Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

Citation: Sobhakumari, V.P., & Mohanraj, K. (2024). Application of Genomic In Situ Hybridization (GISH) and tandem repeat sequence amplification for identification of *Erianthus* – *Saccharum* introgression. *Caryologia* 77(2): 49-55. doi: 10.36253/caryologia-2670

Received: April 08, 2024

Accepted: September 24, 2024

Published: November 10, 2024

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

ORCID
VPS: 0000-0003-4165-6826

Application of Genomic *In Situ* Hybridization (GISH) and tandem repeat sequence amplification for identification of *Erianthus* – *Saccharum* introgression

Valiya Purakkal Sobhakumari*, Krishnasamy Mohanraj

Crop Improvement Division, ICAR-Sugarcane Breeding institute, Coimbatore-641 007, Tamil Nadu, India
*Corresponding author. E-mail: vpsobhakumari@rediffmail.com

Abstract. In our experiment a F1 hybrid (GU (04)-28-EO2) obtained from *Erianthus procerus* (IND 90-776) x *Saccharum officinarum* (PIO 96-435) was crossed with a commercial variety, Co 06027. Resulted BC1 hybrid (GU 12-25) was crossed with a commercial cane Co 12009. From this cross ten BC2 progenies were selected and analysed for introgression of *Erianthus* genome into *Saccharum*. F1 resulted from 2n+n chromosome transmission and was having the whole 40 chromosomes of *E. procerus* in it. The BC1 and BC2 resulted from n+n transmission. The introgression of *E. procerus* chromosomes into BC2 ranged from 8-10. Amplification of *Erianthus* specific tandem repeat (ESTR) sequences was successfully utilized in identification of genuine hybrids of *E. procerus* x *Saccharum*. No recombination events between *Erianthus* X *Saccharum* could be observed in F1, BC1 and BC2 clones. The current study forms a basis for targeted introgression breeding with a different unexploited species of *Erianthus, E. procerus* in sugarcane improvement programme.

Keywords: *Erianthus*, *Saccharum*, introgression, Genomic *in situ* hybridization (GISH), sugarcane, intergeneric hybrid.

INTRODUCTION

Modern sugarcane cultivars (2n=100-130) are developed from interspecific crosses made one century ago among few parent clones of *Saccharum officinarum* L. (2n=80) the sugar producing species and *S. spontaneum* L. (2n=40-128), a wild species. Due to interspecific hybridization involving the frequent utilization of a limited number of parental clones the genetic base has become narrow and the cultivars are showing limited resilience to biotic and abiotic stresses. It has been realized that an efficient method to broaden the genetic diversity for increased productivity and better adaptability as well as for providing more disease resistance the responsible genes of wild relatives have to be transferred through sugarcane breeding.

As one of the most important wild relative of sugarcane, genus *Erianthus* has vital role in contribution of desirable characters to sugarcane culti-

vars. Different species of *Erianthus* have been the focus of several sugarcane breeding programmes as a valuable contributor with many desirable characters like excellent vigour with strong root system, high fiber content, good ratooning ability and tolerance to biotic and abiotic stresses (Ram et al., 2001, Jackson and Hentry, 2011, Fekuhara et al., 2013). In spite of importance of different species of *Erianthus* in sugarcane breeding, the major constraint in generating intergeneric hybrids is the cross-incompatibility due to high genetic distance between *Saccharum* and *Erianthus*. Another constrain is the difficulty in distinguishing genuine intergeneric hybrids and self-progeny. The recent development of efficient molecular tools helped in identification of intergeneric hybrids such as PCR based analysis of 5 Sr DNA, SSRs, AFLPs and genomic slot blot hybridization have greatly enabled the identification of intergeneric hybrids *Saccharum* x *Erianthus* (D'Hont et al., 1995, Cai et al., 2005, Aitken et al., 2006, Besse et al., 1997) from *S. officinarum* (female) and *Erianthus* as male as well as its back crossed variant (D'Hont et al., 1995, Cai et al., 2005, Krishnamurthy et al., 2007, Nair et al., 2006, Piperidis et al., 2000, Piperidis et al., 2010). In order to detect the alien chromosomes and chromosomal segments in a putative hybrid, advanced cytological methods are widely used. Genomic in situ hybridization (GISH is a powerful cytological tool for identifying the introgression status of alien chromosomes in sugarcane (Alix et al., 1998, Jakson and Hentry, 2011).

At ICAR-Sugarcane breeding Institute, Coimbatore, India, *Erianthus* introgression programme has been going on for the last two decades and most of the cases the wild species E. arundinaceus has been used as female parent as a source of potentially valuable traits. In the present study we used a different species of *Erianthus* i. e. *E. procerus* as a female parent to cross with *S. officinarum* (male parent). Cytologically and also at the molecular level we analysed the F1 and back cross progenies of *E. procerus* x *S. officinarum*. In sugarcane during back crosses the problem of shy flowering and non-synchronous flowering makes the breeder to use different sugarcane clones rather than using one of their parents. Such type of crossing methods is recognised as modified back crosses and resulted progenies are referred as back cross progenies. Here we analysed ten BC2 progenies of *E. procerus* x *S. officinarum* through Genomic in situ hybridization (GISH) and also analysed the amplification of *Erianthus* specific tandem repeat sequences to confirm its hybridity. The genuine hybrid identification and molecular cytogenetic characterization will be helpful for the planning of the breeding strategies for further utilization of transferred traits from *E. procerus*.

MATERIALS AND METHODS

The plant materials used for the study are ten BC2 progenies of a F1 hybrid, GU 04(28) EO2. This F1 hybrid was derived from a cross involving *E. procerus* (female parent) and *S. officinarum* (male parent). *E. procerus* clone, IND 90-776, was collected from Arunachal Pradesh and *S. officinarum* clone, PIO 96-435, was an atypical clone derived from interspecific cross undertaken at the place of origin. The F1 hybrid, GU 04 (28) EO2, was back crossed with a commercial sugarcane cultivar, Co 06027, and obtained BC1 progenies. Among this a confirmed BC1 clone, GU12-25, was crossed with another commercial variety, Co 12009. From this cross BC2 progenies were raised and analysed for introgression of *Erianthus* chromosomes.

From the BC2 population ten clones were randomly selected and its hybridity has been confirmed by amplifying the *Erianthus* specific tandem repeat (ESTR) sequences (Yang et al., 2019). A PCR reaction mixture was prepared (Table 1) and amplification was carried out on a Master Cycler (Eppendorf-Nexus gradient) using the primer pairs ESTR-F and ESTR-R (ESTR F: 5'-AGGAAGTTATGGTGGTGGAGTAT-3'; ESTR R: 5'_CGCCATTCCTATTGC-3'). The PCR programme was performed as follows: Pre denaturation at 94°C for 3 min, 34 cycles of 94°C for 1min, 55°C for 35 sec, 72°C foe 30s and 72°C for10 min. PCR products were run in the 1.5% agarose gel electrophoresis

Single budded cuttings of *E. procerus*, *S. officinarum*, F1, BC1, and BC2cloes were collected from the experimental fields of ICAR-Sugarcane breeding institute and planted in the pots. Root tips were collected after 15 days of planting and the somatic chromosome number has been determined according to Sobhakumari and Asmita, 2014.

For GISH analysis of BC2 progenies the mitotic chromosome preparations were performed as described by Sobhakumari et al. (2020). The mitotic slides were freeze dried in liquid nitrogen and dehydrated by dipping in ethanol. These slides were stored in moisture free

Table 1. PCR reaction mixture.

Component	Volume (µl)
Distilled water	15.0
10xPCR buffer	2.0
dNTP mix (10mM)	1.0
ESTR F (primer)	0.5
ESTR R (Primer)	0.5
DNA (50ng/µl)	0.5
Taq enzyme (5U/µl)	0.5

slide boxes in room temperature. For GISH analysis the genomic DNA from *E. procerus* was isolated, fragmented to 500-1000bp size and labelled with biotin-16dUTP (thermo Scientific-USA) and used as GISH probe. The methodology followed for GISH analysis was as described previously by Sobhakumari et al., 2021. The hybridization mixture consists of 50 ng of labeled probe of *E. procerus*, 50% deionized formamide, 10% dextran sulphate, 0.5ng of labeled salmon sperm DNA. After post hybridization washes and FITC incubation the slides were mounted in Vectasheild (Vector labs, UK) mounting medium with DAPI (4,6-diamino 2-phenylindole). GIAH signals were captured using an Axioscope A1 imager fluorescent microscope with Axicam 202 (Carl Zeiss, Gottingen, Germany). Images were processed using Zen 3.0 software (Carl Zeiss, Gottingen, Germany). For each clone 10-15 cells in metaphase were analysed to calculate the number of *E. procerus* chromosomes.

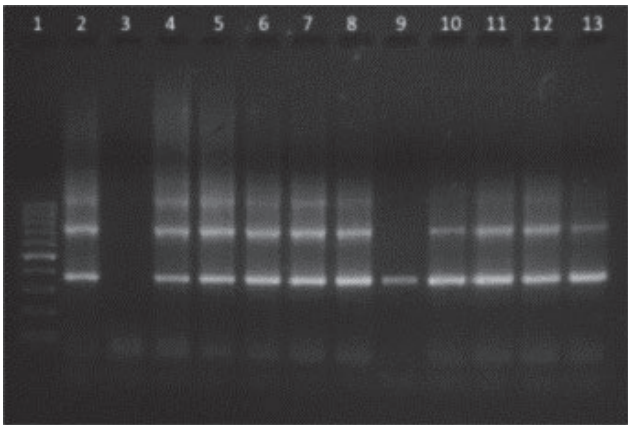

Figure 1. Electrophoretogram of ten BC2 progenies with their parents for amplification of ESTR primer in genomic DNA: 1) 100bp ladder, 2) GU 12-25 (Female parent). 3) Co 12009 (Male parent), 4) GU 19-222 5) GU 19-223 6) GU 19-224 7) GU 19-225 8) GU 19-226 9) GU 19-227 10) GU 19-228 11) GU 19-230 12) GU 19-231 13) GU 19-234.

RESULTS AND DISCISSION

We reported for the first time the chromosome composition of fertile *E. procerus* x *S. officinarum* F1, BC1 (F1 x sugarcane cultivar, Co 06027), and BC2 (BC1 x sugarcane cultivar, Co 775) hybrids *via* genomic *in situ* hybridization (GISH) (Sobhakumari et al., 2020). In this study we utilized a BC1 progeny, GU 12-25 with *20 E. procerus* chromosomes to raise BC2 clones. From the BC2 population obtained from GU 12-25 x Co 12009, a set of clones were randomly selected for the classical and molecular cytogenetic analysis. Identifying the genuine hybrids in the intergeneric hybrid population of *Erianthus* x *Saccharum* is difficult due to the high selfing rate (Besse et al., 1997). As a preliminary evaluation of these BC2 clones for confirmation of its hybridity, PCR amplification was done using *Erianthus* specific 5S rDNA sequences. The results showed that 5S rDNA sequence amplification was not obtained in some of the genuine hybrids (data not shown). As 5S rDNA has one locus per set of basic chromosomes, it is present only in few chromosomes in the *Erianthus* genome. Due to unequal segregation and elimination of *Erianthus* chromosomes at different stages, the advanced back cross progenies may not inherit the chromosome that carry the 5S rDNA loci, this may be the reason for not getting amplification of 5S rDNA sequences in BC2 progenies. This showed that *Erianthus* specific 5S rDNA sequences may not be reliable for the identification of genuine hybrid progenies. Hence the *Erianthus* specific tandem repeat sequences (ESTR) reported by Yang et al. (2019) was used as a marker to confirm the hybridity of randomly selected BC2 progenies. This marker was reported earlier as an *E. arundinaceus* specific marker and it was showing hybridization sites in the sub telomeric regions at one or both ends of 60 chromosomes of *E. arundinaceus* during FISH experiment (Yang et al., 2019). For the first time this marker is used as a hybrid identification tool in the progenies of *E. procerus* x *Saccharum*. In *E. procerus* parent as well as in its true progenies it has amplified successfully around 380bp (Fig. 1). The amplification was not obtained in the sugarcane varieties. The intergeneric population generated from *E. arundinaceus* x *S. officinarum* was initially confirmed with isozyme markers (Deng et al., 2002). However, they could not identify the two hybrids because of absence of banding patterns in different parents. Following this work many reports have come to confirm genuine hybrids with SSR markers, 5S rDNA sequences and internal transcribed spacer (ITS) sequences etc. (Cai et al., 2005a, Cai yet al., 2005b, Zheng et al., 2004). In 2019 Yang et al. reported the AGPR 52/53 sequences for identification of hybrids from *Saccharum* spp. and *E. arundinaceus* and the same sequences worked well in our experiments to identify *E. procerus* x *S. officinarum* backcross hybrids with modifications in the PCR reaction mixture and programme.

In recent years many cytogenetic research has been carried out on chromosome transmission of different generations of intergeneric hybrids involving *Saccharum* and *E. arundinaceus* (Wu et al., 2014, Huang et al., 2015, Piperidis et al., 2000, 2010, Yang et al., 2019). From these reports it was found that elimination of chromosomes appeared to be a common and non-random event during

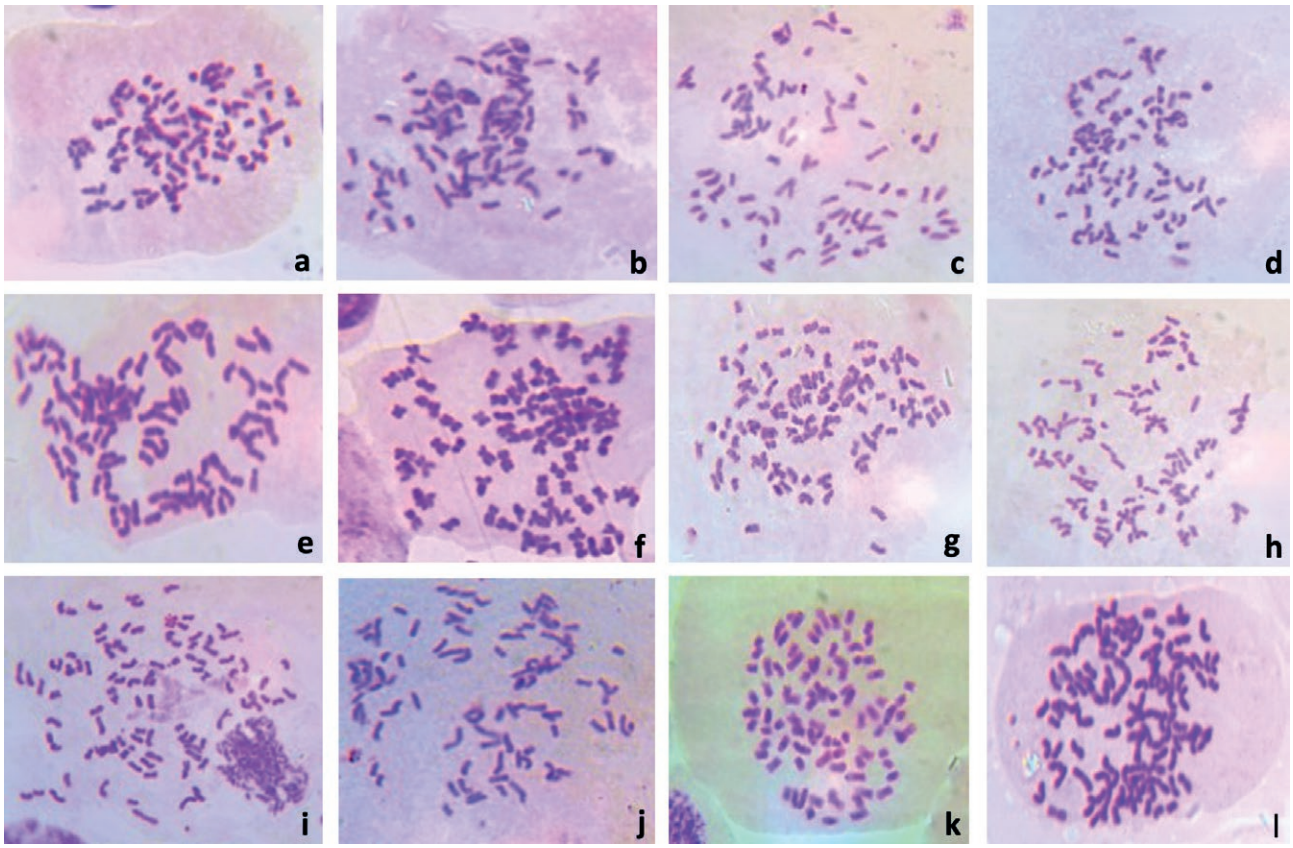

Figure 2. Somatic chromosome number of different clones in BC2 generation and its parental clones: a) GU 19-222 (2n=102), b) GU 19-223 (2n=100), c) GU 19-224 (2n=92), d) GU 19-225 (2n= 100), e) GU 19-226 (2n=92), f) GU 19-227 (2n=92), g) GU 19-228 (2n=100), h) GU 19-230 (2n=96), i) GU 19-231 (2n=96), j) GU 19-234 (2n=88), k) GU 12-25 (2n=92) (Female parent l) Co 12009 (2n=104) (Male parent).

intergeneric hybridization in *Saccharum*. We analysed by genomic in situ hybridization (GISH) the chromosome composition in three generations of *E. procerus* x *Saccharum* intergeneric hybrids: F1 (*E. procerus* x *Saccharum*), BC1 (F1 x sugarcane cultivar) and BC2 (BC1 x sugarcane cultivar). Classical cytological studies in ten BC2 clones showed that the 2n number ranged from 88-102 (Fig. 2). In our earlier *in situ* hybridization studies, it was reported that the F1, GU (04)28 EO2, was showing 2n+n chromosome segregation with 40 chromosomes of *E. procerus* (Sobhakumari et. al., 2020). BC1, GU 12-25, was contained 20 *Erianthus* chromosomes with n+n transmission. When GU-12-25 crossed with a commercial variety, Co 12009, we could raise BC2 population from which 10 clones were randomly selected for GISH analysis with *E. procerus* biotin labelled probe. We found that out of 10 BC2 clones studied two clones were with 8 *E. procerus* chromosomes and only one clone with 9 *E. procerus* chromosomes whereas the majority of them were with 10 *E. procerus* chromosomes (7 clones) (Fig. 3). These results revealed that the number of *E. procerus* chromosomes in transmission of BC1 to BC2 progenies were approximately reduced by half, but we also observed the transmission where reduction was less than half. The details of the parentage, somatic chromosome number and *E. procerus* introgression pattern in F1, BC1 and BC2 generations of *E. procerus* x *S. officinarum* are given in Fig. 4.

In earlier reports GISH analysis allows the visualization of recombination between the species of *Saccharum* i.e., *S. officinarum* and *S. spontaneum* (D'Hont et al., 1996). Different frequencies or abilities of different chromosomes to form homologous recombination among *S. spontaneum* and *S. officinarum* was reported by Wang et al., in 2021. These species are closely related and they showed interspecific recombination. In spite of that in our study the GISH result from F1, BC1 and BC2 clones did not reveal chromosome exchange between *Erianthus* and *Saccharum* chromosomes. The absence of recombination may be due to the genetic distance between *Erianthus* x *Saccharum* which did not allow the gene transfer by chromosome pairing and chiasma formation

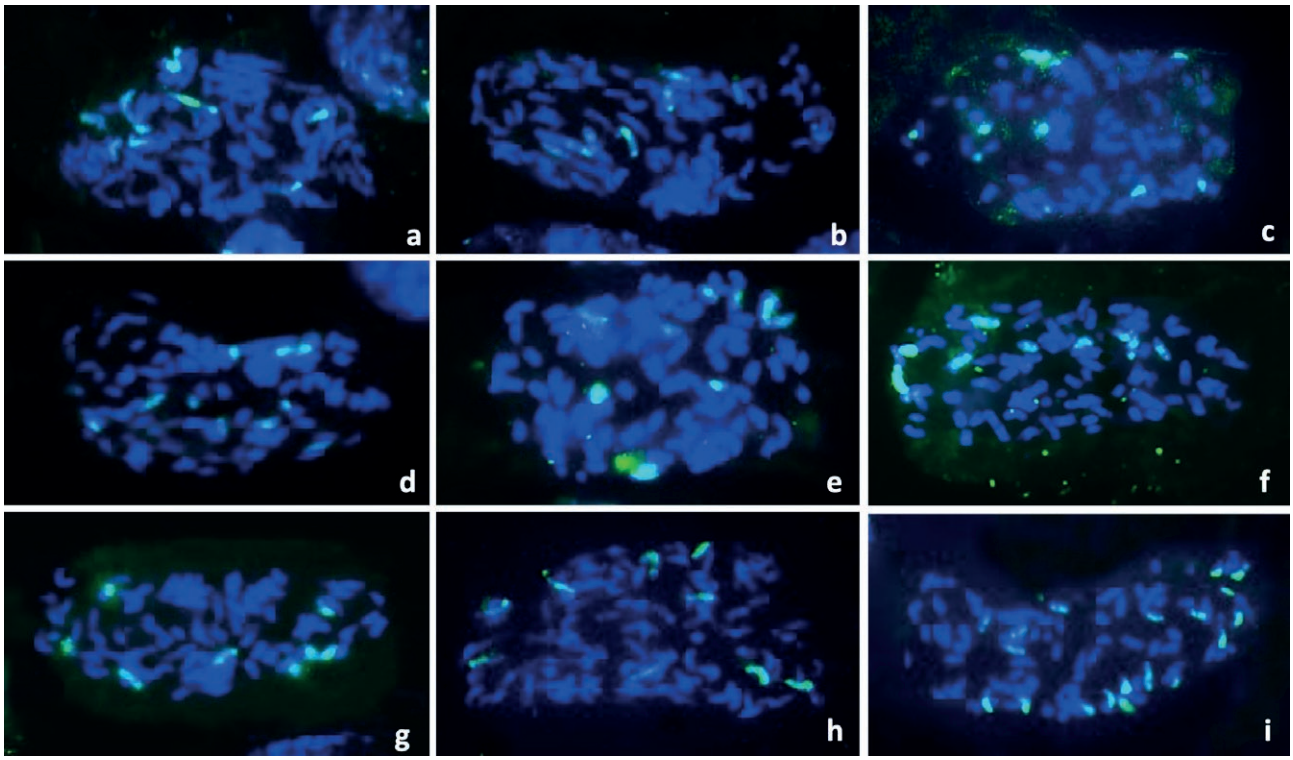

Figure 3. GISH analysis of BC2 (a-h) clones and its female parent (i): a) GU 19-222, b) GU 19-223, c) GU 19-224, d) GU 19-225, e) GU 19-226, f) GU 19-227, g) GU 19-228, i) GU 19-231, j) GU 12-25.

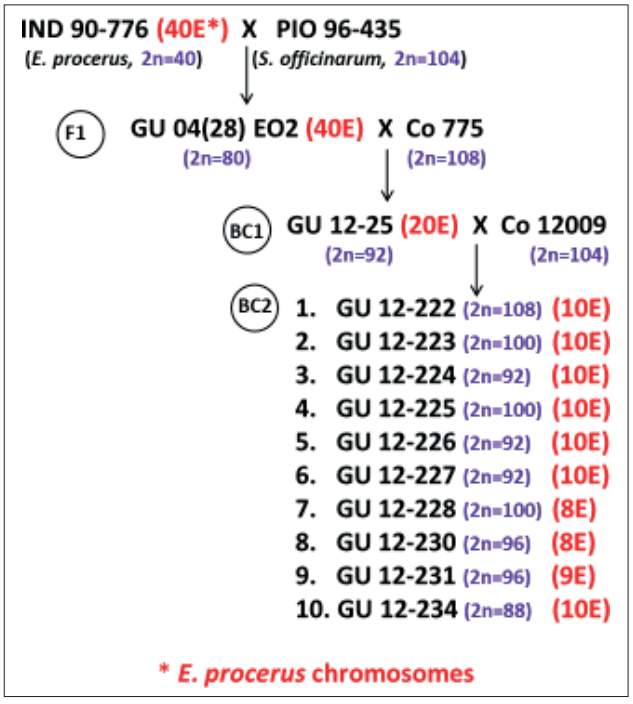

Figure 4. The parentage, somatic chromosome number and *E. procerus* introgression pattern in F1, BC1 and BC2 generations of *E. procerus* x *S. officinarum*.

(Piperidis et al., 2000, 2010). In wheat the role of 'pairing homeologous 1' (ph1) gene has been described in preventing the pairing between related genera (Hauhar and Chibbar, 1999). In sugarcane no such genes have been reported so far.

Chromosome transmission in introgressed population without inter chromosomal exchange/ recombination revealed that *E. procerus* genome has been introgressed into sugarcane cultivars only by whole chromosomes. Such chromosomes in the advanced back cross generations are potential source for gene sequencing and SNP marker production after sorting out them separately. Further back crossing with commercial clones improves the cane traits and juice quality in *Erianthus* x *Saccharum* hybrids along with the biotic and abiotic stress tolerance.

ACKNOWLEDGEMENTS

The authors are grateful to the Director, ICAR-Sugarcane Breeding Institute, Coimbatore, India, for the encouragement and providing facilities to conduct the study. The authors also acknowledge the funding support of the project (EEQ/2019/000124) awarded by

the Science and Engineering Research Board (SERB), Department of Science and Technology, New Delhi. India.

REFERENCES

Aitken K, Li J, Wang L, Qing C, Fan YH. 2006. Characterization of intergeneric hybrids of *Erianthus rockii* and *Saccharum* using molecular markers. Genet. Resour. Crop Evol. 54: 1395–1405. https://doi.org/10.1007/s10722-006-9124-2

Alix K, Paulet F, Glaszmann JC, D'Hont A. 1999. Inter-*Alu* like species-specific sequences in *Saccharum* complex. Theor. Appl. Genet. 99: 962–968. https://doi.org/10.1007/s001220051403

Besse P, McIntyre CL, Burner DM, Almeida CG. 1997. Using genomic slot blot hybridization to assess intergeneric *Saccharum* x *Erianthus* hybrids (*Andropogoneae - Saccharinae*) Genome 40: 428–432. https://doi.org/10.1139/g97-057

Cai Q, Aitken K, Fan YH, Piperidis G, Jackson P, Mclntyre CL. 2005b. A preliminary assessment of the genetic relationship between *Erianthus rockii* and the "*Saccharum* complex" using microsatellite (SSR) and AFLP markers. Plant Science 169: 976–984. https://doi.org/10.1016/j.plantsci.2005.07.002

Cai Q, Aitken K, Deng HH, Chen XW, Fu C, Mcintyre CL. 2005a. Verification of the introgression of *Erianthus arundinaceus* germplasm into sugarcane using molecular markers. Plant Breeding 124: 322–328. https://doi.org/10.1111/j.1439-0523.2005. 01099.x

D'Hont A, Rao, PS, Feldmann P, Berding N, Glaszmann JC. 1995. Identification and characterization of sugarcane intergeneric hybrids, *Saccharum officinarum* and *Erianthus arundinaceus*, with molecular markers and DNA *in situ* hybridization. Theor. App. Genet. 91: 320–326. https://doi.org/10.1007/BF00220894

D'Hont, A, Grivet L, Feldmann S, Rao PS, Berding N, Glaszmann JC. 1996. Characterization of the double genome structure of modern sugarcane cultivars (*Saccharum* spp.) by molecular cytogenetics. Mol. Genet. Genomics. 250: 405–413. https://doi.org/10.1007/BF02174028

Deng ZH, Zhang MQ, Lin WL, Cheng F, Zhang CM, Li YC, Lai LP, Lin YQ, Chen RK. 2010. Analysis of disequilibrium hybridization in hybrid and backcross progenies of *Saccharum officinarum* × *Erianthus arundinaceus*. Agric. Sci. China 9:1271–1277. https://doi.org/10.1016/S1671-2927(09)60216-9

Fukuhara S, Terajima Y, Irei S, Sakaigaichi T, Ujihara K, Sugimoto A, Matsuoka M. 2013. Identification and characterization of intergeneric hybrid of commercial sugarcane (*Saccharum* spp. hybrid) and *Erianthus arundinaceus* (Retz.) Jeswiet. Euphytica 189: 321–327. https://doi.org/10.1007/s10681-012-0748-3

Huang Y, Wu J, Wang P, Lin Y, Fu C, Deng Z, Wang Q, Li Q, Chen R, Zhang M. 2015. Characterization of Chromosome Inheritance of the Intergeneric BC_2 and BC_3 Progeny between *Saccharum* spp. and *Erianthus arundinaceus*. PLoS ONE 10(7): e0133722. https://doi.org/10.1371/journal.pone.0133722

Jackson P, Henry RJ. 2011. *Erianthus*. In: Kole, C. (ed.). Wild Crop Relatives: Genomic and Breeding Resources, Industrial Crops. Springer Verlag, Berlin-Heidelberg. pp. 97–107. https://doi.org/10.1007/978-3-642-20450-010

Jauhar PP, Chibbar RN. 1999. Chromosome mediated and direct gene transfers in wheat. Genome 42(4): 570-583. https://doi.org/10.1139/gen-42-4-570

Krishnamurthi M, Sekar S, Rajeswari S, Kawar PG. 2007. Introgression of *Erianthus* for the development of commercial sugarcane cultivars. Proc Int Soc of Sugar Cane Technol. 26: 678–680.

Nair NV, Selvi, A, Sreenivasan TV, Pushpalatha KN, Mary S. 2006. Characterization of intergeneric hybrids of *Saccharum* using molecular markers. Genet. Resour. Crop Evol. 53: 163–169. https://doi.org/10.1007/s10722-004-1810-3

Piperidis C, Christopher MJ, Carroll BJ, Berding N, D'Hont A. 2000. Molecular contribution to selection of intergeneric hybrids between sugarcane and the wild species *Erianthus arundinaceus.* Genome 43: 1033–1037. https://doi.org/10.1139/g00-059

Piperidis N, Chen J, Deng H, Wang L, Jackson P, Piperidis G. 2010a. GISH characterization of *Erianthus arundinaceus* chromosomes in three generations of sugarcane intergeneric hybrids. Genome 53: 331–336. https://doi.org/10.1139/G10-010

Ram B, Sreenivasan TV, Sahi BK, Singh N. 2001. Introgression of low temperature tolerance and red rot resistance from *Erianthus* in sugarcane. Euphytica 122: 145–153. https://doi.org/10.1023/A:1012626805467

Sobhakumari VP, Asmita D (2014) Cytogenetics and performance analysis of pre-breeding hybrids of *Saccharum officinarum* and S*accharum spontaneum.* J. Sugarcane Res 4: 33–39.

Sobhakumari VP, Mohanraj K, Mohanaprabha M, Mathumathi R. 2021. Establishment of GISH and multi-color GISH techniques to simultaneously discriminate different genomes in *Erianthus procerus* x *Saccharum officinarum* introgressed clones. J. Sugarcane Res. 11: 172-179. https://doi.org/10.37580/JSR.2021.2.11.172-179

Sobhakumari VP, Mohanraj K, Nair NV, Mahadevaswamy HK, Ram B. 2020. Cytogenetic and Molecular approaches to detect alien chromosome introgression and its impact in three successive generations of *Erianthus procerus* × *Saccharum*. *Cytologia* 85(4): 341–346. https://doi.org/10.1508/cytologia.85.341

Wang K, Cheng H, Han J, Esh A, Liu J, Zhang Y, Wang B. 2022. A comprehensive molecular cytogenetic analysis of the genome architecture in modern sugarcane cultivars. Chromosome Res. 30(1):29-41. https://doi.org/10.1007/s10577-021-09680-3

Wu J, Huang Y, Lin Y, Fu C, Liu S, Den, Z, Li Q, Huang Z, Chen R, Zhang R. 2014. Unexpected inheritance pattern of *Erianthus arundinaceus* chromosomes in the intergeneric progeny between *Saccharum* spp. and *Erianthus arundinaceus*. PLoS One 9: e110390. https://doi.org/10.1371/journal.pone.0110390

Yang S, Zeng K, Chen K, Wu J, Wang Q, Li X, Deng Z, Huang Y, Huang F, Chen R, Zhang M. 2019. Chromosome transmission in BC_4 progenies of intergeneric hybrids between *Saccharum* spp. and *Erianthus arundinaceus* (Retz.) Jeswiet. *Sci Rep* 9: 2528. https://doi.org/10.1038/s41598-019-38710-8

Zheng X, Zhang M, Qiwei L, Fangye L, Zuhu D, Rukai C, Yehou Y. 2004. Utilization and characterisation of the genuine intergeneric hybrids from the cross of *Saccharum* and *E. arundinaceum* (2): molecular identification of genuine hybrids from the cross of *Saccharum* and *E.arundinaceum*. Molecular Plant Breeding 2: 35–42.