Theor Appl Genet (1994) 87:843–853

THEORETICAL AND APPLIED GENETICS

Phylogenetic analysis of chloroplast restriction enzyme site mutations in the *Saccharinae* Griseb. subtribe of the *Andropogoneae* Dumort. tribe

B. W. S. Sobral[1], D. P. V. Braga[2,*], E. S. LaHood[2], P. Keim[2]

[1] California Institute of Biological Research, 11099 North Torrey Pines Road, Suite 300, La Jolla, CA 92037, USA
[2] Department of Biology, Northern Arizona University, Flagstaff, AZ 86011-5640, USA

Received: 14 January 1993 / Accepted: 17 May 1993

Abstract. Chloroplast (cp) DNA from 32 genotypes representing eight genera and 19 species from the *Andropogoneae* tribe was analyzed using 15 restriction enzymes and Southern hybridization with 12 cpDNA probes that span the complete rice chloroplast genome. Six of the genera, *Saccharum*, *Miscanthus*, *Erianthus*, *Narenga*, *Eccoilopus*, and *Sclerostachya*, are part of the *Saccharinae* subtribe, whereas the other two, *Zea* and *Sorghum*, were used as outgroups. *Narenga*, *Miscanthus*, *Erianthus*, and *Sclerostachya* are presumed to have been involved in the evolution of *Saccharum officinarum* ("noble" or high sucrose sugarcane) via *S. spontaneum* and *S. robustum*. Southern hybridization with the rice cpDNA probes surveyed approximately 3% of the *S. officinarum* 'Black Cheribon' genome and yielded 62 restriction site mutations (18 informative) that were analyzed using cladistic parsimony and maximum likelihood. These site mutations placed the 32 genotypes into nine different chloroplast groups; seven from within the *Saccharinae* subtribe and the two outgroups (maize and *Sorghum*). Phylogenetic inferrence under various assumptions showed that the maternal lineages of *Narenga*, *Miscanthus*, *Sclerostachya*, and *Saccharum* formed a monophyletic group. This group displayed little variation. On the other hand, 5 of 6 *Erianthus* species and *Eccoilopus longisetosus* formed a separate group. The 'Old World' *Erianthus/Eccoilopus* chloroplast was very different from that of the rest of the '*Saccharum* complex' members and was slightly more related to that of *Sorghum bicolor*. Placement of these *Erianthus/Eccoilopus* genotypes was, therefore, in conflict with analyses based on morphology. Surprisingly, *Erianthus trinii*, a New World species, had the same restriction sites as did one *Miscanthus sinensis*. One *Miscanthus* sp. from New Guinea that has a very high chromosome number (2n = 192) had the same restriction sites as the majority of the *Saccharum* genus, suggesting that introgression between these genera occurs in the wild. The *Saccharum* genus was separated into two clades by single site mutation: one containing *S. spontaneum*, and the other containing all of the remaining *Saccharum* species and all 8 commerical hybrids (from various regions of the world). A physical map of the chloroplast of *Saccharum officinarum* 'Black Cheribon' was constructed using 5 restriction enzymes.

Key words: Sugarcane – *Saccharm* – Evolution – Cytoplasmic inheritance – Restriction mapping – Cladistics – Parsimony – Dollo parsimony – Wagner parsiomony – Maximum likelihood – *Saccharum* complex

Introduction

The grass family is one of the most important plant families providing both grasslands that occupy a third of the world's surface and cereal crops upon which humans depend for food (Clayton and Renvoize 1986). The *Andropogoneae* tribe of the grass family is one of the largest, most specialized (Celarier 1956), and most taxonomically defined of the grass tribes (Hartley 1958; Clayton and Renvoize 1986). Within the grasses (Clayton and Renvoize 1986) this tribe is considered to be advanced, and widespread polyploidy has been

Communicated by A. R. Hallauer
* *Permanent address:* Centro de Technologia Copersucar, Caixa Postal 162, 13.400 Piracicaba, S.P., Brazil.
Correspondence to: B. W. S. Sobral

observed (Stebbins 1956; Clayton and Renvoize 1986). The tribe is divided into three subtribes: the *Saccharinea/Germainiinae*, the *Sorghinae*, and the *Ischaeminae/Dimeriinae/Coicinae* (Clayton and Renvoize 1986). The *Saccharinae* are thought to be the most primitive subtribe because both spikeletes of the pair are fertile and rachis internodes are unspecialized (Hartley 1958; Celarier 1956; Clayton and Renvoize 1986). The *Saccharinae* are also typically tall and hygrophilous, which is considered to be the ecologically primitive condition (Hartley 1958). Within the *Saccharinae*, there are two groups of genera. The first group has a panicate inflorescence, thought to represent the primitive condition (Clayton and Renvoize 1986), and seems to radiate from the genus *Saccharum*. The second group of genera has a digitate inflorescence and consists of a number of divergent lines radiating from the genus *Eulalia* (Clayton and Renvoize 1986). It would appear, then, that much might be learned about the evolution of the *Andropogoneae* and speciation in polyploid complexes by studying the *Saccharinae*, in particular *Saccharum* and its relatives.

The *Saccharum* genus includes the agronomically important *S. officinarum* ("noble" sugarcane) genotypes, which have been cultivated because of their high sucrose content for thousands of years. A comprehension of the taxonomy and evolution of *Saccharum* and its relatives has been difficult because of the widespread occurrence of polyploidy and the added complication of facultative vegetative reproduction. In addition, as with the systematics of many cultivated plants, over-classification for practical reasons is common, resulting in additional confusion. Mukherjee (1957) introduced the term '*Saccharum* complex' to represent the grouping of *Erianthus* (Old World species, sometimes collectively known under the invalid generic designation *Ripidum*), *Sclerostachya*, *Narenga*, and *Saccharum* together because of (1) an overlapping geographic range in the Indo-Burma-China border region, (2) their capacity to produce fertile F_1 offspring, and (3) the observation of synchronous flowering in some of the overlapping range. This complex is thought by many (reviewed in Roach and Daniels 1987) to represent the shared gene pool from which *S. officinarum* evolved, although there are other views (for example, see Grassl 1977). On the basis of pedicel modifications and presence or absence of awn, Mukherjee (1957) considered *Sclerostachya* and *Narenga* to be more primitive than *Saccharum* or *Erianthus*, and *Erianthus* more primitive than *Saccharum*. Celarier (1956) mainly on the basis of cytological observations, concluded that *Eccoilopus* and *Miscanthus* represented the most primitive genera within the *Andropogoneae* and that *Saccharum*, *Erianthus*, *Narenga*, and probably *Sclerostachya* were de-rived forms. Daniels et al. (1975) revised the complex to include part of the genus *Miscanthus* (section *Diandra*; Keng 1957) because the other genera did not possess long callus hairs and hairs below the panicle, which they considered required botanical chacteristics for *Saccharum* to have emerged from this extended breeding pool. It is noteworthy that a modern view of grass systematics considers the genus *Saccharum* to include *Erianthus* and *Narenga*, *Miscanthus* includes *Sclerostachya*, and *Eccoilopus* is included in *Spodiopogon* (Clayton and Renvoize 1986). This trend to consolidate genera appears to have begun with the work of Bor (1960).

Morphological characters, on which much of the modern systematics of the tribe depends, have been suggested by some to be frequently homoplastic or convergent (Systma et al. 1991; Gottlieb 1988; for an opposing view, see Donoghue and Sanderson 1992). Although this assumption remains unproven, it can be envisioned to be possible in groups of plants in which extensive reticulate evolution and polyploidy occurs. In our view it would be most enlightening to study all existing data sets. For example, rDNA and isozyme analysis of various members of the *Saccharum* complex has shown that: (1) *S. spontaneum* genotypes are the most variable of *Saccharum* species, (2) the single *Erianthus* and *Miscanthus* genotypes studied are unique, and (3) *S. robustum* genotypes are more variable than *S. officinarum*, although not all of the variation observed in *S. officinarum* is explained by the *S. robustum* genotypes studied (Glaszmann et al. 1989, 1990). Molecular genetic data has also been used to demonstrate that *S. spontaneum* 'SES 208' behaves like an autopolyploid because of random chromosome pairing and assortment (da Silva et al. 1993; Al-Janabi et al. 1993). Burnquist (1991), using random nuclear restriction fragment length polymorphisms (RFLPs), showed that (1) *Erianthus* species are closely related to one another and very distant from *Saccharum* species, (2) *S. spontaneum* is the most variable of the *Saccharum* species, and (3) *S. rubustum* and *S. officinarum* genotypes are closely related. Molecular data may be particularly useful to help test hypotheses that have been formulated from the analysis of traditional characters such as morphology and cytology. A better understanding of evolution and speciation in polyploid complexes would be useful, given that a very large number of plant species are polyploid (Soltis et al. 1992). As an initial step toward understanding phylogenetic relationships within the postulated base of the *Andropogoneae* and the origin of domesticated sugarcane cultivars, we studied the maternal lineage of genera in the postulated base of the *Saccharinae*, the *Saccharum* complex. Genetic variation within the non-recombining, uniparentally inherited, haploid chloroplast genome of members of the *Saccharum*, *Narenga*, *Sclerostachya*, *Miscanthus*, *Erianthus*, and *Eccoilopus* was investigated. Using *Sorghum bicolor* and *Zea mays* as outgroup species, we

Table 1. Plant genotypes and their origins

Species	Group[a]	Genotype	Origin[b]	Cytol[c]	Source[d]	Type[e]
Eccoilopus longisetosus		US 57-11-2	India	30	Houma	E/E
Erianthus arundinaceus	Ripidium	SES 288	Khemipur, India	30	Houma	E/E
	Ripidium	'Mardon'	Pakistan	U	Houma	E/E
E. bengalense	Ripidium	Imp 2886	U	U	Houma	E/E
E. procerus	Ripidium	Kalimpong	U	40	Houma	E/E
E. ravennae	Ripidium	SES 372	Dharam, India	40	Houma	E/E
E. trinii	New World	US 65-14	Argentina	U	Houma	M
Miscanthus sinensis		Zebrinus	U	38	TAES	M
Miscanthus sp.		NG 77-193	New Guinea	192	Houma	S
Narenga porphyrocoma		US 58-4-1	India	30	TAES	N
Saccharum barberi	Saretha	Chunnee	Uttar Pradesh, India	U	TAES	S
	Saretha	Katha	Punjab, India	90, 91, 92	Houma	S
	Nargori	Nargori	Bihar, India	124	TAES	S
S. edule	New Guinea	NH 70-23	New Hebrides	U	TAES	S
S. officinarum		Black Cheribon	Java	80	TAES	S
		NG 57-72	New Guinea	80	TAES	S
		NG 51-131	New Guinea	80	TAES	S
S. robustum	Sanguineum	NG 28-218	Sepik River, NG	70	TAES	S
S. sinense	Pansahi	Uba nanquim	U	U	TAES	S
	Pansahi	Chuk Chee	U	U	TAES	S
S. spontaneum		Coimbatore	Madra, India	64	TAES	Ss
Sclerostachya fusca		US 58-5-2	India	30	Houma	Sf
S. bicolor		Sweetchew	Breeding	20	TAES	Sb
Zea mays		AP 271 (Sweet)	Breeding	20	APG	Zm
Commerical hybrids		EK28	(POJ 100 × EK 2)	80	TAES	S
		POJ 100	(*B. hitam* × Loethers)	89	TAES	S
		POJ 2878	(POJ 2364 × EK 28)	119, 120	TAES	S
		SP 70-1143	(IAC 4865 × unknown)	U	TAES	S
		CP 70-324	(CP61-39 × CP57-614)	U	TAES	S
		Co 206	(Ashy Mauritius × *S. spontaneum* India)	U	TAES	S
		CP 65-357	U	U	TAES	S
		CP 70-321	(CP61-39 × CP57-614)	U	TAES	S

[a] Group, Non-taxonomic grouping used by sugarcane biologists. Generally related to cytological or geographic observations
[b] Origin, Original place of collection (Artschwager 1954; Brandes et al. 1939; Moriya 1940; Price 1968; Panje and Babu 1960). U, Unknown
[c] Cytol, 2n chromosome number (Moriya 1940; Panje and Babu 1960; Price 1957; Burner 1991; Mohan and Sreenivasan 1983)
[d] Source refers to the place from which we obtained a sample. TAES, Texas Agricultural Experiment Station, Weslaco Tex.; Houma, USDA Sugarcane Laboratory at Houma, La.; APG, American Plant Growers
[e] Type, Cytoplasmic type as determined by RFLP analysis (this work): S, *Saccharum*; *S. spontaneum*; Sb, *Sorghum bicolor*, N, *Narenga porphyrocoma*; M, *Miscanthus sinensis*, Sf, *Sclerostachya fusca*; Zm, *Zea mays*; E/E, *Erianthus/Eccoilopus*, as shown in trees

applied cladistic parsimomy (Hennig 1965; Farris 1977) and maximum likelihood (Edwards and Cavali-Sforza 1963; Felsenstein 1973) methods to generate maternal phylogenetic hypotheses.

Materials and methods

Plant materials and DNA manipulations

Plant genotypes and their origins are listed in Table 1. Identification of restriction site variation in chloroplast DNA (cpDNA) was accomplished by the hybridization of cpDNA clones to total DNA from each genotype. Twelve recombinant cpDNA clones from rice kindly provided by Dr. M. Sugiura and are described in Shimada et al. (1989) and Shimada and Suiguira (1991). These clones represent the entire chloroplast genome of rice (see Fig. 1). One microgram total DNA, extracted using the protocol of Honeycutt et al. (1992), was digested with 21 different restriction enzymes (*Alu*I, *Bam*HI, *Bsp*106, *Xmn*I, *Mbo*I, *Xba*I, *Hin*dIII, *Bst*NI, *Sty*I, *Alu*I, *Bcl*I, *Hin*FI, *Eco*RV, *Hin*dII, *Rsa*I, *Eco*RI, *Dra*I, *Dde*I, *Ssp*I, *Nsi*I, and *Pst*I) according to the supplier's directions (Stratagene Cloning Systems, La Jolla, Calif.). Restriction enzymes were selected to (1) preferentially cut AT-rich target sites because introns are generally more AT-rich and frequently evolve more rapidly than exons (Wolfe et al. 1989; Barbier et al. 1991) and (2) maximize the number of fragments revealed per experiment within the resolving capabilities of agarose gel electrophoresis. Restriction fragments were size fractionated in agarose gels, followed by capillary transfer to a nylon membrane (Maniatis et al. 1982). Recombinant DNA probes were radio-

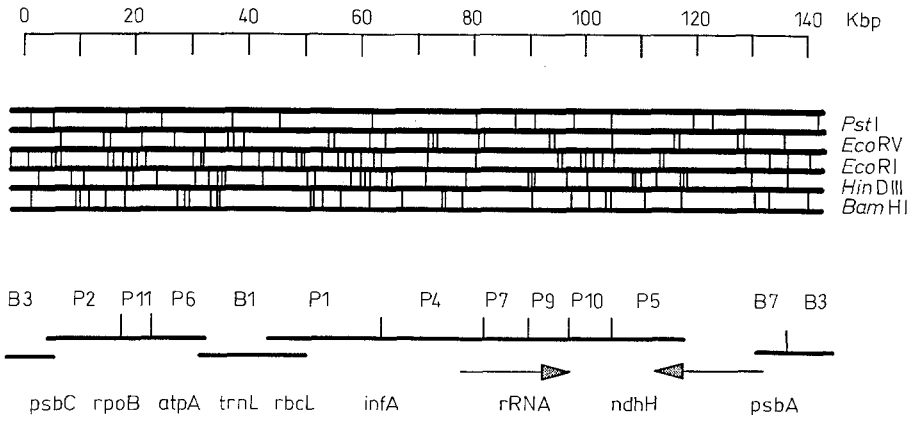

Fig. 1. Restriction map of the 'Black Cheribon' chloroplast genome. Restriction enzyme sites were ordered within the *S. officinarum* 'Black Cheribon' chloroplast genome by hybridization between rice chloroplast probes and double digests of total DNA. The positions of selected genes and the inverted repeat (*arrows*) are inferred from the sequence of the homologous rice probes (Shimada et al. 1989; Shimada and Suguira 1991)

actively labelled by random priming (Feinberg and Vogelstein 1983) and hybridized to the DNA on nylon membranes in an aqueous cocktail at 65 °C as described by Keim et al. (1992). Overnight autoradiography revealed fragments homologous to the probes.

Character definiton

We considered the character to be the presence (scored as 1) or absence (scored as 0) of a clearly defined restriction site. The low level of variation among genotypes in this study allowed restriction fragment patterns to be interpreted to derive individual restriction site characters and character states. Independence of characters was confirmed by examining restriction fragment patterns from adjacent rice probes with the same restriction enzyme. Occasionally a character was detected with more than one probe. In these cases, the most informative probe was used and the other eliminated from the character set. Length mutations (i.e., insertions or deletions) could also lead to non-independent characters being observed from a common probe, but with different restriction enzymes. Therefore, every polymorphic restriction pattern was compared to other restriction enzyme patterns observed with the same probe. Such polymorphic fragments were compared for identical distribution among the genotypes. In no cases were identical distributions found, thereby ruling out the existance of length mutations in our character set. Our scoring method is called "site occurrence analysis" (SOA) (Bremer 1991). The character set we analyzed is shown in Table 2.

Physical mapping of the chloroplast

Restriction enzyme mapping of the *S. officinarum* 'Black Cheribon' chloroplast genome was accomplished by digesting total DNA with 5 different enzymes (*Bam*HI, *Pst*I, *Eco*RV, *Hin*dIII, and *Eco*RI) in all possible double-digest combinations. Digested DNAs were separated by agarose gel electrophoresis, blotted, and analyzed by Southern hybridization using the rice cpDNA clones (see Fig. 1).

Phylogenetic analyses

Phylogenetic hypotheses were inferred using either PHYLIP v 3.42 (Felsentein 1989) or PAUP v 3.0s (Swofford 1991).

Results

Analysis of restriction site mutations

The cpDNA diversity within the 32 genotypes was estimated using 15 restriction enzymes. Southern hybridization with 12 rice cpDNA probes was carried out. For all probe-enzyme combinations, 604 restriction fragments were reliably detected in *Saccharum officinarum* 'Black Cheribon' (reference genotype). This represents approximately 2.5% (approximately 3.0 kilobase pairs) of this genome. Sixty-two restriction sites were polymorphic in this study (Table 3), representing a polymorphism frequency of 9.7% across all of the taxa studied. If no assumptions were made about the ancestral states (i.e. "ancestor = unknown"), then 18 of 62 polymorphisms were synapomorphous and

Table 2. Character matrix[a]

Erianthus/Eccoilopus	10001	00011	11110	00010	00000	00011	01101	01000	00011	10001	10001	10101	10
Miscanthus sinensis	00001	?1111	00100	11100	00000	00010	11011	01000	10111	11011	11000	10100	10
Narenga porphyrocoma	00011	10111	00100	11010	00000	00010	11010	0?000	10111	10011	10000	10110	10
Saccharum spontaneum	00001	10101	00100	11010	00000	00010	11010	00001	10111	10011	10000	10100	10
Sclerostachya fusca	00001	10111	00100	11010	00000	00010	11010	01000	10111	10011	10000	10110	10
Sorghum bicolor	10001	00010	10101	11011	01100	01010	11011	0?000	10111	10001	00000	10100	11
Saccharum	00011	10101	00100	11010	00000	00010	11010	00001	10111	10011	10000	10100	10
Zea mays	11100	00011	11010	11000	10010	10101	1010?	1111?	?1000	00100	10110	01010	00

[a] Characters are in the order presented in Table 3

Table 3. Character description

Character	Probe[a]	Enzyme	Fragments observed[b]	
			Site absent	Site present
1	pRB-1	*Bam*HI	12.2	6.5 + 5.7
2	pRB-1	*Bam*HI	13.0	6.4 + 4.4 + 2.2
3	pRB-1	*Bam*HI	13.0	6.4 + 4.4 + 2.2
4	pRB-1	*Dra*I	3.6	1.6 + 2.0
5	pRB-1	*Dra*I	3.6	3.5 + 0.1
6	pRB-1	*Ssp*I	3.2	3 + 0.2
7	pRB-1	*Ssp*I	3.0	2.5 + 0.5
8	pRB-1	*Xmn*I	4.0	3.3 + 0.7
9	pRB-1	*Bsp*106I	11.5	9.5 + 1.8
10	pRP-7	*Bam*HI	3.8	3.4 + 0.4
11	pRP-2	*Hae*III	1.9	1.0 + 0.9
12	pRP-2	*Bst*NI	1.8	1.0 + 0.8
13	pRP-2	*Bam*HI	9.6	7 + 2.6
14	pRP-2	*Nsi*I	4.3	4.2 + 0.1
15	pRP-2	*Sty*I	2.4	2.1 + 0.3
16	pRP-2	*Ssp*I	9.4	8 + 1.4
17	pRP-2	*Ssp*I	5.4	5.2 + 0.2
18	pRP-2	*Ssp*I	8.0	7.9 + 0.1
19	pRP-2	*Dra*I	6.5	5.5 + 1.0
20	pRP-2	*Eco*RI	2.5	2.35 + 0.15
21	pRP-2	*Eco*RI	3.4	2.8 + 0.6
22	pRP-9	*Bst*I	3.8	3.6 + 0.2
23	pRP-5	*Dra*I	5.6	2.9 + 2.7
24	pRP-5	*Dra*I	4.3	4 + 0.3
25	pRP-5	*Dra*I	6.0	2.5 + 3.5
26	pRP-5	*Nsi*I	2.6	?
27	pRP-5	*Bam*HI	12.9	9.4 + 3.5
28	pRP-5	*Bst*NI	2.7	1.8 + 0.9
29	pRP-5	*Bsp106*I	1.5	1.45 + 0.05
30	pRP-5	*Xmn*I	1.6	1.3 + 0.3
31	pRB-7	*Bcl*I	3.1	2.9 + 0.2
32	pRB-7	*Nsi*I	8.5	6.9 + 1.6
33	pRB-7	*Nsi*I	6.9	6.2 + 0.7
34	pRB-7	*Ssp*I	9.4	?
35	pRB-7	*Ssp*I	8.0	4.3 + 3.7
36	pRB-3	*Dra*I	3.9	2 + 1.9
37	pRB-3	*Xba*I	2.6	2.4 + 0.2
38	pRB-3	*Nsi*I	3.8	3.5 + 0.3
39	pRB-3	*Ssp*I	2.4	1.9 + 0.5
40	pRB-3	*Hae*III	2.4	1.8 + 0.6
41	pRB-3	*Bsp*106I	15.0	12.0 + 3.0
42	pRP-1	*Eco*RV	2.5	1.6 + 0.9
43	pRP-1	*Bsp*106I	2.7	2.4 + 0.3
44	pRP-1	*Hin*DIII	3.5	2.5 + 1.0
45	pRP-1	*Bst*NI	2.8	?
46	pRP-1	*Bst*NI	5.1	2.3 + 2.8
47	pRP-1	*Nsi*I	17.0	12.5 + 4.5
48	pRP-1	*Nsi*I	17.0	14.2 + 2.8
49	pRP-1	*Xmn*I	4.2	2.2 + 2.0
50	pRP-1	*Dra*I	1.9	1.6 + 0.3
51	pRP-11	*Bam*HI	10.0	8.9 + 1.1
52	pRP-11	*Ssp*I	2.4	1.2 + 1.2
53	pRP-11	*Hae*III	1.5	1.3 + 0.2
54	pRP-11	*Eco*RI	1.6	0.85 + 0.75
55	pRP-10	*Xmn*I	3.5	?
56	pRP-10	*Bst*NI	3.8	1.8 + 2.0
57	pRP-10	*Bst*NI	1.0	0.8 + 0.2
58	pRP-10	*Hae*III	4.3	1.8 + 2.5
59	pRP-10	*Nsi*I	4.8	4.3 + 0.5
60	pRP-10	*Eco*RI	2.3	1.8 + 0.5
61	pRP-10	*Eco*RI	2.4	2.3 + 0.1
62	pRP-6	*Nsi*I	9.4	9.2 + 0.2

[a] The probe designations are from Shimada et al. (1989)
[b] *Underlined* fragments were not observed and are postulated to be present. "?" Represents the lack of observed polymorphic fragments. Sizes are in kilobase pairs

the remaining 44 were autapomorphous. The polymorphism frequency is much smaller among other taxa, and informative characters become rare in the derived clades of this study. For example, only one polymorphism separates *Saccharum* genotypes from *S. spontaneum* 'Coimbatore' or *Narenga porphyrocoma* from *Sclerostachya fusca* (Fig. 2).

Construction of a restriction map of the chloroplast of 'Black Cheribon'

A chloroplast genome restriction map was constructed using *S. officinarum* 'Black Cheribon' DNA, 12 rice cpDNA clones, and 5 restriction enzymes (Fig. 1). The total size of the *Saccharum* cpDNA genome was estimated to be 129 kilobase pairs (kb) ± 3 kb by summing the sizes of all of the DNA fragments produced by the 5 restriction enzymes used to generate the map. This estimate is similar to the size of rice cpDNA, which has been determined by complete sequencing of the rice chloroplast genome (134,525 bp; Shimada et al. 1989). This map should be useful for future studies that involve the isolation of specific *Saccharum* restriction fragments for cloning or fine detail mapping with other restriction enzymes. The enzymes used to construct the map were also used to screen for polymorphic restriction sites (see below).

Phylogenetic analyses

The 32 gentoypes were reduced to nine distinct groups based on scoring of the 62 polymorphic restriction sites. Each group was given a representative name, as shown in Table 1. In addition, one *Miscanthus sinensis* genotype was dropped from further anlaysis because many of the characters were dubious (not shown). From the eight remaining groups, a member was selected to represent each group of chloroplasts that shared the same restriction site profiles. This member was the one with the fewest number of uncertain scores, when more than one was available to choose from. These are the terminal taxa used in the data file in Table 2 and the trees. Mean pairwise distances, corrected for missing data, are shown in Table 4.

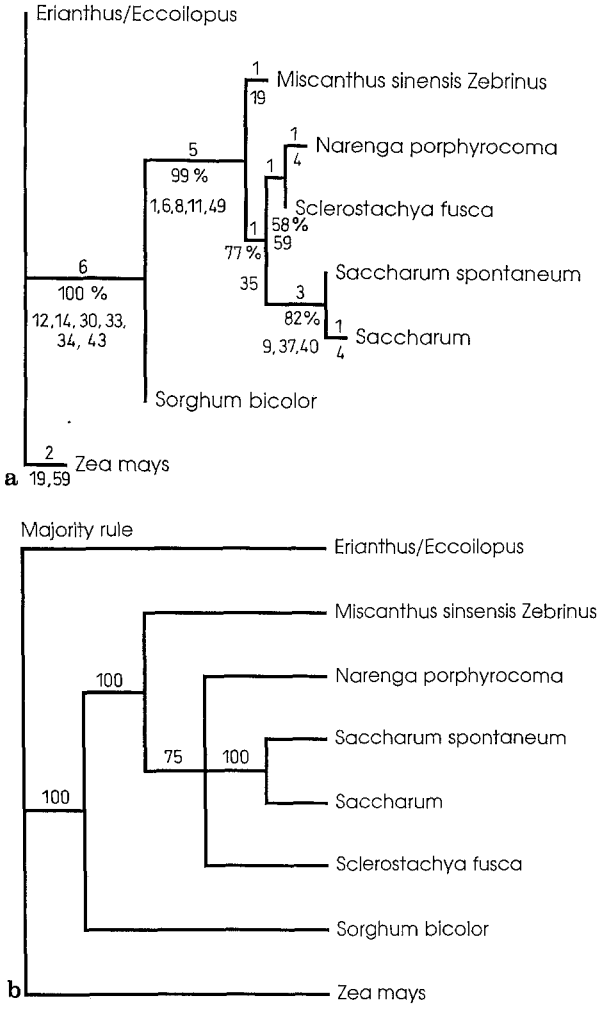

Fig. 2A, B. Wagner trees generated by analysis of 62 polymorphic sites using maize as outgroup taxon and "ancestral states all unknown". **A** Shortest tree (64 steps) decorated with number of steps (informative characters only, *above lines*), 5,000 replicate bootstrap 50% majority rule consensus information (*below lines*, in percentage), and informative characters supporting each branch (*below lines*). Excluding uninformative characters, the tree had a consistency index of 0.857 and a homoplasy index of 0.143. Character designations are the same as in Table 3. **B** Fifty percent majority rule consensus of four trees (shortest and one more step) showing how many of the four trees supported each clade

The Dollo assumption (i.e., that convergent or parallel gains of derived conditions are not allowed) may be considered too rigorous for restriction site data (Sankoff et al. 1983; Alberts et al. 1992). One way around this problem is to assign costs for the transformation of each character state (Sankoff 1975; Alberts et al. 1992). We first used Wagner parsimony (Kluge and Farris 1969; Farris 1970), which permits free reversibility such that changes in character states in either direction are equally probable. The Wagner tree contained 64 steps and is shown in Fig. 2A. Its topology is not significantly different from a Dollo tree (not shown). The only difference between Wagner and Dollo trees is the positioning of very closely related ingroup taxa, namely *Narenga* and *Sclerostachya*: in Dollo trees (not shown) these taxa are usually placed ancestral to the two *Saccharum* taxa, whereas in Wagner trees (Fig. 2A, B) these two taxa form a sister clade in relation to *Saccharum* and *S. spontaneum*. By assigning costs of transformation to the restriction sites, using stepmatrices, we investigated the effect of transformational weightings of 1.1:1 through 2.3:1 (with 0.1:1 increments), and 5:1, on tree topology. Such weightings have been advocated because the costs of transformation are a function of effective nucleotide substitutions in the molecule under study (DeBry and Slade 1985; Alberts et al. 1992). Assignment of a 1.3:1 cost did not alter the Wagner topology, and bootstrap confidence intervals were very similar to Wagner bootstrap confidence intervals (5,000 replicates, not shown). Weighting transformation costs at 2.3:1, as proposed by Alberts et al. (1992) for chloroplast *rbcL* DNA sequence data, yielded the same topology as the Dollo tree (not shown). This topology also was robust under 5,000 bootstrap resamplings (not shown). Interestingly, weighting transformation costs at 5:1 affected tree topology, putting *Erianthus*, maize, and *Sorghum* into a sister clade with respect to the *Saccharum* complex taxa without changing the relationships of the remaining *Saccharum* complex taxa (not shown).

Assumptions about the ancestral state can influence tree topology because it is from the ancestor that PAUP assesses the costs of transformation (Alberts et al. 1992). The Wagner trees shown in Fig. 2 were generated using the "ancestral states unknown" assumption, but changing this to "ancestral states all zero" did not affect tree topology (not shown). The only case in which changing the ancestral state assumption changed the tree topology slightly is the 5:1 weighted tree (not shown). In this case, assigning "ancestral states to all zero" gave rise to a tree in which *Miscanthus sinensis* was connected directly to the root, as was the "*Saccharum* complex clade" (*Narenga*, *Schlerostachya*, *Saccharum*, *S. spontaneum*) and the "outgroup clade" (*Erianthus*, *Sorghum*, maize).

Maximum likelihood analysis of the data was carried out using the RESTML program from PHYLIP (Felsenstein 1989) with run parameter settings for 6-bp, 5-bp, and 4-bp target sites, ancestral states unknown, and 10,000 bootstrap replicates. The resulting tree was identical to the Wagner tree shown in Fig. 2, and the confidence limits placed by bootstrapping were very similar (not shown). Neither variation in target site size nor use of maximum likelihood per se altered tree topology.

Table 4. Mean pairwise intertaxon distances

Taxon	1	2	3	4	5	6	7	8
1. *Erianthus/Eccoilopus*	–	0.344	0.328	0.339	0.306	0.328	0.355	0.542
2. *Miscanthus sinensis*	–	–	0.133	0.148	0.115	0.283	0.164	0.690
3. *Narenga porphyrocoma*	–	–	–	0.066	0.016	0.262	0.049	0.655
4. *Saccharum spontaneum*	–	–	–	–	0.065	0.262	0.016	0.678
5. *Sclerostachya fusca*	–	–	–	–	–	0.246	0.081	0.627
6. *Sorghum bicolor*	–	–	–	–	–	–	0.279	0.707
7. *Saccharum*	–	–	–	–	–	–	–	0.695
8. *Zea mays*	–	–	–	–	–	–	–	–

Because many characters in our data are differences observed between *Z. mays* and the ingroup taxa, we also ran analyses in which this taxon was omitted and *Sorghum bicolor* was used as the outgroup taxon. This did not change the topology of the ingroup, but forced *Erianthus* to assume a derived condition with respect to *Sorghum* and shortened the tree (not shown). We also tried using both *Sorghum* and maize as outgroup taxa, but under these conditions no tree could be found in which the ingroup taxa formed a monophyletic group under the Dollo assumption. This situation could be resolved either by making *Erianthus* an outgroup taxon or by eliminating *Erianthus* form the analysis. When *Erianthus* was included as an outgroup taxon, a clade was formed with *Erianthus*, maize and *S. bicolor* (not shown).

Discussion

We have shown that the genera *Narenga*, *Sclerostachya*, and *Saccharum* form a closely related monophyletic group with respect to their chloroplast genomes. These genera are members of the proposed '*Saccharum* complex' (Mukherjee 1957; Daniels et al. 1975). In contrast, *Erianthus* species, also a proposed part of the *Saccharum* complex, were found to have significantly different chloroplast genomes (Table 4). This result is of interest because modern taxonomy of the *Andropogoneae* considers *Erianthus* and *Narenga* to be part of the *Saccharum* genus. *Erianthus* is considered to be a part of *Saccharum* because the divison of the two genera is based on the existence of an awn (*Erianthus*), a division that is considered to be artificial by Clayton and Renvoize (1986). *Narenga* has coriaceous glumes, which are considered simply to be an extreme expression of a trend found elsewhere in the *Saccharum* genus (Clayton and Renvoize 1986). Furthermore, *Eccoilopus*, found to have the same cpDNA as Old World *Erianthus*, is considered to be a part of *Spodiopogon* because separation of the genera was based solely on the toughness of the rachis (Clayton and Renvoize 1986). Finally, our results show the *Saccharum* species (as defined by sugarcane breeders: see Roach and Daniels 1987) that have been used by humans for sugar production have the same chloroplast restriction sites, and the only variation found within the *Saccharum* genus was in *S. spontaneum*.

Monophyly of the *Narenga-Saccharum-S. spontaneum-Sclerostachya* group was well supported, although higher order structure within this group of genera is tentative because of the small number of differences observed (Fig. 2, Table 3). Bremer (1991) has shown that basal branchings that are supported by few characters are the most likely ones to be influenced by different weighting and scoring schemes. The number of polymorphic sites observed between 'Black Cheribon' and maize is only about 10%, which constitues a very small level of variation when compared to other studies. For example, approximately 2% polymorphisms have been found between 14 species of *Triticum* and *Aegilops* (Bowman et al. 1983), and about 10% of sites were polymorphic between seven *Brassica* species (Palmer et al. 1983). The small number of differences observed in this group of chloroplast genomes may be due in part to facultative vegetative reproduction, which these genera are capable of exploiting. The result of long life cycles could be a slow accumulation of mutations because of long "generation times", or reduced nucleotide substitution rates, as has been demonstrated in palms (Wilson et al. 1990). *Sclerostachya* and *Narenga* both have $2n = 30$, and their chromosomes can pair in interspecific hybrids (Nair and Ratnambal 1965). In addition, homology between *Saccharum* chromsomes and five *Sclerostachya* chromosomes has been shown in interspecific hybrids and backcross progeny (Parthasarathy 1953). Further sampling of *Narenga*, *Sclerostachya*, and especially of the cytologically and morphologically variable *S. spontaneum* is required before the maternal phylogeny of these ingroup species can be understood. In

addition, the targeting of hypervariable regions for DNA sequencing might reveal additional polymorphisms that could be useful in separating the closely related taxa.

One of the most unexpected results of our investigation is the large difference observed between the *Erianthus/Eccoilopus* chloroplasts and those of the other taxa proposed to be in the *Saccharum* complex. Separation of the maternal lineage of *Erianthus/Eccoilopus* genotypes from the ingroup species was clearly supported by a large number of characters (Fig. 2, Table 3). We believe that there is adequate sampling (5 species of *Erianthus* representing different cytological types, plus 1 *Eccoilopus* representative; Tables 1, 5) to suggest that the chloroplast genome of *Erianthus* has a significantly different evolutionary history than the rest of the complex. A divergent chloroplast genome would not preclude *Erianthus* species from introgressing with other genera and thereby participating in the evolution of New Guinea forms of *Saccharum* (Roach and Daniels 1987) and being included in the *Saccharum* genus by modern taxonomic classifications (Clayton and Renvoize 1986). However, given the weight of nuclear data, such as isozymes (Glaszmann et al. 1989), rDNA RFLPs (Glaszmann et al. 1990), nuclear RFLPs (Burnquist 1991), preliminary arbitrarily primed PCR data (R. J. Honeycutt and B. W. S. Sobral, unpublished), and data from the present investigation, we feel that gentoypes of *Erianthus* have gone through a significantly different evolutionary history than genotypes of *Saccharum* and that the split in their lineages must have occurred early in the evolution of the subtribe. Pairwise distances (Table 4) showed that the *Erianthus/Eccoilopus* chloroplast was more related to the *Sorghum* chloroplast than to those *Saccharum* complex members, as did one tree (5:1 weighting, not shown). We note that *Sorghum* chloroplasts display intraspecific variation (Duvall and Doebley 1990), and we only investigated 1 genotype of 1 species. We also note that *Erianthus* is the only genus proposed to be in the complex that has New World distribution, although the 1 New World species we studied had the same cpDNA type as did 1 *Miscanthus* species. In addition, because modern taxonomy of the *Andropogoneae* places *Eccoilopus* as a part of the *Spodiopogen* genus (*Saccharinae*), it should be interesting to include more representatives of both genera to see what the relationship is and whether this particular *Eccoilopus* genotype is a good representative of the genus.

Miscanthus sp. 'NG 77-193' has an unusually high chromosome complement (2n = 192), suggesting introgression with other genera in New Guinea. Chloroplast analysis revealed that it has the same restriction fragment site distribution as sugar-producing *Saccharum* species (Table 4), suggesting that introgression may have occurred with a *Saccharum* genotype. Surprisingly, *Erianthus trinii*, a New World species of this genus, has the same cytoplasm as *M. sinensis* 'Zebrinus' (Table 4), again suggesting some type of introgression or chloroplast capture. Grassl (1974) suggested that the 'Eumiscanthus' section of *Miscanthus* was a product of introgression between some species of *Eulalia* (*E. fastigiata*?) with 2n = 18 and some primitive member of the *Saccharinae* with 2n = 20, to yield 2n = 38 *Miscanthus* Eumiscanthus species such as *M. sinsensis*. Given this hypothesis, it would be interesting to check the chloroplast of *Eulalia* species as well as to sample additional *M. sinensis* genotypes. Because the species of *Miscanthus* that are implicated in the origin of *S. officinarum* are from the *Diandra* section (2n = 40; Roach and Daniels 1987; Grassl 1974), such as *M. rufipilus*, it may not be surprising that *M. sinensis* Zebrinus has a different chloroplast, even if little intraspecific variation is found upon further sampling. Grassl (1974) also suggested that 2n = 40 species of *Miscanthus* might have 20 chromosomes from the 'Eumiscanthus' section and 20 chromosomes from another closely related genus, *Imperata*. We are extending these studies to include representatives of *Imperata* and section *Diandra* of *Miscanthus*.

Our observations suggest that introgression within the *Saccharum* complex may occur in the wild, as has been postulated to explain the origin of many of the species in the complex (Grassl 1974; Roach and Daniels 1987). Alternatively, there is the possibility of mis-identification of some genotypes in the World Collection. We had positive morphological identification for the materials used in our investigation, but it was based on vegetative characteristics only. If we rule out mis-classification, our results suggest that caution must be exercised in interpreting results from an analysis of few representatives of each species because variation within the species may occur. It also suggests that for these plants the maternal phylogeny may not be a good indicator of organismal phylogeny.

Our analysis of 16 genotypes of *Saccharum* from diverse geographic locations representing 5 species (as defined by sugarcane breeders; Roach and Daniels 1987) and 8 interspecific hybrids (*S. officinarum* × *S. spontaneum* crosses, in most cases, with subsequent backcrossing to the maternal parent) revealed a single chloroplast variant within the genus, that of the only *S. spontaneum* genotype analyzed (Table 4). *S. spontaneum* is a highly variable species: its geographic distribution is by far the widest of all members of the genus (as defined by sugarcane breeders; Roach and Daniels 1987); its chromosome numbers vary from 2n = 40 to 2n = 128 (Panje and Babu 1960); isozymes and nuclear rDNA are the most variable of the genus (Glaszmann et al. 1989, 1990); and it has a wide range of morphological variation. *S. spontaneum* has been

long thought to be the primary species within the genus, and the one from which *S. robustum* and, ultimately, *S. officinarum* were derived, potentially through introgression with other members of the complex, during migration southward from the Indo-Burma-China border region which has been postulated to be the center of radiation and diversity for *Saccharum* and many members of the complex (Brandes et al. 1939; Roach and Daniels 1987). Because of the high variability of *S. spontaneum*, it is premature to suggest that there is only one cytoplasmic type. We plan to extend our studies to include a variety of other cytological, geographical, and morphological types. In particular, 2n = 80 forms of *S. spontaneum* from New Guinea will be included to assess whether they are the cytoplasmic donors of the remaining *Saccharum* species. These will also be compared to 2n = 40 forms of *S. spontaneum* from India, which are suggested to be their progenitors (Grassl 1974).

Contrary to *S. spontaneum*, in *S. officinarum* we believe adequate sampling exists to suggest that all high-sucrose-producing *Saccharum* species very likely have only one cytoplasmic type, as defined herein. We surveyed a fairly large number of genotypes from diverse species and diverse geographic locations (Table 1). In addition, DNA sequence data from mitochondrial and chloroplast loci did not reveal any polymorphisms within the sugar-producing *Saccharum* species or between *S. officinarum* and *S. robustum* (Al-Janabi and Sobral, in preparation). These genotypes represent various domesticated forms of the *Saccharum* genus and the proposed progenitor species, *S. robustum* (Roach and Daniels 1987; Grassl 1974, 1977), suggesting the some type of cytoplasmic bottleneck occurred during migration southward to New Guinea.

There are important breeding and economic implications to the lack of cytoplasmic diversity in the cultivated forms of sugarcane (Manglesdorf 1983), not the least of which is a lack of genetic diversity in the field. However, because of the promiscuity of the genera in the complex, alternative cytoplasms could be introduced readily by crossing with other species and genera as females. Even the well-established agronomic practice of "nobilization" (repeated backcrossing to the *S. officinarum* or "noble" parent) need not be changed, except that the recurrent female parent needs to be *S. spontaneum* so that the new cytoplasm is present in the progeny. Nobilization is based on 2n + n transmission of genomes in *S. officinarum* × *S. spontaneum* crosses; however, Price (1957) has shown that 2n + n transmission also occurs in *S. spontaneum* × *S. officinarum* crosses, suggesting that nobilization should be possible with recurrent backcrossing to *S. spontaneum* as a female parent. More detailed studies of the chloroplast of various genotypes of *S. spontaneum* are underway; these should allow breeders to select different chloroplasts to be studied with respect to agronomic performance (Al-Janabi and Sobral, unpublished data). The main problem that breeders might have to face is to find good pollen parents within the commercial genotypes. From a phylogenetic perspective, it is interesting that *S. officinarum* genotypes do not display 2n + n transmission (or n + 2n transmission) when crossed with *S. robustum* genotypes, in which normal n + n transmission is observed (Price 1957).

In summary, we have shown that monophyly of the *Saccharum-S. spontaneum-Narenga-Sclerostachya* is well-supported by chloroplast analysis and that these chloroplast genomes are very closely related. In addition, *Erianthus/Eccoilopus* had a distanct chloroplast genome and seems to be more closely related to *Sorghum* and maize. Introgression seems to have occurred in *Miscanthus* genotype. All New Guinea forms of *Saccharum* as well as all of the commercial hybrids studied had the same chloroplast restriction sites, suggesting a bottleneck in the evolution or selection of high-sucrose producing forms of *Saccharum* and its closest relatives.

Acknowledgements. We thank Michael McClelland (CIBR, La Jolla Calif.) for friendship, opportunities, suggestions, and understanding; James Irvine (Texas Agricultural Experiment Station, Weslaco) for guidance and morphological identification of the plants before DNA extraction; Elizabeth Kellogg (Harvard Arboretum) for guidance; Rhonda Honeycutt (CIBR, La Jolla Calif.) for extraction of genomic DNAs and countless critiques of the manuscript, William Burnquist (Copersucar, SP, Brazil) for arranging for DPVB to conduct laboratory experiments; and Craig Hobart (CIBR, La Jolla Calif.) for technical assistance. We also thank the many unnamed sugarcane scientists from various countries that helped us locate and sample many of the species in this study. This work was supported by a grant from the International Consortium for Sugarcane Biotechnology to BWSS and is dedicated to sugarcane biologists thoughout the world, in particular one A. J. Manglesdorf, *in memorium*; may future generations listen! PK and ESL were supported in part by USDA grant 92-373000-7523 to PK. DPVB was supported in part by fellowship no. 460439/92.8 from the Brazilian National Research Council (CNPq).

References

Alberts VA, Mischler BD, Chase MW (1992) Character-tate weighing for restriction site data in phylogenetic reconstruction, with an example from chloroplast DNA. In: Soltis PS, Soltis DE, Doyle JJ (eds) Molecular systematics of plants. Chapman and Hall, New York, pp 369–403

Al-Janabi SM, Honeycutt RJ, McClelland M, Sobral BWS (1993) A genetic linkage map of *Saccharum spontaneum* (L.) 'SES 208'. Genetics 134:1249–1260

Artschwager E (1954) A taxonomic study of *Saccharum sinense* Roxb. and *S. barberi* Jeswiet. USDA Tech Bull No. 1089, Washington D.C.

Barbier P, Morishima H, Ishihama A (1991) Phylogenetic relationships of annual and perennial rice: probing by direct DNA sequencing. Theor Appl Genet 81:693–702

Bor NL (1960) The grasses of Burma, Ceylon, India and Pakistan. Pergamon Press, London

Bowman CM, Bonnard G, Dyer TA (1983) Chloroplast DNA variation between species of *Triticum* and *Aegilops*: location of the variation on the chloroplast genome and its relevance to the inheritance and classification of the cytoplasm. Theor Appl Genet 65:247–262

Brandes EW, Sartoris GB, Grassl CO (1939) Assembling and evaluating wild forms of sugarcane and closely related plants. Proc 6th Int Soc Sugarcane Technol Congr: 128–154

Bremer B (1991) Restriction site data from chloroplast DNA for phylogenetic reconstruction: is there only one accurate way of scoring? Plant Syst. Evol 175:39–54

Burner DM (1991) Cytogenetic analyses of sugarcane relatives (*Andropogoneae*: *Saccharinae*). Euphytica 54:125–133

Burnquist WB (1991) Development and application of restriction fragment length polymorphism technology in sugarcane (*Saccharum* spp.) breeding. PhD thesis, Cornell University, Ithaca N.Y.

Celarier RP (1956) Cytotaxonomy of the *Andropogoneae*. I. Subtribes *Dimeriinae* and *Saccharinae*. Cytologia 21:272–291

Clayton WD, Renvoize SA (1986) Genera *Graminum*: grasses of the world. Kew Bulletin Additional Series XIII, Her Majesty's Stationary Office, London

daSilva J, Sorrells ME, Burnquist WL, Tanksley SD (1993) Sugarcane (*Saccharum spontaneum*) genome analysis by means of restriction fragment length polymorphisms. Genome 36:782–791

Daniels J, Smith P, Paton N, Williams CA (1975) The origin of the genus *Saccharum*. Sugarcane Breed Newsl 36:24–39

DeBry RW, Slade NA (1985) Cladistic analysis of restriction endonuclease restriction maps within a maximum-likelihood framework. Syst Zool 34:21–34

Donoghue MJ, Sanderson MJ (1992) The suitability of molecular and morphological evidence in reconstructing plant phylogeny. In: Soltis PM, Soltis DE, Doyle JJ (eds) Molecular systematics of plants. Chapman and Hall, New York, pp 340–368

Duval MR, Doebley J (1990) Restriction site variation in the chloroplast genome of *Sorghum* (Poaceae). Syst Bot 15:472–480

Edwards A, Cavali-Sforza L (1963) The reconstruction of evolution. Ann Hum Genet 27:105

Farris JS (1970) Methods for computing Wagner trees. Syst Zool 34:21–34

Farris JS (1977) Phylogenetic analysis under Dollo's law. Syst. Zool 26:77–88

Feinberg AP, Vogelstein B (1983) A technique for radiolabelling DNA restriction fragments to a high specific activity. Anal Biochem 132:6–13

Felsenstein J (1973) Maximum likelihood and minimum-step methods for estimating evolutionary trees from data on discrete characters. Syst Zool 22:240–249

Felsenstein J (1989) PHYLIP-Phylogeny Inference Package. Cladistics 5:164–166

Glaszmann JC, Noyer JL, Fautret A, Feldmann P, Lanaud C (1989) Biochemical genetic markers in sugarcane. Theor Appl Genet 78:537–543

Glaszmann JC, Lu YH, Lanaud C (1990) Variation of nuclear ribosomal DNA in sugarcane. J Genet Breed 44:191–198

Gottlieb LD (1988) Towards molecular genetics in *Clarkia*: gene duplications and molecular characterization of PGI genes. Ann Mo Bot Gard 75:1169–1179

Grassl CO (1974) The origin of sugarcane. Sugarcane Breed Newsl 34:10–18

Grassl CO (1977) The origin of sugar-producing cultivars of *Saccharum*. Sugarcane Breed Newsl 39:8–33

Hartley W (1958) Studies on the origin, evolution, and distribution of the *Gramineae*. I. The tribe *Andropogoneae*. Aust J Bot 6:116–128

Hennig W (1965) Phylogenetic systematics. Annu Rev Entomol 10:97–116

Honeycutt RJ, BWS Sobral, P Keim, Irvine JE (1992) A rapid DNA extraction method for sugarcane and its relatives. Plant Mol Biol Rep 10:66–72

Keim P, Beavis W, Schupp J, Freestone R (1992) Evaluation of soybean RFLP marker diveristy in adapted germplasm. Theor Appl Genet 85:205–212

Keng YL (1957) *Claves Generum* et *Specierum Graminearum Primarium Sinicarum*. Peking

Kluge AG, Farris JS (1969) Quantitative phylogenetics and the evolution of anurans. Syst Zool 18:1–32

Manglesdorf AJ (1983) Cytoplasmic diveristy in relation to pests and pathogens. Sugarcane Breed Newsl 45:45–49

Maniatis T, Fritsch E, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Mohan N, Sreenivasan TV (1983) Chromosome number in the genus *Erianthus*, Michx. (Poaceae) of Indonesian Arquipelago. Cell Chromosome Res 6:14–16

Moriya A (1990) List of chromosome numbers in the genus *Saccharum* and related genera. Jpn J Genet 16:126–136

Mukherjee SK (1957) Origin and distribution of *Saccharum*. Bot Gaz 119:55–61

Nair MK, Ratnambal MJ (1965) Pachytene analysis in *Narenga* × *Sclerostachya* hybrid. Proc 12th Int Soc Sugarcane Technol Congr:875–877

Palmer JD, Shields CR, Cohen DB, Orton TJ (1983) Chloroplast DNA evolution and the origin of amphidiploid *Brassica* species. Theor Appl Genet 65:181–189

Panje RR, Babu CN (1960) Studies in *Saccharum spontaneum* distribution and geographical association of chromosome numbers. Cytologia 25:152–172

Parthasarathy N (1953) Chromosome elimination in *Saccharum*. Nature 168:383–384

Price S (1957) Cytological studies in *Saccharum* and allied genera. III. Chromosome numbers in interspecific hybrids. Bot Gaz 144–159

Price S (1968) Chromosome transmission by *Saccharum robustum* in interspecific crosses. J Hered 59:245–247

Roach BT, Daniels J (1987) A review of the origin and improvement of sugarcane. In: Copersucar Int Sugarcane Breed Workshop. Copersucar, SP, Brazil, pp 1–32

Sankoff D (1975) Minimal mutation trees of sequences. Siam J Appl Math 28:35–42

Sankoff D, Morel C, Cederegen RJ (1983) Simultaneous comparison of three or more sequences related by a tree. In: Sankoff D, Krustal B (eds) Time warps, string edits, and macromolecules: the theory and practice of sequence comparisons. Addison-Wesley, Reading, Mass., pp 253–263

Shimada H, Suiguira M (1991) Fine structural features of the chloroplast genome: comparison of the sequenced chloroplast genomes. Nucleic Acids Res 19:983–995

Shimada H, Whittier RF, Hiratsuka J, Maeda Y, Hirai A, Sugiura M (1989) A physical map and clone bank of rice (*Oryza sativa*) chloroplast genome. Plant Mol Biol Rep 7:284–291

Soltis DE, Soltis PS, Milligan BG (1992) Intraspecific chloroplast variation: systematic and phylogenetic implications. In: Soltis PS, Soltis DE, Doyle JJ (eds) Molecular systematics of plants. Chapman and Hall, New York, pp 117–150

Stebbins GL (1956) Cytogenetics and evolution of the grass family. Am J Bot 43:890–905

Swofford DL (1991) PAUP: Phylogenetic analysis using parsimony, version 3.0s Computer program distributed by the Illinois Natural History Survey, Champaign, Ill.

Systma KJ, Smith JF, Berry PE (1991) Biogeography and evolution of morphology, breeding systems, flavonoids, and chloroplast DNA in the four Old World species of *Fuchsia* (Onagraceae). Syst Bot 16:257–269

Wilson MA, Gaut B, Clegg MT (1990) Chloroplast DNA evolves slowly in the palm family (*Arecaceae*). Mol Biol Evol 7:303–314

Wolfe KH, Sharp PM, Li WH (1989) Rates of synonomous substitution in plant nuclear genes. J Mol Evol 29:208–211