Title	Characterisation and maintenance of the Australian sugarcane mapping populations.
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EXECUTIVE SUMMARY

There were two major aims to this project. The first was to identify markers linked to major diseases of sugarcane that were difficult and expensive to select for. The second objective was to determine the cross-transferability of markers by testing the association between markers and traits in other germplasm. Both of these aims have been successfully addressed.

Fiji Disease virus (FDV) and Pachymetra are two major diseases of sugarcane. Both require laborious and expensive screening in the field or glasshouse and consequently, only limited numbers of clones can be screened. Thus, both of these traits are candidates for molecular markers. Successful identification of molecular markers associated with these diseases could enable indirect selection for resistance to be undertaken, in the absence of screening, or with reduced screening, for the diseases themselves.

Two populations, Q117 x 74C42 and Q96 x Q178, were developed by Dr Nils Berding (BSS038) and each contained more than 200 progeny. Both populations had been previously scored for numerous sugar-related and agronomic traits and marker-trait associations (QTLs) identified for all traits in the Q117 x 74C42 population. One objective in this project was to see if QTLs identified in Q117 x 74C42 would detect the same traits in the Q96 x Q178 population. Unfortunately, in the process of mapping in this second population, it was discovered that the population was a mixture of several very small, different populations. This project objective could not be completed as planned, but was modified, as discussed below.

In addition to the approximately 300 markers already scored on the Q117 x 74C42 population (CTA024), a further 1100 amplified fragment length polymorphism markers (AFLPs), simple sequence repeat (SSR) markers and resistance gene analogue (RGAs) were scored. The first two types were used as they are PCR-based, reliable, easy to generate, and the type of markers currently being used in other sugarcane maps. RGAs are potential candidate genes for disease resistance. Unfortunately, during the amalgamation of the marker information, it became apparent that the replanted progeny clones had become “renumbered” in the field. It was thus not possible to combine the two data sets, and consequently, new maps were developed for the Q117 x 74C42 population using just the AFLP, RGA and SSR data. The 1100 markers were used to develop two parental maps. The Q117 map contains 407 markers in 75 linkage groups, while the 74C42 map contains 447 markers in 84 linkage groups.

The parents of the Q117 x 74C42 population are rated a 4.5 and a 5 for Pachymetra, and a 5.3 and a 1.5 for FDV, respectively. Two hundred progeny were scored for Pachymetra. Four genomic regions were identified as significantly associated with Pachymetra, with two derived from each parent. It was not possible to determine whether these regions were the same regions in both parents. Despite 3 years of attempts, it has not been possible to obtain FDV scores for the population. However, the population was affected by brown rust and brown rust reaction scored. Four different genomic regions were identified as associated with brown rust.

To confirm and determine the transferability of these markers, and measure the frequency of these genomic regions in relevant sugarcane germplasm, the markers were screened over 154 sugarcane varieties and elite clones, the Elite Clone Set

(ECS). Within the ECS, 44 clones were direct descendants of Trojan with 33 of the 44 descended from crosses between Trojan and Co475. The 44 clones were called the Trojan Validation Set (TVS). To validate the regions associated with Pachymetra and brown rust, identified in the Q117 x 74C42 populations, selected markers were screened across the ECS.

Only 3 of the 4 regions associated with Pachymetra could be validated. Of these only one region was still significantly associated with Pachymetra in both the TVS and ECS and it is associated with susceptibility. A second genomic region and an additional minor genomic region were significantly associated with susceptibility to Pachymetra in the TVS. As Trojan is susceptible to Pachymetra (rating of 8), the TVS is most appropriate for markers associated with susceptibility. Thus, 3 genomic regions have been validated for susceptibility to Pachymetra. One minor region is still significantly associated with resistance, but cannot be validated in the TVS. Each of the 4 regions explained between 5 and 9% of the variation in Pachymetra reaction in the Q117 x 74C42 population.

Of the four regions associated with brown rust, none of the regions remained significantly associated with brown rust in the TVS. One minor genomic region was still associated with brown rust resistance in this set. One other minor genomic region was significantly associated with resistance in the ECS. These two regions explained between 8 and 16% of the variation in brown rust resistance in the Q117 x 74C42 population.

The ECS was screened with a total of 1780 markers (1599 AFLPs and 181 SSR markers). New marker trait associations were sought for Pachymetra, Brown Rust and FDV. For Pachymetra, 14 new markers were identified. Some of these markers were present in Trojan and remained significantly associated with Pachymetra in the TVS. Two new markers were identified as associated with Brown Rust. One of the markers was present in Co475 (rated a 9) and was highly significantly associated with rust susceptibility in the TVS. Of special interest was the 4 markers identified as associated with FDV. Two were associated with resistance and 2 with susceptibility. One of the four markers was present in Co475 (rated a 9) and it was highly significantly associated with susceptibility to FDV in the TVS. Another marker, present in both Trojan (rated a 1) and Co475 (rated a 9) was weakly associated with FDV in the TVS.

These studies have demonstrated the feasibility of using association and pedigree-based approaches to both validate and identify markers associated with important traits. Using this combined approach, we have confirmed the identification of several regions associated with Pachymetra, Brown Rust and FDV which could be used to select for increased resistance to these diseases. Future research will focus on identifying new markers, assessing the impact these markers could have on the breeding program, and, as most of these markers are AFLPs, converting the markers to other simpler PCR-based markers to facilitate their use in marker-assisted selection in sugarcane.

BACKGROUND

The complexity of the sugarcane genome necessitates a very large number of markers to obtain reasonable genome coverage. It is an aneuploid, polyploid, interspecific hybrid. Most sugarcane varieties have between 100-130 chromosomes. Consequently there are large (approx. 9-13) and presumably varying numbers of homologous and homoeologous chromosomes. Given the size of our mapping populations (usually <300), only single dose markers segregating 1:1 in the population (or 3:1 if in both parents) can be mapped. Thus, in order to map within a homology group, multiple and linked single-dose markers need to be identified for each member of that chromosome homology group. In practice, it would appear that each map needs to be in excess of 500 markers to obtain the number of linkage groups equivalent to the estimated number of chromosomes. However, even with 1000 markers, the estimated genome coverage is only ~50% (Hoarau et al, 2002, TAG) indicating that while each chromosome has been identified, only a small part of each chromosome has been tagged. Consequently, QTL analyses are undertaken with only a portion of the genome and only a limited proportion of the genes.

Molecular markers have the potential to greatly assist plant breeders through marker-assisted selection, fingerprinting of varieties, characterisation of germplasm etc. Many traits of interest to sugarcane breeders are difficult to select for, such as resistance to Pachymetra and Fiji Disease virus. Both require laborious and expensive screening in the field or glasshouse and consequently, only limited numbers of clones can be screened. Thus, both of these traits are candidates for selection with the aid of molecular markers. Successful identification of molecular markers associated with these diseases could enable indirect selection for resistance to be undertaken, in the absence of screening, or with reduced screening, for the diseases themselves. For markers to be successfully implemented in breeding programs, the marker-trait association has to be real and the marker has to be effective in relevant germplasm. Once the robustness of the association has been confirmed, the marker may require modifying to enable large numbers of assays to be undertaken. Finally, a cost and benefit analysis needs to be done. Does the cost of screening with the marker provide sufficient benefit to the program to warrant its use? This project also aimed to address the first two issues.

As part of CTA047, we have collected DNA from 154 Australian sugarcane varieties and elite clones, termed the Elite Clone Set (ECS). This set of germplasm was selected to represent the genetic diversity present within Australian sugarcane. Within the 154 clones, 44 are direct descendents of Trojan (parent or grandparent), with 33 of the 44 clones derived from a cross between Trojan and Co475. The 44 clones have been termed the Trojan Validation Set (TVS). As Q117 itself is a grandprogeny from Trojan x Co475, the TVS can be used to validate markers that derive from Q117 in the Q117 x 74C42 population. In addition, both the ECS and TVS can be used to identify new marker-trait associations. This latter approach, termed association or pedigree mapping, is currently successfully carried out in markers studies in animals and humans, where it is not possible to generate large numbers of progeny from single sets of parents. The advantages of association /pedigree mapping are many. It uses trait information generated routinely as part of the breeding program, it doesn't require extensive trait analysis of large and specific populations, it uses current genotypes, it provides information on the frequency of specific markers within the germplasm and it isn't limited to the genetic variation within a specific cross.

OBJECTIVES

The project aimed to:

1. Maintain the two sugarcane populations at both Meringa and Bundaberg to safeguard the survival of this valuable resource.

Achieved:
The populations were initially grown at Meringa. Both populations were also transferred to Bundaberg and have since been maintained at both sites. However, as discussed below, serious problems have arisen with both populations. The Q117 x 74C42 population was not maintained in integrity – it appears that during replanting, clonal numbers were mixed. For the Q96 x Q178 population, molecular marker screening of these lines suggested that the "population" was actually a mixture of 3 separate populations.

2. Identify markers linked to important traits such as resistance to Fiji disease and Pachymetra.

Achieved:
Markers have been identified as linked to these two diseases using both map-based and association-based approaches. The latter approach was developed within this project as it has not been possible to obtain FDV ratings in the field. The markers identified are associated with both resistance and susceptibility to Pachymetra and FDV. In addition, markers have been identified as associated with Brown Rust.

3. Determine the transportability of these markers across sugarcane genotypes and measure the predictive value of the markers as tags for Fiji disease and Pachymetra resistance in other crosses.

Achieved:
The markers identified using map-based and association-based approaches have been screened across alternative germplasm to determine the transportability of the markers and to validate the association between the marker and the trait. The germplasm selected was not suitable for validating all markers identified. Nevertheless, markers associated with all three traits have been identified and validated in alternative germplasm, confirming the robustness of the association.

4. Test the cross transferability of markers identified in CTA024 linked to major QTLs for CCS, fibre, Brix, Pol, moisture, stalk number and stem wax in a second sugarcane cross.

Not Achieved:
The second sugarcane cross, Q96 x Q178, was found to be a mixture of 3 separate crosses. There was insufficient progeny in any one of the crosses to enable reliable information to be obtained. Further work with this second population was not undertaken.

5. Confirm and quantify more accurately the value of the markers identified in CTA024 by evaluating the linkage in the entire Q117 x 74C42 progeny set of approximately 300 individuals.

Not achieved:
Marker screening of the entire progeny set suggested that the population had been incorrectly replanted such that progeny clone numbers of the original population did not match progeny clone numbers of the newly planted material. Thus, markers were not able to be screened over the additional progeny as they couldn't be correctly identified.

6. Develop an improved sugarcane map by inclusion of more markers, especially microsatellite markers which have been mapped in other sugarcane maps, to enable better comparison of map location of markers and QTLs, and to enable better isolation of useful information from other maps.

Achieved:
In addition to the approximately 300 markers already available for the Q117 x 74C42 cross (CTA024), a further 1100 markers were screened over the 192 progeny. However, due to progeny number errors, the two data sets could not be combined and the new maps were developed with the 1100 markers. These 1100 markers consist of AFLP, RGAs (RFLPs) and SSR markers. The Q117 map is comprised of 407 markers in 75 linkage groups, while the 74C42 map contains 447 markers in 84 linkage groups.

METHODOLOGY

Phenotyping of Q117 x 74C42 population and the Elite Clone Set

Pachymetra and Brown Rust ratings were obtained for 192 progeny of the Q117 x 74C42 population. For Pachymetra, 100 progeny were assessed each time by Dr Rob Magarey at Tully, using standard BSES practices. Figure 1 illustrates the distribution of Pachymetra ratings for one set of 100 progeny. For Brown Rust, 100 progeny each year for 2 years, and a second 100 progeny for 1 year, were planted at Woodford as part of a FDV trial. There was insufficient FDV disease, but good opportunistic infections of Brown Rust. Brown Rust was scored using standard BSES practice. Figure 2 illustrates the distribution of Brown Rust ratings for one set of 100 progeny of the Q117 x 74C42 population and, for comparison, Brown Rust ratings for progeny from the French population, R570.

Pachymetra, Brown Rust and FDV ratings for clones in the ECS were obtained from the BSES Ltd. Database.

Genotyping of Q117 x 74C42 population and the Elite Clone Set

One hundred and ninety-two progeny of the Q117 x 74C42 population was genotyped using AFLP, SSR and RGA markers (Table 1). For AFLPs, 30 primer pairs were used to generate more than 850 markers, using the method described in Aitken et al (2004). For SSRs, 40 primer pairs were used to generate approximately 180 markers markers, using the method described in Aitken et al (2004). For RGAs, 57 RGAs were isolated (CL McIntyre et al, pers. comm.). The RGAs were screened for polymorphism, using the techniques described in the CTA024 Final Report. Thirty-one polymorphic RGAs were identified and screened across the192 progeny, generating 50 simplex markers. In addition, 8 sugarcane RFLP probes were used to generate a further 20 simplex markers.

The 154 clones in the ECS were screened with 19 AFLP primer pairs and 14 SSR primers, of which 12 and 10, respectively, were used in map construction, using the same techniques as above.

The polymorphic AFLP markers were identified by 6 letters taken from the *Eco*RI and the *Mse*I primers followed by a number or a capital letter to identify the marker. The SSR markers were identified by ms + the microsatellite number + a band number. Only clear segregating bands were scored as present or absent.

Linkage and QTL analysis

Within the Q117 x 74C42 population, all scored markers were tested for 1:1 segregation (or 3:1 for simplex markers present in both parents) using the Chi-squared test. Simplex markers were incorporated into linkage groups using MapManager QTXb16 (Meer et al. 2002). QTL analysis was also undertaken using MapManager QTXb16.

For the ECS, all scored markers were incorporated into “linkage groups” prior to undertaking single-factor analysis (regression analysis) using MapManager QTXb17.

Figure 1 – Pachymetra resistance ratings for 100 progeny of the Q1 (Q117 x 74C42) population in 2000

Resistance Rating (1 = Resistance; 9 = Susceptibility)

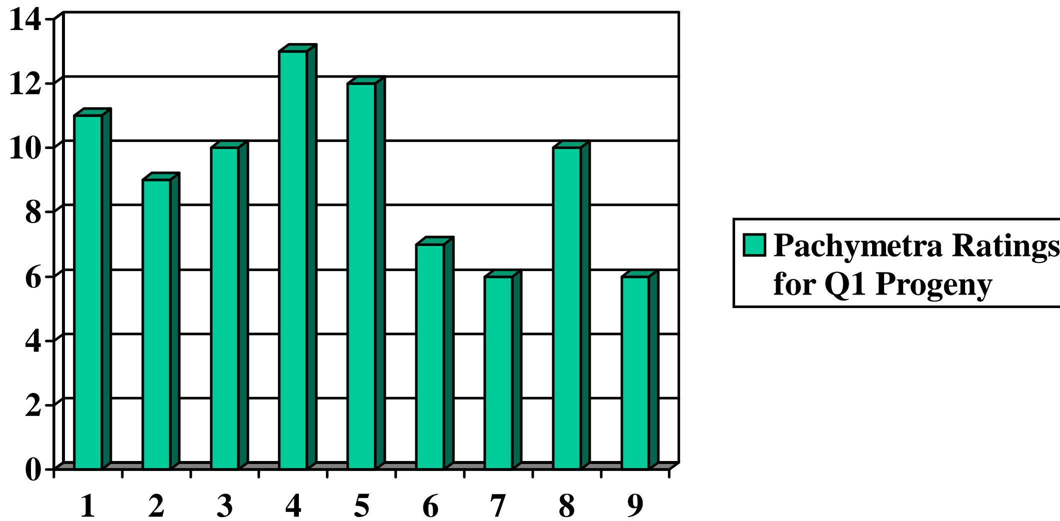
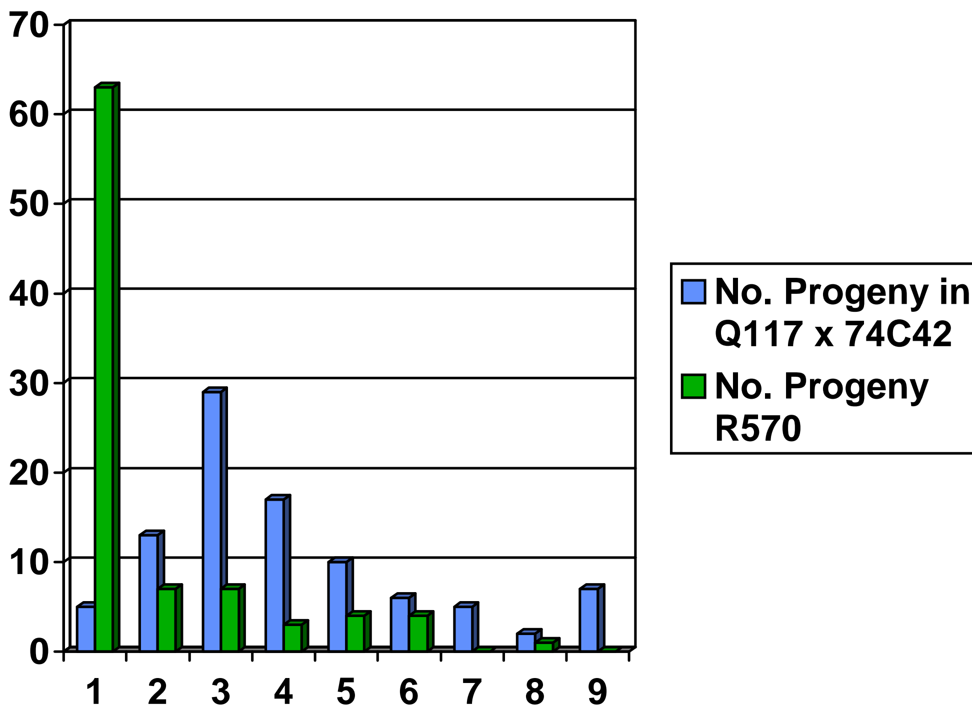


Figure 2. Rust resistance ratings for 100 progeny from Australian (Q1 – Q117 x 74C42) and French (R570) sugarcane populations.

Resistance Rating (1 = Resistant; 9 = Susceptible)



OUTPUTS

1. Development of sugarcane maps within a biparental cross

Almost 1100 single-dose markers have now been scored in the Q1 population. This number of markers is the equivalent of the best Australian wheat map and the best international sugarcane map of R570 (Hoarau et al, 2001) However, our population is biparental as compared to the R570 population which is a selfed population. Consequently, we have had to construct both male and female maps.

The female (Q117) and male (74C42) maps have 407 and 445 single dose markers, respectively (Table 1). There are also 245 single -dose markers which were present in both parents and hence segregate in a 3:1 ratio; these markers have also been included in our maps. Note also that many more markers were scored in our population, but due to the complexity of the sugarcane genome and the size of the Q1 population, only single-dose markers segregating 1:1 (single dose in one parent only) or 3:1 (single-dose in both parents) can be utilized in map construction, unlike most other crops which are diploid where all segregating markers can be used.

Table 1 summarises the map statistics for each map in our population, including the number of markers generated by each marker type (from the number of primer pairs or probes). It also lists the number of SSR primers and RGA and RFLP probes in common between the male (74C42) and female (Q117) maps that can be used as reference points for map alignment. Using the software package MapManager, these markers have been incorporated into linkage groups.

Table 2 summarises the status of the Q117 and 74C42 maps. Our female map (Q117) now has 75 linkage groups, of which 53 contain more than 2 markers. There are 46 unlinked markers. Our male map (74C42) has 84 linkage groups, with 55 containing more than 2 markers and there are 58 unlinked markers. Although most linkage groups contain only 2 markers there are now a reasonable number of linkage groups with more than 2 markers.

Table 1 – Number of markers scored in Male (74C42) and Female (Q117) maps

	SSRs (# primers)	AFLPs	RGAs (# probes)	RFLPs (# probes)	TOTAL
74C42	67 (36)	350	21 (14)	7 (4)	445
Q117	66 (35)	313	22 (16)	4 (3)	407
3:1 Markers	45 (28)	191	7 (5)	2 (2)	245
Common Primers/Probes	(30)		(8)	(1)	

Table 2 – Current status of Q117 and 74C42 maps.

No. of markers per Linkage Group	Q117 (Female Map)	74C42 (Male Map)
Unlinked	46	59
2	22	29
3	19	16
4	8	8
5	6	5
6	5	9
7	3	6
8	1	3
9	2	0
10	2	1
11	1	2
12	1	2
13	3	1
14	1	0
18	0	2
25	1	0

2. Identification and validation of markers linked to FDV, Pachymetra and Brown Rust using map-based QTL and association-based mapping approaches

a. Identification of markers using map-based approaches

Pachymetra resistance

Using all of the simplex markers, 4 different genomic regions were identified as associated with Pachymetra in the Q117 x 74C42 population. These regions were consistently identified over two years at $P<0.01$. Table 3 lists the markers identified as associated with Pachymetra resistance over 2 years. Of the 14 markers identified, 13 were AFLP markers and one was an SSR.

FDV

One hundred progeny were planted at the BSES Ltd Pathology Farm each year for 3 years. However, there has been insufficient FDV disease each 3 years. Alternative methods have been attempted to find markers associated with FDV (see below).

Brown rust resistance

Again, using all simplex markers scored in the Q117 x 74C42 population, four genomic regions were identified as associated with brown rust. These regions were significantly associated with common rust resistance over 2 years data at $P<0.01$. Five other markers, including one unlinked marker, were also significantly associated with brown rust resistance over both years. Table 4 lists the markers identified as associated with brown rust resistance. Of the 22 markers identified, 16 were AFLPs, 3 were SSRs (M851, M39 and M1237) and 3 were resistance gene analogues (RGAs) (22.1.5, T2-2, and 40.1.16).

Table 3. Markers associated with PACHYMETRA resistance, including their Linkage Group, Map Origin (male, female or 3:1), and % variation explained of each marker.

Marker	Linkage Group and Map	P (2001)	% Variation (2001)	P (2002)	% Variation (2002)
Aacctg.29	2 F	0.00955	8	0.02228	4
Acgcta.10	2 F	0.00829	8	0.02255	5
Acccta.11	2 F	0.00764	8	0.04343	4
Acccta.21	2 F	0.00471	9	0.3597	4
Acacta.9	22 F	0.00453	10	0.00959	6
M44.5	22 F	0.00018	16	0.00169	9
Acgcag.3	22 F	0.00049	14	0.00169	9
Acccag.11	22 F	0.0063	9	0.00269	9
Acccag.4	22 F	0.00146	13	0.00296	9
Acgctg.2	5 M	0.00552	9	0.02582	5
Acactt.10	5 M	0.00217	11	0.02078	5
Aggcac.3	5 M	0.00794	8	0.01089	6
Aggcac.8	42 M	0.03514	5	0.02689	5
Xacgctg.5	15 3:1	0.00695	9	0.0046	8

Table 4. Markers associated with BROWN RUST resistance, including their Linkage Group, Map Origin (male, female or 3:1), and % variation explained of each marker.

Marker	Linkage Group and Map	P (2001)	% Variation (2001)	P (2002)	% Variation (2002)
Acactg.16	U	0.0107	7	0.00447	8
Actcat.14	13F	0.0106	7	0.02780	5
Accctc.18	13F	0.01237	7	0.03639	5
40.1.16	13F	0.01204	7	0.02133	6
Acccta.15	18F	0.03003	5	0.02288	6
Aacctc.12	18F	0.04989	4	0.00447	8
M851.3	34F	0.00563	33	0.03733	18
Aggctc.20	60F	0.00999	7	0.00545	8
Accctc.20	5 M	0.00954	7	0.03178	5
22.1.5a	10M	0.01537	7	0.03682	5
T2-2	10M	0.01233	7	0.01540	7
Aacctg.19	10M	0.01515	6	0.01673	6
Acactg.22	10M	0.01179	6	0.02326	6
Agccag.4	10M	0.01179	7	0.02326	6
Aggcac.18	10M	0.01653	6	0.03180	5
Aagctc.6	10M	0.00599	8	0.01289	7
Acacta.29	10M	0.00943	7	0.02687	5
Aagcac.20	23M	0.0128	11	0.00413	9
Actcac.15	23M	0.00740	8	0.03178	5
M39.5	30M	0.00073	16	0.00020	18
M1237.4	43M	0.02221	6	0.02136	6
Agcctc.12	43M	0.01179	7	0.01985	6

b) Validation of markers identified as associated with Pachymetra resistance and Brown Rust resistance using map-based QTL analysis

All marker-trait associations require investigation in alternative genetic material. This is because some may be statistical artifacts, while others may be real but only identify genomic differences found in the plant material studied and thus of limited value in plant breeding programs.

In this project, we have used two different approaches to validate the markers identified earlier in the project using map-based QTL analysis (Tables 1 and 2). These include:

(i) Validation in clones related to one of the parents of the Q1 (Q117 x 74C42) mapping population
(ii) Validation in clones distantly related to the Q1 mapping population.

In collaboration with another SRDC-funded project (CTA047), DNA from 154 varieties and elite clones was isolated (Elite Clone Set). These lines were selected to cover the range of genetic diversity within Australian sugarcane varieties and parental lines. Of these 154 lines, Pachymetra resistance and Brown Rust resistance ratings were available for 130 and 72 lines, respectively. FDV ratings were also available for 111 of the clones.

From Tables 3 and 4, nine AFLP primer pairs were selected as generating markers in the 4 genomic regions associated with Pachymetra resistance and 4 genomic regions associated with Brown Rust resistance. All 4 SSR primers from Tables 3 and 4 were also selected. The nine AFLP primer pairs and 4 SSR primer pairs were screened over the 154 elite clones, and the markers identified as associated with Pachymetra resistance or Brown Rust resistance in the Q1 population were scored in the Elite Clone Set. Using the software package MapManager, associations between these markers and resistance in the Elite Clone Set were examined.

(i) **Validation of Q1 markers in descendents of Trojan**.

As the Australian sugarcane breeding programs have followed a proven cross concept, many of these lines have parents and grandparents in common. Examination of the pedigrees of the Elite Clone Set revealed that Trojan was an important parent in many of the pedigrees. Of the 154 clones, 44 were descendents of Trojan and 33 were descendents of the cross between Trojan and Co475 (Table 5). The female parent of the Q1 population is Q117. The parents of Q117 are Q77 and 58N829 while Trojan and CO475 are parents of 58N829. Of the 44 clones descended from Trojan, Pachymetra and Brown Rust resistance ratings were available for 44 and 33 of the 44 clones, respectively. Trojan and Co475 are rated an 8 and a 9 for Pachymetra, respectively, and a 2 and a 9 for Brown Rust. Thus, this validation set is of most use for validating markers identified in the Q1 mapping population that are associated with Pachymetra susceptibility and Brown Rust resistance or susceptibility markers. Tables 6 and 7 summarise the results of the validation experiment.

Table 5 – The 44 clones from the 154 Elite Clones Set that are related to Trojan, and their Pachymetra and Brown Rust resistance ratings.

Clone	Relationship withTrojan	Pachymetra	Brown Rust	FDV
58N - 829	Progeny (Trojan x Co475)	2.9	-	7
67N - 3184	Grand Progeny (Trojan x Co475)	6	5	5
77N - 792	Grand Progeny (Trojan x Co475)	3.3	-	2
Cassius	Progeny (Trojan x H49-104)	3	5	5
Co475		9	9	8.5
Q107	Grand Progeny (Trojan x Co475)	7.3	5	7
Q113	Grand Progeny (Trojan x Co475)	4.3	1	6.7
Q115	Grand Progeny (Trojan x Co475)	5.7	6	7
Q117	Grand Progeny (Trojan x Co475)	4.9	6	5
Q120	Grand Progeny (Trojan x Co475)	4.7	1	8
Q121	Grand Progeny (Trojan x Co475)	7.9	3	4
Q122	Grand Progeny (Trojan x Co475)	5.8	-	4.5
Q124	Grand Progeny (Trojan x Co475)	4.4	1	4.4
Q127	Grand Progeny (Trojan x Co475)	3	1	6.5
Q133	Grand Progeny (Trojan x Co475)	7.5	-	8.5
Q135	Grand Progeny (Trojan x Co475)	6.2	2	1.5
Q136	Grand Progeny (Trojan x Co475)	5.8	1	3.3
Q137	Grand Progeny (Trojan x Co475)	7.5	1	3
Q138	Grand Progeny (Trojan x Co475)	2.4	3	2.5
Q141	Grand Progeny (Trojan x Co475)	6	1	1.3
Q142	Grand Progeny (Trojan x Co475)	7.3	9	1.7
Q145	Grand Progeny (Trojan x Co475)	7	-	1.4
Q149	Grand Progeny (Trojan x Co475)	5	-	1.5
Q153	Grand Progeny (Trojan x Q28)	6	1	1
Q154	Grand Progeny (Trojan x Co475)	2.5	5	1.5
Q157	Grand Progeny (Trojan x Co475)	7	1	2
Q158	Grand Progeny (Trojan x Co475)	4	4	2.7
Q159	Grand Progeny (Trojan x Co475)	8	-	3
Q161	Grand Progeny (Trojan x CPH139)	4	-	3.7
Q162	Grand Progeny (Trojan x Co475)	1.5	1	1.3
Q166	Grand Progeny (Trojan x Co475)	4.5	-	1
Q167	Grand Progeny (Trojan x Co475)	4	-	5.3
Q172	Grand Progeny (Trojan x Co475)	5	-	3.5
Q179	Grand Progeny (Trojan x Co475)	6.8	9	1
Q191	Grand Progeny (Trojan x Co475)	2.3	1	2
Q196	Grand Progeny (Trojan x Co475)	2.8	1	1
Q197	Grand Progeny (Trojan x Co475)	5.5	1	1.5
Q198	Grand Progeny (Trojan x Co475)	6.3	-	5.3
Q63	Progeny (Trojan x CP29-116)	9	5	1
Q67	Progeny (Trojan x Q27)	8.3	5	8
Q83	Progeny of Co475, Grand Progeny (Trojan x Q27)	8	5	4.8
Q90	Progeny (Trojan x Co475)	6.2	9	6.5
Q96	Grand Progeny (Trojan x CP29-116)	7	3	1.5
Q99	Progeny (Trojan x Co475)	-	5	1
Trojan		8	2	7

For Pachymetra resistance, seven of the original 14 significant markers (Table 1) were markers also present in either Trojan or Co475 and 4 originated from Q117, a descendant of Trojan. All 7 markers were scored in the 44 descendants of Trojan. Of the seven markers, only two remained significantly associated (P<0.01) in this validation set. Marker ACC-CTA21 originated from Q117 and was associated with susceptibility to Pachymetra in the Q1 population. It is also associated with susceptibility to Pachymetra in this validation set and at a more significant level. Thus, this marker appears to have been validated. The remaining Q1 marker, ACG-CTG5, was heterozygous in both parents. The marker is present in Trojan and it is highly significantly associated with resistance to Pachymetra in this validation set. Trojan has a small amount of resistance, which may be being tagged by this marker, or this resistance region is poorly expressed in Trojan in comparison to some of its descendants. Thus, this marker appears to have been validated also. An alternative explanation for the latter marker is that its association with Pachymetra resistance is co-incidental, that it is not near a gene for Pachmetra resistance but lies on a genomic region that has been inherited by many of the Trojan descendents that happen to have inherited resistance to Pachymetra from an unknown source(s). It thus reflects the close relationship and corresponding shared chromosomal regions in most Australian sugarcane varieties.

Table 6. Validation of PACHYMETRA resistance markers identified in the Q1 population, in descendents of Trojan or the Elite Clone Set.
(NS – not significant P<0.01)

Q1Marker	Linkage Group and Map	Trojan				Elite Clone Set	
			T/C MM?	No. Clones with MM /44	t-test	No. Clones with MM /130	t-test
Aacctg.29	2 F	S	-	-	-	-	-
Acgcta.10	2 F		T	8	0.11	39	0.0088
Acccta.11	2 F		C	20	0.102	79	0.00014
Acccta.21	2 F		C	5	0.0089	NS	NS
Acacta.9	22 F		-	-	-	-	-
M44.5	22 F		T	27	0.80	NS	NS
Acgcag.3	22 F		-	-	-	-	-
Acccag.11	22 F		-	-	-	-	-
Acccag.4	22 F		-	-	-	-	-
Acgctg.2	5 M	S	T	26	0.068	NS	NS
Acactt.10	5 M		-	-	-	-	-
Aggcac.3	5 M		-	-	-	-	-
Aggcac.8	42 M	S	C	22	0.018	NS	NS
Xacgctg.5	15 3:1	R	T	20	0.000036	55	0.00016

For Brown Rust resistance, eight of the original 22 markers were present in either Trojan and/or Co475. These markers were scored in the Trojan validation set (Table 4). Of the 8 markers, only marker MS39-5 was still weakly significantly associated with Brown Rust resistance, but as the significance of the association was lower than the association in the Q1 population, this marker may not be reliable. One explanation for the lower significance level is that Co475, rated a 9 for Brown Rust, also had the marker. Thus, clones could have inherited the marker from either Trojan or Co475. This suggests that this region is associated with rust resistance, but that this marker is not a good indicator for resistance. In addition, this marker originates from the male parent in the Q1 population, 74C42. While the parents of 74C42 are known, the grandparents are not and so this validation set may not be suitable for the validation of this marker. Thus, none of the Brown Rust resistance markers identified in the Q1 population have been reliably validated.

Table 7. Validation of BROWN RUST resistance markers identified in the Q1 population, in descendents of Trojan or the Elite Clone Set.
(NS – not significant $P<0.01$)

Marker	Linkage Group and Map	Trojan				Elite Clone Set	
			CT MM ?	No. Clones with MM /33	t-test	No. Clones with MM /72	t-test
Acactg.16	U		C	14	0.31	NS	NS
Actcat.14	13F		-	-	-	-	-
Accctc.18	13F		-	2	-	NS	NS
40.1.16	13F		-	-	-	-	-
Acccta.15	18F		C	21	0.61	NS	NS
Aacctc.12	18F		CT	22	0.63	NS	NS
M851.3	34F		-	2	-	NS	NS
Aggctc.20	60F		C	16	0.17	31	0.0087
Accctc.20	5 M		T	24	0.92	NS	NS
22.1.5a	10M		-	-	-	-	-
T2-2	10M		-	-	-	-	-
Aacctg.19	10M		-	-	-	-	-
Acactg.22	10M		T	17	0.92	NS	NS
Agccag.4	10M		-	-	-	-	-
Aggcac.18	10M		-	1	-	NS	NS
Aagctc.6	10M		-	-	-	-	-
Acacta.29	10M		-	-	-	-	-
Aagcac.20	23M		-	-	-	-	-
Actcac.15	23M		C	16	0.17	NS	NS
M39.5	30M		CT	20	0.029	NS	NS
M1237.4	43M		-	30	0.48	NS	NS
Agcctc.12	43M		-	2	0.46	NS	NS

(ii) **Validation of Q1 markers in clones distantly related to the Q1 population**.

The second validation approach that we have used it that of association-mapping. Of the 154 lines in the Elite Clone Set, Pachymetra resistance and Brown Rust resistance ratings were available for 130 and 72 lines, respectively. All 154 lines were screened with the nine AFLP and 4 SSR primer pairs to measure the extent of the association between the markers identified as associated with Pachymetra or Brown Rust resistance in the Q1 population and this broader set of germplasm. The results are also listed in Tables 6 and 7.

For Pachymetra resistance, of the seven markers screened, three gave significant results and two of the three markers were linked onto the same linkage group. All three markers were similarly or more significantly associated in the Elite Clone validation set than in the Q1 population. One of the markers, marker X-ACG-CTG5, was also significantly associated in the Trojan validation set. This result is additional evidence of the association between this marker and Pachymetra resistance.

For Brown Rust resistance, only one of the 13 markers was significantly associated in this validation set and at a similar significance level to the level in the Q1 population. This result supports the association between this marker and Brown Rust resistance.

B. Use of association- based approaches to identify markers associated with target traits.

Association mapping, the screening of distantly related germplasm with DNA markers and the identification of marker associated with traits, can also be used for the identification of marker-trait association. Such associations would require subsequent validation in alternative germplasm. In an earlier Milestone Report, we had discussed our difficulties in obtaining FDV ratings for our Q1 mapping population, and reported on a successful pilot project using the association mapping approach to identify markers associated with FDV. We were therefore interested in identifying new marker-trait associations using the 154 Elite Clone Set for FDV, and also Pachymetra resistance and Brown Rust resistance.

A total of 19 AFLP primer pairs and 14 SSR primer pairs were screened over the 154 elite clones. These primer pairs generated 1599 and 181 markers, respectively. New marker-trait associations were sought for Pachymetra, Brown Rust and Fiji Disease Virus resistance using the software package, MapManager.

a) New markers associated with Pachymetra resistance

Seventeen markers were identified as associated with Pachymetra resistance at $P<0.001$ (Table 8), with a further 68 markers identified at $P<0.01$ (data not shown). Of the 17, three had markers present in too few or too many clones to be regarded as reliable markers. Of the remaining 14, nine were associated with resistance to Pachymetra and five with susceptibility.

Table 8. Markers associated with Pachymetra, Brown Rust and FDV, identified using association-based mapping approaches.

Trait	Marker	P	No. Clones with MM		Comment	P in Trojan Validation Set	T/C MM?
PACHYMETRA	ACG-CTC31	0.00001	73/154	R	Robust	0.0051	T
	AAC-CTC80	0.00002	73/154	S	Robust	0.00034	
	ACC-CTA23	0.00014	79/154	S	Robust	0.079	C
	ACG-CTG19	0.00016	55/154	R	Robust	0.000097	T
	M286-3	0.00016	34/154	S	OK	0.12	
	ACA-CTA11	0.00018	42/153	R	OK	0.0023	T
	AAC-CTA24	0.00024	32/153	S	OK	0.013	T
	ACC-CAC20	0.00037	48/154	R	OK	0.44	C
	ACC-CTA27	0.00038	9/154	S	Unreliable - Too few with MM	DND	
	AAC-CTA49	0.00038	14/154	S	Unreliable - Too few with MM	DND	
	M39-1	0.00038	56/154	R	Robust	0.032	C
	AAC-CTA50	0.0005	32/153	R	OK	0.000051	
	ACA-CTA96	0.00054	40/153	R	OK	0.0061	
	AGC-CTC3B	0.00082	56/154	R	Robust	Unreliable - Too few with MM	
	ACA-CTG3	0.00084	54/154	R	Robust	Unreliable - Too few with MM	
	ACC-CTC10	0.00094	119/154	S	OK	0.013	T
	AAC-CTA1	0.00099	132/153	S	Unreliable - Too many with MM	DND	T,C
RUST	ACT-CAG5	0.00005	64/154	R	Robust	0.00069	
	AGC-CTG17	0.00028	145/154	R	Unreliable - Too many with MM	Unreliable - Too many with MM	T
	AGC-CTC53	0.00052	50/154	S	Robust	0.000089	C
	ACG-CAG26	0.00065	14/154	S	Unreliable - Too few with MM	0.0051	C
FDV	AGC-CTG54	0.00001	42/154	R	OK	0.006	

	ACA-CTA55	0.00021	34/153	R	OK	0.014	
	ACG-CTA27	0.00038	101/154	S	OK	0.000035	C
	ACT-CAC47	0.00062	92/154	S	Robust	0.087	C,T
	ACG-CTA28	0.00065	25/154	S	Unreliable - Too few with MM	0.072	

In an attempt to validate these 14 markers, they were examined more closely in the Trojan validation set. Trojan is rated an 8 and Co475 a 9 for Pachymetra and thus this validation germplasm is more useful for validating markers for susceptibility to Pachymetra.

Five of the 14 markers were associated with susceptibility to Pachymetra; two of these markers were present in too few of the Trojan validation set to be analysed further. The remaining 3 markers were associated with susceptibility, although only one of the markers was strongly associated.

Of the 9 markers associated with resistance to Pachymetra, five markers were also present in Trojan and/or Co475. These markers were still associated with resistance in this validation set, with one marker, ACT-CTG19 identified previously, very highly significantly associated with resistance. Thus, this marker may be associated with the small amount of resistance present in Trojan, or this resistance source is poorly expressed in Trojan compared to some of its descendants, or the marker may not be associated with resistance, but may reflect the inherent relatedness of Australian sugarcane.

b) New markers associated with Brown Rust resistance

As Trojan is rated a 2 for Brown Rust resistance and Co475 is rated a 9, this validation set is useful for markers associated with both resistance and susceptibility to Brown Rust.

Four markers were identified as associated with Brown Rust resistance at $P<0.001$ (Table 8), with a further 32 markers identified at $P<0.01$ (data not shown). Of the four, two had markers present in too few or too many clones to be regarded as reliable markers.

The remaining two markers were examined more closely in the Trojan validation set. One of the markers was associated with resistance to Brown Rust and was still highly significantly associated with resistance in the Validation Set. The second marker was present in Co475 and was very highly associated with susceptibility in the validation set. Thus, both of these markers appear to have been validated.

c) Identification of markers associated with FDV

Five markers were identified as associated with FDV resistance at $P<0.001$ (Table 8), with a further 34 markers identified at $P<0.01$ (data not shown). Of the five, one had markers present in too few clones to be regarded as reliable.

The remaining four markers were examined more closely in the Trojan validation set. Trojan is rated a 1 for FDV, while Co475 is rated a 9. Thus this validation set is useful for validating markers associated with both resistance and susceptibility to FDV. Two of the four markers were significantly associated within the Trojan validation set, one with resistance and one with susceptibility. The latter marker was also present in Co475 and the level of

significance of the association increased. Thus, this latter marker appears to have been validated in this data set.

3. Development of association mapping approaches to identify and validate markers in sugarcane

As described in Outputs 1 and 2 above, we have successfully used association mapping approaches to validate previously identified marker-trait associations and to identify new marker-trait associations.

INTELLECTUAL PROPERTY

Nil

EXPECTED OUTCOMES

- Successful identification of markers associated with major diseases of sugarcane will allow the usefulness of markers in sugarcane breeding to be evaluated.

- Successful demonstration of the value of association mapping approaches should result in the incorporation of this approach into future marker identification and validation approaches

FUTURE RESEARCH NEEDS

Future activities should focus on:

- Identification of specific pedigrees suitable for validating markers associated with key sugarcane traits. The small pedigree evaluated in this project was not suitable for validating all of the disease-associated markers identified in the Q1 population or using the association mapping approach. Therefore, other pedigrees need to be identified for validation purposes of these and other markers.
- The establishment of specific segregating populations to validate some marker-trait associations
- The development of graphical genotypes for key Australian sugarcane lines. Only a small number of meioses, and consequently a small number of recombination events, are thought to have occurred since the production of modern sugarcane varieties. This may mean that large regions of the genome have been co-inherited by related varieties, which will have implications (positive and negative) on the use of mapping and association mapping approaches to identify new marker-trait associations. If too many regions of the genome have been co-inherited, then many spurious marker-trait associations will be identified. On a more positive note, once a “real” marker-trait association is identified, the marker can be loosely linked to the trait as the genomic region co-inherited is likely to be large. We need to develop graphical genotypes of some of our important lines in order to obtain a picture of the recombination history of sugarcane, and to determine the overall size of co-inherited fragments.

- The conversion of AFLP markers linked to priority traits to a more transferable, easy to use, PCR-based marker system.
- An enhanced statistical capability to utilise pedigree information in the identification and validation of marker-trait associations. We need to investigate the usefulness of animal and human marker software which are based on pedigree and/or association mapping approaches.
- Evaluation of the potential impact of these markers on resistance in the breeding program vs. the cost of implementing their use. We need to know more about factors which may affect the impact of markers in the breeding program, such as their use in selection of parents, as an early screening tool, as a late screening tool, the amount of variation in a trait associated with each marker, the value of different traits etc.

RECOMMENDATIONS

There are numerous recommendations arising from this project.

- A combined QTL and association mapping approach appears to be the most efficient means of identifying and validating marker-trait associations
- We need to understand more about the impact of selection on Australian sugarcane clones. This will impact on the ability to detect marker-trait associations and the closeness of the marker required it to be used as an indirect selection tool.
- We need to investigate the usefulness of animal and human marker software which are based on pedigree and/or association mapping approaches.
- We need to investigate the potential impact of markers as a selection tool within the sugarcane breeding program, including different approaches to their use, the amount of variation explained vs. the value of the trait, the cost of their implementation.

LIST OF PUBLICATIONS

McIntyre.L., Aitken, K., Berding, N., Casu, R., Drenth, J., Jackson, P., Jordan, D., Piperidis, G., Reffay, N., Smith, G., Tao, Y., and Whan, V. Identification of DNA markers linked to agronomic traits in sugarcane in Australia. Proceedings 24th International Sugar Cane Technologists (Brisbane, 17-21 September, 2001) p 560.

McIntyre, C.L., Drenth, J., Knight, D., Jordan, D., Casu, R. and Manners, J. Comparative mapping of resistance gene analogues in sorghum and sugarcane In: Plant Breeding for the 11th Millenium, J.A.McComb (ed), Proceedings 12th Australasian Plant Breeding Conference (Perth, 15-20 September, 2002) p 96-99

Jordan, DR, Casu, RE, Besse, P, Carroll, BC, Berding N, and McIntyre, CL. Markers associated with stalk number and suckering in sugarcane co-locate with tillering and rhizomatousness QTLs in sorghum. Genome (submitted).

Publications in preparation.

1. McIntyre, CL, Casu, RE, Drenth, J, Knight, D, Whan, VA, and Manners, JM. Resistance gene analogues in sugarcane and sorghum and their association with QTLs for disease resistance.
2. McIntyre, CL, Whan, VA, Jackson, M, Croft, B, Magarey, R, Smith G, and Berding, N. Identification and validation of markers associated with disease resistance in sugarcane using map- and association- based approaches.

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