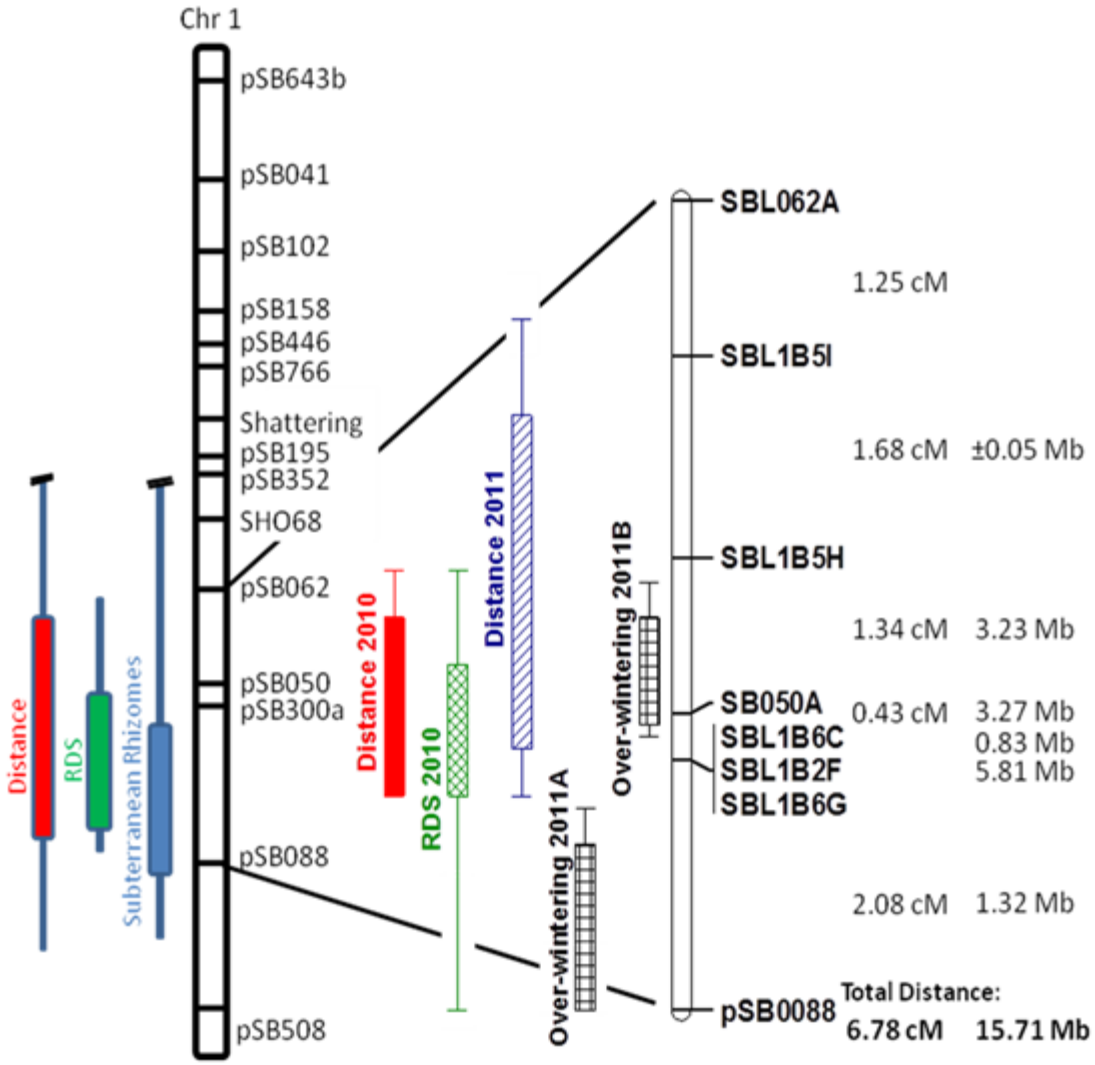

Figure 1 Chromosome 1 QTL map. On the left hand side is the full map of sorghum chromosome one (linkage group C) including QTLs as reported by Paterson et al. (1995). The remainder of the figure is an enlargement of a section of chromosome 1 between markers pSB062 and pSB0088 (the chosen region of analysis for this study). Calculated map distances in centiMorgans along with actual physical distances from the *S. bicolor* genome are displayed next to the figure. QTLs marked "Over-wintering 2011A" and "Over-wintering 2011B" come from the same data collected in March 2011.

Table 3. Significant QTL regions

Trait Name	Position (cM)	Left Marker from Peak	Right Marker from Peak	LOD	PVE* (%)	Additive Effect	Dominance Effect
Ln2010Dist	4	SBL1B5H	SB050A	2.81	10.72	-0.09	0.49
Ln2010RDS	4.5	SB050A	SBL1B6C	2.99	14.18	-0.73	0.09
Ln2011Dist	3.5	SBL1B5H	SB050A	2.63	38.25	-0.57	0.57
Over-wintering2011B	4.1	SBL1B5H	SB050A	3.91	11.53	0.00	0.45
Over-wintering2011A	6.1	SBL1B6G	pSB0088	7.09	25.32	-0.03	0.64

*Phenotypic variation explained

Single marker analysis was also performed on the data and indicated that SB050A and SBL1B5H were the most significant markers related to the phenotypes measured (see table 4). The marker pSB0088 was also significant for 2010 RDS distance and over-wintering in 2011.

Table 4 Significant markers from single marker analysis

Trait Name	Marker Name	Position (cM)	LOD	PVE* (%)	Additive Effect	Dominance Effect
Ln2010Dist	SBL1B5H	2.96	2.43	9.05	-0.21	0.24
Ln2010Dist	SB050A	4.30	4.80	17.07	-0.30	0.42
Ln2010Dist	pSB0088	6.84	2.41	8.98	0.11	0.61
Ln2010RDS	SB050A	4.30	2.19	7.75	-0.70	-0.31
Ln2011Dist	SBL1B5H	2.96	2.38	28.96	-0.62	0.35
Over-wintering 2011	SBL1B5H	2.96	5.95	19.14	-0.05	0.45
Over-wintering 2011	SB050A	4.30	5.67	18.33	-0.16	0.40
Over-wintering 2011	pSB0088	6.84	6.87	21.74	-0.02	0.58

*Phenotypic variation explained

Discussion

Spring re-growth and rhizometousness

The Paterson et al. (1995) study from which our material was derived was conducted during a comparatively mild winter when the minimum temperature was only -4° C. They reported a 92.2% survival rate (Paterson et al. 1995). In the present study, we reported a survival rate of 25.2% during the first season, in families selected for their overwintering ability, which allowed a very powerful discrimination for understanding the relationship between overwintering and rhizome growth. The statistical significance of the regression models in this study indicated that rhizome production has at least some effect (up to 33% under the given conditions) on after winter re-growth. This leaves an additional 67% of the variation in over-wintering unaccounted for by our models. There are several possible reasons for this unexplained variation.

One reason may be that our measurements did not account for all rhizome growth. Rhizome phenotyping was based entirely on aboveground indicators of rhizomes. This means that our method was unable to detect rhizomes that had not sent out shoots. Another possible cause for this variation is that the presence of rhizomes is not the only factor influencing over-wintering. For example, rhizome depth has been implicated to play a large role in winter survival because of soil insulation (Warwick et al. 1986). Rhizome depth measurements were not taken during this study in order to minimize plant disturbance and maximize screening of perennialism.

Various approaches and technologies may help to address rhizome phenotyping problems in the future. One promising method is ground penetrating radar (GPR). Used

extensively in geosciences, this technology would allow the researcher to pass over the rhizomes from above ground and take detailed readings of their depth, length, and diameter. This method was attempted within the current study using a SIR-300 (Geophysical Survey Systems, Inc., Salem, NH), but the results were not useful because of various technological constraints. The main problem with using GPR was that the soil in which the plants were growing was high in clay content. GPR works well with dry sandy soils because they interfere less with the radar signals. In this study, the clay content of the soil was so high that rhizomes could not be distinguished from clay soil. There are also constraints on the size of the object that can be resolved using this method. In this study, we determined that a rhizome would have to be at least 1 cm in diameter in order to be resolved by the GPR equipment. Our incomplete surveying suggested that most rhizomes were smaller than 1 cm although some entries had rhizomes larger than 1cm. It is our opinion that rhizomes in our population could have been detected using GPR if the plants were grown in very sandy soils.

Other factors that may influence over-wintering include anti-freeze proteins, carbohydrate fractions (Whitmire 2011), and root-ball or crown resistance to the cold. Both of these possibilities are very attractive for plant breeding because they would allow a plant to re-grow in a clumping habit without having the spreading habit and weediness potential created by rhizomes. Because all aboveground plant material died during the 2010-2011 winter, and most died in the more mild 2011-2012 winter, we concluded that if anti-freeze proteins such as dehydrins or others exist in our material, they must be present and/or effective in only the below ground plant structures. Cold

resistance within the crown also seems unlikely because most RDS (measured in the spring) came from areas other than the crown of the plant. Root-ball resistance to cold cannot be ruled out because rhizomes and root-ball material are generally closely associated and entangled. The fact that all overwintering plants had rhizome derived shoots of some kind is an indication that rhizomes may be necessary for over-wintering in this cross.

QTLs associated with rhizome growth and perennialism

The QTLs found in this study can be used to indicate a smaller QTL region than previously identified for these traits which should aid in the future discovery of the actual genes responsible for rhizome phenotypes. Assuming that all of our phenotyping methods measured traits that are associated with the same gene or group of genes we can combine the regions we discovered into one large rhizome growth region. Looked at in this way, our QTL's indicate that genes associated with rhizome growth are most likely to be found within an area of 6.5 Mb located between markers SBL1B5H and

SBL1B6C. One QTL for over-wintering was also found within the region between SBL1B6G and pSB0088. Using 1 LOD intervals (as shown in figure 1) and excluding the over-wintering QTL (which may be a separate region altogether) gives an area of interest around 14Mb or 3.8cM, a slight reduction from that reported previously (Paterson et al. 1995).

Both our results and those reported by Paterson et al (1995) show 1 LOD intervals for RDS growth to be smaller than those for RDS distance. Under the assumption that both of these phenotypes are accounted for by the same gene(s) we might conclude that RDS distance is a more variable and thus less accurate way of measuring rhizome growth than is RDS. Separating the data by year, however, yields a different result. When QTL regions from 2010 are compared with each other there is little difference between the 1-LOD regions for RDS and RDS distance (RDS distance is only slightly larger). Looking at the 2-LOD regions we see the opposite trend with RDS being much larger than RDS distance. The 2011 data (including the nearly significant QTL for RDS) also shows a similar trend to the 2010 data (see figure 2).

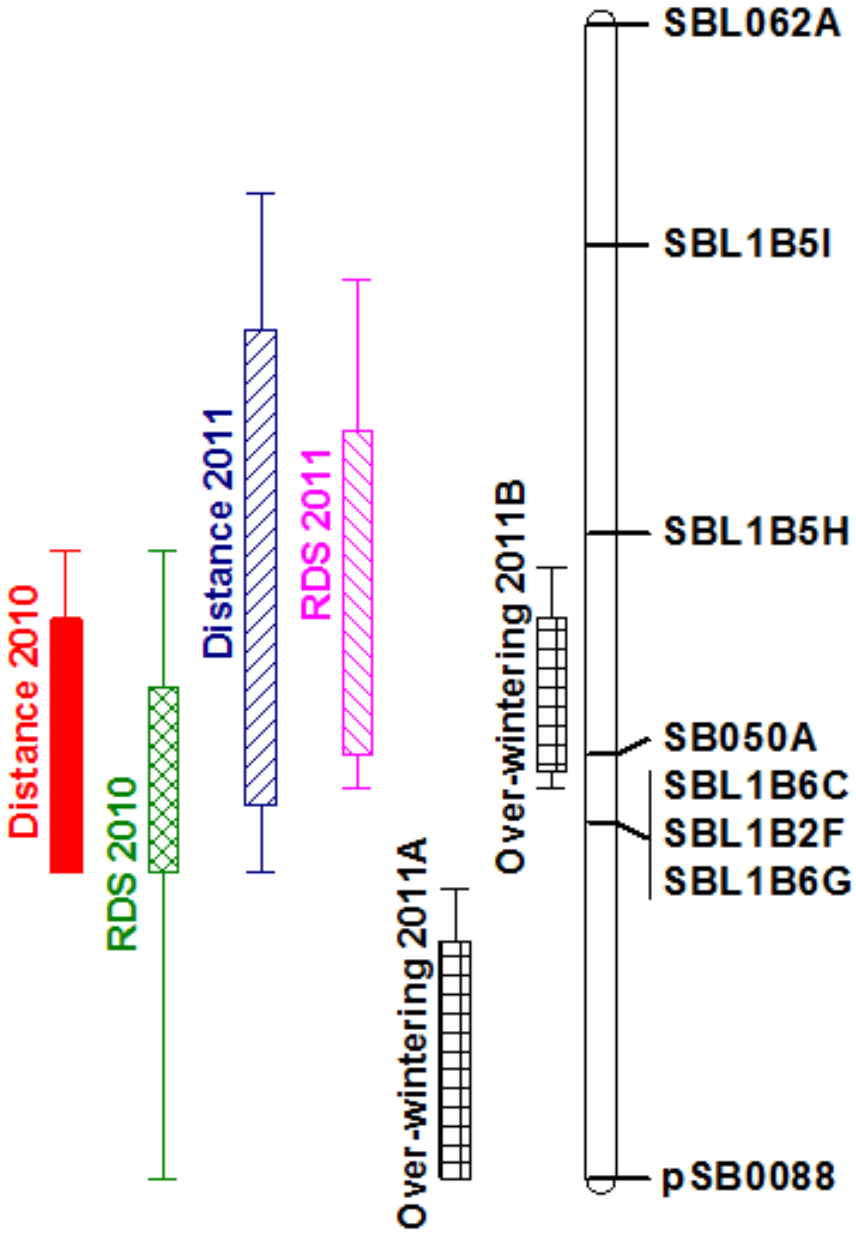

Figure 2 Identified QTL regions including non-significant RDS 2011.

The difference in location and the size of the region between the two years is also useful to consider. Although the actual QTL (LOD peak) positions for the two years are very similar to each other, the 1 and 2 LOD intervals are very different. The 2010 1-LOD intervals are centered over and area around the marker SBL050A while those for 2011 are centered on SBL1B5H. The 2011 regions are also appreciably larger than the same regions from the 2010 data. There are several differences between the phenotyped populations for the two years of data that may explain this discrepancy. One explanation is that all 130 plots, some with rhizomes and some without, were included in the analysis for 2010 while, to maintain a normal distribution, only those that survived the following winter were included for 2011. This means that every plant in the 2011 data had at least some rhizome growth. It also means that the 2011 data only included 33 plots. Because the 2010 data includes lines that did not survive the winter and lines that had little to no rhizome growth, it may have more power towards predicting the regions associated with the presence or absence of rhizome growth. The larger population size of the 2010 data should also give it more statistical power than the data from 2011 which would allow it to pinpoint a smaller interval with more confidence.

The two over-wintering QTL regions are also of great interest. One of them (labeled Over-wintering 2011B), is directly in line with the other regions found to be associated with rhizome growth. It may be that the correlation of this region with over-wintering is simply a result of rhizome growth being highly correlated with over-wintering ability. The co-location of these QTLs is further evidence to support the

hypothesis that rhizomatousness is the most important factor in determining a sorghum plant's ability to over-winter.

The second region associated with over-wintering is also an interesting case. The 1 and 2-LOD confidence intervals associated with the over-wintering QTLs do not overlap with each other indicating that these two regions may be distinct from each other rather than being one large QTL region. Using the 1-LOD intervals this second region would also be considered distinct from any of the rhizome related regions. If in fact the causal gene(s) for this region are distinct from those of the rhizome region it is possible that this region represents the contribution of something other than rhizome growth to sorghum over-wintering ability. Because the 2-LOD region of one of the rhizome QTL's (RDS 2010) overlaps with the entire Over-wintering 2011A QTL, our data cannot rule out the possibility that this QTL represents the same causal gene(s) as the other identified QTLs in the study.

One interesting note from this study was the finding of segregation distortion at all loci. This distortion was towards the non-rhizomatous parental allele (see table 12 in the Appendix). This was unexpected because the population as a whole had not experienced selection towards this parent. One possible explanation for this is that the adapted nature of the non-rhizomatous parent created secondary selection. Considering the proximity of the grain shattering allele to our region of interest this is a very likely explanation (see figure 1). Linkage disequilibrium within this chromosome and our area of interest have been reported previously (Chittenden et al. 1994). Another possibility is that the causal gene(s) within our area of interest interact epistatically with other rhizome

related genes and may therefore be present in both *S. bicolor* and *S. propinquum*, but only functioning when other genes (which exist only in *S. propinquum*) are present. If this is the case then it will be important for future research to focus on not only on our region of interest, but also on additional regions found in previous studies.

For future research, we suggest that larger populations should be used to increase the number of crossover events and the resolving ability of the map. These populations could be derived from selfing individuals heterozygous for this and/or other QTL regions. We also recommend that SNP markers be used in order to more closely flank the QTL region. Other useful avenues that could be employed include transcriptome analysis to locate candidate genes in the area, and then utilization of knock out or knock down procedures to identify which of these genes may control rhizome growth.

Conclusion

In this study, we narrowed the QTL interval from an area of about 16Mb to an area of 12Mb (calculating from the 1-LOD threshold of the 2010 data only) this is a 25% reduction. However, more research is needed to identify the gene(s) and causal polymorphisms responsible for over-wintering in sorghum. We suggest that research in this area continue while using more exact and accurate ways of measuring rhizome growth, larger populations, and higher density SNP genotyping and/or transcriptome analysis.

CHAPTER IV

CONCLUSIONS

The results of this study agree with the traditional scientific consensus that rhizome growth is important and probably necessary for cold climate over-wintering in sorghum. This research also suggests that although the presence of rhizomes appears to be necessary for over-wintering it does not guarantee over-wintering ability. The number of RDS and the distance those shoots have traveled from a plant are good indicators of over-wintering ability but they only predict about 33% of the variability within re-growth. More research, perhaps using a very tightly controlled environment, is needed in order to determine what traits and genes are responsible for the remaining variation in over-wintering ability in sorghum.

A previously identified QTL region related to rhizomatousness was confirmed and more narrowly defined. This QTL region, which this study suggests may actually be two distinct regions, appears to have an effect on the number of RDS and well as the distance they grow from the plant. It is also correlated with over-wintering ability which is a novel finding in this particular QTL region.

Future research in this region and other over-wintering QTL regions may allow the isolation of genes which contribute to and/or cause the over-wintering phenotype. If one or more of these genes can be identified they can be used by scientists and plant breeders to better understand, control, and use rhizome growth and over-wintering in cultivated species. These genes could then be transferred to cultivated sorghum using

marker assisted selection in order to create new cold tolerant sorghum varieties for food, forage, and bioenergy production.

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APPENDIX

Figure 3 First and second season field study pictures.

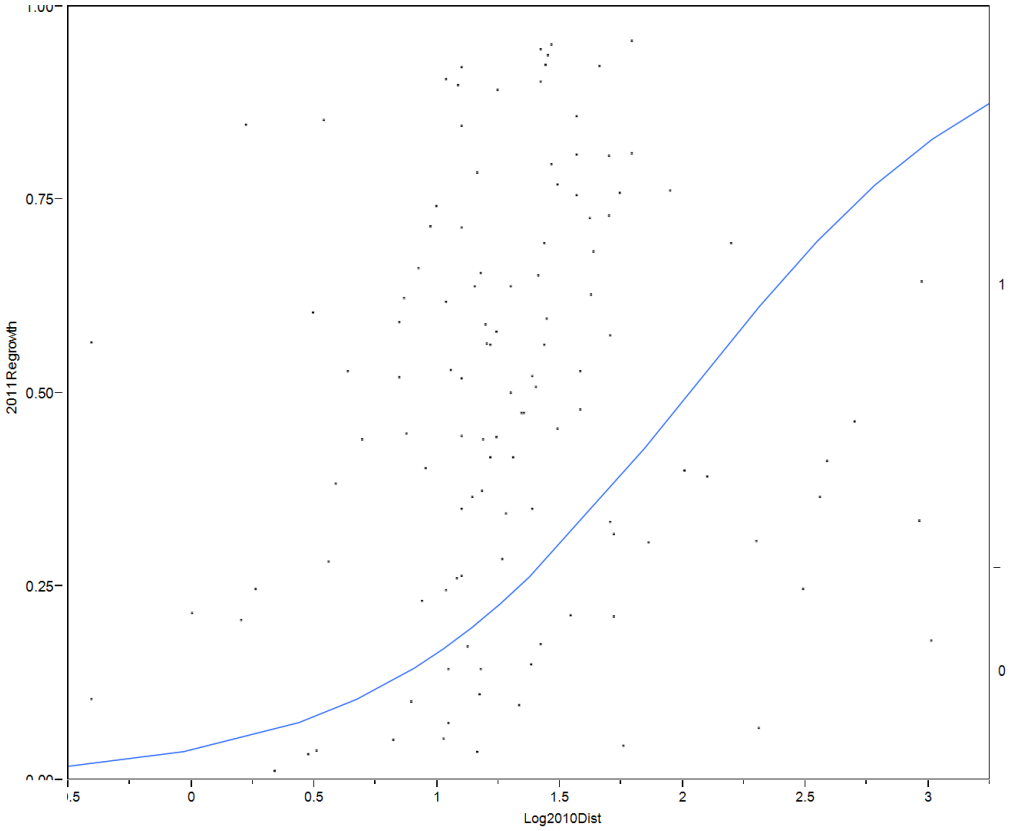

Figure 4 2010 RDS distance correlated with 2011 re-growth. Figure created using JMP® 9 (JMP® 2011).

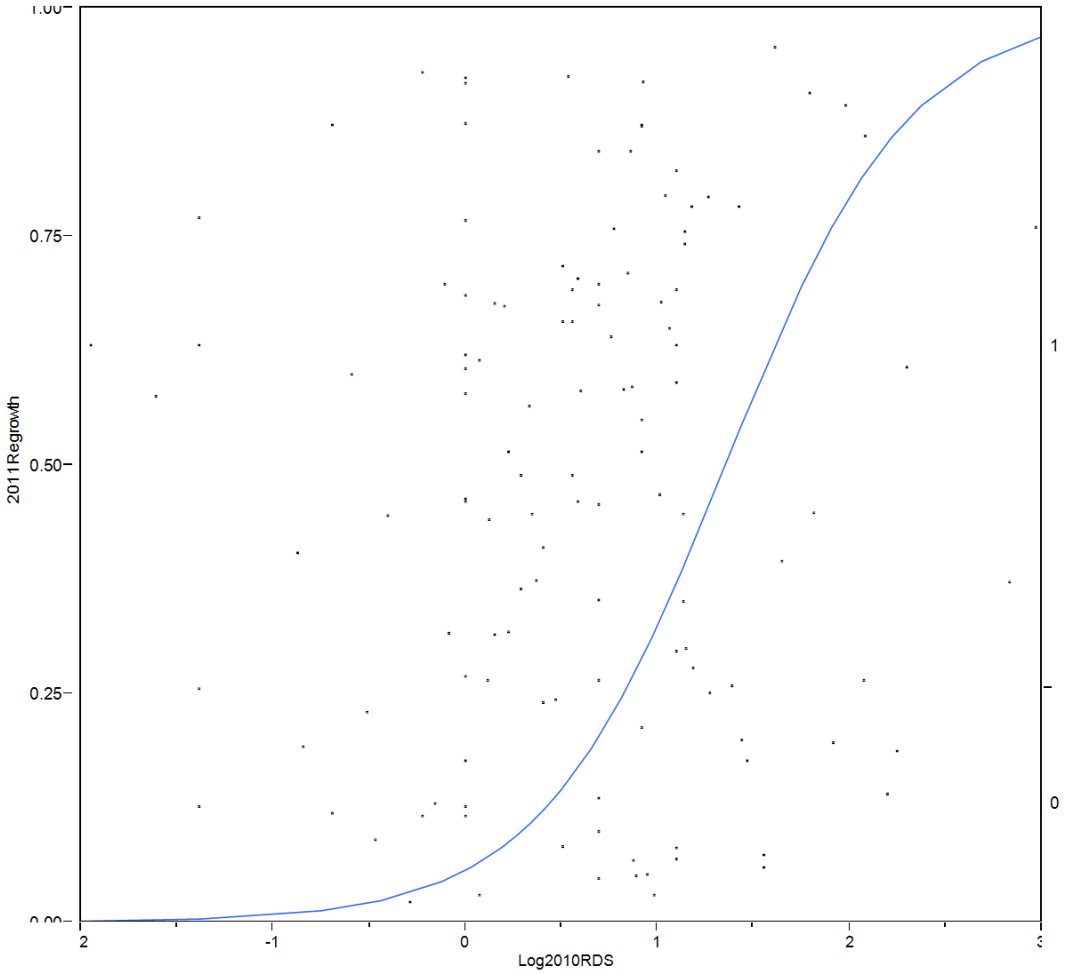

Figure 5 2010 RDS count correlated with 2011 re-growth. Figure created using JMP® 9 (JMP® 2011).

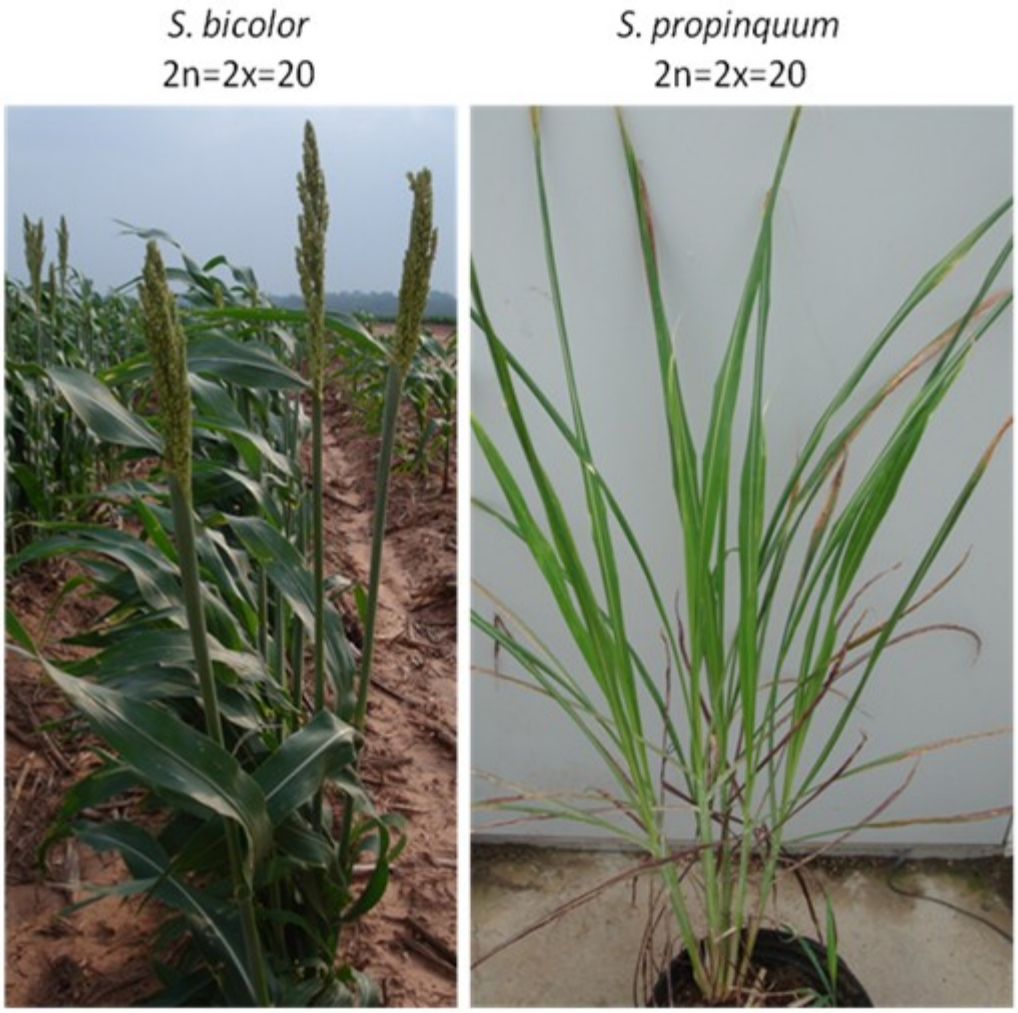

Figure 6 The two sorghum species that were the original parents of the material used in this study.

Figure 7 Plot layout of the greenhouse study

Figure 8 A plant from the greenhouse study displaying a rhizomatous phenotype

Table 5 Basic statistics of trait data

Trait Name	Sample Size	Mean	Variance	StdError	Skewness	Kurtosis	Minimum	Maximum	Range	W-test	P-value
Log2010Dist	118	1.31	0.36	0.60	0.25	1.45	-0.41	3.01	3.42	0.95	0.00
Log2010RDS	125	0.59	0.76	0.87	-0.19	0.67	-1.95	2.97	4.92	0.98	0.28
2011Log(n+1)RDS	32	2.07	1.26	1.12	0.41	-0.80	0.69	4.56	3.87	0.93	0.04
2011LogDist	32	2.98	0.87	0.93	-0.03	-1.14	1.47	4.57	3.10	0.94	0.12
2012LogRDS	26	2.89	1.60	1.27	-0.87	0.93	0.00	5.20	5.20	0.88	0.01
2012LogDist	26	3.66	0.43	0.66	-0.51	-0.44	2.28	4.61	2.32	0.95	0.22
GHMax	124	5.72	3.67	1.92	-0.06	-0.25	1.00	10.00	9.00	0.95	0.00
GHAvg	124	4.67	2.08	1.44	0.49	0.93	1.00	10.00	9.00	0.97	0.05
2011Over-wintering	129	0.25	0.19	0.43	1.17	-0.64	0.00	1.00	1.00	0.52	0.00
2012Over-wintering	32	0.81	0.16	0.40	-1.60	0.56	0.00	1.00	1.00	0.48	0.00

Table 6 Correlation between markers

Marker ID	Marker Name	1	2	3	4	5	6	7	8
1	SBL062A	1	0.5401	0.5622	0.4207	0.4593	0.2053	0.4112	0.0297
2	SBL1B5I	0.5401	1	0.4676	0.2897	0.409	0.2994	0.4123	0.1974
3	SBL1B5H	0.5622	0.4676	1	0.4632	0.3974	0.1754	0.3642	0.1766
4	SB050A	0.4207	0.2897	0.4632	1	0.4965	0.1603	0.3654	0.1452
5	SBL1B6C	0.4593	0.409	0.3974	0.4965	1	0.4051	0.6647	0.1512
6	SBL1B2F	0.2053	0.2994	0.1754	0.1603	0.4051	1	0.5234	0.2547
7	SBL1B6G	0.4112	0.4123	0.3642	0.3654	0.6647	0.5234	1	0.2685
8	pSB0088	0.0297	0.1974	0.1766	0.1452	0.1512	0.2547	0.2685	1

Table 7 Basic marker information

Marke rName	Position	Size(2)	Size(1)	Size(0)	Size(-1)	Chi-Square	Pr>ChiSq
SBL062A	0.00	80.00	39.00	4.00	7.00	65.56	0.00
SBL1B5I	1.26	99.00	27.00	0.00	4.00	104.57	0.00
SBL1B5H	2.96	99.00	22.00	4.00	5.00	99.92	0.00
SB050A	4.30	100.00	11.00	4.00	15.00	121.46	0.00
SBL1B6C	4.73	102.00	25.00	0.00	3.00	111.14	0.00
SBL1B2F	4.73	115.00	6.00	0.00	9.00	171.65	0.00
SBL1B6G	4.73	88.00	21.00	0.00	21.00	96.64	0.00
pSB0088	6.84	95.00	14.00	5.00	16.00	104.57	0.00

Table 8 Trait correlation matrix

Trait	Log2010 Dist	Log2010 RDS	Log2011 Dist	Log(n+1)2011RDS	Log2012 RDS	Log2012 Dist
Log2010Dist	1	0.3631	0.5157	0.5529	0.4424	0.3655
Log2010RDS	0.3631	1	0.1813	0.1147	-0.0932	-0.1887
Log2011Dist	0.5157	0.1813	1	0.8739	0.3686	0.4466
Log(n+1)2011 RDS	0.5529	0.1147	0.8739	1	0.368	0.473
Log2012RDS	0.4424	-0.0932	0.3686	0.368	1	0.8303
Log2012Dist	0.3655	-0.1887	0.4466	0.473	0.8303	1

Table 9 Goodness of fit tests

Shapiro-Wilk Test for normality of residuals		
Residual	W	Prob<W
Log2010Dist	0.973391	0.1304
Log2010RDS	0.974303	0.1255
Log2011Dist	0.903658	0.0412
Log(n+1)2011RDS	0.91623	0.073
Log2012Dist	0.915066	0.1057
Log2012RDS	0.872185	0.0193
GHAvg	0.972483	0.0892
GHMax	0.973676	0.1058
Pearson ChiSquared for binomial distribution		
Binary trait	X2	Prob>X2
2011Over-wintering	5.168141	0.0755
2012Over-wintering	0.667162	0.7164

Table 10 LOD scores between all markers

Marker Name	1	2	3	4	5	6	7	8
SBL062A	0.00	18.13	20.23	17.05	19.63	16.49	16.68	9.63
SBL1B5I	18.13	0.00	20.20	21.43	25.89	27.69	22.28	16.68
SBL1B5H	20.23	20.20	0.00	21.05	22.59	20.78	18.52	13.51
SB050A	17.05	21.43	21.05	0.00	23.80	21.38	20.55	15.02
SBL1B6C	19.63	25.89	22.59	23.80	0.00	28.30	24.38	16.68
SBL1B2F	16.49	27.69	20.78	21.38	28.30	0.00	24.99	20.52
SBL1B6G	16.68	22.28	18.52	20.55	24.38	24.99	0.00	15.73
pSB0088	9.63	16.68	13.51	15.02	16.68	20.52	15.73	0.00

Table 11 Recombination frequencies between markers

Marker Name	1	2	3	4	5	6	7	8
SBL062A	0	0.0126	0.0042	0.0172	0.0042	0.0175	0.0045	0.0381
SBL1B5I	0.0126	0	0.0169	0.0087	0	0	0	0.0231
SBL1B5H	0.0042	0.0169	0	0.0134	0.0083	0.0175	0.0095	0.0441
SB050A	0.0172	0.0087	0.0134	0	0.0043	0.019	0.005	0.0357
SBL1B6C	0.0042	0	0.0083	0.0043	0	0	0	0.0231
SBL1B2F	0.0175	0	0.0175	0.019	0	0	0	0.019
SBL1B6G	0.0045	0	0.0095	0.005	0	0	0	0.0211
pSB0088	0.0381	0.0231	0.0441	0.0357	0.0231	0.019	0.0211	0

Table 12 Marker data summary

Marker Name	Chromosome	Position	Size(2)	Size(1)	Size(0)	Size(-1)	Chi-Square	Pr>ChiSq
SBL062A	1	0	80	39	4	7	68.76	0
SBL1B5I	1	1.26	99	27	0	4	99	0
SBL1B5H	1	2.96	99	22	4	5	87.62	0
SB050A	1	4.3	100	11	4	15	88.62	0
SBL1B6C	1	4.73	102	25	0	3	102	0
SBL1B2F	1	4.73	115	6	0	9	115	0
SBL1B6G	1	4.73	88	21	0	21	88	0
pSB0088	1	6.84	95	14	5	16	81	0

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