Developing cytogenetic and molecular tools to improve selection for soil-borne pathogen resistance in wild hybrids (2013/358)

Final Report submitted to Sugar Research Australia

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Sugar Research Australia (SRA)

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SRA Research Project Final Report

SRA Project Code	**2013358**
Project Title	Developing cytogenetic and molecular tools to improve selection for soil-borne pathogen resistance in wild hybrids
Key Focus Area in SRA Strategic Plan	KFA1
Research Organisation(s)	Sugar Research Australia Limited, CSIRO
Chief Investigator(s)	Nathalie Piperidis
Project Objectives	• Develop cytogenetic/ molecular marker-based methods to select potentially high-value progeny from the introgression program involving *Erianthus*- and *S. spontaneum*-derived clones, resistant to nematodes, Pachymetra and smut. • Short-circuit traditional breeding by performing early selection on progeny based on molecular markers rather than traditional resistance screening. • Add value to the SRA-CSIRO introgression program as this project will allow using a molecular test to quickly identify clones with the desired genes within the breeding program. • To modernize traditional breeding by providing a better understanding of the sugarcane genome and the evolution of introgression chromosomes through selection so breeders can make more informed selection decisions and better targeted crossing. • Combined with project BSS344, produce varieties combining smut resistance with both nematode and improved Pachymetra resistance will increase productivity on affected soils.

Section 1: Executive Summary

In Australia, sugarcane has been grown for more than 100 years now as a monoculture without much fallow cropping. Consequently, the heavily cultivated areas had no chance to reduce the levels of soil-borne pathogens. Soil health is a major concern for the Australian sugar industry.
Large economic losses caused by soil-borne pathogens such as nematodes and Pachymetra severely affect the Australian sugarcane industry every year. In 2013, it was estimated that the losses caused by those two pathogens reached $80 and 100 million/ annum respectively. The best way to address Pachymetra is with resistant varieties. Following the Smut outbreak in 2005/ 2006, growers were well-resourced with high yield performance/ smut resistant varieties such as $Q200^{A}$, $Q208^{A}$, and $KQ228^{A}$; however, these varieties have only intermediate resistance to Pachymetra. There has been a large investment in introgression projects in recent years in Australia to address these issues and introduce new resistance genes into the breeding program to produce better varieties with resistance to targeted disease.
SRA has been involved in two ACIAR projects in collaboration with CSIRO and China to characterize clones from the *S. spontaneum* introgression population as well as *Saccharum*/ *Erianthus* hybrids This sugarcane relative (part of the *Saccharum* complex), *Erianthus arundinaceus*, is highly drought tolerant, almost immune to Pachymetra root rot and is highly resistant to nematodes. Some advanced backcross clones retain high levels of resistance to root knot and lesion nematodes, and Pachymetra root rot. Clones from the introgression populations have been used in this project to initiate the development of molecular marker-based screening methods. The Australian Sugar Industry is looking for enhanced Pachymetra resistance., This project aimed to characterize more *Erianthus* hybrids for the breeders to make better-informed crosses and to find tools/ methods to improve the breeding program efficiency in combining Smut and Pachymetra resistance as well as Nematode resistance in sugarcane. ".

This project ultimately targeted SRA breeding and molecular breeding teams as an end user to provide a marker based screening method for Pachymetra, Nematode and Smut resistance. Collaborations were formed between the cytogenetics laboratory in Mackay, two majors groups within SRA, the pathology team led by Barry Croft and the breeding team led by George Piperidis, and with Karen Aitken leading the molecular marker group at CSIRO. The rationale for this project was based on newly acquired BC3-*Erianthus* hybrids had less than the basic chromosome number of 10 *Erianthus* chromosomes as revealed by Genomic *In Situ* Hybridization (GISH). This implied that each of the *Erianthus* chromosomes within the BC3 clones studied are different from each other and are specific to one homology group (HG) of sugarcane. The main objective of this project was to develop a highly efficient and economical PCR test that could be used to identify each of the 10 basic *Erianthus* chromosomes.

The second objective of the project was to use the results of project 2011344 (ratings on Pachymetra and nematodes for these clones) and expanded in this project to rate more of the *Erianthus*-BC3 population, and identify Quantitative Trait Loci (QTLs) for Pachymetra and Nematode resistance. Once the set of specific primers is validated and QTLs are revealed we should be able to associate a particular chromosome based on the region of interest for the resistance to disease with a simple PCR test and screen new clones.

We also aimed to apply the same approach to clones from the *S. spontaneum* population to develop a set of *S. spontaneum*-specific primers in order to screen more cultivars. If a particular HG/ chromosome has some association with resistance to the soil-borne disease, the primer for this particular HG will be used for screening.

The project deliverable are

- Characterisation of *Erianthus* hybrids by cytogenetics, leading to knowledge of chromosome complement, and chromosome transmission through generations.
- A set of *Erianthus*-specific primers able to identify the number of *Erianthus* chromosomes and their assignation to each of the 10 basic *Erianthus* chromosomes.
- A set of specific *spontaneum* SSR primers for the developed from the clones of the introgression population.
- Characterization of genetic regions or QTLs harbouring disease resistance associated to Pachymetra, Nematode and Smut in the *Erianthus* and *spontaneum* introgression populations.
- If QTLs have been revealed for the diseases targeted in this project we will initiate the development of marker assisted selection protocols for early routine screening of all introgression populations

This project aimed to deliver tools to enhance and facilitate the traditional screening for two soil-borne diseases and potentially smut resistance. This could help to select new varieties from the introgression program with new sources of resistance to soil-borne pathogens and could improve productivity of the sugarcane industry. The beneficial impact of producing more resistant varieties will ripple throughout the industry by reducing the disease levels in fields, resulting in increased yields and lifting profits.

Section 2: Background

This project has been developed based on existing knowledge and results from previous projects.
The two wild species *E. arundinaceus* and *S. spontaneum* have many valuable traits, including high vigour, drought, waterlogging and frost tolerance and resistance to diseases. More than 20 years ago, ten *E. arundinaceus* clones were rated highly resistant (almost immune) to Pachymetra root rot (Magarey and Croft, 1996). Preliminary testing of some clones from crosses using the wild species from China had shown that they have

potential for high yield in small plot trials (Foreman *et al.,* 2007) and resistance to nematodes (Stirling *et al.,* 2011).
Out of all soil-borne diseases, Pachymetra root rot (Magarey and Croft, 1996), root knot (*Meloidogyne javanica*) and root lesion nematodes (*Pratylenchus zeae*) (Blair and Stirling, 2007) are the most important and they are a major contributor to sugarcane yield decline in Australia.

Two introgression populations: The *Erianthus* introgression population have been developed from an ACIAR project in collaboration with CSIRO and China. The BC3 clones used in the project were derived from several small populations with approximately five different BC2 parents and elite cultivars. On a cytogenetic level, the pedigree of the population have been characterized at a chromosome level and results have been published in Genome (Piperidis *et al*., 2010). The *Erianthus* hybrids in Australia are the most extensively characterised hybrids in the world. The *S. spontaneum* population is a BC1 consisting of approximately 400 clones which have been characterised for yield and disease resistance (Pachymetra and smut). Some of these clones have also been characterized by cytogenetics.

Molecular analysis of both population. Genetic maps were developed at CSIRO for both introgression populations. Genetic maps will be used to search for genetic traits related to the targeted diseases. Molecular markers have also been developed previously in correlation to some of the *Erianthus* chromosomes to verify the feasibility of this project and our results – published in Piperidis *et al*., 2012- were very encouraging and showed great prospects for the design of primer pairs.

Screening for the disease: This is part of Barry Croft project's 2011344. Pot trials were used in this project in order to identify resistant vs. susceptible individuals to nematodes in Woodford (Pratylenchus as well as root knot), Pachymetra trials were done at SRA Tully and Smut trials were done in Woodford. The data generated from these trials are combined with molecular data to detect genetic regions of resistance to disease. Previous research on nematode and Pachymetra showed great prospects in isolating resistance genes essential to this project (Stirling *et al*., 2011).

One of the gaps in knowledge for this project is inherent to the complexity of the sugarcane genome. In 2013 we did not have a sugarcane genome sequence, and indeed even today the sugarcane sequence is yet to be revealed. This is an important challenge to face in order to develop primer pairs but we aim to overcome this by using the genome sequence of the closest diploid relative, *Sorghum*. To transpose the methodology from the *Erianthus* introgression population to the *S. spontaneum* introgression population we will need to be able to differentiate each *S. spontaneum* chromosome copy of the same homology group which will not be easy according to the enormous diversity in the multiple alleles for each homology groups in the sugarcane genome.
Another gap in sugarcane is the difficulty in the detection of QTLs or regions associated with certain traits. To date in sugarcane, only one trait is known to be controlled by a single major gene, the rust gene Bru1.

Section 3: Outputs and Achievement of Project Objectives

Wild relatives of sugarcane have many traits of interest such as resistance to major diseases.
For example, some clones of *Erianthus arundinaceus* have been shown to be highly resistant or immune to Pachymetra root rot (Magarey and Croft 1996) and also resistant to Nematodes (Stirling *et al.* 2011). Before 2000, many attempts were made to introduce *Erianthus* germplasm into Australian varieties but all the F1s produced were sterile. With the ACIAR project involving SRA/ CSIRO and China, we finally had access to fertile *Erianthus* hybrids and also to a *S. spontaneum* introgression population. Results from project 2011344 showed that some of the families/ clones have potential for good yield and revealed some *Erianthus* and *S. spontaneum* backcross clones with high levels of resistance to Pachymetra and Nematode.

This project built on the results from project 2011344 and on the cytogenetic characterisation of the *Erianthus* hybrids. The main objective was to create a set of simple molecular markers with specificity to *Erianthus* vs. *Saccharum* chromosomes and test the possibility of associating one specific marker to one specific *Erianthus* chromosome from the BC3 mapping population. Disease screening of more clones was necessary to build/ achieve this objective as in project 2011344 all the screening was conducted to identify the more resistant clones while this project required the whole spectrum of resistance in order to find regions associated to the resistance itself. GISH results coupled to QTLs will be the initial step for the development of a method that can be used with disease screening results to develop a fast, easy and economical screening test for germplasm resistant to Pachymetra root rot, smut and Nematodes.

3.1 Development of specific primer pairs

Erianthus specific primer pairs

Because the sugarcane genome or the *Erianthus* genome sequences are not yet available, we used the *Sorghum* genome as it is the closest known diploid relative to sugarcane. Primers were designed from the whole genome sequence database of *Sorghum* available on the internet, specifically to amplify the introns of genes located on each of the 10 syntenic chromosomes which expectantly increase the polymorphism rate for the amplified sequences.
https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Sbicolor
The targeted region to amplify was approximately 300-1000bp and was identified using "Phytozome 10.3", (http://phytozome.jgi.doe.gov/pz/portal.html#). To ensure our targeted regions were located at a unique locus in the *Sorghum* genome we blasted the sequence back against the whole *Sorghum* genome sequence. However, even though the region of the sequence has a unique locus in *Sorghum*, this assumption might not be true in the sugarcane genome. We know that due to the high level of polyploidy as well as the large amount of repeated sequences in the sugarcane genome that it is possible a PCR could amplify regions from different chromosomes. Finally, the primers were designed on each side of the targeted sequence using the "www.Primer3Plus.com" software.

During the length of this project, we have design approximatively 100 primer pairs covering the 10 *Sorghum* chromosomes. To narrow down this number to 10 primer

pairs, we rejected the monomorphic primers as well as the primers for which the markers were too difficult to read. To be selected as suitable *Erianthus* specific, the amplified marker has to be present in the original *E. arundinaceus* parent and the F1, BC1, BC2 and BC3 progenies, and absent in any *S. officinarum* involved or any cultivars used in subsequent crosses. The 14 initial BC3 clones that we used for the screening were derived from seven different crosses with five different *Erianthus* BC2 parents.

In fact, multiple attempts at designing primers pairs were made during this project as some of our results indicated after the first analysis that more markers than chromosomes were revealed for some of the clones studied. This implied that some primers were not chromosome specific. To identify which of the primers amplified duplicated loci we designed more primers to assemble the perfect set of primers where each marker corresponds to one *Erianthus* chromosome. However, many primers were not polymorphic, did not amplify *Erianthus* specific markers or only amplified markers up to the BC1 generation. We have applied different strategies successively in order to resolve this problem.

Firstly, in order to increase chances of amplifying polymorphic markers suitable for our purpose we investigated the possibility of designing primers to genes that contain SSRs identified from the *Sorghum* genome. *Sorghum* being the closest diploid relative to sugarcane it is highly likely that SSRs identified within a gene sequence in *Sorghum* will also be present in the same gene sequence in sugarcane. Designing the primers by incorporating SSR requirement within the primer did not increase our success in obtaining the perfect set of primers.

Unfortunately, the complex genomes of sugarcane and *Erianthus* continued to present an obstacle for our goal of obtaining the set of primers. New resources were needed to address this issue and provide the perfect set of *Erianthus* specific primers. The first issue was that the amplicon generated by the primer pairs was very small in the scale of an entire *Erianthus* chromosome; thus, the amplicon could easily be duplicated along the whole genome and could reflect different loci in the genome.

The second issue encountered during this project relates to the genomic composition of *Erianthus*. *Erianthus arundinaceus* (2n=60) is a hexaploid and therefore has six copies of the same chromosome for each of the 10 homology groups. At each generation of crossing we know that half of the chromosomes are transmitted except between the F1 and the BC1 where all the *Erianthus* chromosomes are transmitted because of the 2n+n transmission. At the 4th generation of crossing, only one of the six alleles for each HG have been transmitted. For the 400 BC3 clones it is not guaranteed that, even though a clone could have an allele x from HG y, that same allele x will be transmitted in every BC3 clone. In fact the BC3 could be receiving any of the 6 alleles from the HG. So we needed to design primers to detect *Erianthus* specific alleles. Finally, we can also argue that up to two allelic copies from the same HG could have be transmitted in lieu of another allele from another HG and it will not be possible to distinguish by molecular markers 2 copy vs 1 of the same allele. This result could be observed when the number of *Erianthus* chromosomes detected by GISH is superior

to the number of *Erianthus*-specific markers detected by the primers. This must be a rare event. We can also add that the diversity (or polymorphism) in the allelic version of a chromosome could have also prevented the primer pairs to detect an allele if the primers were designed on a different allelic version.

In the third year of this project, we took advantage of significant advances in molecular biotechnology and particularly in the development of SNP genotyping. With the emergence of newer and cheaper technologies such as Genotyping by Sequencing (GBS) we hypothesized that GBS could be used to narrow the number of *Erianthus* chromosomes and reveal which homology group (HG) they belonged to. We used results from another project led by Karen Aitken at CSIRO in which GBS was used to generate markers to enhance the mapping of the *Erianthus* BC3 population.
DNA samples were sent to Cornell University as they have experience in working with sugarcane.
Twelve *Erianthus* clones used in our BAC-FISH analysis were included in the study. These clones were chosen to allow a direct comparison between the GBS and molecular marker results. GBS involves the restriction digestion of the sample DNA to reduce the genome complexity followed by ligation of adapters and barcodes then sequencing using Illumina. As the parents and grandparents of the *Erianthus* BC3 populations were included in the analysis *Erianthus*-specific markers could be identified.

Table 1: GBS results on 24 *Erianthus* hybrids (2 BC2 and 22 BC3)

	Sorghum 1	Sorghum 2	Sorghum 3	Sorghum 4	Sorghum 5	Sorghum 6	Sorghum 7	Sorghum 8	Sorghum 9	Sorghum 10	TOTAL	GISH results
QBYC06-30376	xxxxxxxxx	xxxxxxxx	xxxxxxxxx	xxxxxx	xxx	xxxxxx	xxxxx	xxxx	xxxx	xxx	10	11
QBYC06-30296	xxxxxxx	xxxxxxxxxx	xxxxxxx	xxxxxx	xxx	xxxxxx	xxxxx	xxxx	xxxxxx		9	10
KQ08- 6001		xxx		xxxxx		xxxxxx	xxxx	xxxxx			5	5
KQ08-6002		xxx					xxxxx	xxxxx			3	4
KQ08- 6003				x						x	?	6
KQ08- 6004				xxxxxx		x	xxxx				3	1+2*1/2
KQ08- 6005												4
KQ08- 6006												3+ 2*1/2
KQ08- 6007			xxxxxxxx			xxxxxx			xxxxxx		3	3
KQ08- 6008	xxxxxx	xxxxxxx		xxxx	xxx						4	3
KQ08- 6009	xxxxxxxxx	xxxxxxxxxx	x	xxx						x	5	4
KQ08- 6010				x		xxxxxx	xxxx			xx	4	4
KQ08- 6011	xxxxxxxxx	xxxxxxxxx		xxxxx		xxxxxx	xxxx		xxxx	x	6	6
KQ08- 6012		xxxxxxx	xxxxxxx	x	xxx		xxxx		xxxxxx	x	7	6
KQ08- 6013	xxxxxxxxx	xxxxxxx	xxxxxxx	x			xxxxx	xxxx	xxx		7	7
KQ08- 6014	xxxxxxxx		xxxxxxx	x			xxx	xxx			5	5
KQ08-1061		xxxxxxx	xxxxxxxx	xxxxxx				xxxxxx	xxxxxx		5	5
KQ08-1078												6
KQ08-1079												6
KQ08-1238	xxxxxxx		xxxxxxx	xxxxxx			xxxxx		xxxx	x	5 +x	3 +1/2
KQ08-1239	xxxxxxx	xxxxxxxx	xxxxxxxx	x		xxxxxx	xxxxx		xxxxxx		6 +x	6
KQ08-1294												5+ 1/3
KQ08-1339												4 + 1/2
KQ08-1359	xxxxxxx	xxxxxxxxxx		xxxx		x				xx	5	4 + 1/4

Results extracted from the GBS dataset conducted on the *Erianthus* BC3 population are presented in Table 1. Sequenced fragments for clones were aligned according to the *Sorghum* genome, and therefore each 'x' represents the presence of a fragment per chromosome. The frequency of 'x' is related to the accuracy of the prediction for presence or absence of the chromosome. For the clone KQ08-6003 we only have one to two x's and therefore the probability for the chromosome presence to be genuine is low. In green we highlighted the clones where the GBS and the GISH data matched perfectly while in blue we highlighted clones for which the data differs by one unit. No results are

reported for the clones highlighted in orange. Several reasons could explain the absence of data, for example DNA may have been degraded during transport or the reaction was simply unsuccessful.
These results were then compared to the previous marker results generated from the primer pairs.
As reported in milestone 5 we tested several primers for each chromosome and many of them revealed *Erianthus*-specific markers. The problem encountered was that we could not discern which marker amplified the genuine representative of the entire chromosome or the haplotype. Table 2 shows the comparison between the GBS presence/ absence of a fragment and the marker amplified from the most promising primer pairs for each HG. This information allowed us to choose which primer is more likely to amplify a fragment representative of an *Erianthus* chromosome.
The table highlights the issues we encountered with our primers as some of the primers were either completely monomorphic for many clones (Sb1b) or have inconsistent results. The primers highlighted in bold represent the best congruence with the GBS results.

Table 2: GBS and marker data comparison

chromosome No.	Primer names	QBYC06-30376	KQ08-6001	KQ08-6002	KQ08-6004	KQ08-6007	KQ08-6008	KQ08-6009	KQ08-6010	KQ08-6011	KQ08-6012	KQ08-6013	KQ08-6014
1	GBS 1	1	0	0	0	0	1	1	0	1	0	1	1
1	**Sb1a**	**1**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**1**	**0**	**1**	**1**
1	SB1b	1	1	1	1	1	1	1	1	1	1	1	1
2	GBS 2	1	1	1	0	0	1	1	0	1	1	1	0
2	**Sb2a**	**1**	**1**	**1**	**0**	**0**	**1**	**1**	**0**	**1**	**1**	**1**	**0**
2	Sb2b	1	1	1	0	m	1	1	0	1	1	1	1
2	Sb2c	1	0	0	0	0	1	1	0	1	1	1	0
3	GBS 3	1	0	0	0	1	0	x	0	0	1	1	1
3	**Sb3a**	**1**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**1**	**1**	**1**
3	Sb3b	**0**	**0**	**0**	**m**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
4	GBS 4	1	1	0	1	0	1	1	x	1	x	x	x
4	**Sb4a**	**1**	**1**	**1**	**1**	**0**	**1**	**0**	**0**	**1**	**0**	**0**	**0**
4	Sb4b	1	1	0	f	0	1	0	0	1	0	0	0
5	GBS 5	1	0	0	0	0	1	0	0	0	1	0	0
5	**Sb5a**	**1**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**1**	**0**	**0**
6	GBS 6	x	1	0	1	1	0	0	1	1	0	0	0
6	**Sb6a**	**1**	**1**	**0**	**0**	**1**	**0**	**0**	**1**	**1**	**0**	**0**	**0**
7	GBS 7	1	1	1	1	0	0	0	1	1	1	1	1
7	**Sb7a**	**1**	**1**	**1**	**1**	**0**	**0**	**0**	**1**	**1**	**1**	**1**	**1**
7	Sb7b	1	1	1	0	0	0	0	1	1	1	1	1
7	Sb7c	1	0	0	0	1	0	0	1	1	0	0	0

chromosome No.	Primer names	QBYC06-30376	KQ08-6001	KQ08-6002	KQ08-6004	KQ08-6007	KQ08-6008	KQ08-6009	KQ08-6010	KQ08-6011	KQ08-6012	KQ08-6013	KQ08-6014
7	Sb7d	1	0	0	0	0	0	0	0	0	0	1	0
7	Sb7e	1	0	0	0	0	1	1	0	1	1	1	0
8	GBS 8	1	1	1	0	0	0	0	0	0	0	1	1
8	**Sb8a**	**1**	**1**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**1**
8	Sb8b	1	1	1	0	0	0	0	0	0	1	0	0
8	Sb8c	1	0	0	0	0	1	0	0	1	1	1	0
8	Sb8d	1	1	1	0	0	0	0	0	0	0	1	1
8	Sb8e	1	0	0	0	1	0	1	0	0	0	0	1
9	GBS 9	1	0	0	0	1	0	0	0	1	1	1	0
9	**Sb9a**	**1**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**1**	**1**	**0**	**1**
9	Sb9b	1	1	0	0	1	0	0	0	0	0	0	0
10	GBS 10	1	0	0	0	0	0	x	1	x	x	0	0
10	**Sb10a**	**1**	**0**	**0**	**0**	**0**	**0**	**1**	**1**	**0**	**0**	**1**	**1**
10	Sb10b	1	0	0	0	0	0	1	1	0	0	a	1
10	Sb10c	1	0	0	0	1	0	0	0	1	1	1	0
10	Sb10d	1	0	0	0	0	0	1	1	0	0	1	1
10	Sb10e	1	0	0	0	0	0	1	1	0	0	0	1

Some mismatches (highlighted in red) between the GBS data and the specific markers have occurred. Sixty percent of the primers corresponding to HG 2, 3, 5, 6, 7 and 8 are a perfect match between the GBS results and the marker data, while 40 % of the primers corresponding to HG 1, 4, 9 and 10 have one or two mismatches.
Despite our efforts with the GBS experiment, we are still not 100 % certain of the composition of the clones regarding to which HG the chromosomes belong. At this stage, we accumulated three points of comparison but the results still remain inconclusive. We believe that the primers presented in Table 3 are the best possible selection for this project. Without access to the complete sequence of sugarcane we cannot select more primers at this stage as we have exhausted many potential available *Sorghum* sequences. When the sequence of the sugarcane genome becomes available, we will be able to revisit our primer design and hopefully have access to more gene sequences, directly on the species sequence. The blast will automatically reveal the duplicate status of the sequence from a *Saccharum* point of view as we know that *Erianthus* is part of the "*Saccharum* complex". An even better option will be to have access to the sequences of the *Erianthus* genome from the Japanese group led by Dr Shin Irei. Last year we asked them if it was possible to use their data in order to enhance our own primers. So far we have had the green light to access their data but once published we will be able to use the information for our own research.

Table 3: Selected primer pairs

HG	Primers tested	Name of Primer selected

1	16	Sb1a
2	10	Sb2a
3	11	Sb3a
4	4	Sb4a
5	12	Sb5a
6	8	Sb6a
7	5	Sb7a
8	9	Sb8a
9	11	Sb9a
10	7	Sb10a
Total	**93**	**10**

As we are not sure when these genome sequences will be available ultimately we decided to have a fourth check point using the Bacterial Artificial Chromosome - Fluorescent *In Situ* Hybridisation technique or BAC-FISH and combine this with GISH. If successful, this will literally show us *in situ* if a BAC, corresponding to one particular HG is in fact on the targeted chromosome or not. BACs are typically used in FISH to study the genome organisation or to characterize certain chromosomes. Initially the BAC were going to be used in the *S. spontaneum* section of this project. Bacterial artificial chromosomes (BACs) are DNA constructs based on a functional fertile plasmid, used for transforming and cloning in bacteria. The DNA fragments or inserts are usually around 80 to 350 KiloBases (KB).

A very good representation of what we aim to achieve was the work published by Kim *et al.*, 2005.

The experiment used a cocktail of BAC probes to characterize all the chromosomes from *Sorghum*.

As it is a diploid species it was quite straightforward to classify all 20 chromosomes into 10 pairs.

The pattern of signals enables FISH-based recognition of each chromosome pair and associate specific chromosomes with LG. In sugarcane such experiments are still not feasible but we decided to use the same BACs as Kim *et al.*, 2005 in order to identify *Erianthus* chromosomes. Table 4 is a summary of all the BACs requested from the BAC library in Arizona to be tested in our project. We were also able to access the BAC from the CIRAD *Sorghum* BAC library as well as some sugarcane BAC from CSIRO derived from the R570 BAC sequencing project. We are also using the probe pTA71 (or 45S rDNA) to detect the *Sorghum* chromosome 6 as this probe is a very good control for BAC-FISH experiments; it usually works in any condition and is very reliable.

Table 4: BACs name and position on *Sorghum* chromosomes and Q165 HGs

	Sorghum	**Arm location on *Sorghum***	**Q165 HG**	**BAC name**
1	SB1-01	short	4	18 E 9
2	SB1-01	long	4	11 J 16
3	SB1-02	short	8	14 P 24
4	SB1-02	long	8	31 A 18
5	SB1-02	long	8	25 A 20
6	SB1-03	short	3	20 D 7

	Sorghum	Arm location on *Sorghum*	Q165 HG	BAC name
7	SB1-03	long	3	24 D 9
8	SB1-03	long	3	6 L 24
9	SB1-4	short	1	33 K 7
10	SB1-4	long	1	27 I 22
11	SB1-5	short	2	19 C 23
12	SB1-5	long	2	3 H 22
13	SB1-6	short	2	27 I 19
14	SB1-6	long	2	26 E 10
15	SB1-6	long	2	31 H 19
16	SB1-07	short	5	22 E 19
17	SB1-07	long	5	26 C 4
19	SB1-07	long	5	30 J 3
20	SB1-08	short	8	29 A 20
21	SB1-08	long	8	11 B 23
22	SB1-09	short	6	28 L 5
23	SB1-09	long	6	7 D 14
24	SB1-10	short	7	26 O 22
25	SB1-10	long	7	27 O 12

Several factors can affect a good BAC hybridization on sugarcane chromosomes. BACs are small pieces of DNA and their sequences are a repetition of the four regular bases A, T, G and C; BACs could have a very specific hybridization and therefore give a very good signal such as BAC 2N9 in Figure 1. In this picture BAC 2N9 is visualised by two signals (for the 2 chromatids of a chromosome) on eight different homologous metaphase chromosomes of *S. officinarum* Badila. However, the hybridization signal could be unclear when BACs contain too many repetitive sequences and therefore a non-specific hybridization occurs. The whole chromosome can potentially be hybridized and the BAC can show no specificity at all. All BACs have to be tested and confirmed for their reproducibility.

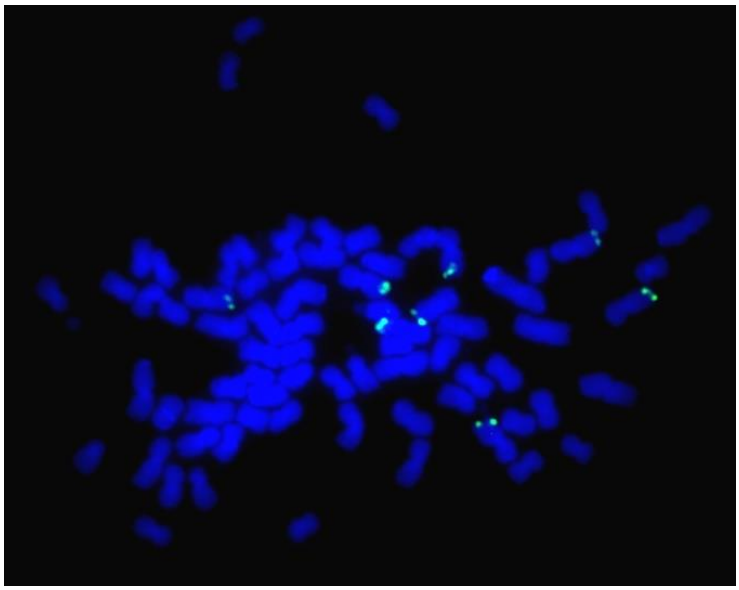

Figure 1: **BAC-2N9 *on S. officinarum* Badila**

Some preliminary results are presented in Table 5. This table shows the comparison of BAC-FISH, GBS and the marker results. We commenced by working with the clone KQ08-6005 because no GBS results were available. We physically mapped all ten chromosomes with BAC probes BAC-FISH results match exactly the marker results.
For clone KQ08-6007, we established that the set of primers revealed exactly the same chromosomes revealed by GBS data; this was also confirmed by BAC-FISH for chromosomes of the HGs 3, 6 and 9.

Results from this work are still in progress. As mentioned previously, BAC-FISH is a very sensitive technique although it is very reliable and produces unambiguous results when it works. We are aiming to confirm/ classify more chromosomes for this project.

The results highlighted in green in Table 5 are those that have been confirmed by GISH/ FISH. The small x are GBS marker results for which only one sequence was revealed and therefore we are not 100 % certain that the marker is genuine. The normal X represents robust GBS data results.

Table 5: Comparison of GBS/ markers and confirmation by BAC-FISH

			Sb1	Sb2	Sb3	Sb4	Sb5	Sb6	Sb7	Sb8	Sb9	Sb10
KQ08-6001	GISH/ FISH	5						X				
	GBS	5		X		X		X	X	X		
	Marker	5		X		X		X	X	X		
KQ08-6002	GISH/ FISH	4						0				
	GBS	3		X				0	X	X		
	Marker	4		X		X		0	X	X		
KQ08-6004	GISH/ FISH	1+ 2*1/2				X		0	X	0	0	
	GBS	2 to 3				X		x	X	0	0	
	Marker	2				X		0	X	0	0	

KQ08-6005	GISH/ FISH	4	0	X	0	0	0	0	0	X	X	X
	GBS	NO DATA										
	Marker	4	0	X	0	0	0	0	0	X	X	X
KQ08-6007	GISH/ FISH	3			X			X			X	
	GBS	3			X			X			X	
	Marker	3			X			X			X	
KQ08-6009	GISH/ FISH	4	X					0				
	GBS	3 to 4	X	X	x	x		0				x
	Marker	3	X	X		X		0				
KQ08-6010	GISH/ FISH	4						X				
	GBS	2 to 4				x		X	X			x
	Marker	3						X	X			X
KQ08-6011	GISH/ FISH	6						X				
	GBS	6	X	X		X		X	X		X	
	Marker	6	X	X		X		X	X		X	
KQ08-6012	GISH/ FISH	6						0				
	GBS	6		X	X	x	X	0	X		X	
	Marker	5		X	X		X	0	X		X	
KQ08-6013	GISH/ FISH	7	X		X			0				
	GBS	6 to 7	X	X	X	x		0	X	X	X	
	Marker	7	X	X	X			0	X	X	X	X

Examples of the BAC-FISH results for KQ08-6005 are shown in Image 2 and Image 3 below.

Image 2 shows the chromosomes of a single cell of KQ08-6005 hybridized with the BAC 33K07 (corresponding to HG4). The chromosomes are dyed in blue (DAPI), and in red there are four *Erianthus* chromosomes. The small green dots represent the hybridization sites of the BAC on the two chromatids of the chromosomes. We can easily count 12 signals which implies that this HG contains 12 haplotypes.

There are no BAC signals on the *Erianthus* chromosomes, we can therefore conclude that none of the four *Erianthus* chromosomes belongs to HG4.

In *Figure 3*, we used BAC 14P24 corresponding to Sb2, (a) represents a *Saccharum* chromosome with 12 BAC signals, (b) represents an *Erianthus* chromosome with no BAC signal and (c) represents an *Erianthus* chromosome with the BAC signal. We can therefore confirm that one of the four *Erianthus* chromosomes belongs to HG2.

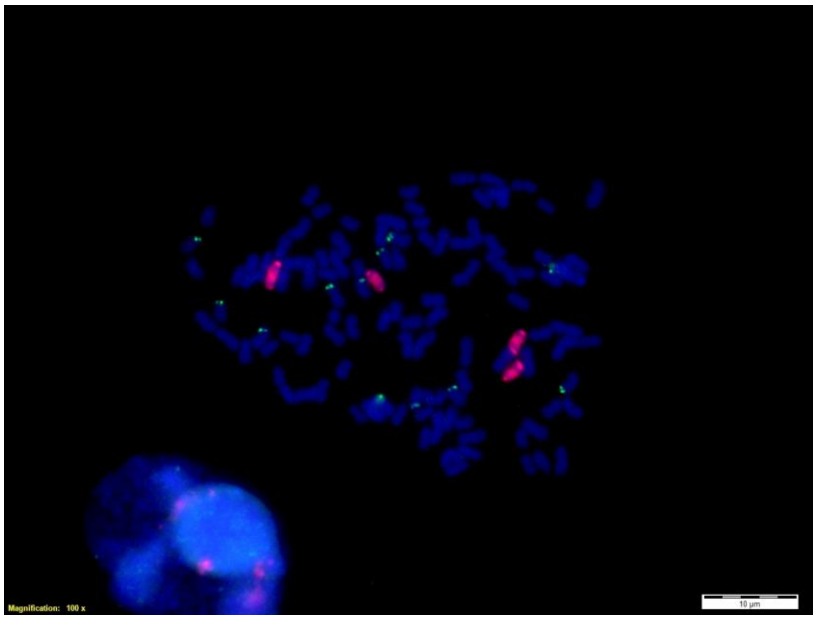

Figure 2: Hybridization of BAC-33K07 to one cell of KQ08-6005

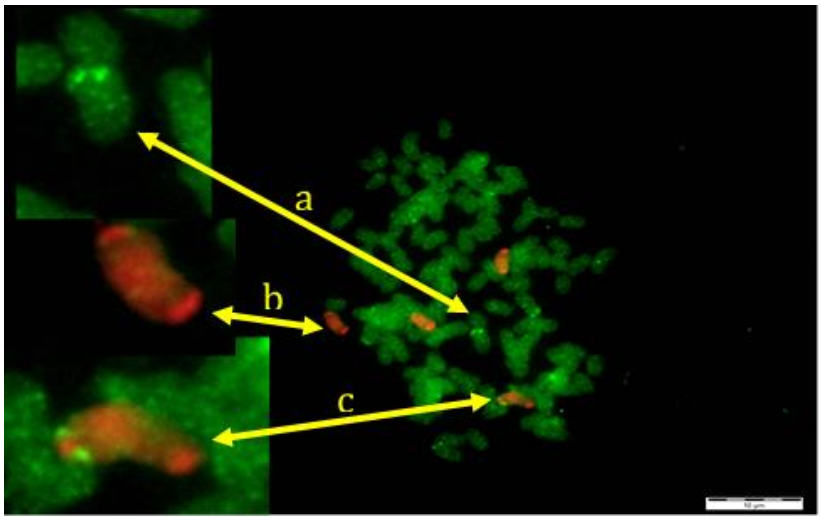

Figure 3: Hybridization of BAC-14P24 to one cell of KQ08-6005

The BAC-FISH experiments, although time-consuming and sensitive, are very important to confirm the results and visually examine the propensity of the markers to target specific chromosomes. This cannot be achieved with the markers alone. Our aim is to continue until at least 10 clones have been tested. Together, the results will help to confirm the probability of our set of primers to be reliable.

S. spontaneum specific primer pairs

We have investigated the development of a simple marker using primers as an efficient/ economical screening test to select germplasm resistant to Pachymetra root rot, Nematodes and Smut. We have identified *S. spontaneum* specific markers (versus *S. officinarum*) using microsatellite markers. Microsatellite markers (SSR) have been used to screen two F1, nine BC1 and eight BC2 from the *S. spontaneum* introgression clones. The pedigree information of these clones is presented in Table 6.

Table 6: F1, BC1, and BC2 pedigree of the *S. spontaneum* introgression population

Generation	Clone	Female	Male
F1	QBYN04-10357	Co419	YN83-157
	YN2002-356	Co419	YN75-1-2
BC1	QBYN04-26003	ROC 25	YN2002-356
	QBYN04-26258		
	QBYN04-26272		
	QBYN04-26239		
	QBYN04-26256		
	QBYN04-26282		
	QBYN04-26034		
	QBYN04-26235		
	QBYN04-26285		
BC2	KQBY07-33512	QBYN04-26003	QS87-7428
	KQBY07-33527		
	KQBY07-34758	QBYN04-26272	QS88-7403
	KQBY07-34782		
	KQBY07-33351	QBYN04-26258	
	KQBY07-33354		
	KQBY07-33413	Q208	QBYN04-26272
	KQBY07-33439		

Twenty-two SSR markers corresponding to the 10 sugarcane basic chromosomes have been used to produce *S. spontaneum* markers, specific to the original *S. spontaneum* parent YN75-1-2 as well as the F1 parent YN2002-356. SSR primers have been screened and selected for their capacity to reveal *S. spontaneum* specific markers. Successful SSR primers reveal a marker in all *S. spontaneum* clones and not in the elite lines Co419 and ROC25 from the pedigree used as control. Approximatively half of the primers did not display a polymorphic pattern usable to identifying specific *S. spontaneum* markers. The presence or absence of these markers is related to the presence or absence or a particular allele for a particular clone. For these clones the marker could be either the evidence of the presence of an entire *S. spontaneum* chromosome but could also be a *S. spontaneum* chromosome recombined with *S. officinarum*.

The preliminary results we produced show that specific primers could be developed, as some primers were polymorphic and the markers were segregating in the population.
Table 7 and Table 8 show preliminary results from the pre-screening for the SSR primers of the F1 and BC1, and the BC2 clones, respectively.

Table 7: Results of SSR screening in the F1 and BC1 clones

	BC1

Sorghum chromosome	Q165 HG	Primer	CO4419	YN75-1-2	ROC25	YN2002-356 (F1)	QBYN04-10357 (F1)	QBYN04-26003	QBYN04-26034	QBYN04-26235	QBYN04-26239	QBYN04-26256	QBYN04-26258	QBYN04-26272	QBYN04-26282	QBYN04-26285
1	4	SSR-Sb1a	0	1	0	1	1	1	1	1	0	1	1	1	1	0
1	4	SSR-Sb1b	0	1	0	1	0	0	1	0	1	0	0	1	1	1
2	8	SSR-Sb2	0	1	0	1	1	1	1	1	0	1	1	1	1	0
2	8	SSR-Sb2*c	0	1	0	1	0	1	0	1	1	0	1	1	0	0
2	8	SSR-Sb2*d	0	1	0	1	0	1	1	1	1	1	1	0	1	1
3	3	SSR-Sb3a	0	1	0	1	0	0	1	0	1	0	0	1	0	0
3	3	SSR-Sb3b	0	1	0	1	0	1	0	0	0	0	0	1	0	1
4	1	SSR-Sb4c	0	1	0	1	0	0	1	1	1	1	0	0	1	1
4	1	SSR-Sb4e	0	1	0	1	0	0	0	1	1	0	1	0	0	0
5	2	SSR-Sb5a	0	1	0	1	0	0	0	1	1	1	1	1	1	0
5	2	SSR-Sb5b	0	1	0	1	0	0	1	0	0	0	0	1	1	1
5	2	SSR-Sb5*a	0	1	0	1	0	1	1	0	1	1	1	1	0	0
5	2	SSR-Sb5*b	0	1	0	1	0	0	0	0	0	0	0	1	1	0
5	2	SSR-Sb5*c	0	1	0	1	0	0	1	1	0	1	0	0	0	1
6	2	SSR-Sb6a	0	1	0	1	0	1	1	1	0	1	0	0	0	0
6	2	SSR-Sb6a	0	1	0	1	0	1	1	1	1	1	0	1	1	1
8	8	SSR-Sb8a	0	1	0	1	1	1	1	0	1	0	1	1	1	0
8	8	SSR-Sb8b	0	1	0	1	0	0	1	0	1	0	1	0	1	1
9	6	SSR-Sb9a	0	1	0	1	0	1	0	1	1	0	0	0	0	1
9	6	SSR-Sb9b	0	1	0	1	0	1	1	0	0	1	1	0	0	0

Table 8: Results of SSR screening in the BC2 clones

Sorghum chromosome	Q165 HG	Primer	CO4419	YN75-1-2	ROC25	YN02-356	QN80-3425	QBYN04-26272	KQB07-34758	KQB07-34783	Q208	QBYN04-26272	KQB07-33439	KQB07-33413	QBYN04-26258	QS88-7403	KQB07-33351	KQB07-33354	QBYN04-26003	QS87-7428	KQB07-33527	KQB07-33512
1	4	SSR-Sb1a	0	1	0	1	0	1	0	1	0	1	0	1	1	0	1	1	1	0	1	1
1	4	SSR-Sb1b	0	1	0	1	0	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0
2	8	SSR-Sb2	0	1	0	1	0	1	0	1	0	1	0	1	1	0	0	0	1	0	0	1
2	8	SSR-Sb2*c	0	1	0	1	1	1	1	1	0	1	1	0	0	0	0	0	1	1	0	1
2	8	SSR-Sb2*d	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	1
3	3	SSR-Sb3a	0	1	0	1	0	1	0	1	1	1	1	1	0	1	0	1	0	0	0	0
3	3	SSR-Sb3b	0	1	0	1	0	1	1	0	0	1	0	0	0	0	0	0	1	0	1	1
4	1	SSR-Sb4c	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	1	SSR-Sb4e	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
5	2	SSR-Sb5a	0	1	0	1	0	1	1	1	0	1	1	0	1	0	0	1	0	0	0	0
5	2	SSR-Sb5b	0	1	0	1	0	1	1	1	0	1	0	1	0	0	0	0	0	0	0	0
5	2	SSR-Sb5*a	0	1	0	1	0	1	0	0	0	1	0	0	1	0	1	1	1	0	1	0
5	2	SSR-Sb5*b	0	1	0	1	0	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0
5	2	SSR-Sb5*c	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	2	SSR-Sb6a	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1
6	2	SSR-Sb6a	0	1	0	1	0	1	1	1	0	1	1	1	0	0	0	0	1	0	1	1

Sorghum chromosome	Q165 HG	Primer	CO4419	YN75-1-2	ROC25	YN02-356	QN80-3425	QBYN04-26272	KQB07-34758	KQB07-34783	Q208	QBYN04-26272	KQB07-33439	KQB07-33413	QBYN04-26258	QS88-7403	KQB07-33351	KQB07-33354	QBYN04-26003	QS87-7428	KQB07-33527	KQB07-33512
8	8	SSR-Sb8a	0	1	0	1	0	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0
8	8	SSR-Sb8b	0	1	0	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0
9	6	SSR-Sb9a	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
9	6	SSR-Sb9b	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	1	1	1	1	0

We have now completed the SSR primer set for *S. spontaneum* specific markers and Table 9 displays the list of the 12 SSR primers and the number of alleles detected. For HG 5 and 8 we have two primers equally robust.

Table 9: SSR primers specific to *S. spontaneum*

***Sorghum* HG**	**SSR primer**	**No. of alleles scored**
1	SSR-Sb1	1
2	SSR-Sb2*	6
3	SSR-Sb3	3
4	SSR-Sb4	4
5	SSR-Sb5	2
5	SSR-Sb5*	3
6	SSR-Sb6	1
7	SSR-Sb7	7
8	SSR-Sb8	2
8	SSR-Sb8*	4
9	SSR-Sb9	3
10	SSR-Sb10	3

Discussion on specific primer sets

We designed two sets of specific primer pairs respectively for the *Erianthus* hybrids population and for the *S. spontaneum* introgression population. To obtain the *Erianthus* specific set it has been a very long process to design primers. We were planning to have the set ready by the first year and we faced many obstacles. Even though we are still debating in using the primer design for HG10, we are quite confident that our set of primers works for most of the BC3 clones. At least 70 % of the time the markers exactly matched the GISH chromosome prediction. We can actually increase this number as we are confident that for some BC3 clones with recombined chromosomes we are still able to reveal the exchanged fragments as if it is a reciprocal recombination the marker will be revealed once for two parts of the chromosome. When the fragment recombined is very small we would obviously struggle to characterize it by markers or BAC-FISH. The other discrepancy can be explained with the same reasoning. For example when the number of markers are less than the number of GISH chromosomes, it could be that some of the chromosomes belong to the same HG. Another case scenario is for example for clone KQ08-1238 where three chromosomes were revealed by GISH and eight markers with the primers. We are pretty confident that the clones studied in cytogenetic lab in Mackay and in the Biotech lab in Brisbane are a mismatch. The last scenario is that

the number of markers is greater that the number of chromosome and this could be explained by the fact that the primer for HG 10 is not the perfect one.

We do know that with more GBS analysis combined with the BAC-FISH we can consolidate our results and if the primer 10 is definitely not the best primer for this HG we will be able to produce another primer for HG10. At the moment we are confident that the set of primer is revealing the number of chromosomes from *Erianthus*. The breeder can now request to use these primers for chromosome identification purposes. The *S. spontaneum* set of primers is actually 100 % functional to detect specific *S. spontaneum* markers as long as it is used with the parents from the introgression population as a reference.

3.2 Cytogenetics data

Erianthus hybrids

GISH experiments revealed the chromosome complement of the *Erianthus* introgression clones, including BC2, BC3 and BC4 hybrids. Over the length of this project, we increased the number of clones studied by GISH and have now tested and characterized 4 BC2, 54 *Erianthus* BC3, and 11 BC4 by GISH. The number of *Erianthus* chromosomes for each clone is shown in Table 10. Many of the clones tested by GISH were selected based on their disease ratings reported in project 2011344. The GISH data will be added to the new version of SPIDNET for easy access by breeders and researchers. The breeders are already using this information to make better-targeted crossing decisions according to the number of *Erianthus* and/or recombined chromosomes present in the clone. This information is used in collaboration with the outcomes of project 2011344; we now know many of the clones are resistant to nematode and Pachymetra, which reinforces the decision-making for crossing.

Table 10: Chromosome composition of *Erianthus* hybrids BC2, BC3 and BC4.

		Clone names	♀ parent	♂ parent	*Saccharum* C.	*Erianthus* C.	Rec.	2n cell
1	BC3	KQ08-1006	QBYC06-30376	Co1007	112	4	1	117
2	BC3	KQ08-1018	Q208	QBYC06-30305	100	2	0	102
3	BC3	KQ08-1029	Q208	QBYC06-30376	113	4	1	118
4	BC3	KQ08-1040	QN80-3425	QBYC06-30260	111	1	0	112
5	BC3	KQ08-1042	QN80-3425	QBYC06-30138	120	1	0	121
6	BC3	KQ08-1044	QN80-3425	QBYC06-30138	116	2	0	118
7	BC3	KQ08-1046	QN80-3425	QBYC06-30138	112	6	0	118
8	BC3	KQ08-1047	QN80-3425	QBYC06-30138	107	5	1	113
9	BC3	KQ08-1049	QN80-3425	QBYC06-30138	113	2	1	116
10	BC3	KQ08-1053	QN80-3425	QBYC06-30138	112	5	0	117
11	BC3	KQ08-1061	QN80-3425	QBYC06-30296	105	5	0	110
12	BC3	KQ08-1074	Q208	QBYC06-30296	110	5	1	116
13	BC3	KQ08-1078	Q208	QBYC06-30296	103	4	0	107
14	BC3	KQ08-1079	Q208	QBYC06-30296	100	6	0	106
15	BC3	KQ08-1080	Q208	QBYC06-30296	112	2	0	114

		Clone names	♀ parent	♂ parent	*Saccharum* C.	*Erianthus* C.	Rec.	2n cell
16	BC3	KQ08-1085	Q208	QBYC06-30305	109	6	0	115
17	BC3	KQ08-1107	QBYC06-30376	Co1007	102	4	0	106
18	BC3	KQ08-1111	QN80-3425	QBYC06-30376	111	6	1	118
19	BC3	KQ08-1134	Q208	QBYC06-30376	106	4	2	112
20	BC3	KQ08-1158	QN80-3425	QBYC06-30296	110	6	0	116
21	BC3	KQ08-1165	QN80-3425	QBYC06-30296	110	5	0	115
22	BC3	KQ08-1186	QN80-3425	QBYC06-30376	115	7	0	123
23	BC3	KQ08-1204	QN80-3425	QBYC06-30376	108	3	0	111
24	BC3	KQ08-1238	QN80-3425	QBYC06-30296	110-114	3	1	115-119
25	BC3	KQ08-1239	QN80-3425	QBYC06-30296	110-112	6	0	116-118
26	BC3	KQ08-1275	QN80-3425	QBYC06-30415	112	4	0	116
27	BC3	KQ08-1281	QN80-3425	QBYC06-30415	105	5	1	111
28	BC3	KQ08-1282	QN80-3425	QBYC06-30415	102	5	1	108
29	BC3	KQ08-1294	Q208	QBYC06-30296	114	6	0	120
30	BC3	KQ08-1323	QN80-3425	QBYC06-30296	106	7	0	113
31	BC3	KQ08-1339	Q208	QBYC06-30296	102-105	4	1	108-111
32	BC3	KQ08-1347	QN80-3425	QBYC06-30415	107	4	1	112
33	BC3	KQ08-1348	QN80-3425	QBYC06-30415	108	6	2	116
34	BC3	KQ08-1359	QN80-3425	QBYC06-30296	116-118	4	1	122-124
35	BC3	KQ08-1384	Q208	QBYC06-30296	109	6	0	115
36	BC3	KQ08-2385	Q208	QBYC06-30296	/	6	0	/
37	BC3	KQ08-2664	Q208	QBYC06-30376	102	4	0	106
38	BC3	KQ08-6001	QN80-3425	QBYC06-30260	112	5	0	117
39	BC3	KQ08-6002	QN80-3425	QBYC06-30260	105-106	4	0	108-110
40	BC3	KQ08-6003	QN80-3425	QBYC06-30296	110-115	7	0	116-121
41	BC3	KQ08-6004	QN80-3425	QBYC06-30296	112-113	1	2	115-116
42	BC3	KQ08-6005	Q208	QBYC06-30296	102-107	4	0	106-111
43	BC3	KQ08-6006	Q208	QBYC06-30376	107	3	2	112
44	BC3	KQ08-6007	Q208	QBYC06-30376	107-108	3	0	110-111
45	BC3	KQ08-6008	Q208	QBYC06-30376	104-107	3	0	107-110
46	BC3	KQ08-6009	QN80-3425	QBYC06-30138	100	4	0	104
47	BC3	KQ08-6010	QN80-3425	QBYC06-30138	109-110	4	0	113-114
48	BC3	KQ08-6011	QN80-3425	QBYC06-30376	103	6	0	109
49	BC3	KQ08-6012	QN80-3425	QBYC06-30376	107-108	6	0	113-114
50	BC3	KQ08-6013	QN80-3425	QBYC06-30415	110-112	7	0	116-119
51	BC3	KQ08-6014	QN80-3425	QBYC06-30415	112-113	5	0	117-118
52	BC2	QBYC06-30296	ROC10	YCE01-86	97	10	0	107
53	BC2	QBYC06-30315	ROC10	YCE01-86	110	11	0	121
54	BC3	QBYC06-30376	ROC20	YCE01-102	100	11	0	111
55	BC2	QBYC06-30415	YCE01-102	ROC10	108	10	0	118
56	BC4	QC12-20001	KQ08-1158	QC02-106	110	1	0	111

		Clone names	♀ parent	♂ parent	*Saccharum* C.	*Erianthus* C.	Rec.	2n cell
57	BC4	QC12-20002	KQ08-1158	QC02-106	102	0	0	102
58	BC4	QC12-20003	QN02-386	KQ08-6004	109	1	0	110
59	BC4	QC12-20004	QN02-386	KQ08-6004	104	1	1	106
60	BC4	QC12-20005	Q240	KQ08-6013	104	4	0	108
61	BC4	QC12-20006	Q240	KQ08-6013	102	2	1	105
62	BC4	QC12-20007	KQ08-1040	QN91-295	NA	1	0	NA
63	BC4	QC12-20008	Q241	KQ08-1158	102	1	0	103
64	BC4	QC12-20009	Q241	KQ08-1158	110	2	0	112
65	BC4	QC12-20011	QN02-386	KQ08-6004	110	0	2	112
66	BC4	QC12-5001	QN02-386	KQ08-6004	108	0	2	110

S. spontaneum introgression clones

The chromosome complement of eleven clones from the *S. spontaneum* introgression has also been investigated by GISH. Even though *S. officinarum* and *S. spontaneum* are very closely related, GISH allows the discrimination of *S. officinarum* and *S. spontaneum* chromosomes as well as recombined chromosomes from the two species as shown in Figure 4.

In this picture *S. officinarum* chromosomes are represented in orange, *S. spontaneum* chromosomes are green and recombined chromosomes are showing different pattern of recombination; recombined chromosomes contained orange and green parts.

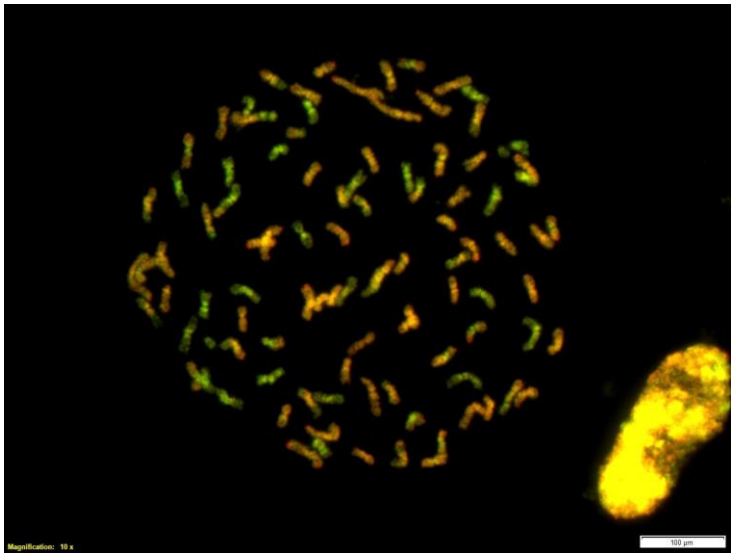

Figure 4: GISH of BC1 clone QBYN04-26003

Seven BC1 clones and four BC2 clones were studied by GISH and the results are presented in Table 11.
For the BC1 clones, the highest number of chromosomes in a 2n cell is close to 100, of which 50 % are *S. officinarum* chromosomes and 50 % are in nearly equal quantity of *S. spontaneum* chromosomes or recombined chromosomes. The composition of these clones differs from a typical cultivar which normally contains 80 % of *S. officinarum*, 10 - 15 % of *S. spontaneum* and 5 - 10 % of recombined chromosomes D'Hont *et al*., 2015. GISH is the only method available to disclose the chromosome composition of sugarcane clones and furthermore to show and reveal recombination between the two species.

Table 11: Chromosome composition of *S. spontaneum* introgression clones

	Female	Male	*S. officinarum*	*S. spontaneum*	Recombined	2n cell
BC1 Clone						
QBYN04-26003	ROC 25	YN 2002-356	53	24	22	99
QBYN04-26258	ROC 25	YN 2002-356	54	24	20	98
QBYN04-26272	ROC 25	YN 2002-356	51-55	21-23	18-21	93-95
QBYN04-26239	ROC 25	YN 2002-356	50-52	27-28	20-24	100-102
QBYN04-26256	ROC 25	YN 2002-356	54	23	23	100

	Female	Male	*S. officinarum*	*S. spontaneum*	Recombined	2n cell
QBYN04-26282	ROC 25	YN 2002-356	55	24	16-17	79
QBYN04-26034	ROC 25	YN 2002-356	51-52	27-28	22-23	99-100
BC2 Clone						
KQBY07-34758	QBYN04-26272	QS88-7403	54	22	20	96
KQBY07-33351	QBYN04-26258	QS88-7403	54-57	20-23	25-28	103-105
KQBY07-33354	QBYN04-26258	QS88-7403	54-55	21-24	20-22	96-100
KQBY07-33413	Q208	QBYN04-26272	57	18	18	93

BAC-FISH was attempted in the third year of this project to reveal the number of *S. spontaneum* chromosomes per HG. A three fluorescence-system is required to achieve this. Fluorochromes are readily available in red and green and are used routinely in GISH experiments in our lab to detect respectively *S. officinarum* and *S. spontaneum* chromosomes. A third fluorochrome is necessary to detect a BAC coupled with the GISH. Unfortunately, Invitrogen has discontinued the provision of a blue fluorochrome for some time now. Recently, another company called ENZO has released a blue fluorochrome but it has not been made available in time for the experiments conceived for this project. We were therefore unable to produce any data relating to the number of *S. spontaneum*/ recombined chromosomes per HG for the introgression clones. We can only estimate that for the BC1 and BC2 each HG is comprised of five to six *S. officinarum* chromosomes, two to three *S. spontaneum*, and two to three recombined chromosomes between the two species. This estimation is made possible from the GISH results showed that for the BC1 and the BC2 have a ratio of 50/50 respectively for the *S. officinarum* and the *S. spontaneum* / recombined chromosomes.

Discussion on cytogenetics data

Cytogenetics are invaluable for this project and are the starting point to evaluate the *Erianthus* clones.

We have nearly 70 clones characterized by GISH which give the breeders very important information for the crossing. Along with the ratings information on many BC3 clones the breeding team can make inform decisions when choosing clones as parents for their priority crosses. Project 2011-344 reported that the resistance present in the wild species tended to decrease with each successive backcross generation and this is obviously due to the expected decrease of *Erianthus* chromosomes. Nevertheless some BC3 clones performed even better than some commercial clones. For example the BC3 KQ08-1040 had an average rEGV of 10.23, had TCH and CCS better or equal to the standard. His rating for Smut was 2 but for Pachymetra it was 5 and he is also susceptible to Nematode. We now know that this BC3 has only one *Erianthus* chromosome so we need to understand where the resistance come from and it is actually unlikely that it is coming from the *Erianthus* genome. On another hand the clone KQ08-1006 showed great resistance to Pachymetra and has a least four entire chromosomes from *Erianthus*. This clone could be a very good candidate for introgression within the breeding program. The GISH data for the *S. spontaneum* introgression BC1 and BC2 were actually really informative as no one had any idea of their chromosomal composition. Even though we could not apply BAC-FISH/ GISH to the clone currently we could

estimate the number of the *S. spontaneum* for the BC1 and BC2 and it was clear that there is no significant difference between the BC1 and the BC2.
All data from the cytogenetics work should be available in the new version of SPIDNET.

3.3 Marker traits association (MTA)

All the results for MTA were generated by Karen Aitken at CSIRO from all the data collected for three years for this project.

Erianthus hybrids

a- Pachymetra screening

The *Erianthus* introgression populations used for this analysis were generated from one initial F1 from a cross between Badila (*S. officinarum*) and HN92-77 (*Erianthus arundinaceus*). From this initial F1 population seven BC3 populations were generated that were used in this analysis. The total number of progeny was 261 generated from predominately these seven crosses listed in Table 12.

Table 12: A list of the crosses used to generate the *Erianthus* population used in this analysis

BC1 cross	Female parent (BC2)	Male parent (BC2)
ROC10 x YCE01-86	KQ228	QBYC06-30305
ROC10 x YCE01-86	Q208	QBYC06-30296
ROC10 x YCE01-86	QN80-3425	QBYC06-30296
ROC10 x YCE01-86	Q208	QBYC06-30305
YCE01-102 x ROC20	Q208	QBYC06-30376
YCE01-102 x ROC20	QN80-3425	QBYC06-30376
YCE01-102 x ROC20	QBYC06-30376	Co1007

Because we were interested in the *Erianthus* part of the genome, markers were generated that were inherited from the *Erianthus* parent only. As the BC3 population contained from 1 to 10 *Erianthus* chromosomes with little recombination, much fewer markers are needed to identify associations. In total with all the markers generated in this project plus additional markers generated from AFLP and SSR markers in previous projects, a total of 79 single dose markers was used to generate consensus linkage maps of the *Erianthus* chromosomes. The single dose markers formed seven linkage groups with from 2 to 37 markers. The clones were screened for Pachymetra resistance using the standard method over a number of years and the score and rating were averaged across years using standards present in each year. Using single regression analysis, makers linked to Pachymetra resistance were identified (Table 13 and Table 14).

Table 13: Markers inherited from *Erianthus* for average score for Pachymetra resistance

Linkage group	Marker	Percent variation explained	P-value	Effect
Unlinked	Accctc-k	2	0.03628	-0.38
Group1a	Acacag-d	4	0.00091	-0.31
Group1a	Acacag-j	4	0.0009	-0.31
Group1a	Accctc-g	2	0.01294	-0.25
Group1b	Actcat-b	6	0.00011	0.61
Group1b	m22680b	6	0.00007	0.54
Group1b	Actcat-i	2	0.01079	0.32

Group1c	m11b	3	0.00659	-0.34
Group1c	XM2456096b	4	0.00166	-0.37
Group1c	Aggcac-i	2	0.03909	-0.19
Group1c	Accctc-b	2	0.01077	-0.27
Group1c	Aggcac-a	3	0.00359	0.27
Group2a	m15940	2	0.04351	0.18
Group2a	Accctc-j	2	0.04091	0.19
Group2a	chalcofc1i	2	0.02163	0.21
Group2a	Acacag-k	2	0.03396	0.2
Group2b	Acacag-a	6	0.00005	-0.39
Group2b	Accctc-c	4	0.00197	-0.34
Group3	Actcat-g	2	0.01992	-0.23
Group3	Actcat-j	2	0.01057	-0.28
Group3	Actcat-d	2	0.01079	-0.25
Group5	Actcat-h	2	0.02036	-0.21
Group5	Max3	3	0.00868	-0.24
Group5	Actcat-c	3	0.00679	-0.25
Group7	chalcofc13i	3	0.00451	-0.34

Table 14: Markers inherited from *Erianthus* for average rating for Pachymetra resistance

Linkage group	Marker	Percent variation explained	P-value	Effect
Unlinked	Accctc-k	2	0.02913	-0.98
Group1a	Acacag-d	6	0.00003	-0.95
Group1a	Acacag-j	6	0.00008	-0.9
Group1a	Accctc-g	5	0.00049	-0.85
Group1b	Actcat-b	3	0.00369	1.12
Group1b	m22680b	3	0.00577	0.92
Group1c	m11b	3	0.00261	-0.92
Group1c	XM2456096b	2	0.01487	-0.7
Group2	Acacag-a	7	0.00001	-1.02
Group3	Actcat-j	2	0.03067	-0.57
Group3	Actcat-d	2	0.02012	-0.55
Group6	m1190	2	0.04622	0.44
Group7	chalcofc1in3c	4	0.00082	-0.97

Both positive and negative associations were identified using both average rating and score.

a- Root Lesion nematode (RLN) screening

Again, only a relatively small number of samples from this population were screened for RLN resistance. In total 96 individuals were screened over a number of years. The data were adjusted across trials and used for this analysis. In total six markers were associated with increased susceptibility to RLN located on three regions of the genome (Table 15).

Table 15: Markers inherited from *Erianthus* that had a significant effect on Pf/ Pi for root lesion nematode

Linkage group	Marker	Percent variation explained	P-value	Effect
Unlinked	Actcat-d	9	0.00303	5.25
Group3	Accctc-m	7	0.00829	4.68
Group3	Actcat-k	5	0.03191	3.83
Group3	cir21	5	0.03306	3.81

Group9	Accctc-f	6	0.03394	4.32
Group9	Aggcac-e	11	0.00112	6.29

b- Root knot Nematode (RKN) screening

Again due to the difficulties associated with screening for RKN only 70 progeny from the *Erianthus* BC3 population have been tested. These seventy clones were screened in a number of trials and the data were adjusted using standards. Using single factor regression a number of markers were significant for RKN with both increased and decreased resistance identified (Table 16). Due to the small number of clones screened these results need to be verified in a larger population.

Table 16: Markers identified with significant association with RKN

Linkage group	Marker	Percent variation explained	P-value	Effect
Unlinked	Agg/cac-d	7	0.02911	-8.66
Group1	Act/cat-d	7	0.02314	-7.91
Group1	Agg/cac-h	10	0.00635	10.14
Group1	XM2456096a	6	0.03046	9.05
Group5	Agg/cac-l	13	0.00188	9.96
Group5	Agg/cac-f	12	0.00341	9.82
Group5	SB8019930a	9	0.00924	8.42
Group8	Aca/cag-g	7	0.02691	-7.34
Group8	Agg/cac-c	13	0.00183	-9.88

c- Screening for Smut

In total 92 progeny from the BC3 population were screened for smut resistance using the standard method. The progeny were screened in a number of trials and the data adjusted and used for regression analysis with the 70 *Erianthus* specific markers. One region was identified that was associated with resistance (Table 17). With such a small number of progeny screened further work on a larger population is needed to verify these associations.

Table 17: *Erianthus* specific markers linked to smut resistance

Linkage group	Marker	Percent variation explained	P-value	Effect
Unlinked	Aca/cag-o	5	0.03897	0.81
Group1	acc/ctc-b	5	0.02278	-1.22
Group1	Act/cat-g	9	0.00384	-1.51
Group1	Act/cat-d	8	0.00504	-1.54
Group1	Acc/ctc-l	6	0.02113	1.11
Group4	Agg/cac-f	10	0.00204	1.46
Group4	Agg/cac-l	6	0.01972	1.1
Group4	SB8019930a	10	0.00178	1.46

S. spontaneum introgression clones

a- Screening for Pachymetra

The BC1 population was created from a cross between ROC25 and YN2002-356. YN2002-356 is an F1 generated from a cross between a *S. spontaneum* from China YN75-1-2 and a commercial variety Co419 which was developed in Coimbatore, India. This

population was screened in groups of clones for Pachymetra resistance over a period of five years (Table 18).

Table 18: Trials and progeny numbers screened for Pachymetra resistance

Trial name	Number of individuals screened
RRF11-76	11
RRF12-78	50
RRF13-80A	45
RRF13-80B	77
RRF14-81B	48
RRF14-82A	92
RRF14-82B	31
RRF15-83A	47
RRF15-83B	15
RRF15-84B	108
Total	**524**

While having small numbers of progeny screened over a number of years is not ideal, having standard clones in each trial allows the data to be combined for analysis. Taking into account replication of clones screened the final population with trait data was 274 individuals. This project took advantage of previous marker work carried out on this same population. Individual trials were analysed for marker trait associations but as the more robust and consistent effects are of more importance in a breeding program only the final analysis of adjusted data over all trials is presented here.

Marker trait associations identified inherited from YN2002-356

In total 705 single dose markers were identified that were inherited from YN2002-356. These were generated using SSR, AFLP and DArT markers. They were grouped into 89 linkage groups which contained from 2 to 42 markers.
Using single factor regression analysis on the combined and adjusted disease resistance data a total of 51 markers were identified that were linked to disease resistance (Table 19).

Table 19: Markers inherited from YN2002-356 that are associated with Pachymetra resistance

Linkage group	Marker	Percent variation explained	P- value	Additive effect
Unlinked	AGCcaa80c	1	0.04993	0.43
Unlinked	AACcac234S	1	0.04457	0.44
Unlinked	ACCcta187S	3	0.00806	-0.59
Unlinked	ACCctc314c	2	0.0182	0.52
Group7	ACTcac218S	2	0.0305	0.48
Group11	CscPth-429755	1	0.04603	-0.44
Group14	Accctc4S	1	0.04807	-0.43
Group14	SscPth-418185	2	0.04765	-0.45
Group14	SscPth-427331	2	0.0283	-0.51
Group16	ACTcac235B	4	0.00048	-0.76
Group16	Accctc62c	4	0.00101	-0.72
Group16 (Ch06)*	CscPtb-429427	5	0.00052	-0.77
Group16 (Ch03)	CscPtb-426528	6	0.00006	-0.89
Group16 (Ch06)	CscPtb-426249	5	0.00044	-0.79

Linkage group	Marker	Percent variation explained	P- value	Additive effect
Group16 (Ch03)	CscPtb-426792	5	0.00033	-0.8
Group16 (Ch06)	CscPtb-424328	5	0.00035	-0.8
Group16 (Ch06)	CscPth-429427	5	0.00059	-0.77
Group16 (Ch03)	CscPth-426792	4	0.00112	-0.73
Group16 (Ch06)	CscPth-424328	5	0.0004	-0.79
Group16 (Ch06)	CscPth-426249	4	0.00155	-0.71
Group16 (Ch05)	CscPth-385124	3	0.00575	-0.65
Group20	ACCctt285S	2	0.03171	0.47
Group29	cir35lS	2	0.03181	0.47
Group30	ACCcag24c	2	0.00998	-0.56
Group30	Accctc19c	2	0.03508	-0.46
Group30	CscPtb-430137	2	0.01311	-0.54
Group30	CscPtb-435663	2	0.01311	-0.54
Group30	CscPtb-436735	2	0.03156	-0.47
Group30	CscPth-436735	2	0.03699	-0.46
Group30	SscPth-167845	2	0.01567	-0.53
Group30	36buqbc	2	0.01881	-0.52
Group30	cir52bc	2	0.00974	-0.57
Group30	smc1120-191B	2	0.04111	-0.45
Group34	Accctc43c	2	0.02314	0.5
Group34	CscPth-189523	2	0.02397	0.51
Group34	CscPth-258319	2	0.04126	0.46
Group36	AACcta91B	2	0.03968	-0.45
Group52	AAGcat61c	1	0.04813	-0.44
Group52	CscPtb-382107	2	0.01181	-0.55
Group52	CscPth-382107	2	0.01263	-0.56
Group52	36buqoc	2	0.01452	-0.53
Group59	AACcat222S	2	0.03136	-0.47
Group59	cir30gS	2	0.03508	-0.46
Group64	smc2055bc	2	0.01006	0.56
Group64	cir56cc	3	0.00582	0.6

Note: Number in brackets is the sequence alignment to the *Sorghum* genome at less than e-20
The one major effect that is inherited from YN02-356 is linked to resistance to Pachymetra on linkage group 16 and explained 6 percent of the variation.

Marker trait associations identified inherited from ROC25

DArT, SSR and AFLP markers generated 755 single dose markers inherited from ROC25. These markers formed 98 linkage groups with from 2 to 22 markers each. Using single factor regression analysis on the combined and adjusted disease resistance data, a total of 62 markers were identified that were linked to disease resistance (Table 20).

Table 20: Marker trait associations linked to Pachymetra disease for average rating inherited from ROC25

Linkage group	Marker	Percent variation explained	P- value	Additive effect
Unlinked	ACGcta122	2	0.03444	-0.2
Unlinked	AGGcta125S	2	0.00937	0.25
Unlinked	AGCctt194C	2	0.02012	0.22
Unlinked	AGCcag68	2	0.03541	-0.2
Unlinked	cir64dC	2	0.02898	0.21
Unlinked	ACTctg75	2	0.02424	-0.22
Unlinked	ACCcta317C	2	0.01903	-0.23

Linkage group	Marker	Percent variation explained	P- value	Additive effect
Unlinked	AAGcag312C	2	0.01679	0.23
Unlinked	scPtb-429690	2	0.03815	0.2
Group3	scPtb-424589	2	0.015	-0.23
Group5	36buql	2	0.02815	0.21
Group5	scPtb-428034	3	0.00583	0.26
Group5	scPtb-418347	3	0.00815	0.25
Group5	scPtb-253647	3	0.00721	0.25
Group5	smc179j	3	0.00235	0.29
Group5	cir36a	4	0.00126	0.3
Group5	ACGctt207B	2	0.03581	0.2
Group10	scPtb-429904	1	0.04942	0.19
Group10	scPtb-420563	2	0.03293	0.21
Group10	scPtb-424332	2	0.0424	0.2
Group16	AGCctt334	2	0.03842	0.2
Group19	ACGcta323S	2	0.02856	-0.21
Group19	scPtb-368251	7	0.00002	-0.4
Group19	scPtb-424622	6	0.00005	-0.38
Group19	scPtb-430016	7	0.00001	-0.42
Group19	scPtb-436658	7	0.00001	-0.41
Group19	scPtb-429538	7	0.00001	-0.41
Group19	scPtb-424253	7	0.00001	-0.42
Group19	scPtb-425034	7	0.00001	-0.43
Group19	scPth-424622	7	0.00001	-0.42
Group19	scPth-430016	6	0.00003	-0.4
Group19	scPth-436658	7	0	-0.44
Group19	scPth-429538	7	0.00001	-0.41
Group19	scPth-425034	8	0	-0.44
Group19	smc21gS	10	0	-0.49
Group19 (Ch08)	scPtb-418729	9	0	-0.48
Group19 (Ch05)	smc1047-157	10	0	-0.49
Group19	Actcat25B	11	0	-0.52
Group19	Accctc34	7	0.00001	-0.42
Group19	ACCctc190	6	0.00005	-0.39
Group19 (Ch09, Ch07, Ch04)	scPtb-386175	8	0.00001	-0.43
Group24	scPtb-428269	2	0.03087	-0.21
Group24	scPtb-430089	2	0.03573	-0.21
Group25	AAGcaa106	2	0.01159	0.24
Group31	ACTcaa330	3	0.00756	-0.26
Group31	scPtb-428481	2	0.01637	0.23
Group31	scPth-428481	2	0.02232	0.22
Group32	AGGctc83B	2	0.01806	-0.23
Group45	ACTcag130S	1	0.04691	-0.19
Group45	AGCctc142	2	0.02055	-0.22
Group53	cir26b	1	0.0437	-0.19
Group62	scPtb-385124	2	0.01307	-0.25
Group62	scPth-385124	2	0.02467	-0.23
Group65	scPth-368251	8		-0.44
Group68	ACActc40	2	0.02038	0.22
Group68	cir33fC	2	0.02527	0.21
Group68	Actcat14C	3	0.00499	0.26

Linkage group	Marker	Percent variation explained	P- value	Additive effect
Group68	cir64b	2	0.02422	0.21
Group68	ACCcta177C	1	0.0468	0.19
Group75	AAGcag276C	2	0.03079	0.21
Group78	cir29b	2	0.0345	0.2
Group90	36buqhC	2	0.03008	-0.21

One major region inherited from ROC25 on linkage group 19 was linked to increased resistance and explained a maximum of 11 percent of the variation (Table 20). If the markers that were most significant for increased resistance were combined in the population then progeny that contained both markers (CscPtb-426528 and smc1047-157) had a disease rating of 4.54 compared to progeny that lacked both markers which had an average rating of 6.29 (Table 21). This is a decrease in disease rating of 27.8 %. These markers have been converted to high throughput markers in project 2015/025 to screen some introgression clones within the introgression program at SRA.

Table 21: Average effect for disease rating for two markers inherited one from each parent

Marker		Smc1047-157	
		0	1
CscPtb-426528	0	6.3 (66)	5.7 (68)
	1	5.9 (55)	4.5 (77)

Note: No. of progeny in brackets

b- Screening for Root Lesion nematode (RLN)

The method for screening root lesion nematodes is still being optimized and is very labor intensive. For this reason only 73 clones were screened for this pest over a number of years. The same markers were used for the analysis as in the Pachymetra screen. The small population size results in an over estimation of marker effects. These results should be verified in a larger population.
Using Pf/ Pi (final nematode population/ initial nematode population) as an indicator of resistance to root lesion nematodes a number of regions were identified that increased and decreased resistance (Table 22).

Table 22: Markers inherited from YN2002-356 that had a significant effect on Pf/ Pi for RLN

Linkage group	Marker	Percent variation explained	P-value	Effect
Unlinked	ACTctg88B	6	0.02748	-8.66
Unlinked	ACAcag67c	6	0.03137	8.69
Unlinked	ACGctc109S	7	0.02251	9.02
Unlinked	cir8hS	7	0.02313	8.91
Unlinked	ACCcag27c	6	0.03256	-8.64
Unlinked	AGCctt96c	15	0.00051	13.51
Unlinked	Accctc48S	8	0.01234	10.13
Group1	SscPth-254168	7	0.02831	-8.87
Group1	SscPth-426700	6	0.04012	-8.11
Group1	SscPth-254503	6	0.04012	-8.11
Group1	SscPth-420303	6	0.04012	-8.11
Group1	SscPtb-426700	6	0.04012	-8.11
Group1	SscPtb-254503	6	0.04012	-8.11
Group1	SscPth-420100	6	0.04012	-8.11
Group1	SscPth-36804	6	0.04012	-8.11
Group3	ACGcta88S	6	0.03192	8.47
Group3	ACCcat87S	6	0.03049	8.44
Group3	SscPth-39683	9	0.0099	9.99
Group3	SscPth-42545	9	0.0099	9.99
Group3	SscPth-41892	9	0.0099	9.99
Group16	ACActg101B	7	0.02562	9
Group25	Aggcac10S	9	0.00822	-10.25

Linkage group	Marker	Percent variation explained	P-value	Effect
Group25	cir25aS	9	0.01085	-9.87
Group25	ACCcat213S	8	0.01322	-9.61
Group28	ACGcag271c	16	0.00039	13.99
Group48	ACGctg301S	8	0.01217	-9.93
Group48	AACcta261S	6	0.03072	-8.43
Group65 (Ch03)	cir32-197S	6	0.04057	-8.01
Group65 (Ch03)	cir35eS	7	0.02441	-8.78
Group65 (Ch03)	smc2055eS	11	0.00343	-11.27
Group65	ACCcta361S	10	0.00462	-10.93
Group65	ACCctc171S	10	0.00462	-10.97
Group65	Accctc38S	9	0.00863	-10.17
Group85	smc1232fc	5	0.04954	-7.68
Group102	Actcat11aB	8	0.04933	9
Group105	ACActa75S	8	0.01752	8.82

Although only 36 of the 705 markers (only slightly more than you would expect by chance) inherited from YN2002-356 were identified with a significant effect on the trait, there was one region on group 65 that appears to increase resistance to RLN. Another unlinked marker appears to decrease resistance (Table 22).
More markers inherited from ROC25 showed an effect on Pf/ Pi for RLN (Table 23). Again the effects are an over estimation due to small population size and again both positive and negative effects were identified.

Table 23: Markers inherited from ROC25 that had a significant effect on Pf/ Pi for RLN

Linkage group	Marker	Percent variation explained	P-value	Effect
Unlinked	AGCctc345	8	0.01598	9.36
Unlinked	AGCctg188S	5	0.04291	-7.95
Unlinked	AGCctg85C	6	0.02845	-8.64
Unlinked	AAGcta241S	9	0.01086	-9.87
Unlinked	cir16b	7	0.02378	-8.81
Unlinked	AAGctg111C	6	0.02875	8.66
Unlinked	ACCcaa86C	7	0.02393	-8.8
Unlinked	AGCcag252S	7	0.01764	9.42
Unlinked	AGCctg128B	9	0.00786	-10.57
Unlinked	ACTcaa317	6	0.03255	6.93
Unlinked	ACGcaa109C	7	0.0184	-7.63
Unlinked	scPth-385208	5	0.04793	7.75
Group5	scPtb-424686	9	0.00754	10.66
Group5	scPth-417576	8	0.01221	9.83
Group5	scPth-385780	8	0.01221	9.83
Group5	scPth-253102	8	0.01221	9.83
Group24	scPtb-382719	6	0.03127	-8.54
Group24	smc16aC	7	0.01872	-9.21
Group24	smc1232cC	7	0.01872	-9.21
Group32	ACActc9	6	0.03963	-8.39
Group36	scPth-428316	6	0.03658	-8.45
Group36	cir25b	8	0.0123	-10.06
Group36	scPtb-426651	8	0.0123	-10.06
Group36	scPth-426651	8	0.0123	-10.06
Group36	scPth-384093	8	0.01278	-10.17

Linkage group	Marker	Percent variation explained	P-value	Effect
Group43	AGGcat194	6	0.03548	8.4
Group46	scPth-369686	7	0.01887	-9.17
Group46	scPth-257096	6	0.03285	-8.39
Group53	scPtb-367451	6	0.03492	-8.33
Group55	Agccta4	8	0.01293	10.15
Group55	scPtb-430071	8	0.01103	10.29
Group55	scPtb-429605	8	0.01713	9.69
Group55	Accctc59	5	0.04583	7.81
Group60	scPtb-428126	6	0.04085	8.36
Group60	scPtb-214655	5	0.04861	7.71
Group60	scPth-214655	5	0.04861	7.71
Group60	ACTcag130S	9	0.01078	9.89
Group70	AACcac260C	9	0.01136	10.05
Group78	scPth-382165	6	0.04311	8.34
Group79	scPth-368425	5	0.04348	8.17
Group83	scPth-128704	6	0.03605	-8.35
Group83	scPth-416806	9	0.01254	-9.9
Group87	cir56bC	6	0.03412	-8.36
Group97	ACCctt184	10	0.00476	10.89
Group97	Agccta42	8	0.01223	9.83

c- Screening for Root knot Nematode

A total of 73 clones from the same BC1 population was screened for root knot nematode resistance over the years from 2011 to 2015. Plants were screened over a number of trials to obtain an accurate rating for RKN. The data were adjusted across trials and used for single factor regression analysis. Both positive and negative effects were identified inherited from YN2002-356 parent (Table 24).

Table 24: Markers inherited form YN02-356 that had a significant effect on trial score for RKN

Linkage group	Marker	Percent variation explained	P-value	Effect
Unlinked	cir54eS	7	0.02352	28.46
Unlinked	ACGctg279S	8	0.01447	30.68
Unlinked	ACCctc408c	6	0.0351	26.82
Unlinked	ACCcag27c	5	0.04819	-25.31
Group2	CscPtb-383575	7	0.0254	-28.65
Group2	cir42bc	8	0.01628	-31.42
Group3	ACAcaa150S	6	0.03841	-26.27
Group3	SscPtb-426700	5	0.04583	-25.19
Group3	SscPtb-254503	5	0.04583	-25.19
Group3	SscPth-426700	5	0.04583	-25.19
Group3	SscPth-254503	5	0.04583	-25.19
Group3	SscPth-426079	5	0.04853	-25.06
Group3	SscPtb-426079	8	0.01426	-30.82
Group3	cir54gS	5	0.04845	-24.94
Group3	ACTctg88B	7	0.02497	-28.22
Group9	SscPtb-205691	6	0.04029	26.4
Group9	SscPtb-428031	5	0.04472	25.59
Group13	Accctc4S	7	0.02175	-29.1
Group13	ACActc19S	7	0.01765	-29.88

Linkage group	Marker	Percent variation explained	P-value	Effect
Group18	SscPth-428187	5	0.04526	26.48
Group26	AGCctt117S	7	0.02415	29.56
Group27	ACCcat87S	6	0.03601	26.69
Group27	smc179hS	5	0.04557	-25.39
Group33	ACCcag24c	7	0.0186	-29.51
Group36	AGCctt96c	8	0.01484	31.59
Group48	SscPth-384735	9	0.01133	-31.86
Group48	SscPth-427259	6	0.0285	-27.71
Group48	SscPth-419002	7	0.01948	-29.48
Group48	ACCcaa167S	6	0.04048	-25.82
Group48	ACGcta208S	6	0.0481	-24.82
Group49	AGCcag112S	7	0.01753	30.83
Group58	BscPth-385786	6	0.03016	27.28
Group60	smc21aS	8	0.01616	-30.62
Group62	SscPth-418352	8	0.01153	32.1
Group62	SscPth-384589	10	0.00493	35.37
Group62	SscPth-420495	16	0.00045	43.7
Group62	ACCcaa319S_1	13	0.0013	39.97
Group62	AAGcaa282S	7	0.01846	29.56
Group62	ACGctc137S	7	0.02343	28.52
Group62	Aggcac27S	7	0.02346	28.47
Group62	ACTctg251S	10	0.00534	35.06
Group68	SscPtb-416667	5	0.04923	25.04
Group74	AACcta214c	7	0.02952	-27.23
Group79	cir14cS	6	0.03321	-28.51
Unlinked	cir54eS	7	0.02352	28.46
Unlinked	ACGctg279S	8	0.01447	30.68

One region of the YN2002-356 genome seems to be associated with increased susceptibility to RKN on linkage group 62. A similar result was identified with the single marker regression for markers inherited from ROC25 (Table 25).

Table 25: Markers inherited from ROC25 linked to RKN resistance or susceptibility

Linkage group	Marker	Percent variation explained	P-value	Effect
Unlinked	ACGcta122	10	0.00562	34.87
Unlinked	AACcta293C	6	0.03254	27.32
Unlinked	AGGctg157C	7	0.01937	-29.39
Unlinked	Accctc28C	6	0.03536	-28.5
Unlinked	ACTctg75	9	0.00831	-33.64
Unlinked	smc75-159	6	0.04186	-26.55
Unlinked	ACTctc179	8	0.01598	-31.8
Group1	cir12-316	6	0.04193	-26.7
Group2	cir30b	7	0.02125	-29.04
Group2	scPtb-385676	11	0.00379	-36.68
Group2	scPth-385676	10	0.00674	-34.15
Group3	smc16fC	9	0.01068	32.05
Group3	smc1232hC	7	0.01934	29.55
Group5	Accctc49aC	14	0.00128	40.55
Group21	AGGcta114S	6	0.03771	26.49
Group27	ACTcac271	7	0.0223	-29.95

Linkage group	Marker	Percent variation explained	P-value	Effect
Group29	scPth-370141	6	0.03232	27.68
Group29	scPtb-253140	6	0.03232	27.68
Group34	scPtb-424633	6	0.02919	28.01
Group34	scPtb-425361	7	0.01769	30.07
Group34	ACActc6S	5	0.04563	25.74
Group38	AACcat99C	6	0.03358	-25.68
Group43	scPtb-251623	6	0.03396	26.74
Group49	smc1825b	7	0.01849	-29.53
Group50	scPth-435272	6	0.04978	-23.49
Group50	smc16aC	6	0.02737	-27.72
Group50	smc1232cC	6	0.0396	-25.92
Group51	scPth-257967	5	0.0488	25.13
Group53	cir26b	9	0.00814	34.47
Group63	AACcta158C	11	0.00435	35.25
Group63	scPtb-257332	10	0.00782	33.89
Group71	scPtb-426268	6	0.03874	-26.86
Group82	ACGcag80C	7	0.02247	29.67
Group89	scPth-384647	5	0.04784	25.26

Although fewer markers were identified inherited from ROC25 there is still evidence that regions of the genome are associated with increased and decreased resistance to RKN.

d- Screening for Smut

In total 290 progeny from the BC1 *spontaneum* population was screened for smut resistance using standard methods over a number of years. The data from the different trials were adjusted and combined for analysis. A large effect QTL was identified inherited from YN2002-356 (Table 26).

Table 26: Marker associations for smut resistance (disease incidence) inherited from YN02-356

Linkage group	Marker	Percent variation explained	P-value	Effect
Unlinked	AAGcaa179S	1	0.03889	-6.15
Unlinked	AACcaa145c_2	7	0	-13.9
Unlinked	cir36dc	2	0.01427	-7.3
Unlinked	ACCcaa376c	2	0.01885	-7
Unlinked	AAGcat97S	1	0.04584	5.97
Group1	ACActc13S	1	0.04538	6
Group15	AACcta261S	2	0.03335	6.34
Group15	SscPth-251728	2	0.0126	7.45
Group15	SscPth-254006	2	0.0126	7.45
Group15	ACCcta139S	1	0.04007	6.14
Group15	SscPth-385428	2	0.01833	7.09
Group20	36buqiS	2	0.00921	7.74
Group20	BscPth-250956	3	0.00629	8.13
Group20	BscPth-254534	2	0.00975	7.7
Group26	ACGcag192S_1	5	0.00017	11.15
Group26	SscPth-253908	4	0.00193	9.77
Group26	SscPth-257508	4	0.00175	9.75
Group26	SscPth-250827	3	0.00396	9.03
Group26	SscPth-189611	4	0.00212	9.63
Group28	AGGctg147S	2	0.02386	6.75

Linkage group	Marker	Percent variation explained	P-value	Effect
Group36	AGCctt96c	3	0.00661	8.16
Group37	SscPth-427357	1	0.03685	6.22
Group37	SscPth-427721	2	0.02963	6.48
Group37	BscPth-367551	2	0.02443	6.71
Group40	cir66jS	2	0.00807	7.91
Group40	cir66hS	2	0.02986	6.47
Group40	ACCctt85S	3	0.00396	8.56
Group41	ACTcag132S	2	0.02687	6.62
Group45	smc21ic	2	0.00984	7.69
Group45	CscPtb-369649	4	0.00136	9.98
Group45	CscPth-369649	4	0.00079	10.46
Group49	scPtb-166852	2	0.03987	6.58
Group49	SscPtb-419128	1	0.04916	6.4
Group53	Agccta7S	20	0	-23.13
Group53 (Ch05)	336bsaS	36	0	-30.52
Group53	ACGcag268S	26	0	-26.1
Group53 (Ch05)	BscPtb-420385	29	0	-27.5
Group53 no hits	SscPth-176304	25	0	-25.38
Group53	ACCcag31S	22	0	-23.85
Group53	Actcat29S	6	0.00001	-12.97
Group53	ACTctt228S	5	0.00049	-10.54
Group54	AGCctt176S	1	0.04305	-6.17
Group61	ACCcaa85c	2	0.03066	6.45
Group62	SscPth-384589	2	0.01604	-7.18
Group62	SscPth-420495	1	0.0436	-6.02
Group62	ACGctc137S	1	0.04396	-6.06
Group62	Aggcac27S	1	0.0425	-6.04
Group71	ACTcag117S	1	0.0454	-5.98
Group81	ACAcag67c	2	0.03597	-6.46

The large effect QTL identified on linkage group 53 was inherited from the *S. spontaneum* ancestor. Associations were also identified inherited from ROC25 that again increased resistance (Table 27).

Table 27: Marker associations for smut resistance (disease incidence) inherited from ROC25

Linkage group	Marker	Percent variation explained	P-value	Effect
Unlinked	cir30a	2	0.02214	6.82
Unlinked	ACCcaa86C	1	0.04345	6.05
Unlinked	AAGcat88S	1	0.04523	-6.01
Unlinked	ACAcag202	2	0.01891	7.07
Group5	Accctc59	1	0.04159	-6.12
Group10 (Ch07)	scPth-418060	9	0	-15.2
Group10 (Ch07)	scPtb-429904	8	0	-14.2
Group10 (Ch07)	scPtb-420563	7	0	-14.07
Group10 no hits	scPtb-424332	8	0	-14.19
Group10 no hits	scPth-424332	6	0.00002	-13.19
Group10 (Ch05)	scPth-417591	6	0.00003	-12.82
Group12	Aggcac13	1	0.04572	5.99
Group14	scPth-128934	1	0.04526	-5.97
Group17	Aggcac12	2	0.01945	-6.99

Linkage group	Marker	Percent variation explained	P-value	Effect
Group17	scPth-384175	1	0.03834	-6.17
Group17	scPtb-427901	2	0.01026	-7.65
Group17	scPtb-426823	2	0.02319	-6.94
Group20	cir18-234	1	0.04873	5.88
Group29	scPth-436050	2	0.04214	-6.42
Group32	scPtb-253140	1	0.0446	5.99
Group39	smc2042g	2	0.01015	7.65
Group39	scPtb-396805	3	0.00731	8.3
Group39	scPtb-253938	3	0.00738	8.33
Group39	scPtb-385642	2	0.01768	7.47
Group39	scPtb-419022	2	0.03028	6.78
Group43	scPth-385208	2	0.01944	-7.32
Group51	Agccta8	2	0.02488	-6.68
Group52	cir16h	2	0.02103	6.89
Group52	scPth-252388	2	0.0382	6.96
Group56	scPtb-424605	2	0.03423	-6.36
Group58	scPtb-424529	1	0.04732	-6.1
Group72	ACCcat127S	1	0.04321	-6.14
Group86	AACcta160	4	0.00161	-9.71
Group87	cir39b	1	0.04065	6.12
Group90	36buqhC	2	0.01745	7.07
Group92	AAGcaa338	2	0.01953	-6.95
Unlinked	cir30a	2	0.02214	6.82
Unlinked	ACCcaa86C	1	0.04345	6.05

A large effect QTL was also identified inherited from ROC25 on linkage group 10 (Table 27) that again increases resistance. When the progeny were investigated for the marker effects of the two markers linked to the main effect in each parent the combination of the presence of both markers decreased disease rating by 73 % (Table 28).

Table 28: Average effect for disease incidence for smut resistance for two markers inherited one from each parent

Marker		scPth-418060	
		0	1
336bsaS	0	43.3 (75)	18.7 (57)
	1	2.4 (66)	1.2 (73)

Note: No. of progeny in brackets

These markers could be used to introgress smut resistance into the sugarcane breeding germplasm. SRA project 2015/025 is converting these markers to SNP markers for use in the introgression program.

Discussion on the marker trait association

The marker trait analysis was conducted over a period of three years and sometime two trials per year were conducted. We only reported data that were revealed over that period of time for better robustness. We are facing a challenge as the number of testing we projected to do were in fact limited by the capacity of testing at SRA. More than one group are doing testing at the same time and the number of clones that we could test was limited. The *Erianthus* population is also very limited by the fact that the whole population is actually an association mapping population composed of small number of individuals from crossings of different parents. The number of individuals is too small for a high level of confidence in the results, but they indicate that the same effects can be detected in different

disease screenings. More progeny need to be screened to verify any results even though we are somehow confident where the p value is near 0.
The *S. spontaneum* introgression population had a better structure for the MTA analysis and we were able to detect two markers associated with Pachymetra resistance derived from the parent ROC25. When progeny contained both markers (CscPtb-426528 and smc1047-157) there was a decrease in disease rating of 27.8 %.
Another large effect QTL was also identified inherited from ROC25 on linkage group 10 that again increases smut resistance. When the progeny were investigated for the marker effects of the two markers linked to the main effect in each parent the combination of the presence of both markers decreased disease incidence by 73 % (Table 28).
These results are very promising and the markers could potentially be used in the breeding program. However, we are currently unable to associate the markers with the HG as we planned to do within the project. Currently we are not be able to use our primers to attempt screening for Pachymetra and Smut as the genetic maps for the *Erianthus* and the *S. spontaneum* BC1 are not dense enough and most LG are unlinked and do not belong to any HG.
Nevertheless, the markers have been converted to high throughput markers in project 2015/025 to screen some introgression clones within the introgression program at SRA.

3.4 Screening with the set of primer pairs

Erianthus hybrids

Molecular screening was done on 136 *Erianthus* BC3 clones and the nine clones corresponding to their pedigrees. Table 29 represents a subset of the results. The clones highlighted in red are *Saccharum* while in green are derived from the *Erianthus* ancestor (HN92-77) and specific markers are specific to *Erianthus* as all the *Saccharum* clones in red deliberately do not possess that marker.

Table 29: Screening of BC3 *Erianthus* hybrids with 10 primer pairs

Primer	Badila	HN92-77	YC96-40	CP84-1198	ROC20	YCE01-102	QN80-3425	QBYC06-30376	QBYC06-30296	KQ08-1009	KQ08-1010	KQ08-1011	KQ08-1012	KQ08-1013	KQ08-1014	KQ08-1015	KQ08-1111	KQ08-1112	KQ08-1186
Sb1a	0	1	1	0	0	1	0	1	1	0	1	1	0	1	0	1	1	0	1
Sb1a	0	1	1	0	0	1	0	0	0	0	1	1	0	0	1	0	0	0	1
Sb2a	0	1	1	0	0	1	0	1	1	1	1	1	0	1	0	1	1	0	1
Sb3a	0	1	1	0	0	1	0	1	0	1	1	1	1	0	0	1	1	1	1
Sb4a	0	1	1	0	0	1	0	1	1	0	0	1	1	1	0	0	1	0	1
Sb5a	0	1	1	0	0	1	0	1	1	0	1	1	0	1	1	1	0	1	1
Sb6a	0	1	1	0	0	1	0	1	1	0	1	0	1	0	0	0	1	0	0
Sb7a	0	1	1	0	0	1	0	1	1	1	1	0	0	0	0	0	1	0	0
Sb7b	0	1	1	0	0	1	0	1	1	1	1	0	0	0	0	0	0	0	0
Sb8a	0	1	1	0	0	1	0	1	1	0	1	1	0	0	0	0	1	1	0
Sb9a	0	1	1	0	0	1	0	1	1	1	0	0	0	1	0	1	1	1	1
Sb10a	0	1	1	0	0	1	0	1	0	0	0	1	0	1	0	1	0	1	1

Table 30 is a summary of the molecular screening combined with the GISH results for those clones that have been tested by GISH. Some of this data has already been confirmed by GBS and BAC-FISH, but it could be important to use GBS data as well as BAC-FISH to validate more of the *Erianthus* clones involved. We also have uncertainties about the results for one of the primer Sb10 (and to a lesser extend to the primer SB01) and again, it would be useful to design a new primer to replace the current one when

more sequence becomes available. It is noteworthy that this primer coincides also with the chromosome where the GBS data was the less informative (only one x).
Table 30: shows the correlation between the number of markers and the number of chromosomes and for most BC3 clones (18/31), there is a match. Interpretation becomes more difficult when there is recombination between the *Erianthus* and *Saccharum* chromosomes. However, for some clones KQ08-6004 and KQ08-6006 the primer pairs are capable in revealing the recombined chromosomes.

Table 30: Correlation between marker No/ Chromosome No

Primer	Badila	HN92-77	YC96-40	CP84-1198	ROC20	YCE01-102	QN80-3425	QBYC06-30376	KQ08-1111	KQ08-1186	KQ08-6011	KQ08-6012	KQ08-1029	KQ08-1134	KQ08-6007	KQ08-6008	QBYC06-415	KQ08-1347	KQ08-1348	KQ08-6013	KQ08-6014	KQ08-1040	KQ08-1046	KQ08-1049	KQ08-1158	KQ08-1061	KQ08-1078	KQ08-1079	KQ08-1238	KQ08-1239	KQ08-1294	KQ08-1339	KQ08-1359	KQ08-6001	KQ08-6002	KQ08-6003	KQ08-6004	KQ08-6005	KQ08-6006	KQ08-6009	KQ08-6010	Total of "1"
Sb1a	0	1	1	0	0	1	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	1	0	1	1	0	0	1	0	0	0	0	0	10
Sb1b	0	1	1	0	0	1	0	0	0	1	1	1	0	0	0	0	1	0	1	0	1	0	0	0	0	0	1	0	0	1	1	0	0	0	1	1	0	1	0	1	0	13
Sb2a	0	1	1	0	0	1	0	1	1	1	1	1	1	1	0	1	1	0	1	1	0	0	1	0	0	1	1	1	0	1	1	0	1	1	1	0	0	0	0	1	0	20
Sb3a	0	1	1	0	0	1	0	1	1	1	0	1	1	1	1	0	1	m	m	1	1	0	1	0	1	0	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	15
Sb4a	0	1	1	0	0	1	0	1	1	1	1	0	0	0	1	1	1	1	0	0	0	m	0	0	m	1	m	1	1	m	1	1	1	1	1	0	1	1	1	0	1	19
Sb5a	0	1	1	0	0	1	0	1	0	1	0	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	0	1	0	0	1	0	24
Sb6a	0	1	1	0	0	1	0	1	1	0	1	0	0	1	1	0	1	0	1	0	0	0	1	0	1	0	0	0	0	1	1	0	0	1	0	0	0	1	0	1	0	13
Sb7a	0	1	1	0	0	1	0	1	1	0	1	1	1	1	0	0	1	0	1	1	1	0	0	1	0	0	1	1	1	1	0	0	0	1	1	1	0	0	1	0	1	19
Sb7a*	0	1	1	0	0	1	0	1	0	0	1	1	1	1	0	0	1	0	1	1	1	0	0	1	0	0	1	1	1	1	0	0	0	1	1	1	1	0	0	0	1	18
Sb8a	0	1	1	0	0	1	0	1	1	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	1	1	1	0	0	1	0	0	1	1	1	0	1	1	1	0	16
Sb9a	0	1	1	0	0	1	0	1	1	1	1	1	0	0	1	0	1	0	1	1	0	0	1	0	1	1	1	0	1	1	1	1	0	0	0	0	0	1	0	0	0	17
Sb10a	0	1	1	0	0	1	0	1	0	1	0	0	0	1	0	0	1	0	0	1	1	1	1	0	0	0	0	1	1	0	0	0	1	0	0	1	0	1	0	1	1	14
marker total	0	10	10	0	0	10	0	10	8	7	6	6	4	5	5	3	10	3m	7m	6	6	2	6	2	5m	5	7	5	7	7	8	4	5	5	6	5	2	6	4	4	4	198
GISH No. Chrom	0	60	30	0	0	30	0	10	7	7	6	6	4	4+2*1/2	3	3	10	4	6	7	5	2	6	2	6	5	4	6	3	6	6	4	4	5	4	7	2+2*1/2	4	3+2*1/2	4	4	

What is also interesting from Table 30 is that there appears to be a prominent transmission of chromosome 5 with a high number of 24 markers transmitted for this subset of clones. This higher proportion of chromosome 5 is also observed in the whole dataset (data not shown).

Association between primers and disease rating was conducted by a regression analysis of relative trial score of disease on primer score. The relative trial score was calculated at each disease screening trial by the average of disease score of a test clone over the average trial score of all standards in the trial. The impact of population structure on this association study was addressed to some extent by the inclusion of clone type in the analysis for the *Erianthus* population. Because of the small number of primers and even smaller number of significant ones, we did not use a prediction model as the result would not be valid. We also tried to cluster clones by primers to see whether the clones could be grouped by families for example; however, no apparent clusters could be identified, and again it might due to the low number of primers and also close relatedness of most of the clones. On relative trial score, the chromosomes and the p value for the association is as follows:

Pachymetra: Sb5a0 (0.01802), Sb3a (0.02785), Sb6a (0.05398)
RKN: Sb8a (0.04751)
RLN: Sb7a (0.03721), Sb1a (0.005808)
Smut: S8a (0.0005671), Sb1a (0.04807)

The most reliable association was detected for Smut and chromosome 8 and all of the other associations were not significant. It is clear that we underestimated the number of clones necessary for the study to be more robust. The *Erianthus* BC3 population is actually an association mapping population with clones from multiple crosses and this is one of the major factors undermining the relationship between the disease rating and the markers.

For Smut, the distribution of ratings is presented in the Figure 5 and shows the unevenness of the distribution with 50 clones resistant to Smut.

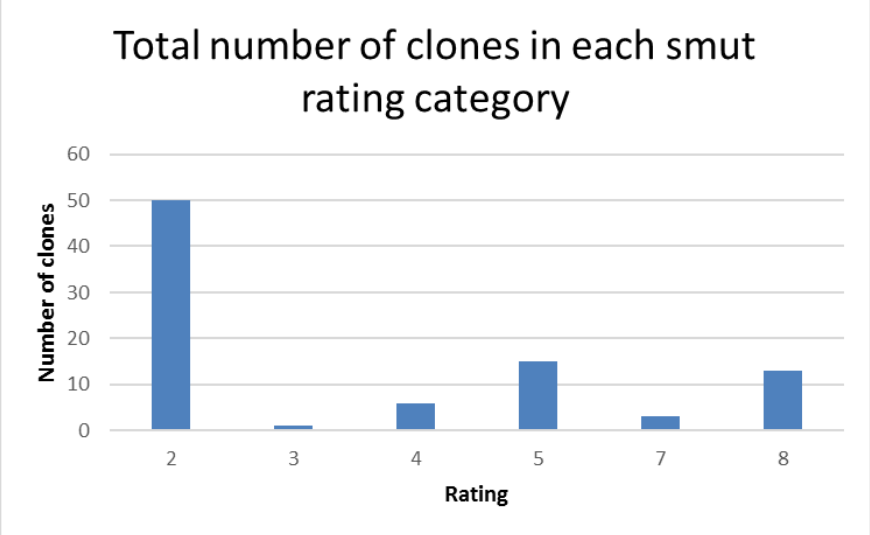

Figure 5: Smut ratings

The results in Table 31 report on the association between chromosome 8 (revealed with the regression analysis) and smut rating. For 58 out of 94 clones that did not have chromosome 8 present, the average smut rating is 1 rating unit less than for the 36 clones that did have chromosome 8.

Table 31: Association between chromosome 8 and smut ratings

Absence or presence of Sb08G019930	Average Smut rating	Number of clones
0	3.31	58
1	4.36	36
Grand Total	3.71	94

Similar analyses were done for Pachymetra, RKN and RLN. Results for Pachymetra are presented below in Figure 2 and Table 32. Results for RKN and RLN were inconclusive (data not shown).
Figure 2 shows the number of BC3 *Erianthus* clones in each of the Pachymetra rating categories and Table 32 shows the associations between Pachymetra rating and combinations of presence and absence of three chromosomes 3, 5 and 6.

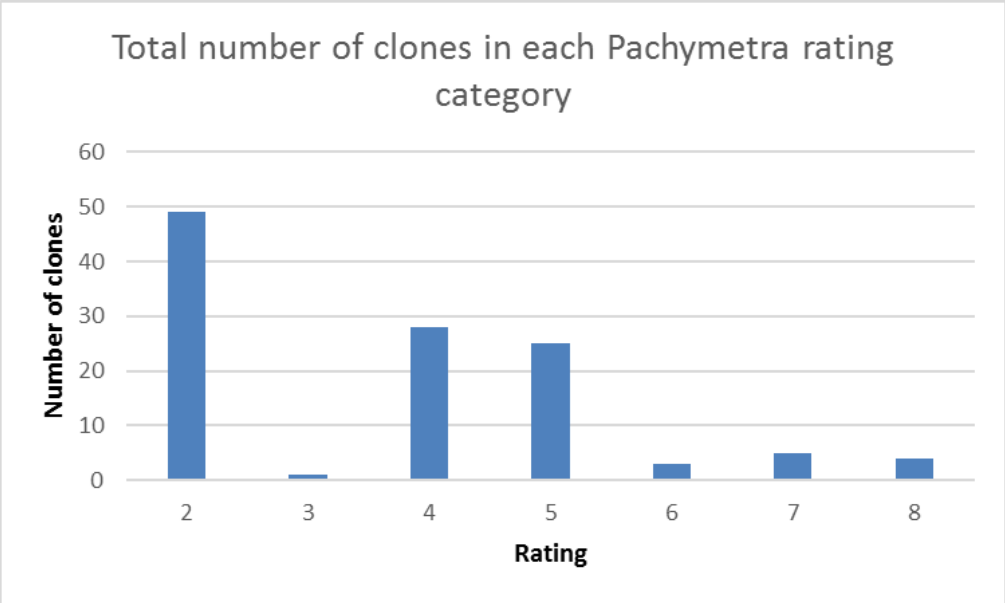

Figure 6: Pachymetra ratings

Table 32: Associations between chromosomes 3, 5, 6 and Pachymetra ratings

Sb3a	Sb5a	Sb6a	Average of Pachymetra ratings	Number of clones
0	**0**	**0**	**3.38**	**13**
0	**0**	**1**	**3.28**	**14**
	0 Total		**3.33**	**27**
0	**1**	**0**	**4.52**	**21**
0	**0**	**1**	**4.60**	**10**
	1 Total		**4.54**	**31**
0 Total			**3.98**	**58**

Sb3a	Sb5a	Sb6a	Average of Pachymetra ratings	Number of clones
1	0	0	3.71	7
1	0	1	2.66	12
	0 Total		3.05	19
1	1	0	3.52	19
1	1	1	3.55	18
	1 Total		3.54	37
Grand Total			3.68	114

Table 32 shows that the absence or the presence of the three chromosomes together give respectively an average rating of 3.385 for 13 counts and an average ratings of 3.556 for 18 counts (in grey). The worst combination of chromosomes for the incidence of the disease is when chromosome 5 is present and 3 and 6 absents, or when chromosome 5 and 6 are both present and 3 absent (respectively 4.524 and 4.6, highlighted in red). On another hand, the best combination of chromosomes is highlighted in blue and the rating for Pachymetra have an average of 2.66. The incidence of the disease decrease by approximatively 1 unit by the absent of chromosome 5 and presence of chromosomes 3 and 6 compared to the total absence or presence of the 3 chromosomes, but decrease by 2 units in the worst case scenario. This results are looking promising but again caution need to be apply here as we only screened a small number of *Erianthus* clones through the three years and again the clones are part small populations from several crosses.

S. spontaneum hybrids

The SSR primer set for *S. spontaneum* is complete and the primers are listed in Table 9. All primers have now been screened in 70 BC2 and BC1 *spontaneum* clones (data not shown). The specific primers have also been screened on another set of approximatively 20 clones (excluding the introgression population) to validate or not that these primers are specific to *spontaneum*. We wanted to test the SSR primers on a complete different source of *S. spontaneum* clones so we used clones such as DACCA, ECL-1-18-85, IK76-3, Tongza to cite only a few. We were able to score specific *S. spontaneum* markers when we submitted these clones to the chosen specific SSR primers. We can report that the screening with the SSR primers has been successful for other clones from another *S. spontaneum* background.

As we mentioned before no BAC-FISH work to date has shown the number of alleles per clone specific to *spontaneum* as this will require a tri-fluorescence system that currently is not working in sugarcane.

We are nevertheless anticipating that, due to the complexity of the *Saccharum* genome and in particular the complex composition of introgression clones, the primers selected and marker associated will be difficult to trace the specificity of *spontaneum* chromosomes. This issue rests in the multiple alleles present in the introgression clones. The chromosome mixing that occurs at each generation of crossing produces clones with thousands of potential allelic combinations making it almost impossible to trace a particular chromosome. The SSR markers that we developed are probably revealing a particular haplotype of the clone but are not revealing all the haplotypes for the same HG and certainly not across different clones where the segregation is different. On the hand, it is possible to trace a trait associated to a particular SNP for example.

We are now considering that a better method to reveal specific *spontaneum* chromosomes could be by analyzing the introgression *spontaneum* with GBS. When QTL

or regions of interest are linked to the genetic map it should be possible to then sequence that region and subsequently develop a specific marker associated with the trait, as we know that in some cases major QTL for disease resistance is inherited from the wild species *S. spontaneum*.

Nevertheless, the association between primers and disease was conducted by a regression analysis of relative trial score of disease on primer score. The relative trial score was calculated at each disease screening trial by the average of disease score of a test clone over the average trial score of all standards in the trial. Because of the small number of primers and even smaller number of significant ones, we did not use a prediction model as the result would have no real value. On relative trial score, the chromosome and the p value for the association is as follows:

Smut: SSR-Sb3 (0.02776294)
Pachymetra: SSR-Sb3a (0.02889833); SSR-Sb9a (0.05591893)
RKN: SSR-Sb8a (0.045020152); SSR-Sb8b (0.006427256); SSR-Sb2 (0.068212684); SSR-Sb9b (0.025341412)

Again we used the prediction to look back at the ratings and investigate any significant correlation.

For Smut, the distribution of ratings is presented in the Figure 7 and shows the disproportion of the distribution with approximately 60 % of the clones resistant to Smut. Similar results were found for the *Erianthus* analysis.

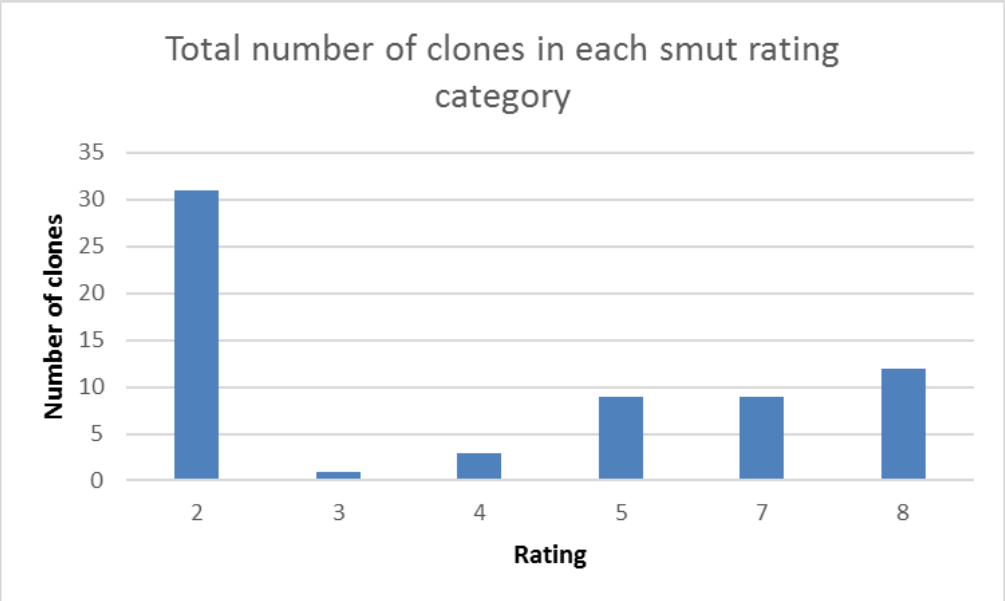

Figure 7: Smut Ratings

Nevertheless the presence of marker SSR-Sb3 seems to increase the incidence of Smut in 27 clones by an increase of 1.2 unit.
Without the SSR, 38 clones have an average incidence of 3.8. The number of clones used in the study seems to be a limiting factor to obtain better and more robust results (limitation due to SRA capacity in disease testing).

Table 33: Association between SSR_Sb3 and smut ratings

Absence or presence of sscir35b	Average of Smut ratings	Number of clones
0	3.81	38
1	5.03	27
Grand Total	4.32	65

For the Pachymetra, two SSR primers were revealed with the regression analysis. Figure 8 shows the ratings for the disease amongst the clones tested.

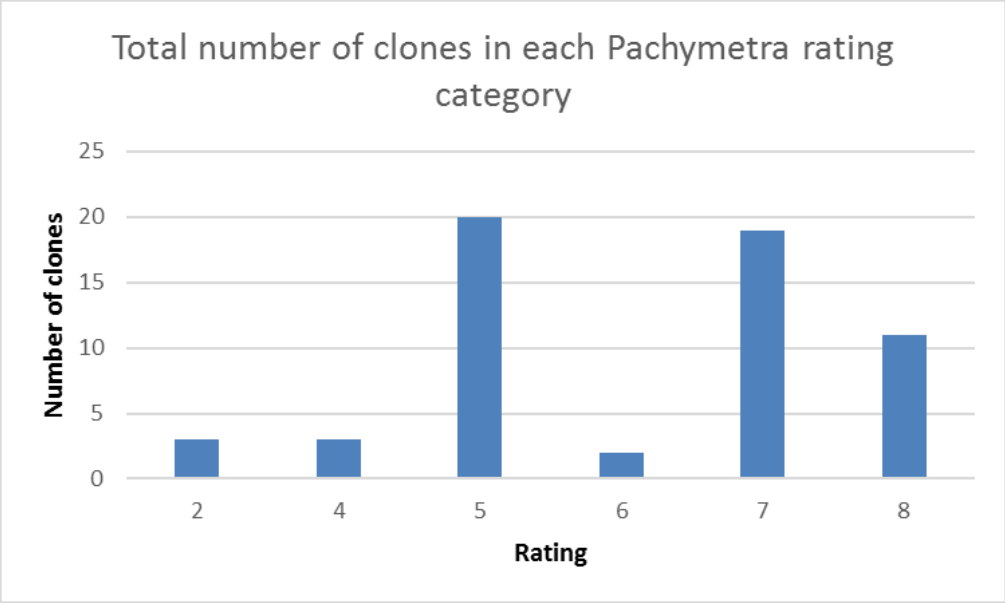

Figure 8: Pachymetra ratings

Table 34: Association between SSR –Sb3a/ SSR-Sb9c and Pachymetra ratings

SSR-Sb3a	SSR-Sb9c	Average of Pachymetra ratings	Number of clones
0	0	6	5
0	1	5.69	26
	-	7	1
0 Total		5.78	32
1	0	6.6	15
1	1	6.09	11
1 Total		6.38	26
Grand Total		6.05	58

Table 34 confirms the probability associated with two SSR primers and the Pachymetra ratings. The p value for the primers was definitely very low with highest probability around 0.2 for SSR-Sb3.

Therefore the association table and showed no real significant difference with the presence or absence of the two primers.

For RKN, Figure 9 shows that most of the clones were rated susceptible or intermediate. Only one clone QBYN04-26030 is resistant to RKN. Consequently we cannot really test the association between the four SSR markers from the regression analysis as there is no segregation for the disease. One clone is not enough to make any conclusion on the combination of chromosome toward the incidence of RKN for the introgression *S. spontaneum* clones.

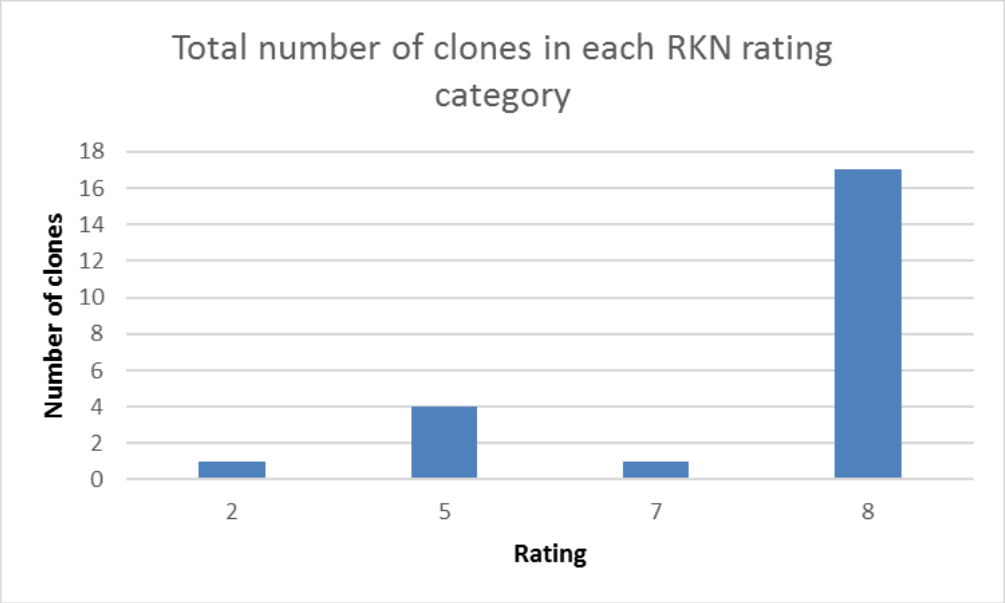

Figure 9: Pachymetra ratings

Discussion on the screening with specific primers

Screening with the specific *Erianthus* primers have two mains functions. Firstly we can predicted the number of chromosome from each BC3 *Erianthus* clones. Secondly the primers can reveal the HG for each chromosome of the BC3. Knowledge of the HG for the chromosome is important when we have information of region of interest for disease resistance. The marker trait analysis have reveal some region of interest for Pachymetra, Smut and Nematode but no QTLs large enough was identified with an *Erianthus* origin. We were hoping that the screening results will be sufficient to detect potential association between the markers and the disease ratings but so far it is inconclusive. We realise that the number of clones tested, 136 out of 400 of the *Erianthus* clones, is too low and if we can continue the investigation for this project we might be able to screened the whole population with our primers and reveal genuine associations. This was the same situation for the *S. spontaneum* primers and for none of the disease we were able to reveal any genuine association as the number of clone tested was too low. Nevertheless we did reveal through the marker traits association at least four regions of interests/ markers linked to Pachymetra and Smut disease resistance.
These regions were not linked to any of the HG but they are being transform in project 2015-025 in high-throughput markers to be implemented in the breeding program.

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Section 4: Outputs and Outcomes

The project has delivered cytogenetics data for approximatively 50 *Erianthus* hybrids as well as 11 BC1 and BC2 clones from the *S. spontaneum* introgression population. These data will be stored in the new version of SPIDNET and have already been used by breeders to assist in decision-making for crossing with introgression material. This project has also delivered ratings for root knot and lesion nematodes, Pachymetra root rot and Smut resistance for many introgression clones over a period of three years. This data is stored in the SRA plant breeding database SPIDNet.

Two sets of primers have been produced to screen *Erianthus* and *S. spontaneum* introgression clones. The primer information will also be stored in SRA database and will be accessible for breeders and researchers.

Major regions of interest have been identified for Pachymetra and Smut in this project. The markers are currently being transformed in high-throughput markers in project 2015-025 for potential use in the SRA introgression program.

One introgression clone derived from *Erianthus arundinaceus,* KQ08-1006, has good potential to be used as a parent in further crossing. This clone has resistance to Pachymetra and has five *Erianthus* chromosomes. Another clone from the introgression *S. spontaneum* (QBYN04-26030) has shown great potential but this time for its resistance to root knot nematode. The introgression plant breeders have been using some of the introgression clones for some time now in the core breeding program.

This project has collaborated closely with projects 2011344, 2014053 and 2015025 as well as the core-breeding program.

Section 5: Intellectual Property (IP) and Confidentiality

The *Erianthus* and *S. spontaneum* specific primers sequence information has not been disclosed. This information should be considered confidential until publication.

The regions of interest identified in this project will also remain confidential until further work and verification. These markers have great potential for the Australian sugar industry and should be protected until we have full awareness of their value.

Section 6: Industry Communication and Adoption of Outputs

The main message from this project is that the introgression of wild species in our Australian germplasm could unravel new sources of disease resistance for Pachymetra, Smut, and Nematodes. Data and information generated in this project have been used by other projects and this uptake and use of data has the potential to produce new varieties with enhanced traits and/or different genes through new sources of germplasm. The project will be featured in the next cane connection. A paper will probably be presented next year at the ASSCT conference.

Section 7: Environmental Impact

This project had no immediate impact on the environment. However, if new varieties are produced in the future with new sources of resistance to soil-borne pathogens there is potential for a positive environmental impact through an improvement in soil health from an increase in the number of ratoons.

Section 8: Recommendations and Future Industry Needs

1- Molecular breeding is a relatively new field for the sugarcane community and it has proven very effective for many other crops already such as rice, barley, and wheat. This project was one of the first in sugarcane to attempt a simple method to select clones with molecular markers. We did not completely achieve the main objective for this project but we were able to produce good data and deliver it over to the breeders. We also produced data that are currently being utilized in other projects. It is recommended that the markers from the primers should be transformed into an appropriate SNP platform to combine the ten primers.

2- Introgression breeding is an important focus for the Australian industry and many growers have shown increasing interest in this area. It is important for SRA to continue to fund projects in this area to ensure that benefits to the industry can be realized.

3- The clones KQ08-1006 and QBYN04-26030 showed good potential for resistance to Pachymetra and nematodes, respectively. We hope to send these clones to Meringa for crossing. They could perform really well as parents.

Section 9: Publications

No publications have been published yet.