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Molecular investigation of the genetic base of sugarcane cultivars

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Abstract Molecular diversity was analysed among 162 clones of sugarcane using DNA restriction fragment length polymorphism (RFLP). One hundred and nine of them were modern cultivars of interspecific origin; most of them were bred in Barbados or in Mauritius. Fifty three were from *Saccharum officinarum* species, which is the major source of genes in modern cultivars, prevailing over the part of the genome incorporated from the wild species *Saccharum spontaneum*. Twelve low-copy nuclear DNA probes scattered over the genome were used in combination with one or two restriction enzymes. A total of 399 fragments was identified, 386 of which were polymorphic. Each sugarcane clone displayed a high number of fragments per probe/enzyme combination, illustrating the polyploid constitution of the genome. Among the *S. officinarum* clones, those from New Guinea had the largest variability and encompassed that present among clones collected from the Indonesian Islands and those known to have been involved in the parentage of modern cultivars. This is in agreement with the hypothesis that New Guinea is the centre of origin of this species. The clones from New Caledonia formed a separate group and could correspond to *S. officinarum* clones modified through introgression with other members of the '*Saccharum* complex'. Despite the low number of *S. officinarum* clones used for breeding cultivars, more than 80% of the markers present in the whole *S. officinarum* sample were also found in modern cultivars due probably to a high heterozygosity related to polyploidy. Among the cultivars, the two main groups, originating from Barbados and Mauritius, were clearly separated. This appeared essentially due to *S. spontaneum* alleles present in Mauritian cultivars and absent in Barbadan ones, probably in relation to the regular use of early generation interspecific hybrids in the breeding program employed in Mauritius.

Key words *Saccharum officinarum* · Sugarcane cultivars · Polyploid · Genetic diversity · RFLP

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Introduction

Sugarcane is a large grass cultivated in tropical and intertropical regions. It belongs to the genus *Saccharum* L. of the family Poaceae within the tribe Andropogoneae. The genus *Saccharum* is characterised by both a high ploidy level and aneuploidy, and formally comprises six species: *S. spontaneum* L., *S. robustum* Brandes and Jesweit ex Grassl, *S. officinarum* L., *S. barberi* Jesw., *S. sinense* Roxb. and *S. edule* Hassk. An extended gene pool of the genus known as the '*Saccharum* complex' (Mukherjee 1957; Daniels and Roach 1987) is composed, in addition to *Saccharum* species, of allied genera including *Erianthus* Mickx. sect. *Ripidium* Henrard, *Miscanthus* Anderss. sect. *Diandra* Keng, *Narenga* Bor. and *Sclerostachya* (Hack.) A. Camus. Members of the '*Saccharum* complex' are thought to have arisen through polyploidisation and hybridisation events.

Two major *Saccharum* species, *S. officinarum* and *S. spontaneum*, have contributed to the origin of current sugarcane cultivars. *S. officinarum* is known as the

sugar-producing species or 'noble' cane (Bremer 1961) and is generally characterised by a chromosome number of 2n = 80 (Bremer 1930; Li and Price 1967; Price and Daniels 1968; Jagathesan et al. 1970). It is thought to be derived from the 2n = 80 forms of the wild species *S. robustum* in New Guinea, the centre of origin (Brandes 1958). From there, clones of *S. officinarum* would have first been dispersed to the surrounding islands and later to various regions that became the current sugar-producing countries. *S. spontaneum* is a wild species that shows great variability and a wide range of chromosome numbers from 2n = 40 to 128 (Sreenivasan et al. 1987). It produces little sugar but is adaptable to different environments and is resistant to several diseases. It has a large distribution from Japan and New Guinea to the Mediterranean and Africa, with India as its centre of origin (Daniels et al. 1975). *S. sinense* (2n = 81–124) and *S. barberi* (2n = 111–120) are two 'species' thought to be derived from natural hybridisation between *S. officinarum* and *S. spontaneum* in China and India, respectively (Price 1968). *S. edule* is a minor group of sterile canes, supposedly derived from intergeneric hybridisation (Daniels and Roach 1987). Beyond the efforts of botanists to describe it as a taxonomic unit, the genus *Saccharum* can be viewed as a complex swarm with a diversity related to ploidy levels superimposed with interspecific or intergeneric hybridisation.

Until the end of the 19th century, *S. officinarum*, together with *S. barberi* and *S. sinense*, provided most of the commercially grown cultivars. Following the discovery of sexual fertility and stimulated by disease outbreaks, the first man-made interspecific hybrids were produced in Java and India, involving essentially *S. officinarum*, *S. spontaneum* and *S. barberi*. Restoration of the high-sugar-producing type was obtained through repeated backcrossing of the hybrids to *S. officinarum*. During this procedure, known as 'nobilisation', *S. officinarum*, usually used as the female parent, transferred 2n chromosomes to the F_1 and BC_1, whereas *S. spontaneum* transferred n chromosomes (Bremer 1961). It is from the BC_2 onward that the chromosome transmission becomes normal. Therefore, nobilised clones are characterised by a high chromosome number (2n = 100–130), with roughly 80% of the genome derived from *S. officinarum*, and the remainder from *S. spontaneum* either directly or via *S. barberi* (Price 1957; Roach 1969). This break-down of the genome into two distinct and clearly identified sources is supported by fluorescent in situ hybridisation experiments: using typical *S. officinarum* and *S. spontaneum* genotypes as probe DNA, it is possible to classify the chromosomes into two parental compartments when cultivars and *S. barberi* clones are surveyed (D'Hont et al. 1996, in preparation). Interspecific intrachromosomal recombination can be observed in modern cultivars. The interspecific hybrids have proven to be a major breakthrough in sugarcane improvement, solving some of the disease problems and presenting an unexpectedly high yield, high ratooning, and adaptability (Roach 1972). Modern sugarcane cultivars are derived largely from intercrossing of these first nobilised hybrids. The number of initial parental contributors is very small (Arcenaux 1967) and is a matter of concern among sugarcane breeders.

Various molecular studies have been conducted to assess germplasm diversity within the genus *Saccharum*. Isozyme analyses (Glaszmann et al. 1989) and restriction fragment length polymorphism (RFLP) using cytoplasmic heterologous probes (D'Hont et al. 1993), as well as ribosomal DNA (Glaszmann et al. 1990) and low-copy nuclear sequences (Burnquist et al. 1992; Lu et al. 1994 a, b), have been performed. The results obtained were in accordance with the taxonomic and phylogenetic schemes based on morphological, cytological, as well as biochemical, data (Daniels and Roach 1987) and revealed a limited variability within *S. officinarum*. However, these analyses were conducted on only a small number of clones. In addition to the evaluation of molecular diversity within the *Saccharum* genus, several genetic linkage maps were constructed for ancestral species (da Silva et al. 1995) and for cultivars (D'Hont et al. 1994; Grivet et al. 1996), using either random PCR or nuclear probes.

The present study analyses the diversity existing within a sample of cultivars from different breeding stations and of clones labelled as *S. officinarum* from the area of origin and diversity of this species. Due to the predominance of *S. officinarum* in the genome of cultivars, and to the importance of the agronomic characters inherent in this species, it is important to evaluate the diversity existing within this species and the proportion of this diversity present in the cultivars.

Materials and methods

Plant material

The material analysed consisted of 53 clones of *S. officinarum* L. and 109 cultivars, which are listed in Tables 1 and 2 respectively.

The clones of *S. officinarum* are divided into four groups. One group, referred to as the geographical New Guinea group (Iryan Jaya plus Papua New Guinea), was composed of clones collected from different parts of this island, which is regarded as the centre of origin of *S. officinarum*. Two other groups consist of clones collected from various islands of Indonesia (Molokai, Sulawesi, Kalimantan) and from the Pacific Islands (Fiji and New Caledonia) respectively. The fourth group consists of clones involved in the genealogy of modern cultivars, as described by Arcenaux (1967), and are referred to as genealogy clones. These clones usually correspond to old cultivars of uncertain geographic origin. Three clones which have largely contributed to the genome of Mauritian cultivars were also included.

Cultivars originated mainly from two breeding stations located in Barbados and Mauritius. Cultivars from other breeding stations and some of the first interspecific hybrids mainly from India and Java, present in the genealogy of most modern cultivars, were also included.

Table 1 Chromosome number, prospecting area, collection site and some markers characteristics of the *S. officinarum* clones studied

Clones	Chromosome number	Origin[a]	Collection source	Nf/c[b]	RM[c]	UM[d]	UMC[e]
Genealogy							
Cristalina	80	New Guinea	Montpellier	5.44	3	0	0
Loethers	–	–	Montpellier	4.86	3	0	0
Bandjermasin Hitam	80	–	Montpellier	5.43	1	0	0
EK28	–	–	Montpellier	–	–	0	0
Kaludai Boothan	80	–	Montpellier	5.71	1	0	0
Vellai	80	–	Montpellier	5.62	4	0	0
Mauritius Guingan	–	–	Montpellier	4.78	2	0	0
Fiji 24	80	–	Montpellier	7.52	14	0	0
Black Cheribon	80	New Guinea	Montpellier	6.14	0	0	0
Badila	80	New Guinea	Montpellier	5.50	6	0	0
Korpi	80	–	Mauritius	6.05	7	0	0
Mp131	80	Mauritius	Mauritius	6.05	3	0	0
Bambou S	–	Mauritius	Mauritius	6.14	0	0	0
Bambou Rose	80	Mauritius	Mauritius	7.57	24	7	2 (MF-HF)
New Guinea							
28 NG 285	80	New Guinea	Florida	5.00	5	1	1 (LF)
28 NG 288	80	New Guinea	Australia	7.19	24	6	1 (LF)
51 NG 113	80	New Guinea	Mauritius	6.19	9	1	0
51 NG 142	80	New Guinea	Mauritius	5.62	5	0	0
51 NG 153	80	New Guinea	Mauritius	5.67	2	0	0
57 NG 11	80	New Guinea	Mauritius	6.14	5	0	0
57 NG 16	80	New Guinea	Australia	6.05	3	0	0
57 NG 30	80	New Guinea	Barbados	5.71	3	0	0
57 NG 52	80	New Guinea	Australia	5.57	7	1	1 (HF)
57 NG 57	80	New Guinea	Mauritius	5.52	1	0	0
57 NG 68	80	New Guinea	Mauritius	5.86	1	0	0
57 NG 123	80	New Guinea	Mauritius	5.67	2	0	0
57 NG 198	80	New Guinea	Mauritius	6.00	16	5	3 (LF)
77 NG 66	80	New Guinea	Mauritius	7.24	25	10	10 (MF-HF)
77 NG 142	80	New Guinea	Mauritius	5.48	3	2	0
IJ 76-291	–	Iryan Jaya	Australia	5.14	0	0	0
IJ 76-316	–	Iryan Jaya	Australia	6.95	14	0	0
IJ 76-325	–	Iryan Jaya	Australia	5.77	4	0	0
IJ 76-432	–	Iryan Jaya	Mauritius	5.68	2	0	0
IJ 76-447	–	Iryan Jaya	Australia	7.36	11	3	3 (MF-HF)
IJ 76-468	–	Iryan Jaya	Australia	5.41	3	2	0
IJ 76-521	–	Iryan Jaya	Florida	7.18	23	3	2 (HF)
Indonesian Islands							
IS 76-116	–	Sulawesi	Florida	4.91	2	0	0
IS 76-117	–	Sulawesi	Mauritius	5.64	6	1	0
IS 76-203	–	Sulawesi	Mauritius	5.5	3	0	0
IS 76-226	–	Sulawesi	Australia	4.95	2	0	0
IK 76-33	–	Kalimantan	Australia	5.33	1	0	0
IK 76-95	–	Kalimantan	Australia	4.52	4	0	0
IM 76-244	–	Molokai	Australia	7.00	11	3	2 (MF)
Pacific Islands							
Fiji 23	–	Fiji	Australia	5.18	1	0	0
Fiji 38	80	Fiji	Australia	5.10	6	2	0
Fiji 44	–	Fiji	Florida	5.45	7	1	0
Fiji 45	80	Fiji	Guadeloupe	5.32	3	1	1 (LF)
Fiji 63	–	Fiji	Florida	4.77	3	0	0
NC-24	95	New Caledonia	Florida	5.64	6	0	0
NC-29	95	New Caledonia	Florida	5.64	6	0	0
NC-30	107-114	New Caledonia	Florida	7.32	18	3	1 (LF)
NC-42	84	New Caledonia	Florida	5.73	11	1	0
NC-99	80	New Caledonia	Florida	5.32	3	0	0

[a] The origin indicates the areas where the clones have been prospected. Three clones, originating from Mauritius, correspond to intraspecific *S. officinarum* hybrids

[b] Nf/c: average number of markers per probe/enzyme combination

[c] RM: number of markers present in less than five *S. officinarum* clones

[d] UM: number of markers present in a single *S. officinarum* clone

[e] UMC: number of markers present in only one *S. officinarum* clone and also present among cultivars. Their frequency in cultivars is indicated by: LF = low frequency (<5 cultivars), MF = moderate frequency (5–10 cultivars), HF = high frequency (<10 cultivars)

Table 2 List of cultivars studied, together with their original breeding station, their collection source and marker characteristics

Clones	Origin	Collection source	Nf/c[a]
Mauritius			
M134/32	Mauritius	Mauritius	7.00
M213/40	Mauritius	Mauritius	7.24
M241/40	Mauritius	Mauritius	7.88
M147/44	Mauritius	Mauritius	7.08
M31/45	Mauritius	Mauritius	7.08
M202/46	Mauritius	Mauritius	7.27
M93/48	Mauritius	Mauritius	6.96
M305/51	Mauritius	Mauritius	6.46
M356/53	Mauritius	Mauritius	8.00
M13/56	Mauritius	Mauritius	7.15
M377/56	Mauritius	Mauritius	6.54
M351/57	Mauritius	Mauritius	7.54
M555/60	Mauritius	Mauritius	7.00
M907/61	Mauritius	Mauritius	8.00
M574/62	Mauritius	Mauritius	6.85
M2173/63	Mauritius	Mauritius	6.92
M376/64	Mauritius	Mauritius	7.19
M657/66	Mauritius	Mauritius	5.73
M1156/66	Mauritius	Mauritius	6.69
M3035/66	Mauritius	Mauritius	7.62
M695/69	Mauritius	Mauritius	7.77
M292/70	Mauritius	Mauritius	6.00
M1205/70	Mauritius	Mauritius	7.42
M1557/70	Mauritius	Mauritius	7.38
M298/71	Mauritius	Mauritius	7.15
M1030/71	Mauritius	Mauritius	7.23
M1236/71	Mauritius	Mauritius	6.77
M50/75	Mauritius	Mauritius	7.04
M134/75	Mauritius	Mauritius	6.62
M536/75	Mauritius	Mauritius	7.27
M791/75	Mauritius	Mauritius	8.08
M1077/75	Mauritius	Mauritius	7.73
M1176/77	Mauritius	Mauritius	6.62
M52/78	Mauritius	Mauritius	7.15
M624/78	Mauritius	Mauritius	7.65
M725/78	Mauritius	Mauritius	7.15
M1658/78	Mauritius	Mauritius	7.58
M2077/78	Mauritius	Mauritius	7.38
M2229/80	Mauritius	Mauritius	8.23
Barbados			
B3337	Barbados	Mauritius	7.76
B34104	Barbados	Mauritius	6.77
B37161	Barbados	Barbados	6.92
B41211	Barbados	Barbados	7.13
B41227	Barbados	Barbados	6.54
B45151	Barbados	Barbados	6.83
B4744	Barbados	Barbados	7.17
B51129	Barbados	Montpellier	7.12
B52107	Barbados	Barbados	6.58
B54142	Barbados	Barbados	5.91
B5992	Barbados	Montpellier	7.08
B62163	Barbados	Barbados	7.00
B63118	Barbados	Barbados	7.17
B69379	Barbados	Montpellier	6.84
B69566	Barbados	Montpellier	7.52
B70532	Barbados	Montpellier	6.92
B74541	Barbados	Barbados	6.63
B77602	Barbados	Barbados	6.88
B79474	Barbados	Barbados	7.71
B8008	Barbados	Montpellier	7.64
B80251	Barbados	Barbados	6.46
B80689	Barbados	Barbados	7.38
Barbados			
B82139	Barbados	Montpellier	7.36
B82238	Barbados	Barbados	6.21
B83345	Barbados	Barbados	6.71
BJ7015	Barbados	Barbados	5.50
BJ7452	Barbados	Barbados	7.00
BJ7465	Barbados	Barbados	7.42
BT73686	Barbados	Barbados	6.42
BT74209	Barbados	Barbados	6.46
DB63113	Barbados	Barbados	6.38
DB7047	Barbados	Barbados	6.13
DB7160	Barbados	Barbados	6.75
DB75159	Barbados	Barbados	6.71
DB7869	Barbados	Barbados	7.08
Australia			
Q96	Australia	Mauritius	8.54
Q90	Australia	Australia	6.58
Q110	Australia	Australia	5.58
Q117	Australia	Australia	6.24
Q124	Australia	Australia	6.14
Q138	Australia	Australia	7.44
Q141	Australia	Australia	6.36
Q155	Australia	Australia	7.60
Reunion			
R472777	Reunion Island	Mauritius	6.69
R570	Reunion Island	Mauritius	7.42
R575	Reunion Island	Mauritius	7.58
R576	Reunion Island	Mauritius	7.46
R577	Reunion Island	Mauritius	7.50
R578	Reunion Island	Mauritius	7.73
R579	Reunion Island	Mauritius	7.04
Other cultivars			
SP70-1284	Brazil	Montpellier	9.12
SP71-6163	Brazil	Montpellier	7.44
SP79-1011	Brazil	Montpellier	8.44
N12	South Africa	Montpellier	7.84
N14	South Africa	Montpellier	7.40
N53216	South Africa	Montpellier	7.72
FR832035	Guadeloupe	Montpellier	7.24
ROC5	Taiwan	Montpellier	7.16
CP44-101	USA	Australia	6.48
First interspecific hybrids			
Co213	India	Mauritius	8.35
Co281	India	Mauritius	7.88
Co290	India	Mauritius	7.96
Co312	India	Mauritius	7.46
Co6415	India	Montpellier	7.92
H32-8560	Hawaii	Mauritius	8.00
NCo310	India	Mauritius	7.23
NCo376	India	Mauritius	8.15
POJ2878	Java	Mauritius	7.73
PT4352	Java	Mauritius	7.08
S17	Taiwan	Mauritius	7.00

[a] Nf/c: the average number of fragments per probe/enzyme combination

Clones were obtained from the world collection at Canal Point, Florida, USA, the working collections of WICSCBS (West Indies Central Sugar Cane Breeding Station) in Barbados, BSES (Bureau of Sugar Experiment Stations) in Australia, MSIRI (Mauritius Sugar

Industry Research Institute) in Mauritius, and CIRAD (Centre de coopération Internationale en Recherche Agronomique pour le Développement) in Guadeloupe and Montpellier, France.

DNA isolation and restriction enzyme analysis

Sugarcane leaves were freeze-dried and total DNA was extracted according to the protocol of Hoisington (1992). Ten micrograms of DNA was restricted with either *Dra*I, *Hin*dIII or *Sst*I. The restricted DNA was purified by phenol/chloroform extraction and DNA fragments were separated in 0.8% TAE agarose gels at 1.7 V/cm for 24 h. After de-purination in 0.25 N HCl for 10 min, gels were Southern blotted onto a Nylon membrane (Hybond N+) in 0.4 N NaOH. Pre-hybridisation (at least 4 h at 65°C) was carried out in 5 × SSC, 0.2% SDS (w/v), 1 × Denhardt's and 0.1 mg/ml of sheared salmon-sperm DNA. Hybridisation (overnight at 65°C) was performed with the same solution as for pre-hybridisation plus 10% dextran sulphate (w/v) and the labelled probes.

Membranes were washed for 5 min in 2 × SSC, 0.5% SDS (w/v) at room temperature, two-times for 30 min in 0.5 × SSC, 0.1% SDS (w/v) at 65°C and two-times for 30 min in 0.1 × SSC, 0.1% SDS (w/v) at 65°C, and exposed to X-ray film (Fuji NIF New RX) at − 80°C for 5 days with one intensifying screen.

DNA probes

Twelve sugarcane probes were used, including both genomic DNA (SSCIR and SG probes) and cDNA (CDS) probes. SSCIR probes come from the sugarcane genomic library developed from the *S. spontaneum* clone 'SES 278' (Grivet et al. 1996). SG and CDS probes were kindly provided by Drs. J. da Silva and W. Burnquist (Copersucar, Brazil), Dr. P. Moore (USDA-HSPA, USA) and Dr. M. Sorrells (Cornell University, USA; da Silva et al. 1993). The probes have been mapped on the genome of the sugarcane cultivar 'R570' (Grivet et al. 1996), and at least one probe per linkage group was chosen in order to cover all the sugarcane chromosomes and to yield hybridisation patterns with strong well-resolved bands. Ten of the twelve probes are present in one copy in the genome and two may be duplicated on two different linkage groups. The probes were labelled with ^{32}P αdCTP (ICN Pharmaceuticals, Inc.) using the Amersham Megaprime labelling kit.

Statistical analysis

Cluster analysis

The genetic similarity between clones was calculated for all possible pairwise comparisons using the Dice index (Dice 1945; Nei and Li 1979) according to the formula:

$$Sij = 2\ Nij/(Ni + Nj),$$

where Sij is the measure of the genetic similarity between sugarcane clones i and j, Nij is the number of bands in common between i and j, and Ni and Nj are the number of bands in i and j, respectively, with regard to all probe/enzyme combinations considered. This analysis was performed using the software package NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System, Rohlf 1993). The similarity matrix was imported into DARwin software (Perrier and Jacquemoud, CIRAD software in preparation) and a dendrogram (hierarchical ascending classification, HAC) was generated based on the dissimilarity values (1 − Sij) and using the UPGMA (unweighted pair group mean arithmetic) clustering method.

Multivariate analysis

Each RFLP band was scored as 1 for presence and 0 for absence and entered onto an Excel spread sheet. Factor analyses of correspondences (FAC) (ADDAD 1985) were performed on the binary matrix after disjunction of the variables in order to give the same weight to the different clones. This factorial analysis treats qualitative data; several independent axes are identified that sequentially account for the largest part of the remaining variation. These axes are linear combinations of the markers, and each clone can be located along the axes. The loading of the markers on the axes measures the discriminative power of these markers. When a few clones are strongly differentiated from the others due to specific bands, the main axes of the FAC will be largely determined by these markers and may not resolve the rest of the clones. Therefore, very rare markers (with a frequency below 5%) were not taken as active variables in order not to unbalance the analysis. For the same reason, very frequent markers (with a frequency over 95%) were not considered as active. As for the markers, it is also possible to locate 'inactive' individuals along these axes by calculating their co-ordinates *a posteriori*. In this case, markers specific to the inactive individuals are also considered as inactive variables.

Results

Polymorphism

The RFLPs were revealed using 12 probes, each in combination with one or two restriction enzymes.

The *S. officinarum* clones 'Bambou S' from Mauritius and 'Black Cheribon' from Guadeloupe displayed exactly the same profiles with all probe/enzyme combinations, as did 'NC-24' and 'NC-29' from the world collection in Florida. 'Bambou S' and 'NC-29' were arbitrarily discarded from further analyses. All other clones presented a specific multilocus RFLP pattern.

Each probe/enzyme combination revealed between 8 and 40 restriction fragments within the population analysed. A complete set of data was obtained for 21 probe/enzyme combinations, out of a possible 24, applied to both *S. officinarum* clones and cultivars, revealing 13 monomorphic and 386 polymorphic markers. This low number of monomorphic markers was also observed when *S. officinarum* clones and cultivars were considered separately. The frequency of the markers identified in the whole population was calculated (Fig. 1). Their distribution in each frequency class between 0.1 and 1.0 was not homogeneous: 27% of the markers were present in less than eight individuals (5% of the population) and 68% were present in less than 50 individuals, corresponding to less than one third of the population under survey. This high proportion of rare markers illustrates a large genetic diversity among the material analysed.

The average number of fragments (Nf/c) identified by probe/enzyme combinations for each clone is given in Tables 1 and 2 for *S. officinarum* clones and cultivars, respectively. Due to the polyploid nature of sugarcane, the probes, although of low copy, revealed a large number of markers per clone. A large variation for Nf/c

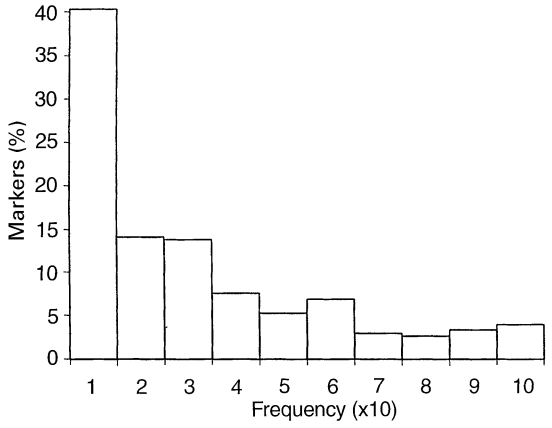

Fig. 1 Frequency distribution of the 399 markers revealed with the 21 probe/enzyme combinations among cultivars and *S. officinarum* clones

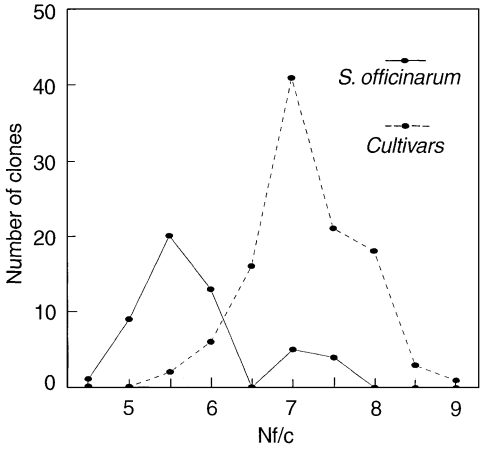

Fig. 2 Distribution of the average number of markers revealed per probe/enzyme combination (*Nf/c*) among cultivars and *S. officinarum* clones

existed between the clones, ranging from 4.52 to 9.12. The cultivars displayed a normal distribution of Nf/c with an average of seven. *S. officinarum* displayed a bimodal distribution for Nf/c with two peaks, one at 5.5 and another at around 7.0–7.5 (Fig. 2).

Intervarietal relationships

Cluster analysis

In order to visualise affinity among the clones, a hierarchical ascending classification (HAC) was performed on the whole sample based on the 386 restriction fragments. The dendrogram produced is presented in Fig. 3. Two main groups could be observed: the first consisted of most of the *S. officinarum* clones, while the second group included cultivars and a few *S. officinarum* clones. One cultivar from Mauritius, 'M657/66', was included in the *S. officinarum* cluster. The structure within the *S. officinarum* cluster was very weak; however, clones from New Caledonia formed a subgroup together with two clones from Fiji. Among the cultivars, a preferential association was observed according to their geographical origin: most Mauritian cultivars were clustered together and were separated from those coming from Barbados. The dendrogram also shows that the global diversity is of similar magnitude among the cultivars and the *S. officinarum* clones.

The *S. officinarum* clones classified with the cultivars were either clones involved in the genealogy of cultivars or clones having a Nf/c parameter higher than 6.5 (second peak in Fig. 2).

Factorial analysis of correspondences (FAC)

A cluster analysis represented by a dendrogram gives a global view of the structure existing within the population under study. A FAC enables selection of a subsample as a reference ('active individuals') to analyse particular aspects of the structure and location of the rest of the samples ('inactive individuals') in relation to this reference. Therefore, three FACs were performed on the 160 clones in order to have a better understanding of the organisation of the variability observed: (1) on the whole sample, (2) taking only reference *S. officinarum* clones as active individuals, and finally (3) taking only cultivars as active individuals.

The first FAC was carried out on 280 active markers and using all 160 clones as active individuals (Fig. 4). The first plane explained 9.80% of the variability. This apparently low percentage is likely to be related to the large number of markers employed. The main trends noted on the dendrogram were also observed here: *S. officinarum* clones were separated from the cultivars on the first axis with, however, no clear cut delimitation between the two groups. Within *S. officinarum*, the clones originating from New Guinea displayed the widest distribution; the clones involved in the genealogy of cultivars also displayed a rather wide distribution. The clones with the most extreme differentiation originated from New Guinea and the Pacific islands (Fiji and New Caledonia) and had high Nf/c. Among the cultivars, the separation of clones according to their two main origins, Mauritius and Barbados, was apparent along the second axis. Moreover, the Mauritian cultivar 'M657/66' was placed together with *S. officinarum* clones as observed on the dendrogram.

It is noteworthy that the seven *S. officinarum* clones closest to the cultivars, based on the FAC and the cluster analysis, are part of the nine clones with a high Nf/c (>6.50). Each presents an important number of markers, rare or even unique among *S. officinarum* clones (Table 1). Several of these markers were present among cultivars, indicating a putative *S. spontaneum* origin. The two other *S. officinarum* clones with a high

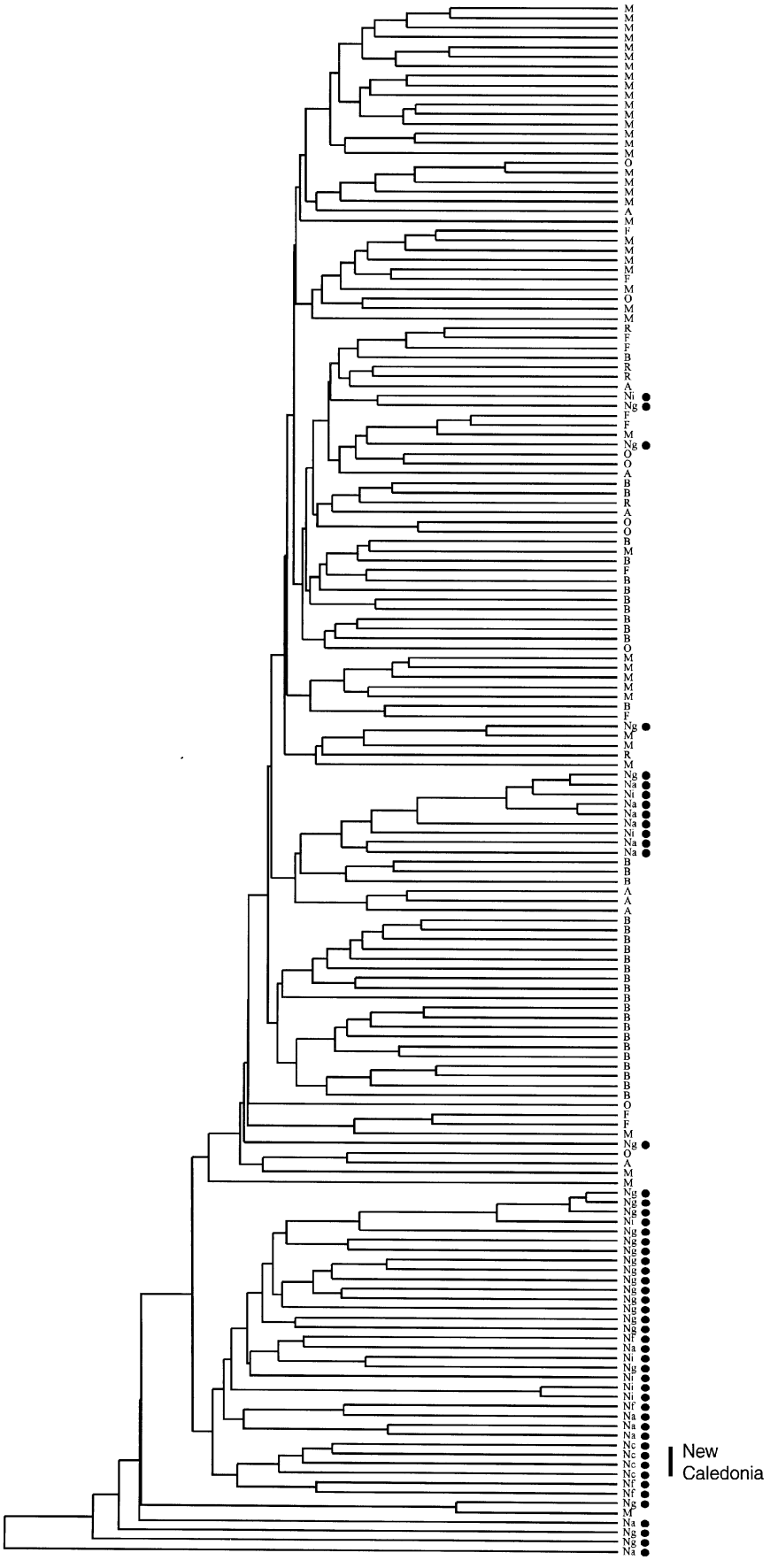

Fig. 3 Hierarchical ascending classification (HAC) among 109 cultivars and 51 *S. officinarum* clones (identified with ●) using 386 RFLP markers identified with 21 probe/enzyme combinations. The station where cultivars have been bred is given as follows: *M* Mauritius; *B* Barbados; *A* Australia; *R* Reunion; *F* first interspecific hybrids mainly from India and Java; *O* cultivars from other breeding stations. The different populations of *S. officinarum* are symbolised by: *Na* clones involved in the genealogy of cultivars; *Ng* New Guinea; *Ni* Indonesian Islands; *Nf* Fiji; *Nc* New Caledonia

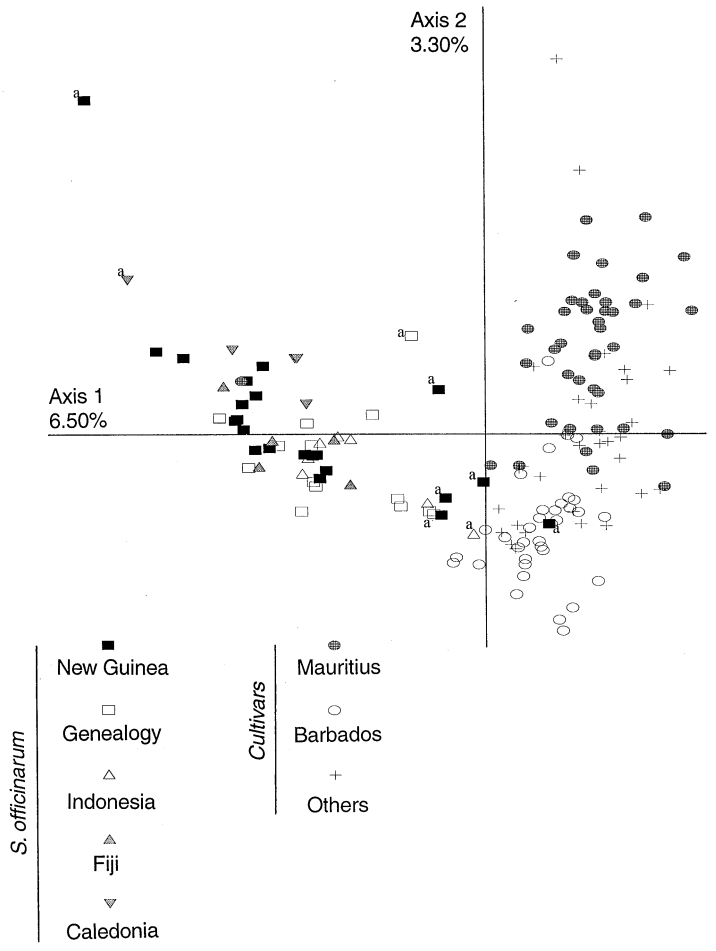

Fig. 4 First plane of a factor analysis of correspondences among 51 *S. officinarum* clones and 109 cultivars using 280 active markers. Symbols labelled with an '*a*' correspond to *S. officinarum* clones with a Nf/c > 6.50

Nf/c had an extreme position on axis one and axis two. They displayed several markers rare among *S. officinarum*, most of them being absent from cultivars (Table 1).

A second FAC was performed using *S. officinarum* as active, and the cultivars as inactive, individuals. This analysis was performed in order to visualise how the *S. officinarum* clones were structured and to evaluate the proportion of the diversity within this species which has been exploited in breeding programs. Given the peculiarity of most of the nine *S. officinarum* clones with high Nf/c, they were considered as inactive individuals. The first plane of the FAC performed on 220 active markers explained 16.60% of the variation (Fig. 5). *S. officinarum* clones from New Guinea were spread along the first axis with, however, most clones clustered together. No particular geographical structure was observed among them, including along subsequent axes of the FAC. This absence of geographical structure was also observed when these clones were analysed alone (data not shown). The clones involved in the genealogy of cultivars and those from the Indonesian islands were also spread along the first axis, their distribution being almost completely encompassed by the distribution of the New Guinea clones. Clones from New Caledonia were separated from the other *S. officinarum* clones analysed along axis two. A few markers predominant or specific to this group were identified. Clones from Fiji were intermediate between those from New Caledonia and New Guinea but closer to the latter. Cultivars projected as inactive individuals formed a large cluster near the centre of the first plane of the FAC. Some *S. officinarum* clones were placed near the cultivars, most of them being involved in the genealogy of cultivars. However, two clones involved in the genealogy of cultivars, many clones from New Guinea and particularly New Caledonian ones, were distant.

Among the nine inactive *S. officinarum* clones with a high Nf/c, the seven presenting high affinity to the cultivars in previous analyses were also clustered together with the latter. The two others, '28 NG 288' and 'NC-30', did not, as already observed on the first FAC.

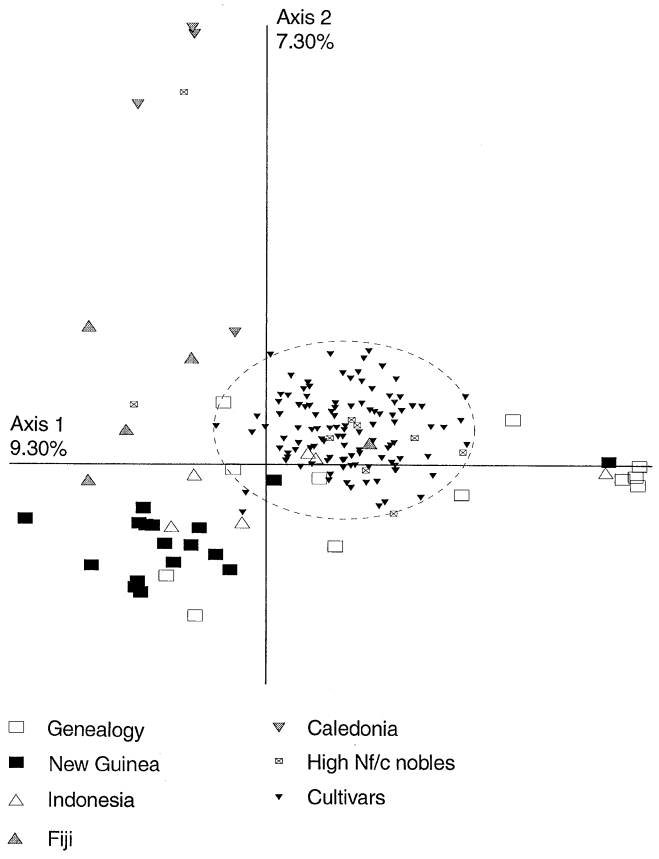

Fig. 5 First plane of a factor analysis of correspondences (FAC) among 51 *S. officinarum* clones using 220 active markers and a projection of 109 cultivars and nine *S. officinarum* clones with a high Nf/c as inactive individuals

Conversely, a third FAC was performed taking only the cultivars as active individuals and the *S. officinarum* clones as inactive individuals in order to evaluate the extent to which the diversity of *S. officinarum* has influenced the diversity among the cultivars (Fig. 6). The two cultivars 'Co213' and 'M657/66' were considered as inactive elements. When considered as actives, the first and second axes were explained mainly by these two individuals thus not allowing resolution of the variability existing within the rest of the cultivars. The first plane accounted for 9% of the variability with the 235 active markers. All the cultivars were homogeneously spread on the first plane. This striking structure, related to the origin of the cultivars, as noted on the dendrogram and the first FAC, was confirmed here: the Mauritian cultivars were on the left half of the plane whereas the cultivars from Barbados were on the right half. The few cultivars from Reunion island were on the same side as those from Mauritius. Australian cultivars, as well as the single cultivar from Guadeloupe, were placed on the Barbadan side. Cultivars from Brazil and South Africa were in an intermediate position. Among the first interspecific hybrids, the four clones from India were located together with the Mauritian cultivars. The

Table 3 Distribution of markers in the material surveyed

Materials	No. of clones	% Markers
S. officinarum	51	100
Genealogy	12	67
New Guinea	17	88
Indonesia	6	58
Pacific	9	70
Cultivars	109	100
Mauritius	40	90
Barbados	35	86
Reunion	7	67
Australia	8	66
Others	8	72
First hybrids	9	77
Whole sample	160	100
S. officinarum	51	73
		11[a]
Cultivars	109	89
		27[a]
		85[b]

[a] Percentage of markers specific to *S. officinarum* clones or to cultivars
[b] Percentage of *S. officinarum* markers in the cultivars

others were placed on an intermediate position between the Mauritian and Barbadan cultivars but closer to the Mauritian ones. The most discriminative markers between the Mauritian and Barbadan cultivars were identified using their loading on the first axis of the FAC. Thirteen markers were involved: ten were either specific or highly frequent in Mauritian cultivars (present in more than 40% of the cultivars) while three were predominant in Barbadan cultivars (present in more than 50%). These markers were scattered on all the linkage groups except for groups 1 and 9 of the cultivar 'R570' map (Grivet et al. 1996) and most of them were absent in the *S. officinarum* clones surveyed here. They were therefore considered as being specific to *S. spontaneum*. The *S. officinarum* clones, when projected as inactive individuals, were clustered in the centre of the distribution of the cultivars.

Quantitative differentiation

After analysing globally the diversity and the relationship within the different groups, a more-specific analysis of marker distribution was carried out between groups. The nine particular *S. officinarum* clones with a high Nf/c were not considered for the different analyses.

A high proportion of the 386 restriction fragments, comparable between the *S. officinarum* clones and the cultivars, was shared by both groups (Table 3): 62% were in common, 27% were present only in the cultivars and may therefore correspond to markers specific to *S. spontaneum*, and 11% were specific to the

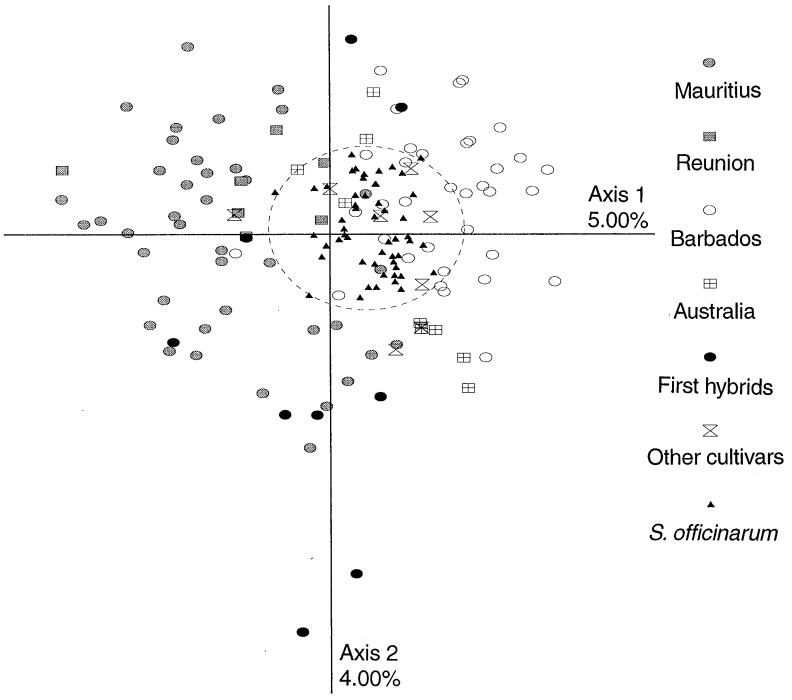

Fig. 6 First plane of a factor analysis of correspondences (FAC) among 109 cultivars using 235 active markers and a projection of 51 *S. officinarum* clones as inactive individuals

S. officinarum clones. Within *S. officinarum*, 88% of the markers were present in the clones from New Guinea, which represents less than half of the *S. officinarum* sample under survey, whereas 67% of the markers were present in clones involved in the genealogy of cultivars. Around 85% of the markers present in *S. officinarum* clones were also shared by cultivars. Eighty two percent of the markers identified within the cultivars were common between the two main origins, Mauritius and Barbados, although we previously observed a separation between them.

The average number of markers per probe/enzyme combination (Nf/c) was calculated for *S. officinarum* clones and cultivars (Tables 1 and 2). The group formed by the majority of *S. officinarum* clones displayed an average Nf/c of 5.50, whereas it was 7.39 for cultivars (Fig. 2). Within cultivars, the average Nf/c for the first interspecific hybrids (7.84) was statistically higher (Student t test, $P = 0.05$) than that observed for Mauritian (7.19) and Barbadan cultivars (6.86). The mean Nf/c was also significantly different between Mauritian and Barbadan cultivars. Among these cultivars no difference of Nf/c was observed between older and more recent cultivars. The Mauritian cultivar 'M657/66', which clustered with *S. officinarum* clones in previous HAC and multivariate analyses, displayed a Nf/c of 5.73, similar to the average Nf/c found for most *S. officinarum* clones. This clone could correspond to a *S. officinarum* clone either misclassified or mislabelled.

The genetic similarity was calculated for all possible pairwise combinations between the clones under survey, and the mean similarity was computed within and between the different groups (Table 4). The similarity within and between *S. officinarum* clones and cultivars was of the same magnitude, the between-groups similarity being the lowest. This is in agreement with the separation observed between *S. officinarum* and cultivars in Fig. 4. It is noteworthy that the mean similarity among cultivars (0.57) has not decreased compared to that among *S. officinarum* clones involved in the genealogy of cultivars (0.56), even though a very polymorphic material, *S. spontaneum*, has been introduced in the cultivars. Within *S. officinarum*, the greatest differentiation was observed among clones from the Pacific Islands. Within the cultivars, the similarity among old cultivars was not different from that observed within the other groups. However, the apparent separation observed between cultivars from Mauritius and Barbados in previous analyses was hardly reflected by the similarity index.

Discussion

Our study revealed a large degree of RFLP polymorphism within the material under survey, only 4% of the markers being monomorphic. This large amount of molecular variation allowed a thorough analysis of the organisation of genetic diversity within *S. officinarum* and the cultivars studied.

Table 4 Mean similarity within and between the different groups of cultivars and *S. officinarum* clones

Type		Cultivars						*S. officinarum*			
		Mauritius	Reunion	Barbados	Australia	First hybrids	Other cultivars	Genealogy	P. New Guinea	Indo. Islands	Pacific
Cultivars	Mauritius	0.59									
	Reunion	0.58	0.59								
	Barbados	0.55	0.55	0.59							
	Australia	0.54	0.56	0.55	0.58						
	First hybrids	0.57	0.59	0.55	0.56	0.59					
	Other cultivars	0.57	0.58	0.58	0.58	0.58	0.63				
	Mean cultivars			0.57							
S. officinarum	Genealogy	0.47	0.47	0.49	0.47	0.46	0.48	0.56			
	P. New Guinea	0.45	0.47	0.45	0.43	0.43	0.46	0.50	0.54		
	Indo. Islands	0.47	0.49	0.48	0.48	0.46	0.48	0.52	0.51	0.55	
	Pacific	0.44	0.44	0.45	0.44	0.42	0.45	0.48	0.48	0.49	0.52
	Mean *S. officinarum*			0.46					0.52		

Considering the *S. officinarum* germplasm, the low number of monomorphic markers identified and the large distribution of the clones on the first plane of the FAC (Fig. 6) suggest that a considerable diversity exists within this species. This result is in contrast with earlier studies based on isozymes (Glaszmann et al. 1989) and nuclear data (Glaszmann et al. 1990; Burnquist et al. 1992; Lu et al. 1994 a) where only a limited diversity were observed. This is probably due to the relatively low number of accessions previously studied. Our data are, however, congruent with the large morphological diversity observed within *S. officinarum*, even for traits considered diagnostic for this species such as stalk thickness and leaf width (Artschwager and Brandes 1958; Jagathesan et al. 1970) as well as sugar content (Roach 1965; Nair et al. 1980).

The distribution of the number of markers per probe/enzyme combination (Nf/c), which can be considered as an indirect estimation of heterozygosity, revealed a discontinuity among the clones labelled as *S. officinarum*. Nine *S. officinarum* clones were identified with a high Nf/c, similar to that observed for the cultivars. Whether this is properly within-species variation is unclear, given the hazy nature of the delimitation between species in the *Saccharum* genus/complex. One hypothesis to account for this characteristic is that they are derived from introgression with another compartment of the complex. Among these clones, seven showing close affinity to the cultivars displayed several possible *S. spontaneum* markers, for they are absent among the other *S. officinarum* clones but present among the cultivars under survey. These clones were 'Fiji 24', 'Bambou Rose', '77 NG 66', 'IJ 76-316', 'IJ 76-447', 'IJ 76-521' and 'IM 76-244'. Only the first two are amongst those known to be involved in the genealogy of the cultivars. The other five, from New Guinea or from the Indonesian Islands, were never reported in the pedigree of any cultivar. Morphological data are available for 'Bambou Rose', '77 NG 66', 'IJ 76-447' and 'IM 76-244' from which DNA has been extracted: 'IM 76-244' and 'IJ 76-447' have large-diameters stalks and broad leaves, two characteristics usually found in clones commonly described as typical *S. officinarum*, whereas 'Bambou Rose' and '77 NG 66' have waxes and fine stalks not observed among typical *S. officinarum*. These seven clones having *S. spontaneum* markers could therefore correspond to hybrids between *S. officinarum* and *S. spontaneum*. First generation (F_1) interspecific hybrids between *S. officinarum* and *S. spontaneum* can occasionally reach commercial status, as was the case for 'Co205' in India, and can arise spontaneously in nature, as was the case for Kassoer, a clone used in early interspecific breeding in Java (Berding and Roach 1987). However, it cannot be completely excluded that these clones are probably cultivars which have been mislabelled as noble clones. The other two clones with a high Nf/c, '28 NG 288' and 'NC-30', are also extreme with respect to the differentiation of the

S. officinarum clones but do not present close affinity to the cultivars: they display several markers rare among *S. officinarum* and the cultivars. Morphological data available for the clone '28 NG 288' differentiate it from typical *S. officinarum* clones for both stalk diameter and leaf width. Clones '28 NG 288' and 'NC-30' could be natural hybrids between *S. officinarum* and a member of the *Saccharum* complex other than *S. spontaneum*. The chromosome number of all these clones with a high Nf/c needs to be checked and in situ hybridisation analysis could be performed in order to clearly determine their genomic constitution. Assuming that the nine clones above might confuse the description of a typical core of *S. officinarum* species, they were deliberately excluded from some of the analyses and from the computation of average diversity parameters aimed at characterising the species *S. officinarum*. This is arbitrary, but it seemed appropriate until other tools can be applied to test their true identity.

New Guinea is considered to be the centre of origin of *S. officinarum*. Clones from this island, representing less than half of the *S. officinarum* clones under survey, encompassed the majority of the markers identified, suggesting that most of the diversity is present in this area. This important diversity is also illustrated by the large, although not uniform, distribution of these clones (Fig. 5). The diversity does not seem related to geographical distribution, since individuals from the west and the east of the island are not separated from each other. *S. officinarum* is a domesticated species which does not exist in the wild. Even though domestication has probably occurred independently in different parts of New Guinea, the absence of geographical differentiation observed is thus probably related to the profuse exchange of material from different subsistence gardens as well as genetic recombination. Current hypotheses suggest that it could have evolved from *S. robustum*, a wild species which is abundant in New Guinea. Some clones of *S. robustum* are slightly sweet and man, aided by animals, could have selected sweet forms for chewing independently in different parts of the island. The involvement of several sources of *S. robustum* could explain the large nuclear genetic diversity observed among *S. officinarum* clones, for the diversity within *S. robustum* is larger than that within *S. officinarum* (Daniels and Roach 1987; Lu et al. 1994 a). The sweetest clones of *S. robustum* would have been maintained in subsistence gardens, and propagated throughout the island. Recombination between these sweet clones and hybridisation with other species, together with selection in successive generations, would have produced high-sucrose, low-fibre *S. officinarum* (Brandes 1958; Warner 1962; Grassl 1974, 1977). This interpretation is corroborated by the observation of numerous small QTLs when sucrose content is analysed in a modern cultivar (J.Y. Hoarau, personal communication).

From Papua New Guinea, *S. officinarum* is thought to have been transported to the east and to the west through human Austronesian migrations. These migrations have been dated between 10 000 and 12 000 BC (Daniels and Daniels 1975; Jennings 1979). The diversity observed among New Guinea clones encompasses the diversity among the Indonesian islands. Contrastingly, to the east, clones from the Pacific Islands, particularly those from New Caledonia, represent a group separated from the New Guinea clones. All but one of the New Caledonia *S. officinarum* clones under survey have a chromosome number higher than 80 (Table 1). The clone with $2n = 80$ chromosomes, 'NC-99', is close to *S. officinarum* clones from New Guinea, whereas the one with the highest chromosome number, 'NC-30' with $2n = 107–114$ chromosomes, is the most distant on the FAC performed when all *S. officinarum* clones were considered as active individuals (data not shown). Two other New Caledonian clones 'NC-42' and 'NC-24' with $2n = 84$ and 95 chromosomes, respectively, appear intermediate between 'NC-30' and the rest of *S. officinarum*. Altogether, the clones from New Caledonia accumulate several markers absent from the rest of the *S. officinarum* and the cultivars analysed. The South Pacific area is reported as being a centre of modification and diversity of *S. officinarum*. Grassl (1967) and Price and Daniels (1968) have reported that *Miscanthus* introgression altered *S. officinarum* in Oceania. Many suspected natural hybrids between *Saccharum* and *Miscanthus* have a high chromosome number (Sreenivasan et al. 1987). The New Caledonian *S. officinarum* clones with a high chromosome number are probably hybrids and could correspond to *S. officinarum* clones modified by *Miscanthus*. Here also, in situ hybridisation analysis could be performed in order to elucidate the true identity of these clones.

The noble clones involved in the genealogy of cultivars seem to well represent the variability existing among *S. officinarum* clones from New Guinea, which probably provides a good representation of the whole diversity present within 'true' *S. officinarum* clones.

The genome of cultivars consists of approximately 80% of *S. officinarum* chromosomes. This is also illustrated by our data. Knowing that the similarity between *S. officinarum* and *S. spontaneum* is relatively low (20%, Lu et al. 1994 a), most of the markers present in the cultivars and *S. officinarum* clones were probably inherited from *S. officinarum*. The high proportion of markers shared by *S. officinarum* and the cultivars illustrates the effect of the nobilisation process. The joint analysis of the cultivars and the *S. officinarum* clones shows that, in general, the cultivars have retained alleles from the different populations of *S. officinarum* under survey: most of the clones involved in the genealogy of the cultivars and some clones from New Guinea and Fiji are close to the cultivars. However, most of the variability within the population from New Caledonia has not been used in the cultivars.

Moreover, although *S. officinarum* clones involved in the genealogy of cultivars represent a subset of the variability existing within *S. officinarum* from New Guinea, not all the variability existing within the New Guinea population has been exploited in the cultivars.

The molecular data confirms the genealogy data to some extent: the clones which occur in the genealogy of many cultivars are close to the cultivars, whereas those which occur in the genealogy of only few cultivars are more distant. There are, however, two exceptions: 'Bandjermasin Hitam' and 'Black Cheribon' are reported as the ancestors of many cultivars but are not particularly close to the latter (Fig. 6). Although relatively few *S. officinarum* clones have played an important role in the genealogy of cultivars, most of the *S. officinarum* markers were present in cultivars probably due to the high ploidy of sugarcane and to the fact that a few other noble clones have also been introduced into breeding programs to a lesser extent. This could also account for the larger *S. officinarum*-derived variability in cultivars than in the ancestors of the main cultivars.

The differentiation observed between the cultivars coming from different breeding stations is surprising knowing the tradition of profuse exchange of parental material between sugarcane breeding stations. This difference is explained by few markers, most of them being specific to or predominant in Mauritian cultivars. The majority of these markers are specific to *S. spontaneum*, suggesting that the main structuring part of the variability among the cultivars is due to alleles inherited from *S. spontaneum*. In both selection stations, MSIRI in Mauritius and WICSCBS in Barbados, as in other stations, almost the same genetic pool has been initially used to create cultivars, thus explaining a relatively low degree of differentiation. However, the first interspecific hybrids created in India are found to be closer to the Mauritian cultivars. A thorough observation of the genealogy shows the frequent occurrence of locally produced hybrids as great grandparents and grandparents in Barbados. In contrast, in Mauritius the first hybrids created in India have been used as parents in a recurrent fashion (Ramdoyal, personal communication), thus explaining the greater affinity of the first Indian cultivars to those of Mauritius. This recurrent use of early generation interspecific hybrids in the Mauritian breeding program could have resulted in cultivars having more *S. spontaneum* markers, which would account for the higher Nf/c observed for Mauritian cultivars as well as for the distinction between cultivars of different origins.

A relation between markers and adaptation would also explain the molecular contrast between the two origins. This can be expected only if a strong linkage disequilibrium exists within the genome, in particular between the markers and the adaptive gene loci. This is possible in sugarcane, since a strong disequilibrium may have arisen with the selection of the first interspecific hybrids, with few meioses (less than ten) having occurred since then. This is a matter of importance for the future application of molecular markers and must be tested by appropriate studies.

Our study offers a basis for rationalising the management of the genetic diversity in sugarcane cultivars. The restricted number of *S. officinarum* clones that contributed to cultivars is a common matter of concern. The large number of bands revealed with single-copy probes is indicative of a high level of heterozygosity, which can generate considerable genotypic diversity in this polyploid background. Therefore, although few, the main noble clones used in the early history of modern sugarcane breeding provided a template with large potential for recombination by breeders. The sample surveyed revealed that even though 73% of the markers were present in *S. officinarum*, only 11% were specific as compared to the cultivars. This 11% of markers illustrates an additional reservoir to widen genetic diversity in cultivars and represents 15% of the *S. officinarum* diversity. Two-thirds are present in typical *S. officinarum* clones and one-third in clones from New Caledonia, which might correspond to another compartment of the '*Saccharum* complex'.

The present work reveals a higher than expected diversity within *S. officinarum*, probably due to the combination of the large number of individuals analysed and the investigation of a large portion of the genome. Most of this diversity is present in the cultivars. However, for the unexplored part, molecular markers can provide a guide for further broadening the genetic base of the cultivars with *S. officinarum*, especially if we are focussing on sugar improvement.

A striking structure was also revealed among the cultivars according to their origin. The other main component of cultivar diversity corresponds to the *S. spontaneum* genome fraction. Our results illustrate the large genetic contribution of this species and provide indications that it may be related to the specialisation of the cultivars. Since this portion of the genome of cultivars is less redundant, and may have contributed to many favourable traits, it is more amenable to QTL analysis and further marker-assisted selection.

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