CYTOGENETIC CHARACTERIZATION OF NEW WILD CLONES OF THE *Saccharum* COMPLEX

Roxana Portieles✉, Raisa Rodríguez and María T. Cornide

ABSTRACT. In order to confirm taxonomic identification, a chromosomal count of new wild clones of *Saccharum* complex was performed and Giemsa C-banding technique was adapted to develop the use of polymorphism patterns as possible chromosome markers. Clones, cytogenetically characterized through these techniques, constituted new accessions representative of *Erianthus* and *Saccharum* genera. Twelve chromosomic patterns were revealed among the C-banding and ten of these presented bands at different positions lengthwise the chromosome. Five chromosomic patterns separated three clonal groups: *Erianthus, Saccharum* and hybrid clones. To determine the hybrid origin of ECL 5-4, ECL 1-29 and ECL 1-31 clones, a Factor Analysis of Correspondence (FAC) was performed. To confirm the discriminative value of the five patterns associated to clonal groups and their within-group uniformity, a test of independence (P<0.01) was used. Effectiveness of use of possible cytogenetical markers in the taxonomic identification was demonstrated.

Key words: karyotypes, chromosome banding, *Saccharum*

RESUMEN. Se realizó el conteo cromosómico de un grupo de clones silvestres del complejo *Saccharum*, para corroborar su identificación taxonómica y se aplicó la técnica de bandeo-C con Giemsa, con el objetivo de emplear los patrones de polimorfismo como posibles marcadores cromosómicos. Los clones citogenéticamente caracterizados a través de estas técnicas, constituyeron nuevas accesiones representantes de los géneros *Erianthus* y *Saccharum*. A partir del bandeo-C, doce patrones cromosómicos fueron revelados entre los clones estudiados, presentando diez de estos, bandas en diferentes posiciones a lo largo del cromosoma. Solamente cinco de estos patrones separaron los clones en tres grandes grupos: clones de *Erianthus*, clones de *Saccharum* y clones híbridos. El origen híbrido de los clones ECL 5-4, ECL 1-29 y ECL 1-31 fue confirmado con el empleo del análisis factorial de correspondencia (FAC) y el valor discriminante de los cinco patrones asociados a los grupos clonales y su uniformidad dentro de los grupos fue confirmado mediante el *test* de independencia (p<0.01). Se demuestra la efectividad del uso de marcadores citogenéticos en la identificación taxonómica de nuevas formas de origen desconocido.

Palabras clave: cariotipos, bandeo de cromosomas, *Saccharum*

INTRODUCTION

Chromosome polymorphisms revealed by C-banding and *in situ* hybridization are improving our understanding of the structure, function, organization and evolution of the genes and genomes in plant species. They have been successfully employed separately or sequentially for karyotype and individual chromosome identification, specially in plants with a high number of small chromosomes such as Arabidopsis, rice, Brassica and potato (1, 2, 3).

These chromosome polymorphisms could provide also markers for the identification of individual chromosomes or chromosome segments to assist the monitoring of introgression of the donor genome and the mapping of the gene linkage groups at chromosome level.

Roxana Portieles, Investigador y Raisa Rodríguez, Investigador Agregado, Centro de Ingeniería Genética y Biotecnología (CIGB), Ave: 31, esquina 158, Reparto Cubanacán, Playa; Dra.C. María T. Cornide, Investigador Titular del Instituto Nacional de Investigaciones de la Caña de Azúcar (INICA), MINAZ, La Habana, Cuba.

✉ roxana.portieles@cigb.edu.cu

Modern sugarcane varieties are interspecific hybrids derived from crosses performed a century ago between the high sugar content species, *Saccharum officinarum* L. and its wild relatives, mainly *S. spontaneum* L, donor of useful characteristics such as vegetative vigor and resistance to disease and abiotic stress (4, 5). To broaden this genetic base for breeding purposes, clones of other genera have been employed, mainly *Erianthus arundinaceus*.

Due to their complex polyploid origin, the introgression programs involving these species are hazardly conducted. The unequal transmission of parental chromosomes to the progenies due to the fusion of unreduced female gametes with reduced male gametes and the subsequent loss of chromosomes are frequently reported.

Saccharum chromosomes are small (1-3 µm) and numerous, so the hypotheses on genome structure and evolution have been mainly established based on the distribution of chromosome numbers. Important results have been reported by molecular cytogenetic studies suggesting basic numbers of 10 for *S. robustum* Brandes&Jeswiet and *S. officinarum* L and 8 for *S. spontaneum* L, as well as the

occurrence of recombination events between chromosomes of the latter two species (6, 7, 8, 9). Up to now, C-banding techniques have not been reported for the taxonomical identification of *Saccharum* and *Erianthus* clones.

The present study is aimed to develop the use of Giemsa polymorphism patterns as chromosome markers for these purposes.

MATERIALS AND METHODS

Plant material. Plant materials studied (Table I) included a group of 20 *Saccharum* and *Erianthus* clones and three clones reported as their putative hybrids (10).

Table I. Classification of the clones studied

Name	Species	Origin
Cristalina	*S. officinarum* L.	New Guinea
Badila	*S. officinarum* L.	New Guinea
28 NG289	*S. robustum* Brandes&Jeswiet	*New Guinea*
51NG 91	*S. robustum* Brandes&Jeswiet	New Guinea
Oshima	*S. sinense* Roxb.	*Japan*
Uba	*S. sinense* Roxb.	*China*
Hatooni	*S. barberi* Jeswiet	*New Guinea*
Kewali	*S. barberi* Jeswiet	*India*
SES 182	*S. spontaneum* L.	India
SES 13	*S. spontaneum* L.	Laos
ECL 1-32	*S. spontaneum* L.	Laos
ECL 3-5	*S. spontaneum* L.	Laos
ECL 3-8	*S. spontaneum* L.	Laos
E. arundinaceus	*Erianthus* sp.	*Laos*
E. sara	*Erianthus* sp.	*Laos*
E. elegans	*Erianthus* sp.	*Laos*
ECL 1-24	*Erianthus* sp.	Laos
ECL 1-30	*Erianthus* sp.	Laos
ECL 6-6	*Erianthus* sp.	Laos
ECL 6-1	*Erianthus* sp.	Laos
ECL 5-4	Putative *Saccharum x Erianthus* hybrid	Laos
ECL 1-29	Putative *Saccharum x Erianthus* hybrid	Laos
ECL 1-31	Putative *Saccharum x Erianthus* hybrid	Laos

Clones were provided by (INICA) (11)

These clones were previously evaluated for the main botanical descriptors and agronomic characters under bank conditions (11) according to Daniels & Roach (12). Their taxonomical identification was confirmed by DNA amplification with the pair of 5S- specific primers (8).

All plant materials were kindly provided by the National Institute for Sugarcane Research (INICA).

Somatic metaphase chromosome preparations. Samples consisting of three stalks per stool and two stools per clone were collected. Basal and medium internodes from each selected stalk were germinated in trays at 32°C in the stove and 1-2 cm long fresh root tips were excised and pretreated in 0.002 % 8-hydroxiquinoline at room temperature for 3 h. Root tips were then washed in distilled water and fixed in a 3:1 solution of ethanol : glacial acetic acid for 24 hours. They were incubated in 1N hydrochloric acid at 60°C for 15 min. and stained in 0.5 % Gomory hematoxylin. Each single root tip was transferred to a glass slide and squashed with drops of 45 % acetic acid solution. Fifty slides with good metaphase chromosome spreads of each clone were examined and photographed through an Olympus Vanox-T microscope.

Giemsa C- banding technique. Root tips were pretreated and fixed as previously described for somatic metaphase preparations. After fixation, root tips were washed in distilled water and squashed in 45 % acetic acid.

The Giemsa C-banding technique was performed as previously described for cereals (13) with modifications testing different concentrations of hydrolysis from 0,05 N HCl to 0,2 N and times from 0.5 to 2 min. Variants of time in Giemsa stain were evaluated from 10 min. to 1,5 hours. At last, suitable hydrolysis concentration and time were resulted of 0,1 N HCl for 1 min., as well as time of Giemsa stain of 1 hour.

Slides with cells in mitotic metaphase, previously examined by a phase contrast microscope, were frozen in liquid nitrogen and the cover glass was removed with a sharp blade. The slides were then air dried and incubated in 0.1N hydrochloric acid at 60°C for 1 min. The preparations were denatured in saturated solution of Barium hydroxide at room temperature for 7 min and washed in running tap water. Samples were incubated in 2x SSC buffer solution at 60°C for 1h and dipped in the 2.5% Giemsa solution (phosphate buffer ph=7) for 10-15 min, followed by washing with tap water. After complete drying, the preparations were mounted in Canada Balsam and observed under an Olympus Vanox-T microscope. Good pro-metaphase chromosome spreads, with a complete diploid chromosome set and without any overlapping or heavily attaching sites were photographed.

Chromosome C-banding patterns were evaluated in a sample of 10 preparations for each clone.

Statistical analysis. Each C-banding pattern was scored as a binary variable denoting 1, for the presence and 0, for the absence.

For each clone, the relative frequencies of these patterns were evaluated and considered as variables. To determine the most discriminative C-banding patterns among the clones of the two genera and their putative hybrids, a Factor Analysis of Correspondence (FAC) (14) was separately performed employing STATITFC (15).

Similarly, a FAC including the totality of clones and the eight polymorphic variables previously selected, was performed to determine the patterns associated to each clonal group and to provide a synthetic representation of their polymorphism.

To confirm the discriminative value of the five patterns associated to clonal groups and their within-group uniformity, the frequencies of each pattern were compared between the three clonal groups and among all clones within each group, by test of independence ($P<0.01$) employing STATISTICA (16).

RESULTS AND DISCUSSION

Somatic metaphase analysis. The somatic chromosome numbers of the clones studied are shown in Table II. Different types of meiotic irregularities have been reported; nevertheless, it is generally accepted that *S. officinarum* L. usually forms 40 bivalents, its somatic chromosome number being 2n=80 (17), with a suspected basic chromosome number of x=10 (18, 6).

Table II. Somatic chromosome numbers in the clones studied

Names	Somatic chromosome numbers
Cristalina	2n=80
Badila	2n=80
28 NG289	2n=90
51NG 91	2n=120
Oshima	2n=100-120
Uba	2n=100-120
Hatooni	2n=100-124
Kewali	2n=120
SES 182	2n=64-80
SES 13	2n=54-64
ECL 1-32	2n=54-80
ECL 3-5	2n=64-80
ECL 3-8	2n=64-80
E. arundinaceus	2n=40-60
E. sara	2n=40-60
E. elegans	2n=40
ECL 1-24	2n=30-40
ECL 1-30	2n=30-40
ECL 6-6	2n=30-40
ECL 6-1	2n=30-40
ECL 5-4	2n=30-40
ECL 1-29	2n=30-40
ECL 1-31	2n=30-40

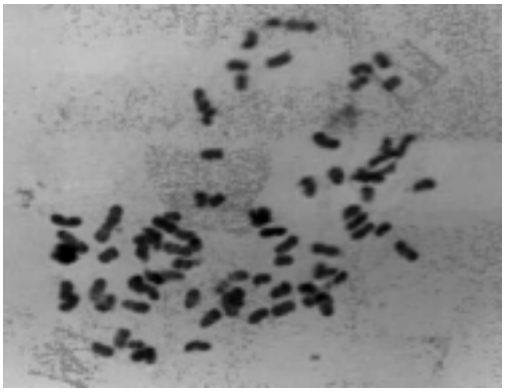
The chromosome number of the other three cultivated species are: *S.sinense* Roxb. 2n=110 to 120, *S.barberi* Jeswiet 2n=82 to 124 (19), and *S.robustum* Brandes&Jeswiet largely encompasses clones with 2n= 60 or 80 but also includes many other forms that may have up to 200 chromosomes (5).

Among the wild species, *S. spontaneum* L. is considered an important genomic component of modern sugarcane varieties. Its chromosome number ranges from 2n=40 to 128 (20) and its proposed basic chromosome number of x=8 (21) was recently supported by cytogenetical molecular studies (7). Based on chromosome numbers, at least three cytotypes and 11 euploid forms are considered in this species (21, 22).

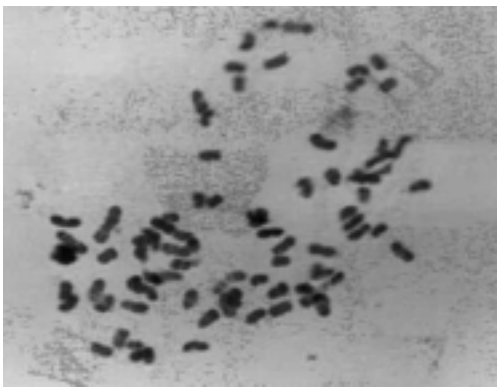
S. spontaneum clones studied could be considered as representatives of euploid forms 54, 64, and 80. Although the most frequent cytotype previously reported in the Eastern region (South Asia and Pacific) is 2n= 80 to 112, the ECL clones (ECL 1-32, 3-5, 3-8) (Figure 1a) could be included in the Central (Indian) cytotype with 2n=40 to 80 (22).

In the present study, the chromosome numbers are coincident with the previous reports in the literature on *Saccharum* clones.

A relatively wide range of somatic chromosome number (2n=20, 30, 40, 50 and 60) has been reported in the genus *Erianthus* (21). Chromosome counts of *Erianthus* clones studied are included in the range. These results confirmed the taxonomic identification of four wild *Erianthus* ECL clones (ECL 1-24, 1-30, 6-6, 6-1) (Figure 1b) and three putative hybrids (ECL 5-4, 1-29 and 1-31), previously reported according to their RFLP nuclear and cytoplasmic polymorphisms and by 5S-specific primer amplifications (23, 10).



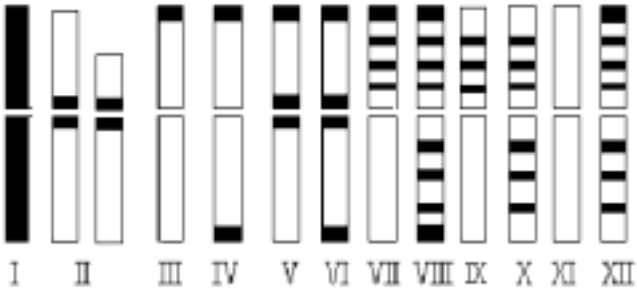
a



b

Figure 1. (a) Karyotype of *Saccharum* complex wild clone: ECL 3-8 (2n=71) 500x, (b) Karyotype of *Erianthus* wild clone: ECL 1-30 (2n=40) 400 x

Giemsa C- banding technique. Twelve chromosomic patterns (I-XII) were revealed among the clones studied (Figure 2). Ten patterns presented bands at different positions lengthwise the chromosome: telomeric (III, IV), interstitial (IX, X), and centromeric (II) bands. A combination of two types of bands were also exhibited: telomeric and centromeric (V, VI), and telomeric and interstitial bands (VII, VIII, XII). Chromosomes evenly and intensely stained (I) and non-stained chromosomes (XI) were also observed.



I-XII C-banding chromosomic patterns (see text)

Figure 2. Schematic representation of the C-banding chromosomic patterns considered in this study

All patterns were polymorphic. A unique variant (XII) was observed in all cells of hybrid ECL 5-4. The patterns II and X are present in all cells of the majority of clones and exhibited within-clone polymorphism. The remaining patterns shown between-clone polymorphism being absent (0) or present (1) in all cells and within-clone polymorphisms due to their intermediate frequency in clonal cells were examined.

Figure 3 shows the results from the FAC performed, considering the variables with the greatest contribution to the total variability (I-X) selected according to previous analyses carried out separately on each clonal group. The first two principal axes of the FAC explained 80 % of the total variability. Five variables (III-VII) were the main contributors to the variability explained by the first axe (E_1) and separate three clonal groups: *Erianthus* (group A) and *Saccharum* (group C) clones in the opposite extremes and hybrid clones (group B) located between them.

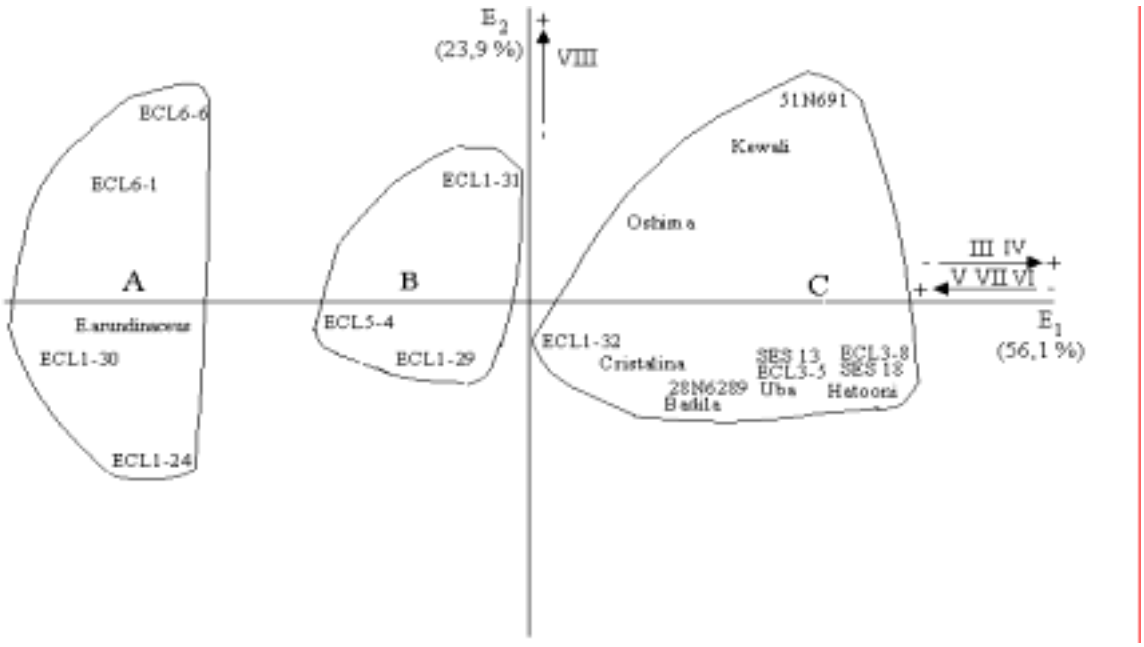
The *Saccharum* karyotype (group C) could be characterized by the presence of chromosomes with only telomeric bands (patterns III and IV) in all cells examined, in opposition to *Erianthus* (group A) where those variants were totally absent. On the contrary, patterns V, VII and VI, exhibiting telomeric and centromeric or interstitial bands, were present in all *Erianthus* clones at high frequencies (>0.50) and were absent or in low frequency in *Saccharum* samples (Figures 4a, 5a and 5b).

All these patterns are present in the hybrid karyotypes (group B) with intermediate frequencies, and thus, located between groups A and C in E_1. These results support previous reports already mentioned, based on molecular polymorphism concerning their putative hybrid origin (Figure 4b).

The presence of pattern VIII was the main variable contributing to the second axe (E_2). This variant was associated to the within-group variability as it was present in only six clones that could be observed in plane (1, 2) above axe E_1.

Comparisons of the relative frequencies of the five banding patterns mainly contributing to group variability confirmed their discriminative value as they were statistically different ($p<0.01$): between the three clonal groups (A-C) for patterns III and IV and between *Saccharum* clones (group C) and the other clones (groups A and B) for patterns V, VI and VII (Table III).

Results of the independence test of the relative frequencies of these patterns among clones within each group were not statistically significant, confirming their discriminative value.



E_1, E_2: two principal axes A-C: *Erianthus*, hybrids and *Saccharum* clonal groups, respectively
III-VIII: relative frequencies of chromosomic patterns III-VIII

Figure 3. Distribution of the clones in plane (1, 2) of a Factor Analysis of Correspondences (FAC)

Figure 4. C-banded, somatic metaphase of the wild clones, (a) *Erianthus* ECL6-6 (2n=40)400x, (b) Putative hybrid ECL1-29 (2n=40) 220x

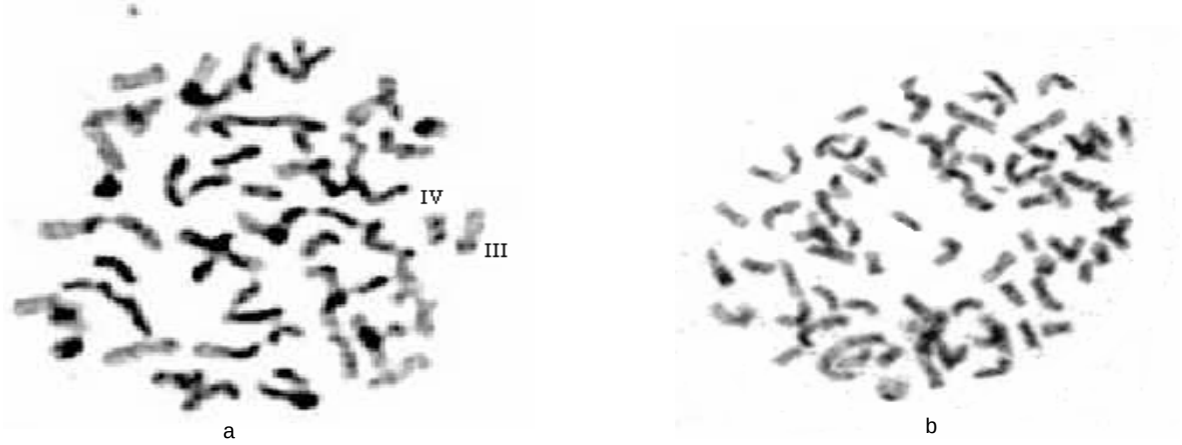


Figure 5. C-banded, somatic metaphase of the tester *Saccharum* complex (a)*S. spontaneum*: SES 182 (2n=68)500X (b) *S. officinarum*: Badila (2n =80)400X

Table III. Results about the comparison test of independent samples between groups

Groups	III	IV	V	VI	VII
A B	**	*	NS	NS	NS
B C	**	**	**	*	**
A C	**	**	**	**	**

*, **: chromosomic pattern frequencies are statistically different between groups for p< 0.05 and <0.01, respectively
NS: non-significant

According to the present results, C-banding polymorphism can be expressed binarily (patterns III, IV), or quantitatively (patterns V-VIII) associated to *Erianthus* and *Saccharum* clones and could be used to assist their taxonomical identification. C-banding patterns can be also associated to individual clones and could be employed jointly with group variants in their identification.

Three hybrids were also successfully separated from representatives studied of both genera by C-banding variants, demonstrating their utility to confirm their origin.

Patterns associated to clonal group differentiation are composed by telomeric (III, IV) bands or their combination with centromeric (V, VI) and interstitial (VII) bands. The presence of patterns with only interstitial bands (IX, X) was not informative for group identification.

REFERENCES

1. Ohmido, N.; Akiyama, Y. and Fukui, K. Physical mapping of unique nucleotide sequences on identified rice chromosomes. *Plant Molecular Biology,* 1998, vol. 138, p. 1043-1052.
2. Dong, F.; Song, J.; Naess, S. K.; Helgeson, J. P.; Gebhardt, C. and Jiang, J. Development and applications of a set of chromosome-specific cytogenetic DNA markers in potato. *Theor Appl Genet.*, 2000, vol. 101, p. 1001-1007.
3. Schwarzacher, T. y Heslop-Harrison, P. Practical *in situ* Hybridization. UK: BIOS Scientific Publishers Limited, 2000.
4. Arceneaux, G. Cultivated sugarcanes of the world and their botanical derivation. *Proc. Int. Soc. Sugar Cane Technol. (ISSCT),* 1965, vol. 12, p. 844-854.
5. Price, S. Cytology of *Saccharum robustum* and related sympatric species and natural hybrids. *US Dept. Agric. Res. Serv. Tech. Bull*., 1965, vol. 1337, 47 p.
6. D'Hont, A.; Rao, P. S.; Feldman, P.; Grivet, L.; Islam-Faridi, N.; Taylor, P. and Glaszmann, J. C. Identification and characterisation of sugarcane intergeneric hybrids, *Saccharum officinarum* x *Erianthus arundinaceus*, with molecular markers and DNA *in situ* hybridisation. *Theor Appl. Genet*., 1995, vol. 91, p. 320-326.

7. D'Hont, A.; Grivet, L.; Feldman, P.; Rao, S.; Berding, N. and Glaszmann, J. C. Characterization of the double genome structure of modern sugarcane cultivars (*Saccharum* spp.) by molecular cytogenetics. *Mol Gen Genet.,* 1996, vol. 250, p. 405-413.
8. D'Hont, A.; Ison, D.; Alix, K.; Roux, C. and Glaszmann, J. C. Determination of basic chromosome numbers in the genus *Saccharum* by physical mapping of ribosomal RNA genes. *Genome,* 1998, vol. 41, p. 221-225.
9. Ha, S.; Moore, P. H.; Heinz, D.; Kato, S.; Ohmido, N. and Fukui, K. Quantitative chromosome map of the polyploid *Saccharum spontaneum* by multicolor fluorescence *in situ* hybridization and imaging methods. *Plant Molecular Biology,* 1999, vol. 39, p. 1165-1173.
10. Coto, O.; Cornide, M. T.; Calvo, D.; Canales, E.; D'Hont, A. and Prada, F. Genetic diversity among wild sugarcane germplasm from Laos revealed with markers. *Euphytica,* 2002, vol. 123, p. 121-130.
11. Pérez, G.; Bernal, N.; Chinea, A.; O'Relly, J. P. and Prada, F. Recursos genéticos de la caña de azúcar. La Habana: Publicaciones IMAGO, 1997.
12. Daniels, J. and Roach, B. T. Taxonomy and Evolution. In: Heinz D. J., ed. Sugarcane Improvement through Breeding. Holland: Elsevier, 1987.
13. Naranjo, T. Identificación de los cromosomas de trigo del triticale "Cachirulo" por homología con los de *Tríticum aestivum*. *An Aula Dei*, 1988, vol. 19, no. 1–2, p. 55-62.
14. Benzecri, J. P. L'analyse des données. Tome II: L'analyse des correspondances. Paris: Dunod, 1973.
15. STATITCF. Version 4.0, 1988.
16. STATISTICA. Release 4.5, Stat Soft, Inc, 1994.
17. Price, S. Cytogenetics of modern sugarcane. *Econ. Bot*., 1963, vol. 17, p. 97-106.
18. Bremer, G. Problems in breeding and cytology of sugar cane. *Euphytica,* 1961, vol. 10, p. 59-78.
19. Heinz, D. J. Sugarcane Cytogenetics. Chapter 15. In: Tsuchiya T & Gupta P K., ed. *Chromosome Engineering in Plants: Genetics, Breeding, Evolution.* Part B. Elsevier, 1991.
20. Babu, C. N. Studies in *Saccharum spontaneum*. Contributions to an "atlas" of chromosome number in *Saccharum spontaneum*. *Proc 3rd All India Conf Sugarcane Research and Development Workers*, Part II, 1957, p. 48-50.
21. Sreenivasan, T. V.; Ahloowalia, B. S. and Heinz, D. J. Cytogenetics. In: Heinz, D. J., ed. *Sugarcane Improvement Through Breeding*. Holland: Elsevier, 1987, p. 211-253.
22. Panje, R. R. and Babu, C. N. Studies in *Saccharum spontaneum* distribution and geographical association of chromosome numbers. *Cytologia,* 1960, vol. 25, no. 2, p. 152-172.
23. Cornide, M. T.; Coto, O.; Calvo, D.; Canales, E.; Prada, F. and Pérez, G. Molecular markers for the identification and assisted management of genetic resources for sugarcane breeding. *Plant Varieties and Seeds,* 2000, vol. 13, p. 113-123.

Recibido: 12 de noviembre de 2002
Aceptado: 2 de junio de 2003