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Exploiting *Erianthus* diversity to enhance sugarcane cultivars

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Abstract Introgression of *Erianthus arundinaceus* into the SRA sugarcane-breeding program has been a goal for researchers for many years. The *Erianthus* genome was finally accessible to sugarcane breeders with the identification in 2005 of the first *Saccharum/Erianthus* fertile hybrids, developed in China. Today, *Saccharum/Erianthus* BC3 and BC4 clones are available in Australia, and *Erianthus*-sugarcane hybrids have been characterised by cytogenetics and investigated for their potential resistance against pachymetra root rot, sugarcane smut and nematodes. Some clones have shown potential as new sources of resistance for incorporation into the SRA breeding program. These hybrids were created from *Erianthus* clones indigenous to China and their reaction to the above diseases is unknown in Australian conditions. In Meringa we also have access to many *Erianthus* clones of Indonesian origin. Some of these *Erianthus* clones have previously shown immunity to pachymetra root rot. In the late 1990s, these Indonesian *Erianthus* clones were used in crossing but no fertile hybrids were ever produced due to an incompatibility between the *Saccharum* and the *Erianthus* genomes. We revisited this untapped source of resistance by utilising the fertile *Erianthus* hybrids derived from China to cross with the Indonesian *Erianthus* of known resistance to pachymetra root rot. Here we report on the early stage results of introgressing Indonesian *Erianthus* into the SRA breeding program.

Key words *Erianthus*, sugarcane hybrids, new germplasm, pachymetra resistance, introgression

INTRODUCTION

Erianthus arundinaceus belongs to the '*Saccharum* complex' (Mukherjee 1957) along with four other interbreeding genera (*Saccharum* L., *Miscanthus* sect. *Diandra* Keng, *Narenga* Bor. and *Sclerostachya* Hack). Many sugarcane-breeding programs around the world have attempted to introduce traits from *E. arundinaceus* because of its excellent vigour, good ratooning ability, and drought and waterlogging tolerance (Berding and Roach 1987). It has also been recognised for its high fibre content and resistance to pachymetra root rot (Magarey and Croft 1996), red rot (Bakshi *et al.* 2001) and nematodes (Stirling *et al*. 2011).

Even though *E. arundinaceus* is classified in the *Saccharum* complex, cross-incompatibility was a major limitation to achieving successful hybrids between *Saccharum* and *Erianthus* and progress was limited during the 1990s (Nagai *et al.* 1991; D'Hont *et al.* 1995; Besse *et al.* 1997; Deng *et al.* 2002; Piperidis *et al*. 2010a). During this time, genuine *Erianthus* hybrids were produced in Australia using the *Erianthus* clones collected from Indonesia (Berding and Koike 1980; Piperidis *et al.* 2000). Ninety-six crosses were made, and 19 of 37 genuine hybrids identified by PCR survived, but only two hybrids ever flowered (Piperidis *et al.* 2000). Thirty crosses were made with these two flowering hybrids, but no seeds were produced suggesting they were sterile. It is likely that the sterility issue was caused by meiotic abnormalities.

Fertile *Erianthus* hybrids were produced in China with locally collected *Erianthus* clones (Deng *et al*. 2002; Cai *et al*. 2005) and, in collaboration with Guangdong Sugar Industry Research Institute, this material became available for crossing with Australian germplasm in 2004. Furthermore, cytogenetic characterisation was performed on at

least four generations of back-crosses (BC) between the F1 and Australian elite cultivars (Piperidis *et al*. 2010a). The number of the 60 *E. arundinaceus* chromosomes was halved at each generation, except for the BC1 as an atypical 2n+n cross transmission was observed. Very few recombination events between *Saccharum* and *Erianthus* chromosomes were found. In the BC3 clones, 1-8 *Erianthus* chromosomes were found and in the BC4 0-3 chromosomes were found.

Erianthus clones from Indonesia are considered immune to pachymetra root rot (B Croft pers. comm.) and we wanted to develop a segregating population with this new source of resistance to that disease. The opportunity to revisit the introgression of immunity to pachymetra root rot from the Indonesian *Erianthus* has been made possible due to the Chinese *Erianthus* hybrids. Our hypothesis was based on the higher chance of obtaining hybrids between pure *Erianthus* from Indonesia and *Erianthus* hybrids (with a Chinese origin), as the latter already contains *Erianthus* chromosomes; therefore, facilitating normal meiosis to produce fertile hybrids.

The development of new germplasm using sugarcane and *Erianthus* with both a Chinese and Indonesian origin is described along with the cytogenetic characterisation of the hybrids. We also report on the resistance to pachymetra root rot of this new hybrid material.

MATERIALS AND METHODS

Plant material

Crosses were made from plants grown in the field (F) and photoperiod houses (PH) at the SRA Meringa (17.068349°S, 145.773849°E). All F crosses were made between May and June in each of 2016 and 2017, and the PH crosses were made between July and September 2017.

Erianthus hybrids from China range from F1, BC1, BC2 and BC3. *Erianthus* clones from Indonesia were chosen based on their diversity documented in previous studies (Besse *et al.*1997; Cai *et al*. 2005). We initially used three different *Erianthus* accessions. These accessions are given with their chromosome composition (if available) in Table 1.

Table 1. *Erianthus* accessions and hybrids used in crossing during 2016 and 2017.

Erianthus hybrids	Generation	Number of chromosomes		
		Erianthus	*Saccharum*	Total
CYC96-40	F1	30	40	70
QBYC05-20866	F1	30	40	70
YCE01-116	BC1	92	30	122
QBYC05-20863	BC1	-	-	NA
QBYC05-20866	BC1	-	-	NA
QBYC05-30881	BC2	-	-	NA
QBYC05-30962	BC2	-	-	NA
QBYC06-30376	BC2	11	100	111
QBYC06-30138	BC2	-	-	NA
QBYC06-30315	BC2	11	110	121
KQ08-1347	BC3	5 + 1 (recombined)	107	113
KQ08-2838	BC3	-	-	NA
KQ08-6003	BC3	7	115	122
KQ08-6013	BC3	7	112	119
KQ08-6014	BC3	5	113	118
Erianthus Indonesia				
IK76-48	Pure species	60	-	60
IK76-79	Pure species	60	-	60
IJ76-370	Pure species	60	-	60

Seedling germination was fast-tracked in 2016 to shorten the time for characterisation and re-crossing in 2017. Our routine germination cycle occurs approximately 9 months after the seed is harvested (here it would have been March 2017). In lieu of waiting the 9 months to germinate the *Erianthus* seed produced, we fast-tracked the seed germination approximately 2 months after field crossing in July 2016. This was to guarantee we could

germinate the progeny from seed produced, test for genuine hybrids, and transplant them to the field so they could be available for the 2017 F crossing season.

In 2017, progeny of interest from the 2016 crossing efforts were also replanted as single-node setts to bulk-up material available for 2018 F crossing, and to preserve the germplasm for future use (instead of relying on one plant per genotype as a seedling).

Microsatellite characterisation

Three microsatellites (simple sequence repeats, SSRs) were used to determine if the progeny were true hybrids and so have chromosomes inherited from both parents. The SSR used were developed for sugarcane screening (Cordeiro *et al.*, 2000) so we had to choose the ones displaying the most polymorphic markers inherited from the *Erianthus* genome. We selected mSSRCir26, mSSRCir36 and mSSRCir41. All markers were generated at CSIRO Brisbane.

GISH procedure

Clones for cytogenetic characterisation were grown in a glasshouse in Mackay (21.14298°S, 149.1837°E) in 20-L pots containing a mixture of 50% vermiculite/perlite and/or in an aeroponic system with liquid fertilizer (Ionic grow by Growth Technology).

The roots used to prepare the protoplast spreads were harvested between October 2017 and November 2018. Protoplast preparation, chromosome spreading and GISH experiments were as described by D'Hont et al., 1996, Piperidis *et al.* (2013, 2014). Treated root tips were spread on microscope slides to expose the chromosomes in the metaphase stage of mitosis.

Genomic DNA was labelled by random priming according to the manufacturer's directions. Labelled DNA consisted of a mixture of double- and single-stranded fragments. We used the BIOPRIME DNA labelling system with Fluorescein 12-d-UTP (FITC, our 'green' fluorescence) to label *Saccharum officinarum,* Badila genomic DNA. Badila was used because the majority, of the *Saccharum* chromosomes are *S. officinarum*, and it also hybridises to *S. spontaneum* chromosomes sufficiently to discriminate between *Saccharum* and *Erianthus* chromosomes (Piperidis *et al.* 2013). Rhodamine 5-d-UTP (rhodamin is our 'red' fluorescence) was used to label genomic DNA from *E. arundinaceus* clone IK76-79. Slides were then counter-stained with a blue dye 4'-6-diamidino-2-phenylindole (DAPI, Vectashield Mounting Media with DAPI) in Vectashield mounting medium. Images were digitally captured with a CCD camera attached to a BX53 Olympus microscope and added to the database by the Olympus Cellsens software. Chromosome counts were made using the free accessible software Image J.

Resistance to pachymetra root rot

We established a trial to determine resistance to pachymetra root rot according to procedures described by Croft (1989) and modified in Croft *et al.* (1998). All clones were visually rated according to the amount of root rot present on a 0-4 basis: 0 = no disease, and 4 = highly diseased.

RESULTS

Crosses made

Results are presented in Table 2. Here we only report on the crosses that produced viable seedlings. In 2016, six F crosses were made, and while four of the families produced seedlings, only two families were kept (and re-propagated in 2017) based on the genuine hybrid status of the progeny. These were crosses 16*219 and 16*276.

SSR markers were used to test 20% of the progeny for families 16*219, 16*276 and 17*282. All progeny were tested for other families that germinated fewer than 10 seedlings. Parent specific markers were scored for the three SSRs and, based on all results, true hybrid progeny were identified. If all tested progeny were genuine hybrids, we then assumed that the entire population of progeny were genuine hybrids.

The 84 surviving clones from cross 16*219 (Table 2) show extensive phenotypic diversity, for example, leaf colour, stalk number, vigour and growth habit all demonstrated high levels of variation. This diversity is shown in Figure 1.

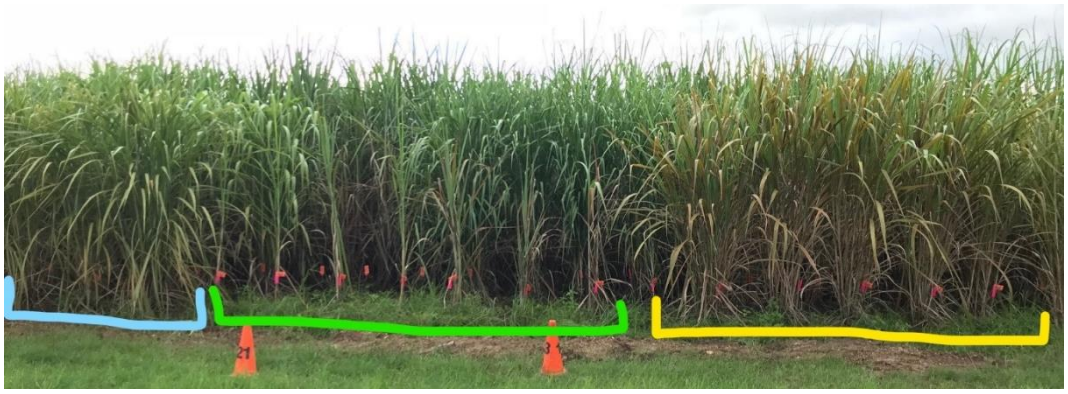

Figure 1. Three clones QBN16-* from the cross 16*219. Three clones are demarcated by different coloured brackets. The clone in the yellow bracket has a high stalk number and yellowish leaves, whilst the clone in the green bracket has very few stalks and poor vigour. The clone in the blue bracket has a reasonable number of stalks and a very dense green-leaf coverage.

In 2017, 19 F crosses were made. From the 10 families that germinated, four cross combinations produced genuine hybrids based on the SSR results, the other six families were discarded. We kept seedlings from four cross combinations based on the SSR results.

Cross 17*281 (in bold in Table 2) is a cross of the second generation where progeny QBN16-10078 (from cross 16*219) was crossed with a high-breeding-value pachymetra-susceptible clone. This specific cross produced one progeny, QBN17-10115, which has successfully incorporated Indonesian *Erianthus* chromosomes (as demonstrated in *Schema 2*), and also demonstrates that its female parent, QBN16-10078, is a fertile *Erianthus* hybrid.

In 2017, 23 PF crosses were made. From the 21 families that germinated, we kept seedlings from five cross combinations based on the SSR results.

Table 2. Field and photoperiod house crosses kept from 2016 and 2017 crossing. Cross 17*281 (in bold) is a second generation between QBN16-10078 (from cross 16*219) and a high breeding value pachymetra susceptible clone.

Cross	F/PF	Female	Male	Number planted to field	GISH
16*219	F	CYC96-40	IK76-48	84 (QBN16-10001 - QBN16-10095)	yes
16*276	F	QBYC05-20866	IK76-48	16 (QBN16-10101 - QBN16-10116)	yes
17*163	F	CYC96-66	IK76-79	2 (QBN17-10112, QBN17-10113)	
17*164	F	CYC96-66	IK76-48	3 (QBN17-10117 - QBN17-10119)	
17*262	F	CYC96-40	IJ76-370	80 (QBN17-10202 - QBN17-10281)	
17*281	**F**	**QBN16-10078**	**QS06-7316**	**1 (QBN17-10115)**	**yes**
17*282	F	CYC96-66	IK76-79	6 (QBN17-10120 - QBN17-10125)	
17*3299	PH	QBYN04-20119	IK76-48	Seedlings	
17*3445	PH	QBYC06-30315	IK76-79	Seedlings	
17*3448	PH	QBYC05-20866	IK76-79	Seedlings	
17*3767	PH	CYC96-66	IK76-48	Seedlings	
17*3823	PH	CYC96-66	IK76-48	Seedlings	
17*3824	PH	KQ08-6003	IK76-48	Seedlings	yes

Microsatellite characterisation

Three SSR (mSSRCir26, 36 and 41) were used to screen approximatively 20% of the progeny of all 13 crosses from Table 2. All markers specific to the female parent CYC96-40 and the male parent IK76-48 were scored for

presence versus absence and progeny with both specific markers are were classified as genuine hybrids (data not shown). The selection of progeny from the first 12 crosses listed in Table 2 were screened with the SSR markers and classified to be genuine hybrids. Even though SSR is an easy method to identify hybrid progeny from a cross, it also has limitations. For example, progeny from cross 17*3824 were checked using SSR markers and only one of the three primers showed that they were genuine hybrids. However, GISH analysis on two of these clones revealed that they were not genuine hybrids but resulted either from selfing of the female parent or from pollen contamination. Therefore, GISH is the only method that can give an unambiguous result to determine the hybrid status of any clone. However, GISH is a highly skilled procedure, requires much more time than running SSR markers and consequently is more expensive.

Cytogenetic characterisation

GISH analyses were performed on a subsample of clones from the cross 16*219, (female parent CYC96-40 and male parent IK76-48). We characterised 14 clones, and the chromosome counts are given in Table 3. Nine of the 14 clones analysed (in bold) revealed a classic n+n chromosomes transmission as shown in Figure 2.

Table 3. Chromosome composition of QBN16 clones. Those in bold resulted from n+n transmission.

Clone	Chromosome origin			2n chromosome number
	S. officinarum	*Erianthus*	Recombined	
QBN16-10008	**20**	**45**	**0**	**65**
QBN16-10010	**20**	**42**	**0**	**62**
QBN16-10019	**20**	**40**	**0**	**60**
QBN16-10021	**20**	**40**	**0**	**60**
QBN16-10024	**18**	**42**	**1**	**60**
QBN16-10028	37	52	2	89
QBN16-10033	**20**	**44**	**0**	**64**
QBN16-10037	**20**	**42**	**0**	**62**
QBN16-10046	**20**	**45**	**0**	**65**
QBN16-10049	**20**	**40**	**0**	**60**
QBN16-10061	40	55	0	95
QBN16-10073	40	50	0	90
QBN16-10078	39	54	1	93
QBN16-10091	40	56	0	96

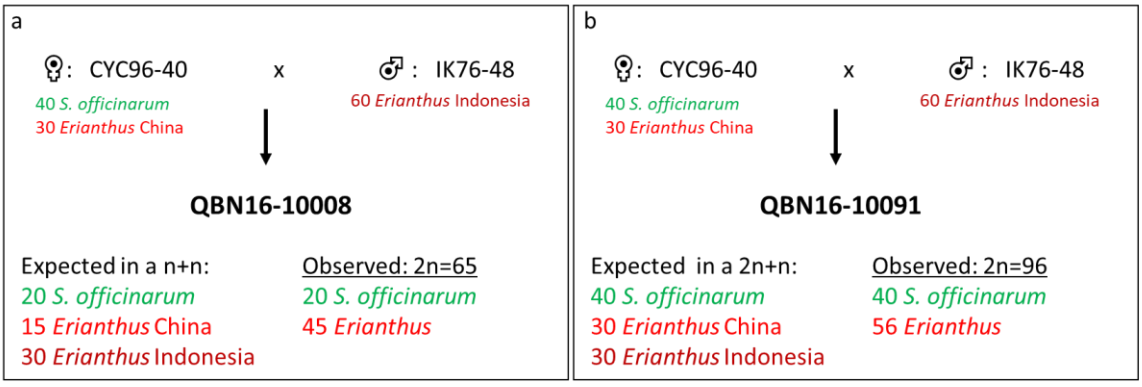

Figure 2. Two types of chromosome transmission observed in the CYC96-40 x IK76-48 cross.

Unexpected results were obtained for 5 of the 14 QBN16-*clones from cross 16*219; the number of chromosomes observed in these clones is in accordance with a 2n+n transmission. This is the first time a 2n+n transmission has been reported involving the *Erianthus* genome without the presence of *Saccharum spontaneum* chromosomes. Figure 1b shows how a 2n+n transmission occurred for clone QBN16-10091. The entire

complement of chromosomes from the female parent is transmitted to the progeny, while the male parent only transmits half of its chromosomes.

GISH characterisation showed that three clones, QBN16-10024, QBN16-10028 and QBN16-10078, have one or two recombined chromosomes between the *Erianthus* and the *Saccharum* genome. Figure 3 shows GISH results for four of the hybrid clones. Clone QBN16-10008 has 42 *Erianthus* chromosomes and 20 *Saccharum* chromosomes following a classic n+n transmission (Figure 3a), whilst clone QBN16-10073 has chromosome numbers that suggest a 2n+n transmission with 50 *Erianthus* chromosomes and 40 *Saccharum* chromosomes (Figure 3b). Clone QBN16-10024 also followed a traditional n+n transmission but has a recombined chromosome between *Erianthus* and *Saccharum* (yellow arrow in Figure 3c), whilst clone QBN16-10078 followed a 2n+n transmission and also display a recombined chromosome (Figure 3d).

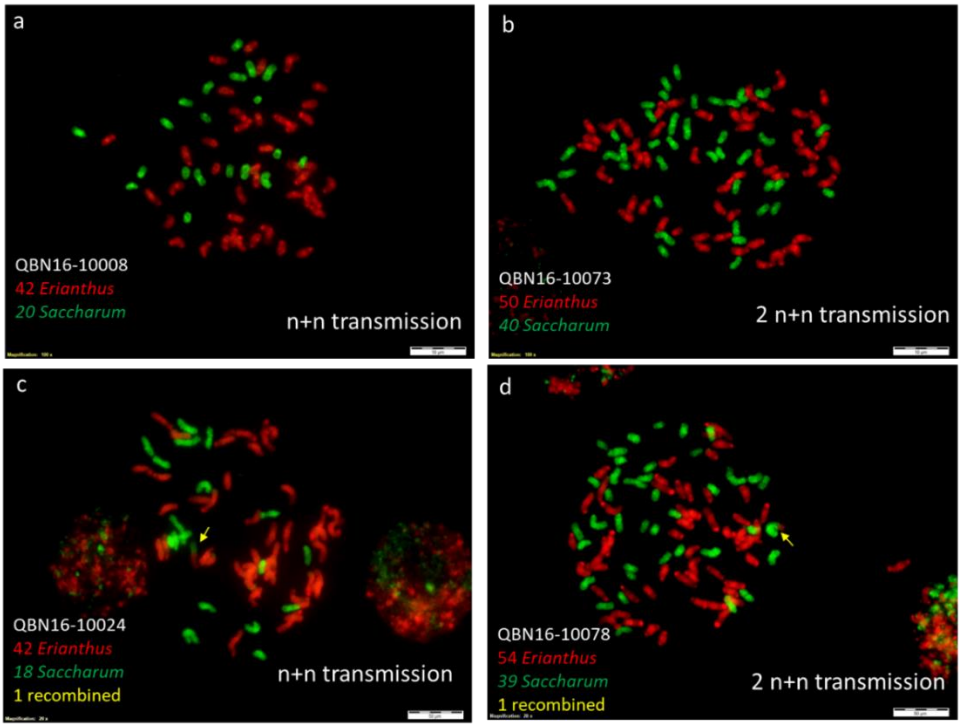

Figure 3. GISH photos of QBN16* clones with *Erianthus* chromosomes in red and *Saccharum* chromosomes in green, recombined chromosomes are shown by an arrow.

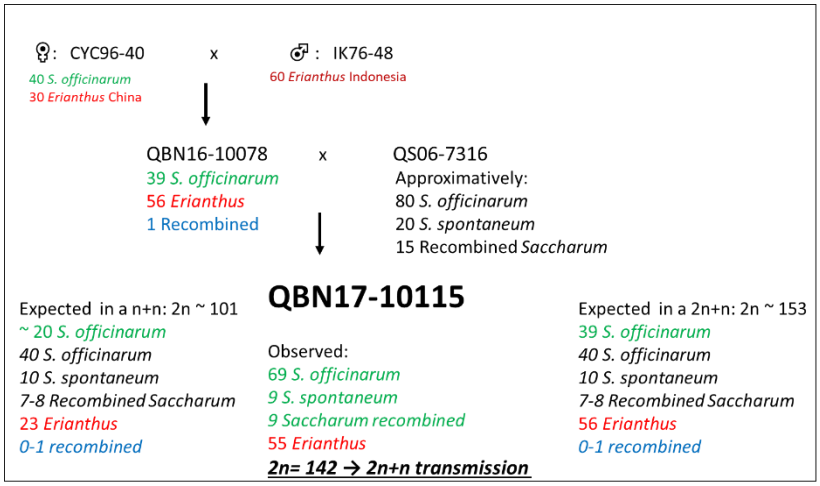

Figure 4. Pedigree of QNB17-10115.

We also characterised clone QBN17-10115 from the F cross 17*281. Its pedigree is shown in Figure 4. This clone is an introgression clone developed by crossing a BC1 *Erianthus*/*Saccharum* hybrid with a sugarcane clone. It has more chromosomes than a classic cultivar (normal range between 110-130) with 2n=142 chromosomes.

Resistance to pachymetra root rot

Most of the progeny from cross 16*219 and 16*276 were sent to the SRA Tully to screen for resistance to pachymetra root rot. Seventy-six progeny were tested in September 2017 and 63 clones (83%) were resistant, eight clones (11%) were intermediate and only five clones (6%) were susceptible to pachymetra root rot (Figure 5). Of the 63 resistant clones, 30 (48%) had a trial score of 0, while for the conventional material (clones in advanced stages of selection) in the same trial, only 26% of the resistant clones had a trial score of 0.

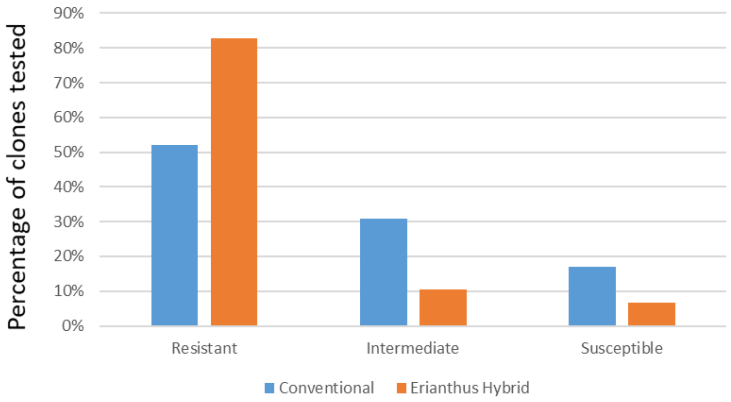

Figure 5. Resistance levels to pachymetra root rot for QBN16* clones and conventional cultivars.

DISCUSSION

Of the two major objectives for this project, the first was to obtain sugarcane hybrid clones with the *Erianthus arundinaceus* from Indonesia with the knowledge that producing genuine fertile hybrids with *Erianthus*, (even though they belong to the *Saccharum* complex), is a difficult exercise as experienced worldwide in several breeding programs. The second was to introgress resistance to pachymetra root rot previously observed in Indonesian *Erianthus* clones, with the possibility of extending this to other important agronomical traits in the future.

We have been able to generate at least 12 crosses that produced true *Erianthus* hybrids. We used a novel approach to introduce *Erianthus arundinaceus* from Indonesia into the Australian sugarcane germplasm, using parents/hybrids that already have *Erianthus* germplasm/chromosomes from China. This strategy has been far more successful than crossing pure Indonesian *Erianthus* with conventional sugarcane cultivars.

In 2016 we obtained one population of 84 clones that contains individuals with either an n+n or a 2n+n chromosome transmission. The total number of chromosomes (2n) for these clones varied between 60 and 96. At the same time we also showed some recombined chromosomes between *Saccharum* and *Erianthus* in a subset of these accessions. The genetic makeup of these clones appears to be very diverse, as is reflected in the chromosome complement as well as phenotypically in the field trial; every one of the 84 clones looks different phenotypically in the field. Another interesting characteristic that has emerged from these 84 clones is that some clones displayed either two-eyes or even three-eyes per node (instead of the usual single-eye) from which either two or three primary stalks germinated. This is another trait that could potentially be associated with stalk number and yield and will be reported in a subsequent paper.

The 2n+n transmission revealed by the GISH analysis is the first report worldwide of a 2n+n transmission occurring in the absence of *S. spontaneum* chromosomes. In our previous paper (Piperidis *et al*. 2010a) the 2n+n transmission with *Erianthus* occurred when *S. spontaneum* chromosomes were present in one of the

parents of the cross. Even more interesting, we identified a second consecutive 2n+n transmission in the second generation, QBN17-10115, where one of the parents (QS06-7316) is a typical elite clone with a proportion of *S. spontaneum* chromosomes and the other parent (QBN16-10078) has only *S. officinarum* and *Erianthus* chromosomes. QBN17-10115 has 142 chromosomes, of which 61% are *Saccharum* and 39% are *Erianthus*. Of the 61% from *Saccharum*, 80% are inherited from *S. officinarum*, 10% from *S. spontaneum* and 10% are recombined chromosomes between the two *Saccharum* species. This is very similar to what has been observed in commercial cultivars (D'Hont *et al*. 1996), except that we have an additional 55 *Erianthus* chromosomes. QBN17-10115 is proof that at least one clone (its female parent, QBN16-10078) from the cross 16*219 is fertile. In addition, it is also resistant to pachymetra root rot, and will be used in further crossing with the intention to introgress that resistance into a sugarcane background. The transmission of the *Erianthus* chromosomes in each generation will be monitored as well as further resistance testing on the derived progeny.

We observed that in most cases a three-SSR system was sufficient to reveal genuine hybrids. In sugarcane, every cultivar or clone of interest has been catalogued with a system of six SSR markers that are polymorphic in *Saccharum* germplasm but are not useful for hybrid detection in *Erianthus* hybrids; in *Saccharum* these SSR have proven to be a very efficient tool for variety identification. For identification of *Erianthus* hybrids, different sugarcane SSR markers were identified, but in one case the results from the SSR markers were ambiguous and we used GISH to confirm the non-hybrid status of the cross. The most important verification method for hybrids is to perform GISH and chromosome counting inherited from each parent. Nevertheless, GISH is time consuming and requires root collection from glasshouse-grown plants. To address this, we are testing the use of SSR markers designed specifically from *Erianthus* by Zhang *et al.* (2017). We hypothesise that they will be more polymorphic and, therefore, more specific to the *Erianthus* genome. We are also interested in finding a way to discriminate between the *Erianthus* chromosomes of different origin (i.e. China versus Indonesia). The current methods of SSR and GISH are not specific enough to do this but there is potential to use SNP markers for this purpose.

The population of 84 clones from 16*219 will be sent for genotyping in order to produce a genetic map of the Indonesian *Erianthus* parent IK76-48. This will allow us to compare with the genetic map produced by KSA of a Chinese *Erianthus* clone, as well as the newly released sequence of the sugarcane cultivar R570 (Garsmeur *et al.* 2018).

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