Gregor Mendel Foundation Proceedings 2007: 16-22.

Substitution of cytoplasm of sugarcane with that of the wild grass *Erianthus arundinaceus*

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Abstract: *Sugarcane is an important agro-industrial crop in the tropical and subtropical parts of the world cultivated for sugar, ethanol and fibre. The sugarcane varieties commercially grown at present are derived from man made interspecific hybrids involving very few Saccharum officinarum L. clones, and thereby with a very narrow cytoplasmic diversity. Due to extensive cultivation of sugarcane varieties with same or very similar cytoplasmic background, the possibility of them becoming susceptible to various diseases and stress factors is high. To enhance the cytoplasmic diversity and to broaden the genetic base, variability could be introduced from other species and related genera. The wild related species S. spontaneum L. and Erianthus species are good candidates for such transfer of cytoplasm to sugarcane. The intergeneric hybrid between E. arundinaceus (Retz.) Jesw. clone IK 76-62 (2n=60) as female and S. spontaneum clone Iritty-2 (2n=64) which was confirmed to have Erianthus cytoplasm by chloroplast DNA polymorphism was used in crosses with sugarcane varieties Co 775 and CoC 671. The progeny raised was also confirmed to have Erianthus cytoplasm and the yield and quality characters of the plants were analyzed. These hybrids were further crossed with sugarcane and the backcross hybrids had Erianthus cytoplasm. These hybrids were having cane characters similar to commercial sugarcane varieties and had the sucrose % juice comparable to sugarcane clones. The chromosome number of the E. arundinaceus x S. spontaneum was 2n=62 and its hybrid with sugarcane varied from 2n=102 to 120. The backcross hybrids with sugarcane had the chromosome number ranging from 2n=102 to 114. The chromosome number of the backcross hybrids attained the level of chromosome number in commercial sugarcane varieties. Many of the backcross hybrids of sugarcane were male sterile, so that it could be easily made use of in crosses as female parent to evolve sugarcane varieties with Erianthus cytoplasm.*

Key words: sugarcane, *Erianthus arundinaceus* x *Saccharum spontaneum*, *Erianthus* cytoplasm, cytoplasmic substitution, chloroplast DNA, male sterility.

INTRODUCTION

Sugarcane is a large grass belonging to the genus *Saccharum* L. of family Gramineae. It is being cultivated in more than 20 million hectares in the tropical and subtropical regions across the world mainly for sugar and ethanol. In India sugarcane is grown in nearly 4.5 million hectares to meet the annual sugar requirement of 22 million tonnes at present in the country. The alternate use of sugarcane as an energy crop is gaining momentum. The ethanol from cane juice for blending with petrol as motor fuel besides that for potable alcohol and as feed stock for the chemical industry, and the cogeneration of electricity from sugarcane biomass necessitates increased sugarcane production in the coming years. Sugar production and productivity in the country experiences wide fluctuations in different years, mainly due to vagaries of nature, which increase the biotic and abiotic stress factors. New sugarcane varieties with resistance or tolerance to the diseases and pests as well as drought, water logging and salinity are being evolved to improve the productivity of sugarcane. The commercial sugarcane varieties under cultivation are derivatives of man made hybrids of *S. officinarum* with the wild *S. spontaneum*. The 'nobilization' as a breeding method involved the backcrossing of the *S. officinarum* x *S. spontaneum* hybrid with *S. officinarum* clone as female parent, thereby having the *S. officinarum* cytoplasm in all the sugarcane varieties. Only three or four *S. officinarum* clones used in Java and India in the early part of the 20th century appear in the pedigree as the cytoplasm donor of almost all the important sugarcane varieties under cultivation across the world. This narrow cytoplasmic diversity in sugarcane poses vulnerability of it to disease epidemics and susceptibility to varying abiotic stress factors (Mangelsdorf, 1983).

The cytoplasm of many of the crop plants such as wheat, maize, rice, oats, barley, sorghum, etc. were substituted with those of their wild related species to study the nuclear-cytoplasmic interactions and to have new genetic combinations with added agronomic features. The most important effect of alien cytoplasm substitution in plants was male sterility and it was effectively utilized in many breeding

programmes. Plant height, flowering time, drought tolerance, heat tolerance, tolerance to iron deficiency, disease resistance and grain quality also were reported to be affected by cytoplasm substitution (Kihara, 1951; Shonnard and Gepts, 1994; Tsunewaki *et al.*, 2002; Liu *et al.*, 2002; Allen, 2005; Hariprasanna *et al.*, 2006; Atienza *et al.*, 2008). The wild species *S. spontaneum* and those belonging to the related genus *Erianthus* are considered to be potential source of genes for biotic and abiotic stress resistance in sugarcane. Bakshi Ram *et al.* (2007) reported that there was no significant contribution of the *S. barberi and S. spontaneum* cytoplasm in hybrids with sugarcane for cane yield and juice quality traits. The present study is on the substitution of the sugarcane cytoplasm with that of *Erianthus arundinaceus*, a cane forming wild robust grass by repeated crossing of sugarcane with an *E. arundinaceus* x *S. spontaneum* hybrid confirmed to have *Erianthus* cytoplasm (Premachandran *et al.*, 2006).

MATERIALS AND METHOD

The *Erianthus arundinaceus* (IK 76-62) x *Saccharum spontaneum* (Iritty-2) hybrid CYM 04-420 (INGR No. 08039; IC 556972) with *Erianthus arundinaceus* cytoplasm was crossed with sugarcane commercial varieties Co 775 and CoC 671 as pollen parent. The seedlings raised in glasshouse were transplanted to field. Selected hybrids from CYM 04-420 x Co 775 and CYM 04-420 x CoC 671 were further crossed with sugarcane varieties Co 775, CoC 671, Co 62198, Co 89029 or BO 130 and the seedlings were raised. All the parental material and the hybrids were clonally maintained at Sugarcane Breeding Institute, Coimbatore.

The morphological features of the hybrids such as plant height, cane diameter, leaf length and leaf width were observed at 8 months and were compared with that of parental material. The plant height was measured from the base to the top visible dewlap. The cane diameter was measured using vernier caliper, at the middle of the 5^{th} internode from the base. Leaf length and leaf width were measured from the third leaf below the topmost visible dewlap leaf. Cane juice quality was determined by hand refractometer (HR); brix reading of juice taken at the middle of the cane with a cane piercer.

The somatic chromosome numbers of the plants were determined from mitotic cells in the root tip. The cane cuttings were planted in sand and root tips from the actively growing roots were taken. The root tips were kept in saturated aqueous solution of α-bromo naphthalene for one hour and were fixed in 3:1 ethanol-acetic acid. After hydrolysis in 1N Hydrochloric acid for about 13 minutes the root tips were stained in leuco basic fuchsin for about 30 minutes. The stained meristem region was excised and squashed in 1% acetocarmine. The chromosome counts were made under the microscope from well spread cells. The meiotic studies were done from pollen mother cells in the immature inflorescence fixed in 3:1 ethanol-acetic acid. The young anthers were smeared in 1 % acetocarmine and pollen mother cells with meiotic division were observed under the microscope. Pollen fertility was determined by squeezing the fully matured anthers in 1:1 glycerine-acetocarmine mixture on glass slides. The fully stained pollen grains were considered fertile and the partially stained and unstained pollen grains sterile.

The cytoplasmic background of the hybrids was determined by polymorphism between *Saccharum* and *Erianthus* at the chloroplast DNA segments *psb*C-*trn*S and *trn*L intron as reported by Premachandran *et al*. (2006). The genomic DNA was isolated from freshly collected young leaves using CTAB method (Sambrook *et al.*, 1989). The chloroplast DNA segment *psb*C-*trn*S was amplified by the primer pairs: F 5'-GGT CGT GAC CAA GAA ACC AC-3'; R 5'-GGT TCG AAT CCC TCT CTC TC-3' (Demesure *et al.*, 1995) as described by Parani *et al*., 2000. The temperature profile consisted of 4 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 63°C and 2 min at 72°C and the final extension for 15 min at 72°C. About 200 ng of PCR product in 10 µL reaction mix was digested with *Hae*III restriction enzyme (GENEI, Bangalore). The restricted product was separated on 1% agarose gel stained with ethidium bromide along with the 1 Kb DNA ladder at 80 m.amps for 2 hours. The chloroplast DNA region *trn*L intron was PCR amplified using a pair of universal primers: F 5'-CGA AAT CGG TAG ACG CTA CG-3'; R: 5'-GGG GAT AGA GGG ACT TGA AC-3' (Taberlet *et al.*, 1991). The PCR profile consisted of 5 min at 95°C, 30 cycles of 15 sec at 94°C, 1 min at 50°C and 1 min at 72°C and the final extension for 10 min at 72°C. The amplified PCR product was digested with *taq*I restriction enzyme (GENEI, Bangalore). The restricted product was separated on 1.5 % agarose gel along with the 100bp DNA ladder at 60 m.amps for 2-3 hours.

RESULTS AND DISCUSSION

The PCR-RFLP of the chloroplast DNA of the hybrids between *E. arundinaceus* x *S. spontaneum* hybrid CYM 04-420 and sugarcane commercial varieties Co 775 or CoC 671 as pollen parent had shown that all such hybrids were with *Erianthus* cytoplasm as that in female parent CYM 04-420. The PCR products of *psb*C-*trn*S and *trn*L intron had products of 1.5 kb and 520 bp respectively. The *psb*C-*trn*S amplified product when restricted with *Hae*III restriction enzyme showed 4 bands, in which *Erianthus* type of restriction pattern had one band at 600 bp whereas in case of *Saccharum* all the bands are less than 400 bp only. In case of *trn*L amplified product, when restricted with *taq*I, out of 4 visible bands, the higher sized band in the case of *Saccharum* is approximately 210 bp, whereas in case of *Erianthus* type of restriction the higher sized band is approximately 160 bp. The backcross hybrids of CYM 04-420 x Co775 or CYM 04-420 x CoC 671 with sugarcane varieties Co 62198, Co 89029, BO 130, etc. also were with *Erianthus* cytoplasm as indicated in the PCR-RFLP of *psb*C-*trn*S and *trn*L intron segments (Fig. 1).

The morphological characters of the parental varieties and the hybrids observed are presented in tables 1 and 2. Plant height and cane diameter of the between *E. arundinaceus* x *S. spontaneum* hybrid CYM 04-420 was low compared to that of the sugarcane clones used in the study, whereas in CYM 04-420 x sugarcane hybrids and (CYM 04-420 x sugarcane) x sugarcane hybrids it was high, nearer to that of the sugarcane parent. The backcross hybrids were having mean cane diameter as that of the sugarcane varieties. The mean leaf length and width of the CYM 04-420 x sugarcane hybrids and the backcross hybrids were also nearer to that of the commercial sugarcane varieties used as parents. This indicated that by further backcrossing with sugarcane varieties the cane morphological characters could be brought at the level of the sugarcane varieties. The Brix was improved by backcrossing the (*Erianthus arundinaceus* x *S. spontaneum*) x sugarcane. The sucrose percentage of juice can be improved further by backcrossing the hybrids with sugarcane.

Most of the CYM 04-420 x sugarcane hybrids and (CYM 04-420 x sugarcane) x sugarcane hybrids were male sterile (Table 4 and Fig. 2). Pistillody, the homeotic transformation of stamen in to pistil was also observed in many hybrids. Only less than 5% of the hybrids had more than 20% fertile pollen. The maximum pollen fertility among the hybrids studied was in (CYM 04-420 x CoC 671) x Co 62198. The female fertility of the hybrids was not affected and seed set was found to be good in all the (*E. arundinaceus* x *S. spontaneum)* x sugarcane hybrids studied. Male sterility is a manifestation of the nuclear-cytoplasmic interaction where the nucleus is brought to an incompatible cytoplasm background. In alien substitution lines of wheat, barley, rice, oats, maize, sorghum, etc. male sterility was observed and in many such crops it was effectively used in hybrid seed production (Kihara, 1967; Tang *et al*., 2007; Hariprasanna *et al*., 2006).

The chromosome number of the *E. arundinaceus* x *S. spontaneum* hybrid CYM 04-420 is 2*n* = 62, resulted from normal reduced (*n*) gametes from both the parents. The chromosome number in progeny from CYM 04-420 x Co 775 (2n = 116) cross ranged from 2n = 102 to 120 and that of CYM 04-420 x CoC 671 (2n = 108) ranged from 2n = 108 to 120 (Table 5). The increase in chromosome number in the progeny than that expected from n + n transmission (31 + 58 = 89 or 31 + 54 = 85) indicated that 2*n* gametes had functioned from either male or female gamete. The chromosome number of the (CYM 04-420 x sugarcane) x sugarcane hybrids ranged from 2*n* = 102 to 120 (Table 5) indicating that only *n* + *n* gametes functioned in the backcross progeny. The range in chromosome number in the backcross progeny is as that observed in commercial sugarcane varieties and hence the use of the *Erianthus* cytoplasm hybrids in crosses with sugarcane may not alter the level of chromosome number in further backcross generations.

The present study has shown that substitution of cytoplasm of sugarcane varieties with that of the wild species *Erianthus arundinaceus* is possible. Male sterility in the hybrids and the backcross hybrids favour the hybridization with sugarcane varieties for repeated backcrossing to develop new sugarcane varieties with *Erianthus* cytoplasm.

Table 1. Morphological and quantitative characters of the parental clones.

Sl. No.	Parent	Plant height (cm)	Cane diameter (cm)	Leaf length (cm)	Leaf width (cm)
1	IK 76-62	286	1.5	151	4.1
2	Iritty-2	200	0.7	100	0.9
3	Co 775	128	2.9	113	6.2
4	CoC 671	165	3.2	129	5.4
5	Co 62198	240	2.2	107	4.9
6	Co 89029	185	2.2	126	4.2
7	BO 130	138	2.6	136	3.4
8	CYM 04-420	110	0.8	88	1.1

Table 2. Mean values of plant morphological characters and hand refractometer Brix in the progeny of hybrids with *Erianthus* cytoplasm

Sl. No	Parent	No. of plants observed	Plant height	Cane diameter (cm)	Leaf length (cm)	Leaf width (cm)	HR Brix (8 months)
1	CYM 04-420 x Co 775	96	134.00	1.64	122.70	3.54	12.60
2	CYM 04-420 x CoC 671	32	149.10	1.71	112.12	3.24	13.90
3	(CYM 04-420 x Co 775)– 871 x BO 130	100	115.64	1.94	126.88	3.52	14.79
4	(CYM 04-420 x Co 775)– 882 x Co 89029	61	126.77	1.93	131.57	3.67	14.74
5	(CYM 04-420 x CoC 671)– 1008 x Co 62198	91	123.85	1.95	132.59	3.13	15.11

Table 3. Chromosome number and pollen fertility in parental clones

S. No	Clone	Chromosome number (2*n*)	Pollen fertility (%)
1	*S. spontaneum* 'Iritty-2'	64	98.1
2	*E. arundinaceus* 'IK 76-62'	60	33.9
3	Co 775	116	82.5
4	CoC 671	108	74.2
5	Co 62198	120	68.0
6	Co 89029	110	73.3
7	BO 130	116	28.7
8	CYM 04-420	62	-

Table 4. Pollen fertility in [(*E. arundinaceus* x *S. spontaneum*) x sugarcane and [(*E. arundinaceus* x *S. spontaneum*) x sugarcane] x sugarcane hybrids

Parentage	Pollen Fertility (%)	No. of plants	Frequency
CYM 04-420 x Co 775	0	32	0.67
	01-10	9	0.19
	10-20	5	0.10
	20-30	1	0.02
	30-40	1	0.02
	>40	0	0.00
	Total	48	
CYM 04-420 x CoC 671	0	12	0.70
	01-10	4	0.24

	10-20	1	0.06
	>20	0	0.00
	Total	17	
(CYM 04-420 x Co 775)-871 x BO 130	0	18	0.72
	01-10	5	0.20
	10-20	1	0.04
	20-30	1	0.04
	>30	0	0.00
	Total	25	
(CYM 04-420 x Co 775)-882 x Co 89029	0	25	0.81
	01-10	5	0.16
	10-20	1	0.03
	>20	0	0.00
	Total	31	
(CYM 04-420 x CoC 671)-1008 x Co 62198	0	10	0.66
	01-10	3	0.20
	10-20	1	0.07
	20-30	0	0.00
	30-40	1	0.07
	>40	0	0.00
	Total	15	

Table 5. Chromosome number in [(*E. arundinaceus* x *S. spontaneum*) x sugarcane and [(*E. arundinaceus* x *S. spontaneum*) x sugarcane] x sugarcane hybrids

Parentage	Chromosome No. (2*n*)	No. of plants observed
CYM 04-420 (2n = 62) x Co 775 (2n = 116)	102	1
	108	4
	110	1
	112	3
	116	2
	118	3
	120	6
Total		20
CYM 04-420 (2n = 62) x CoC 671 (2n = 108)	108	1
	110	4
	112	1
	114	1
	116	1
	120	4
Total		12
(CYM 04-420 x Co 775)– 871 (2n = 102) x BO 130 (2n = 116)	108	1
	110	2
	112	1
	118	1
Total		5
(CYM 04-420 x Co 775)– 882 (2n = 112) x Co 89029 (2n = 110)	102	1
	108	3
	112	1
	114	2
Total		7
(CYM 04-420 x CoC 671)– 1008 (2n = 110) x Co 62198 (2n = 120)	106	1
	110	2
	120	1
Total		4

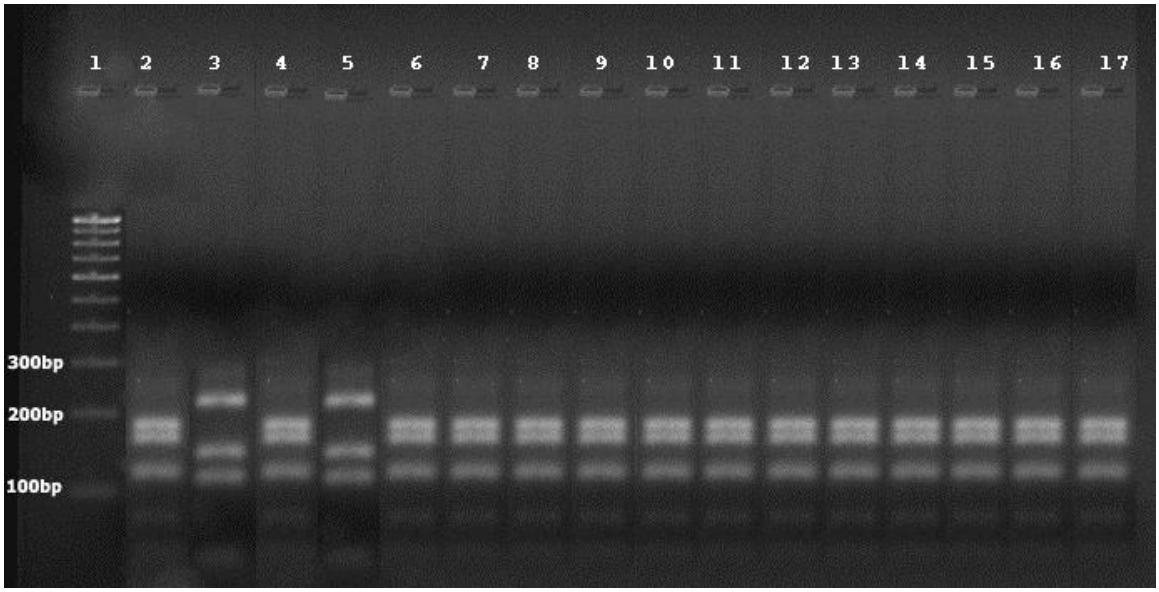

Fig 1: **Restriction pattern of chloroplast DNA segment *trn*L intron with *taq*I in parental clones and hybrids.**

Lane 1: 100bp DNA marker; **Lane 2:** *E. arundinaceus* 'IK 76-62'; **Lane 3:** *S. spontaneum* 'Iritty-2'; **Lane 4:** CYM 04-420 (IK 76-62 x Iritty-2); **Lane 5:** Co 775; **Lane 6:** (CYM 04-420 x Co 775)– 871; **Lane 7 - 11:** (CYM 04-420 x Co 775)– 871x Bo 130; **Lane 12:** (CYM 04-420 x Co 775)– 882; **Lane 13 - 17:** (CYM 04-420 x Co 775)– 882 x Co 89029.

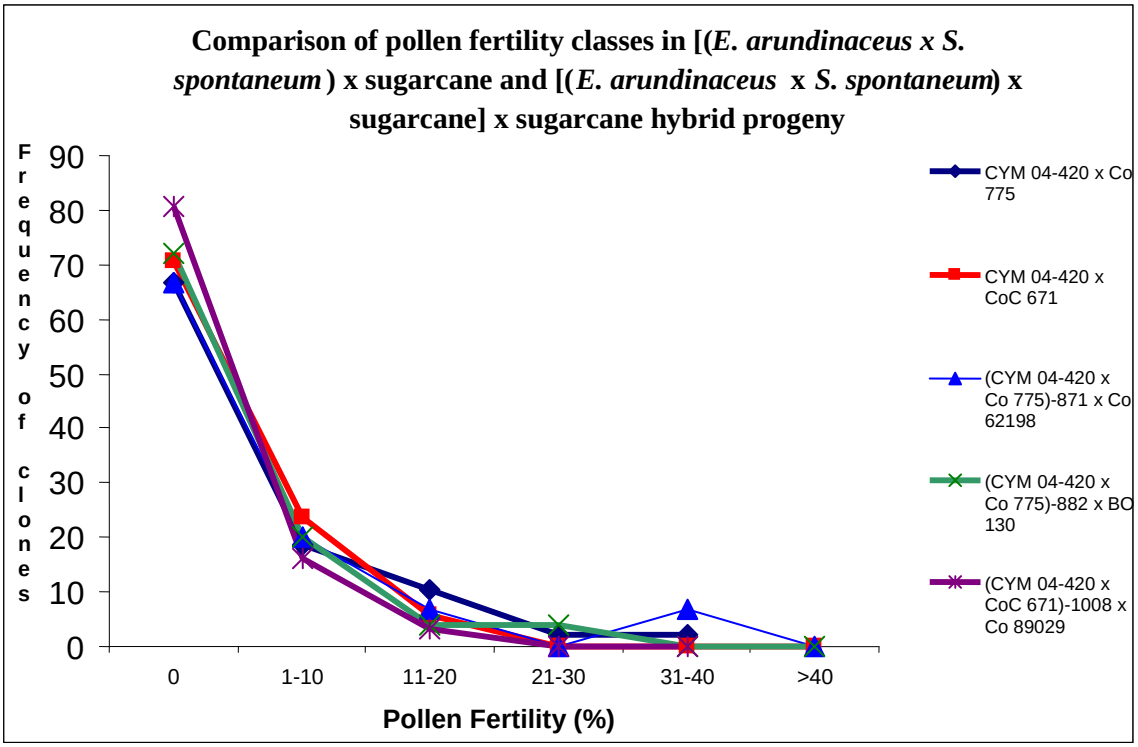

Fig. 2. Comparison of pollen fertility classes in *E. arundinaceus x S. spontaneum* x sugarcane and [(*E. arundinaceus* x *S. spontaneum* x sugarcane] x sugarcane hybrid progeny

REFERENCES

Allen J.O., 2005. Effect of teosinte cytoplasmic genomes on maize phenotype. *Genetics* 169: 863–880.

Atienza S.G., Martin A., Peechioni N., Platani C. and Cattivelli L., 2008. The nuclear-cytoplasmic interaction controls carotenoid content in wheat. *Euphytica* 159: 325 –331.

Bakshi Ram., Nair N.V., Radhakrishnan C.M., Singh N. and Sahi B.K., 2007. Effect of cytoplasmic diversity on performance of sugarcane hybrids. *Indian. J. Genet.* 67(3): 229-231.

Demesure B., Sodzi N. and Petit R., 1995. A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. *Mol. Ecol.* 4: 129-131.

Hariprasanna K., Zaman F.U. and Singh A.K., 2006. Influence of male sterile cytoplasms on the physico-chemical grain quality traits in hybrid rice (*Oryza sativa* L.). *Euphytica* 149:273–280.

Kihara H., 1951. Substitution of nucleus and its effect on genome manifestations. *Cytologia* 16: 177 – 193.

Kihara H., 1967. Cytoplasmic male sterility in relation to hybrid wheat breeding. *Theor. Appl. Gen.* 37 (2): 86-93.

Liu C.G., Wu Y.W., Hou H., Zhang C. and Zhang Y., 2002. Value and utilization of alloplasmic common wheats with *Aegilops crassa* cytoplasm. *Plant Breed.* 121: 407–410

Mangelsdorf A.J., 1983. Cytoplasmic diversity in relation to pests and pathogens. *Sugarcane Breeders' Newsletter* 45: 45-49.

Parani M., Rajesh K., Lakshmi M., Parducci L., Szmidt A.E. and Parida A.K., 2001. Species identification in seven millet species using polymerase chain reaction-restriction fragment length polymorphism of *trnS-psbC* region. Genome 44: 495-499.

Premachandran M.N., Arvinth S. and Lalitha R., 2006. Chloroplast DNA polymorphism in *psb*C-*trn*S and *trn*L intron segments differentiate *Saccharum* and *Erianthus*. *Ind. J. Genet.* 66: 283-286.

Sambrook J., Fritsch E.F. and Maniatis T., 1989. Molecular Cloning- A Laboratory Manual II ed. Cold Spring Harbor, New York.

Shonnard G.C. and Gepts P., 1994. Genetics of heat tolerance during reproductive development in common bean. *Crop Sci.* 34: 1168-1175.

Taberlet P., Gielly L., Patutou G. and Bouvet J., 1991. Universal primers for amplification of three non coding regions of chloroplast DNA. *Plant Mol. Biol.* 17: 1105-1109.

Tang H.V, Pederson J.F., Chase C.D. and Pring D.R., 2007. Fertility restoration of the sorghum A3 male sterile cytoplasm through a sporophytic mechanism derived from sudan grass. *Crop Sci*. 47: 943-950.

Tsunewaki K., Wang G-Z. and Matsuoka Y., 2002. Plasmon analysis of *Triticum* (wheat) and *Aegilops*. 2. Characterization and classification of 47 plasmons based on their effects on common wheat phenotype. *Genes Genet. Syst.* 77: 409–442.