Tree Genetics & Genomes (2012) 8:1345–1358
DOI 10.1007/s11295-012-0520-9

ORIGINAL PAPER

Geographical gradients in the genome size variation of wild coffee trees (*Coffea)* native to Africa and Indian Ocean islands

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Received: 28 November 2011 / Revised: 16 April 2012 / Accepted: 2 May 2012 / Published online: 31 May 2012

Abstract The genus *Coffea*, mainly native to Africa and to the Indian Ocean islands (Mascarocoffea), accounts for 124 species. Genome size data are available for 23 African species. The aim of this study was to assess the genome size of 44 Mascarocoffea species and to investigate possible association with species geographic distribution, stomata traits, and species relationships. 2 C values were measured using flow cytometry. A lyophilization procedure for leaves was tested. The 2 C nuclear DNA content of Mascarocoffea species ranged from 0.96 to 1.41 pg. *Coffea mauritiana* and *Coffea humblotiana* have the smallest genomes and *Coffea millotii* has the largest. Mean 2 C DNA for Mascarocoffea and Africa is 1.19 and 1.43 pg, respectively. The overall DNA values corresponded to two partially overlapped normal distributions: one harboring species from east Africa Mascarocoffea, the other harboring species from west/central Africa. Plotted on a geographical map according to the native origin of species, these values showed a gradient in Madagascar and Africa. Genome sizes increased following a north to southeast gradient in Madagascar and an east to west gradient in Africa. None, or only weak correlations were noted between genome size and stomata parameters. Genetically close species could be highly distinctive in their genome size while divergent species could be similarly sized. The non-random geographic distribution and habitat of species, and the absence of correlation between genome size and genetic relationships, suggest that during *Coffea* genome evolution, both DNA content increase and/or decrease occurred independently in Africa and in the Indian Ocean Islands.

Keywords *Coffea* · Madagascar · Africa · Genome size variation · Flow cytometry

Abbreviations

FL	Fresh leaves
GCL	Guard cell length
HD	Hierarchical clustering
HSD	Honestly significant difference
LL	Leaf length
LyL	Lyophilized leaves
My	Million years
PI	Propidium Iodide
SD	Stomata density

Communicated by D. Grattapaglia

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Introduction

In flowering plants, genome size is highly variable and may dramatically change even between closely related species (Ohri et al. 2004; Bennett and Leitch 2005; Zonneveld et al. 2005) or within species (Kalendar et al. 2000; Greilhuber 2005). This raises questions on the mechanisms of genome size evolution and its role in adaptation and speciation. Several studies have been carried out to gain greater insight into the origin of such variation by assessing correlations with the geographical distribution of species (Bennett 1987) and many biophysical traits such as environmental physical parameters of habitats, e.g., temperature, latitude, and elevation (Knight and Ackerly 2002; Slovák et al. 2009). Ecological factors appear to be more important in shaping genome size at lower divergence levels (Eilam et al. 2007; Dušková et al. 2010) than at higher taxonomic levels (Jakob et al. 2004). In some cases, genome size variation was found to be related to phenotypic and/or life traits (Beaulieu et al. 2007; Knight and Beaulieu 2008). As a contribution to this debate, it was interesting to study a genus characterized by both high species richness and a wide geographical distribution.

The genus *Coffea* belongs to the Rubiaceae family and currently includes 124 species native to Africa, Comoros, Madagascar, Mascarenes, India, Papua New Guinea, and Australasia (Davis et al. 2011). Based on internal transcribed spacer or plastid or both sequences, closely similar geographic clades were obvious from molecular phylogenies (Lashermes et al. 1997; Cros et al. 1998; Maurin et al. 2007 and Anthony et al. 2010) approximately corresponding to west, west-central Africa, east Africa, and Madagascar plus Mascarenes. *Coffea* species from western Indian Ocean islands (currently accounting for almost half the genus) named Mascarocoffea were first identified as caffeine-free species (Chevalier 1947).

West plus west-central African and east African species have several distinctive features such as a strong reproductive barrier (Louarn 1993) and different cytogenetic characteristics (Noirot et al. 2003a; Hamon et al. 2009). Studies on Mascarocoffea morphology and phenology have led to their classification into eight (Chevalier 1947) then six (Charrier 1978) botanical series, namely Humblotianae/Mauritianae from Comoros and Mascarenes, and Garcinoides, Millotii complex, Multiflorae, Subterminales and Verae from Madagascar. All *Coffea* species (restricted in this study to the genus *Coffea*) are diploids with 2n=2x=22 (Bouharmont 1963; Louarn 1972), except for *Coffea arabica,* an amphidiploid with 2n=4x=44 (Bouharmont 1959; Lashermes et al. 1996, 1999). Previous studies performed on fresh leaves revealed 2 C DNA content variations (1.03–1.76 pg) among a set of 23 African *Coffea* species (Noirot et al. 2003a) and two Mascarocoffea species (Cros et al. 1995).

Our objectives were (1) to develop a methodology that permits reliable estimations of genome size and investigations on large panels; (2) to assess the genome size of a broad sample of Mascarocoffea and to investigate possible association with species geographic distribution, leaf parameters, and *Coffea* species relationships.

Material and methods

Plant material

The study includes a total of 416 accessions from 52 populations corresponding to 44 Mascarocoffea species (from Comoros, Mauritius, and Madagascar) and six African species. Leaves from wild Malagasy and Comoros species maintained at the Kianjavato FOFIFA station, Madagascar, were sampled between 2009 and 2010. The six African species used as references were sampled from IRD Montpellier greenhouses (France), while the Mauritius species came from the *Coffea* international collection maintained at the Station CIRAD - Armeflhor de Bassin Martin, Saint-Pierre (Réunion).

Fully developed leaves were sampled from one to 10 trees (depending on the availability) per species or population (when several populations were available). Half of each fresh leaf (FL) sample was lyophilized (LyL) and stored until use for flow cytometry, while the remaining halves were used for stomata measurements. A full description of the analyzed accessions is given in Table 1.

DNA content estimation

DNA content was measured using flow cytometry at the Imagif Cell Biology platform (Gif-sur-Yvette, France) according to Marie and Brown (1993). *Lycopersicon esculentum* cv. Roma (2 C=1.99 pg, 40.0 % GC; Coba de la Peña and Brown 2001) was used as internal standard. About 1 cm^2 LyL of the studied species and one of the internal standards were chopped using a razor blade in a petri dish with 800 μL of cold Galbraith nuclear-isolation buffer (Galbraith et al. 1983), brought to 0.5 % (w/v) Triton X100 and supplemented with 10 mM sodium metabisulfite, 1 % polyvinylpyrrolidone 10,000 and 5 μg/mL RNAse (ref 10109 169001 Roche, Mannheim, Germany). The suspension was passed through a 48-μm mesh nylon filter. The recovered nuclei were stained with 50 μg/ml propidium iodide (PI) (Sigma-Aldrich, Saint-Quentin, France), an intercalating agent.

Lyophilized leaves were just imbibed in the buffer for a few minutes. Three extractions per tree and 10 trees per population were made. When less than 10 trees were available, independent extractions were made in order to have at least 10 measurements.

Table 1 Description of the *Coffea* populations studied and mean 2 C DNA content assessed on nuclei obtained from lyophilized leaves

Main region[a]/ Botanical series	Species	Abbreviation used in figures	Population code	Country/ region	City-Locality of origin	Forest habitat[b]	Number of plants	Mean 2 C DNA±SD (pg)	CV
MAS/Mau	*C. mauritiana* Lam.	MAU	MAU	MAU	Plaine Champagne	humid	5	0.96±0.04 [a]	4.85
MAS/Mau	*C. humblotiana* Baill.	HUMB	A230	COM-CO	Mount Kartala, Grande Comore	humid	5	0.97±0.05 [ab]	4.65
MAS/Mau	*C. myrtifolia* (A. Rich. ex DC.) J.-F.Leroy	MYR	MYR	MAU	Mount Brisé	humid	5	1.07±0.03 [efgh]	3.25
MAS/Mau	*C. macrocarpa* A.Rich.	MAC	MAC	MAU	Le Pétrin	humid	7	1.17±0.04 [nopqrs]	2.37
MAS/Mau	*C. bernardiniana* J.-F.Leroy	BER	BER	MAU	Montagne des Créoles	humid	6	1.23±0.03 [qrstu]	2.59
MAS/Gar	*C. tetragona* Jum. & H.Perrier	TET	A252	MDG/ NW	Behangony (Maromandia)	dry	8	1.07±0.03 [efgh]	2.90
MAS/Gar	*C. mogenetti* Dubard cf.	MOG	A975	MDG/ NW	Vohimarina (ex-Vohémar)	dry	4	1.09±0.02 [ghij]	1.76
MAS/Gar	*C. dubardii* Jum.	DUB	A969	MDG/ NW	Antsiranana (ex-Diégo-Suarez)	dry	9	1.20±0.03 [nopqrst]	2.78
MAS/Gar	*C. heimii* J.-F.Leroy	HEI	A516	MDG/ NW	Antsiranana (ex-Diégo-Suarez) (Sahafary)	dry	10	1.25±0.05 [rstu]	4.04
MAS/Mil	*C. abbayesii* J.-F.Leroy	ABA	A601	MDG/SE	Tolagnaro (ex-Fort-Dauphin) (Isaka-Ivondro)	humid	10	1.25±0.03 [stu]	2.22
MAS/Mil	*C. millotii* J.-F.Leroy (*C. ambodirianensis* Portères)	AMB	A572	MDG/ east	Toamasina (ex-Tamatave) (Ambodiriana)	humid	7	1.27±0.04 [tuv]	2.84
MAS/Mil	*C. richardii* J.-F.Leroy	RIC	A575	MDG/ east	Fenerive-Est (Tampolo)	humid	10	1.28±0.02 [vw]	1.56
MAS/Mil	*C. farafanganensis* J.-F.Leroy	FAR	A208	MDG/ SE	Farafangana (Amboangibe)	humid	10	1.32±0.03 [wxy]	2.47
MAS/Mil	*C. millotii* J.-F.Leroy	MIL1	A721	MDG/ SE	Nosy Varika (Ampasinambo)	humid	10	1.36±0.03 [xyz]	2.21
MAS/Mil	*C. millotii* J.-F.Leroy	MIL2	A222	MDG/ SE	Mananjary (Tolongoina)	humid	8	1.40±0.04 [yz]	3.14
MAS/Mil	*C. millotii*J.-F.Leroy (*C. dolichophylla* J.-F.Leroy)	DOL	A206	MDG/ SE	Nosy Varika (Ampasinambo)	humid	10	1.41±0.04 [z]	2.99
MAS/Mul	*C. arenesiana* J.-F.Leroy	ARE	A403	MDG/C	Moramanga (Ambodivato)	humid	8	1.11±0.04 [hij]	3.52
MAS/Mul	*C. mangoroensis* Portères	MAN1	A401	MDG/C	Ankazobe (Tampoketsa)	humid	3	1.12±0.06 [ijkl]	5.81
MAS/Mul	*C. mangoroensis* Portères	MAN2	A402	MDG/C	Moramanga (Mangoro)	humid	7	1.16±0.03 [mnopq]	2.79
MAS/Mul	*C. sahafaryensis* J.-F. Leroy	SAHA	A978	MDG/NE	Vohimarina (ex-Vohémar)	dry	10	1.16±0.05 [mnopq]	4.20
MAS/Mul	*C. leroyi* A.P.Davis cf. (*C. costei* ined.)	COS	A956	MDG/NE	Sambava	dry	9	1.16±0.03 [mnopq]	2.77
MAS/Mul	*C. leroyi* A.P.Davis cf (*C. tsaratanensis* ined.)	TSA	A730	MDG/ NW	Tsaratanana	gallery	10	1.16±0.03 [mnopq]	3.34
MAS/Mul	*C. ankaranensis* A.P.Davis & Rakotonas.	ANK1	A525	MDG/ NW	Antsiranana (ex/Diego/Suarez) (Andranofohy)	dry	5	1.17±0.07 [nopqrs]	5.66
MAS/Mul	*C. ankaranensis* A.P.Davis & Rakotonas.	ANK2	A808	MDG/ NW	Diego/Suarez (Ankarana)	dry	5	1.17±0.07 [nopqrs]	6.30
MAS/Mul	*C. leroyi* A.P.Davis cf. (*C. daphnoides* ined.)	DAP	A317	MDG/ SE	Ifanadiana (Ranomafana)	humid	1	1.18 [nopqrs]	
MAS/Mul	*C. leroyi* A.P.Davis	AND1	A227	MDG/ SE	Mananjary (Andrambovato/ Tolongoina)	humid	5	1.19±0.04 [nopqrst]	3.64
MAS/Mul	*C. montis-sacri* A.P.Davis	MON	A321	MDG/ SE	Mananjary (Vatovavy)	humid	10	1.19±0.05 [nopqrst]	4

Table 1 (continued)

Main region[a]/ Botanical series	Species	Abbreviation used in figures	Population code	Country/ region	City-Locality of origin	Forest habitat[b]	Number of plants	Mean 2C DNA±SD (pg)	CV
MAS/Mul	*C. perrieri* Jum. & H.Perrier	PER3	A732	MDG/ SE	Mitsinjo	gallery	10	1.19±0.03 ^{nopqrst}	2.77
MAS/Mul	*C. leroyi* A.P.Davis	AND2	A310	MDG/ SE	Mananjary (Vatovavy)	humid	5	1.20±0.04 ^{nopqrst}	3.6
MAS/Mul	*C. perrieri* Jum. & H.Perrier	PER2	A305	MDG/S	Ihosy	gallery	10	1.21±0.04 ^{opqrst}	2.18
MAS/Mul	*C. bertrandii* A.Chev.	BERT	A5	MDG/ SE	Tolagnaro (ex/Fort/Dauphin) (station Nahampoana)	gallery	10	1.22±0.05 ^{pqrstu}	4
MAS/Mul	*C. leroyi* A.P.Davis	LER	A315	MDG/ SE	Ifanadiana (Ambodirafia)	humid	10	1.23±0.07 ^{qrstu}	5.6
MAS/Mul	*C. vianneyi* J.-F.Leroy	VIA1	A20	MDG/ SE	Nosy Varika (Ampasinambo)	humid	5	1.24±0.05 ^{rstu}	3
MAS/Mul	*C. coursiana* J.-F.Leroy	COU	A570	MDG/E	Ambodiriana (Toamasina (ex/ Tamatave))	humid	10	1.26±0.06 ^{tuv}	4.7
MAS/Mul	*C. betamponensis* Portères & J.-F.Leroy	BET	A573	MDG/E	Toamasina (ex/Tamatave) (Ambodiriana)	humid	3	1.26±0.08 ^{tuv}	6.3
MAS/Mul	*C. resinosa* (Hook.f.)Radlk.	RES1	A71	MDG/E	Maroantsetra (Forêt Farakaraina)	humid	10	1.27±0.03 ^{uvw}	2.63
MAS/Mul	*C. vianneyi* J.-F.Leroy	VIA2	A946	MDG/C	Moramanga	humid	10	1.27±0.03 ^{uvw}	2.29
MAS/Mul	*C. perrieri* Jum. & H.Perrier	PER1	A12	MDG/ SE	Tolagnaro (ex/Fort/Dauphin)	gallery	11	1.28±0.04 ^{vw}	3.47
MAS/Sub	*C. boiviniana* (Baill.)Drake	BOI	A980	MDG/NE	Vohimarina (ex-Vohémar)	dry	4	1.00±0.01 ^{abc}	1.34
MAS/Sub	*C. vatovavyensis* J.-F.Leroy	VAT	A830	MDG/ NE	Sambava	humid	11	1.03±0.04 ^{abc}	4.34
MAS/Sub	*C. bonnieri* Dubard	BON	A535	MDG/ NW	Antsiranana (ex/Diégo/Suarez) (Forêt d'Ambre)	dry	10	1.06±0.05 ^{cde}	4.53
MAS/Sub	*C. tsirananae* J.-F.Leroy	TSI	A515	MDG/ NW	Antsiranana (ex/Diégo/Suarez) (Cap d'Ambre)	dry	10	1.06±0.02 ^{cde}	2.18
MAS/Sub	*C. mcphersonii* A.P.Davis & Rakotonas.cf (C. *vohemarensis* ined.)	VOHE	A977	MDG/ NE	Vohimarina (ex-Vohémar)	dry	10	1.08±0.05 ^{efgh}	4.08
MAS/Sub	*C. pervilleana*	PERV	A957	MDG/ NE	Sambava	dry	10	1.12±0.05 ^{ijkl}	4.7
MAS/Sub	*C. jumellei*	JUM	A974	MDG/ NE	Vohimarina (ex-Vohémar)	dry	10	1.14±0.04 ^{lmnop}	3.15
MAS/Sub	*C. sakarahae* J.-F. Leroy	SAK	A304	MDG/S	Ihosy	dry	10	1.15±0.03 ^{lmnop}	2.48
MAS/Sub	*C. ratsimamangae* A.P.Davis & Rakotonas.	RAT	A528	MDG/ NW	Antsiranana (ex/Diego/Suarez) (Sandokoto)	dry	6	1.16±0.05 ^{mnopq}	4.23
MAS/Sub	*C. augagneuri* Dubard	AUG	A966	MDG/ NE	Ambilobe (Anivorano Nord)	humid	10	1.18±0.01 ^{nopqrs}	1.46
MAS/Ver	*C. homollei* J.-F.Leroy	HOM	A945	MDG/SE	Mananjary	humid	3	1.21±0.02 ^{opqrst}	1.81
MAS/Ver	*C. kianjavatensis* J.-F.Leroy	KIA2	A602	MDG/SE	Tolagnaro (ex/Fort/Dauphin) (Isaka/Ivondro)	humid	7	1.23±0.02 ^{qrstu}	1.21
MAS/Ver	*C. kianjavatensis* J.-F.Leroy	KIA1	A213	MDG/SE	Kianjavato/ Mont Vvatovavy	humid	9	1.24±0.02 ^{rstu}	1.65
MAS/Ver	*C. lancifolia* A.Chev. var. *auriculata* J.-F. Leroy	LAN	A320	MDG/SE	Mananjary (Madiorano/ Tolongoina)	humid	10	1.25±0.05 ^{stu}	4.06
ETA	*C. pseudozanguebariae*Bridson	PSE	H	KEN	Shimba Hills (coastal hills)	dry	4	1.23±0.04	3.25
ETA	*C. eugenioides* S.Moore	EUG	DA	KEN	Cheptuyet (Victoria Lake)	humid	6	1.47±0.06	4.08

Table 1 (continued)

Main region[a]/ Botanical series	Species	Abbreviation used in figures	Population code	Country/ region	City-Locality of origin	Forest habitat[b]	Number of plants	Mean 2C DNA±SD (pg)	CV
WCTA	*C. congensis* A.Froehner	CON	CB	CAF	Louma island (Oubangui River)	humid	8	1.54±0.09	5.08
WCTA	*C. canephora* Pierre ex A.Froehner	CAN	BC	CAF	Nana River	gallery	6	1.55±0.08	5.16
WCTA	*C. heterocalyx* Stoff.	HET	JC	CMN	Non available	humid	3	1.81±0.05	2.76
WTA	*C. humilis* A.Chev.	HUM	G	IVO	Taï forest	humid	6	1.84±0.08	4.34

Letters indicate groups of populations that were not significantly different at $p<0.05$ (HSD test)

MAS Mascarocoffea, *Mau* Humblotianae/Mauritianae, *Gar* Garcinoides, *Mil* Millotii, *Mul* Multiflorae, *Sub* Subterminales, *Ver* Verae, *NW* northwest, *NE* northeast, *S* south, *C* center, *E* east, *SE* southeast, *ETA* east Tropical Africa, *WTA* west Tropical Africa, *WCTA* west/central Tropical Africa, *MDG* Madagascar, *MAU* Mauritius, *COM/CO* Comoros

[b] Habitat information from Leroy 1972a, b; Charrier 1978; Davis et al. 2006 and F Rakotonasolo, Royal Botanical Gardens, Antananarivo, Madagascar, ‘ pers. comm.’).

[a] Main region and country code as defined in Brummitt (2001)

The DNA content of 5,000-10,000 stained nuclei was determined for each sample using a CyFlow SL3 flow cytometer (Partec, Sainte-Geneviève-des-Bois, France) with a 532-nm green solid-state laser (100 mW). Fluorescence emission of propidium iodide was collected through a 590-nm Longpass filter. Total 2 C DNA values were calculated on the basis of the linear relationship between fluorescent signals from stained nuclei of the unknown specimen and the known internal standard (DNA content in pg = [sample peak mode / standard peak mode] × 2C standard DNA content) (see Discussion).

Leaf parameters

Transparent nail polish was applied to the lower surface of mature fresh leaves (no stomata were observed on the upper surface) on the middle portion on either side of the midrib. This leaf region contains guard cell lengths (GCL) and stomata densities (SD) comparable to the mean of the entire leaf (Beaulieu et al. 2008). GCL was measured on two prints per leaf and five measurements per print. The SD (number per mm^2) was recorded after counting the number of stomata per field of view in five random fields from two prints out of five fresh leaves per accession. Overall, the measurements concerned five accessions per population. Species values were then calculated from the arithmetic mean of the 50 (5×2×5) individual records. Measurements were recorded under an optical microscope (Leitz Dialux 22 EB) at×200 magnification for GCL and SD. A micrometer was used for measurement calibration.

In addition to stomata records, the leaf length (LL) was measured and the arithmetic mean calculated from five adult leaves per accession picked on the third branching from the top. The arithmetic mean per population was then calculated and used thereafter. All measurements concerned Malagasy species from Kianjavato, Madagascar.

Statistical analyses

Linear regression was used to analyze: (a) correlations between 2 C DNA values from LyL and FL, and (b) relationships between the genome size and stomata parameters. A two-way nested ANOVA model was used to test variations in DNA content estimation due to series (fixed effects) and populations (random effects) nested in series. A three-way nested ANOVA was performed to test variations in physiological parameters (SD, GCL, and LL) due to population/ taxa (fixed effect), accessions (random effects), and leaves nested in accessions (random effects). Comparisons between arithmetic means were further assessed by honestly significative difference (HSD) test.

To gain further insight into the Mascarocoffea structure, a principal component analysis (PCA) was performed using

four variables (LL, GCL, SD, and 2 C DNA) and 44 populations. A hierarchical clustering (HC) was then performed on PCA coordinates using the WARD method. All statistical analyses were performed using R software 2.11.1, (Bretz et al. 2010, http://cran.r-project.org/).

Results

Lyophilized leaves are valid material for 2C DNA estimation

Mascarocoffea lyophilized leaves turned out to be suitable materials for flow cytometric analyses. Most measurements revealed coefficient of variation (CV) values of around 3 %, comparable with those generally obtained from fresh material. Overall, a very high linear correlation was obtained between measurements of FL vs. LyL of a set of eight Malagasy and six African species used as references (R^2=0.96; Fig. 1). Hence, for coffee trees, the relationship between 2 C values obtained from the two data sets (LyL vs FL) was as follows: 2 C value (in pg) LyL=1.052×2 C value FL−0.029. This was used to predict the 2 C DNA values that would be obtained using LyL from data obtained previously with FL of African species (Noirot et al. 2003a), (see Table S1 in additional files).

Mascarocoffea genome size variation

Mascarocoffea genome sizes (2C DNA values) were estimated from 416 accessions, corresponding to 52 populations representing 44 species. DNA content variation between populations within a species was not significant (Table 1). This allowed determination of 2 C DNA values per species by a simple arithmetic mean. Although 2 C DNA values ranged from 0.96 (*Coffea mauritiana*) to 1.41 pg (*C. millotii* ex *Coffea dolichophylla*), discrimination between all species, based only on genome size, was impossible.

The global 2 C DNA value distribution (this study and values calculated from Noirot et al. 2003a, Table S1) was bimodal and was obtained by overlapping two curves, one corresponding to Mascarocoffea and the other to African species (Fig. 2). Highly significant differences in genome sizes between Africa and Madagascar (1.43 and 1.20 pg, $F_{1,75}$=59.5, p<0.0001) were obtained.

Moreover, the Mascarocoffea 2 C DNA mean value was similar to that obtained for east Africa (1.19 and 1.18 pg, respectively) and lower than for west plus west-central Africa (1.50 pg, $F_{1,74}$=147; p<0.0001).

Regarding the habitat, species from dry environments had significantly bigger genomes in Africa (1.21 pg) than in Madagascar (1.13; $F_{1,22}$=5.2; p=0.03). However, comparable mean values were calculated for African species from dry habitat (1.21 pg) and all Malagasy species (1.20 pg, $F_{1,53}$=0.09, p=0.8) or species restricted to humid habitat (1.24 pg, $F_{1,32}$=0.63, p=0.43).

Among Mascarocoffea, significant 2 C DNA value differences were noted between the botanical series ($F_{5,46}$=43.7; p<0.0001). However, only the Mauritianae/Humblotianae plus Subterminales series (mean values: 1.08±0.12 vs 1.10 ±0.06 pg), the Multiflorae (1.20±0.05 pg) and the Millotii complex (1.33±0.06 pg) could be differentiated from one to another (HSD tests highly significant).

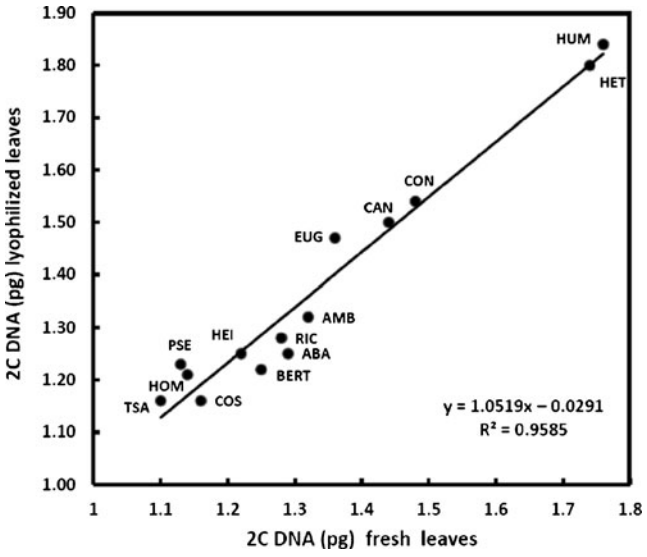


Fig. 1 Linear regression between 2 C DNA content values for lyophilized and fresh leaves (eight from this study and six from Noirot et al. 2003a) of 14 species covering the range of 2 C DNA of African species. *CAN C. canephora*, *CON C. congensis*, *EUG C. eugenioides*, *HET C. heterocalyx*, *HUM C. humilis*, and *PSE C. pseudozanguebariae*

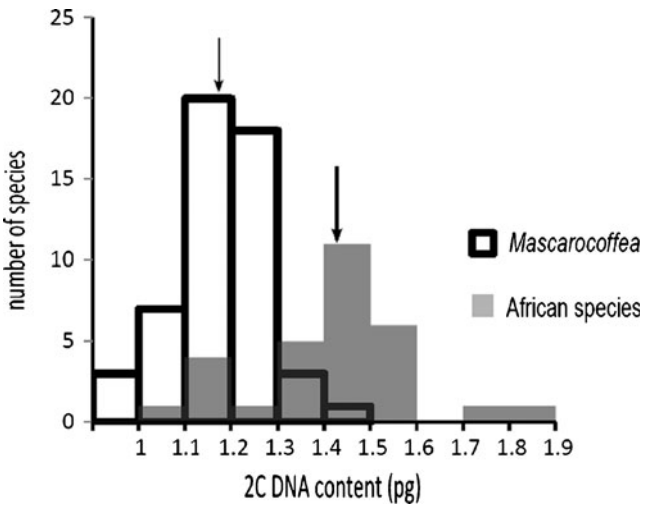


Fig. 2 2C DNA values distribution obtained for the two groups: Mascarocoffea (*open*) and African species (*dark*, from Noirot et al. 2003a normalized to lyophilized leaves according to the regression equation in Fig. 1)

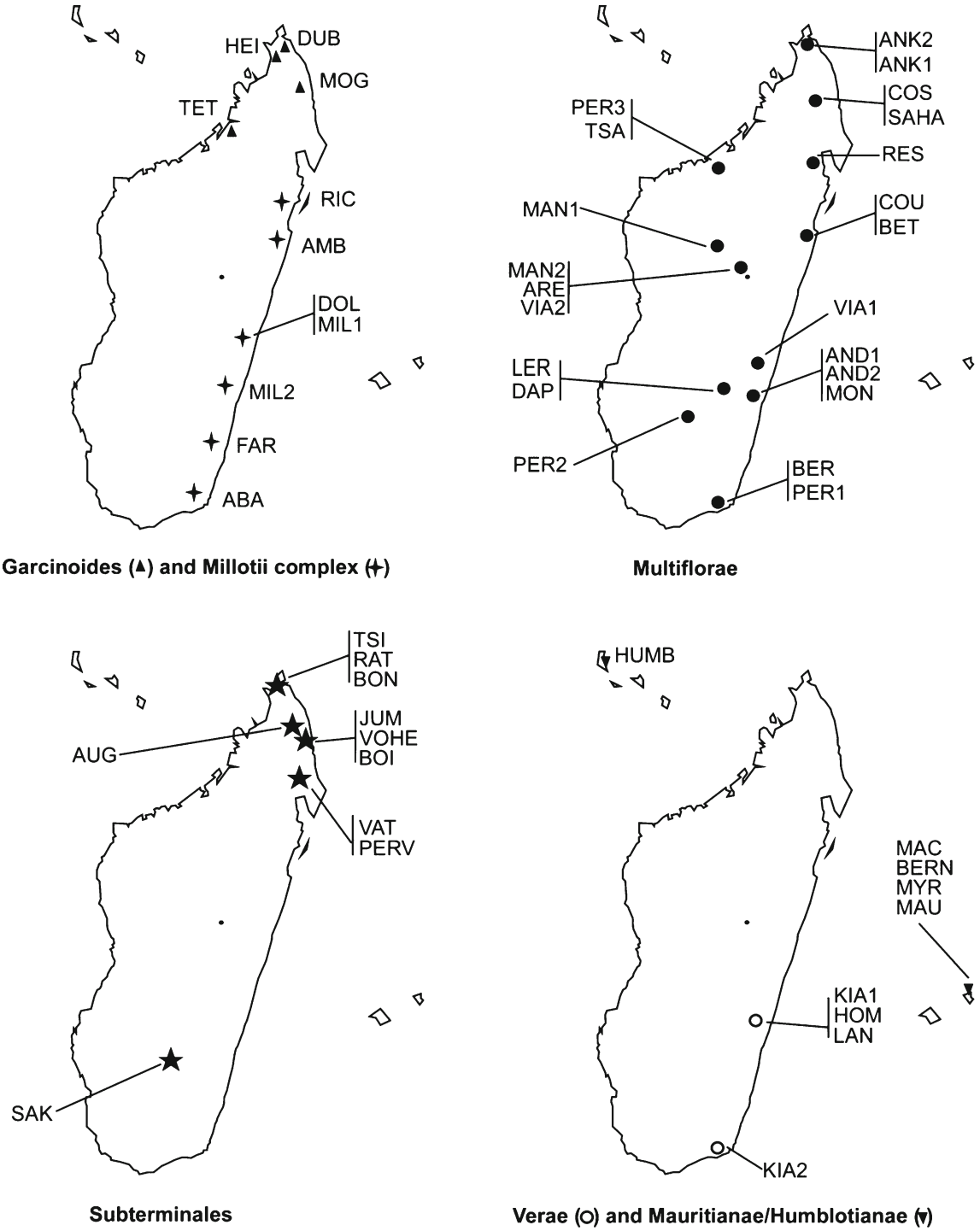
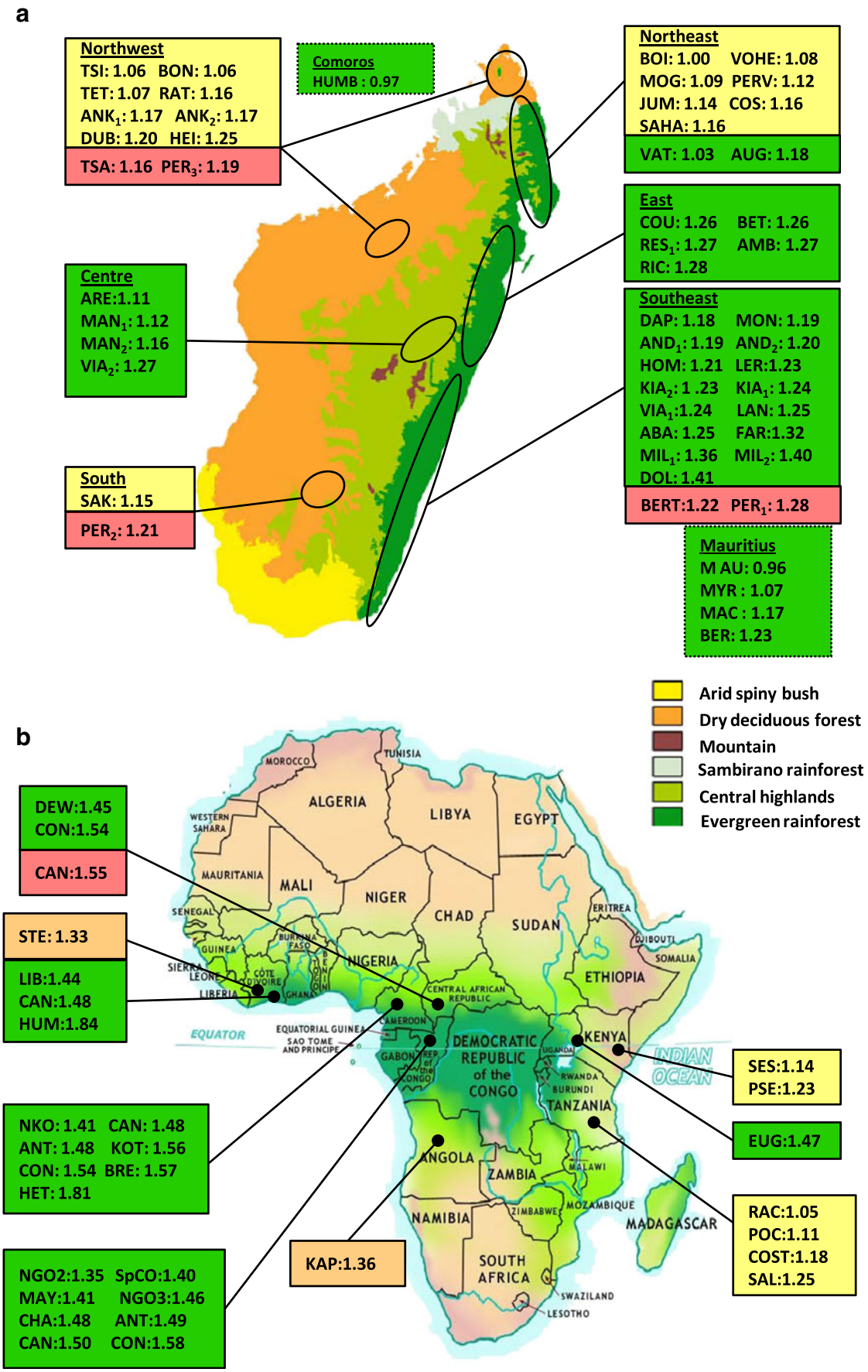


Fig. 3 Geographical distribution of the Mascarocoffea populations studied. The species codes used are as follows: *ABA*: *C. abbayesii*; *AND*: *C. leroyi* ex *C. andrambovatensis*; *ANK*: *C. ankaranensis*; *AMB*: *C. millotii* ex *C. ambodirianensis*; *ARE*: *C. arenesiana*; *AUG*: *C. augagneuri*; *BET*: *C. betamponensis*; *BER*: C. *bernardiniana*; *BERT*: *C. bertrandii*; *BON*: *C. bonnieri*; *BOI*: *C. boiviniana*; *COS*: *C. leroyi* ex *C. costei*; *DAP*: *C. leroyi ex C. daphnoides*; *DOL*:*C. millotii* ex *C. dolichophylla*; *DUB*: *C. dubardii*; *FAR*: *C. farafanganensis*; *HEI*: *C. heimii*; *HOM*: *C. homollei*; *HUMB*: *C. humblotiana*; *JUM*: *C. jumellei*; *KIA*: *C. kianjavatensis*; *LAN*: *C. lancifolia*; *LER*: *C. leroyi*; *MAC*: *C. macrocarpa*; *MAN*: *C. mangoroensis*; *MOG*: *C. mogenetii*; *MAU*: C. *mauritiana*; *MON*: *C. montis-sacri*; *MIL*: *C. millotii*; *MYR*: *C. myrtifolia*; *PER*: *C. perrieri*; *PERV*: *C. pervilleana*; *RAT*: *C. ratsimamangae*; *RES*: *C. resinosa*; *RIC*: *C. richardii*; *SAHA*: *C. sahafaryensis*; *SAK*: *C. sakarahae*; *TET*: *C. tetragona*; *TSA*: *C. perrieri* ex *C. tsaratanensis*; *TSI*: *C. tsirananae*; *VIA*: *C. vianneyi*; *VAT*: *C. vatovavyensis*; *VOHE*: *C. mcphersonii* ex *C. vohemarensis*


a
Northwest
TSI: 1.06 BON: 1.06
TET: 1.07 RAT: 1.16
ANK1: 1.17 ANK2: 1.17
DUB: 1.20 HEI: 1.25
TSA: 1.16 PER3: 1.19
Comoros
HUMB : 0.97
Northeast
BOI: 1.00 VOHE: 1.08
MOG: 1.09 PERV: 1.12
JUM: 1.14 COS: 1.16
SAHA: 1.16
VAT: 1.03 AUG: 1.18
East
COU: 1.26 BET: 1.26
RES1: 1.27 AMB: 1.27
RIC: 1.28
Centre
ARE:1.11
MAN1: 1.12
MAN2: 1.16
VIA2: 1.27
Southeast
DAP: 1.18 MON: 1.19
AND1: 1.19 AND2: 1.20
HOM: 1.21 LER:1.23
KIA2: 1 .23 KIA1: 1.24
VIA1:1.24 LAN: 1.25
ABA: 1.25 FAR:1.32
MIL1: 1.36 MIL2: 1.40
DOL: 1.41
BERT:1.22 PER1: 1.28
South
SAK: 1.15
PER2: 1.21
Mauritius
M AU: 0.96
MYR : 1.07
MAC : 1.17
BER: 1.23
Arid spiny bush
Dry deciduous forest
Mountain
Sambirano rainforest
Central highlands
Evergreen rainforest
b
DEW:1.45
CON:1.54
CAN:1.55
STE: 1.33
LIB:1.44
CAN:1.48
HUM:1.84
NKO: 1.41 CAN: 1.48
ANT: 1.48 KOT: 1.56
CON: 1.54 BRE: 1.57
HET: 1.81
NGO2:1.35 SpCO:1.40
MAY:1.41 NGO3:1.46
CHA:1.48 ANT:1.49
CAN:1.50 CON:1.58
KAP:1.36
SES:1.14
PSE:1.23
EUG:1.47
RAC:1.05
POC:1.11
COST:1.18
SAL:1.25
MOROCCO
TUNISIA
ALGERIA
LIBYA
EGYPT
WESTERN SAHARA
MAURITANIA
MALI
NIGER
CHAD
SUDAN
ERITREA
DJIBOUTI
SOMALIA
SENEGAL
BURKINA FASO
GUINEA
SIERRA LEONE
LIBERIA
CÔTE D'IVOIRE
GHANA
NIGERIA
ETHIOPIA
CENTRAL AFRICAN REPUBLIC
EQUATOR
EQUATORIAL GUINEA
SAO TOME AND PRINCIPE
CAMEROON
GABON
DEMOCRATIC REPUBLIC of the CONGO
KENYA
UGANDA
RWANDA
BURUNDI
TANZANIA
INDIAN OCEAN
ANGOLA
ZAMBIA
MALAWI
ZIMBABWE
MOZAMBIQUE
NAMIBIA
MADAGASCAR
SOUTH AFRICA
SWAZILAND
LESOTHO

◀ **Fig. 4** 2C DNA content distribution. Box color indicates the habitat: in *green*, humid forest; *yellow*, dry forest; *orange*, dry environments (top of hills or shrub savanna); *red*, gallery forest. **a** For Mascarocoffea, species from Madagascar, Comoros and Mauritius, the species codes used are as given in Fig. 3 and Table 1. **b** For African species, data given here are those calculated for lyophilized leaves from Noirot et al. (2003a). The species codes used are as follows: *ANT*: *C. anthonyi*, *BRE*: *C. brevipes*, *CAN*: *C. canephora*, *CHA*: *C. charrieriana*, *CON*: *C. congensis*, *COST*: *C. costatifructa*, *DEW*: *C. liberica* var dewevrei, *EUG*: *C. eugenioides*, *HET*: *C. heterocalyx*, *HUM*: *C. humilis*, *KAP*: *C. kapakata*, *LIB*: *C. liberica* var liberica, *POC*: *C. pocsii*, *PSE*: *C. pseudozanguebariae*, *SAL*: *C. salvatrix*, *SES*: *C. sessiliflora*, *STE*: *C. stenophylla*, *RAC*: *C. racemosa*, SpCO: *C. sp* Congo, *KOT*: *C. sp* Koto, *MAY*: *C. sp* Mayombe, *NGO2*: *C. sp* Ngongo2, *NGO3*: *C. sp* Ngongo3, *NKO*: *C. sp* Nkoumbala. 2 C DNA values are in pg

Geographical distribution of Mascarocoffea genome size

Regarding their biogeography, the Malagasy series were not evenly distributed (Fig. 3). Two (Multiflorae and Millotii complex) had a broad northeast to southeast range. Among Multiflorae species, *Coffea perrieri,* occurring in gallery forest, has the largest but most fragmented distribution resulting from past climatic changes and/or anthropic factors. Two series (Subterminales and Garcinoides) were mainly found in the north, while Verae was restricted to the southeast. Significantly different genomes sizes ($F_{5,41}$= 9.22, p=0.0001) were obtained among Mascarocoffea species. Three clusters were evidenced according to their geographical distribution: northeast (1.12±0.05 pg) plus northwest (1.14±0.06 pg), east (1.27±0.008) plus southeast (1.24±0.09 pg) distinct from one another (HSD test highly significant, p<0.001) and center plus south (1.17±0.07 and 1.18±0.04 pg) with intermediate values, statistically not different from the two clusters mentioned above.

Finally, the results highlighted global trends (Fig. 4a): (a) the smallest genomes were native to Mauritius and Comoros; (b) in Madagascar, 2 C DNA values tended to increase from north to southeast; (c) considering the species habitat reported by Leroy (1972a, b), Charrier (1978), and Davis et al. (2006), (F Rakotonasolo, Royal Botanic Gardens, Antananarivo, Madagascar, 'pers. Comm.'), genomes of the species that occur in dry forests (yellow boxes; Fig. 4a) covered the lower-middle part of the total size variation range, while genomes of species from humid forests (green boxes; Fig. 4a) mainly represented the middle-upper part of the genome size spectrum.

Estimation of leaf parameters and its correlation with 2C DNA content

Differences among species for the three morpho-physiological parameters investigated (SD, GCL, and LL) were highly significant ($F_{48,156}$=15.8, p<0.0001; $F_{44,146}$=51.6, p<0.0001; $F_{40,136}$=31, p<0.0001 respectively). Correlations between morpho-physiological parameters and 2 C DNA content, and relationships among leaf traits were observed. A negative correlation was found between SD and GCL (r=-0.65, p<2.2 10^{-16}; Fig. 5a), but no correlation was obtained between SD and LL. A significant positive correlation was obtained between LL and 2 C DNA content (r=0.48, p<0.0001, Fig. 5b). There was a weak but significant correlation between GCL and 2 C DNA content (r=0.18, p=0.013, Fig. 5c). Despite some marked differences between species in stomata density, no correlation was found with 2 C DNA content.

Mascarocoffea organization

The Mascarocoffea organization was assessed from multivariate analyses based on both leaf parameters and 2 C DNA content. Two components explained 79.9 % of the generalized variance with 43.1 and 36.8 % due to the first and the second component respectively. The differentiation between species with long leaves and large genomes (mostly from southeast) and long guard cell length species (mostly from the north) was clear on the first PCA axis (Fig. 6 insert). While the second axis mainly differentiated species with high stomata density (from east).

An HC (Fig. 6) performed on factorial coordinates revealed three clusters respectively defined by the following general trends: (1) high stomata density and medium-sized genomes (1.21 pg mean value), (2) large stomata guard cells associated with small leaves and genomes (1.13 pg mean value), and (3) long leaves associated with large genomes (1.33 pg mean value). When the geographical origin and habitat of species were reported on the HC, it appeared that species characterized by large stomata and generally small leaves and smaller genomes were mainly from the north and dry habitats, while species with long leaves and the larger genomes were mainly from humid habitats in the southeast. Species with high stomata density were mainly from humid habitats of east and southeast.

Discussion

Lyophilization, a reliable process to assess *Coffea* 2C DNA content

Generally, fresh plant material is requested for 2 C DNA content and/or ploidy estimation. However, as reported by Doležel and Bartoš (2005), this poses a strong limitation upon cytometry of material collected far from laboratories and with very large sampling. Hence, silica-dried material (routinely practiced in the field for sample preservation) has been used (Suda and Trávníček 2006). For plants with high content in phenolics, as coffees, such material is often oxidized and could therefore be inappropriate. For this reason we chose lyophilization.

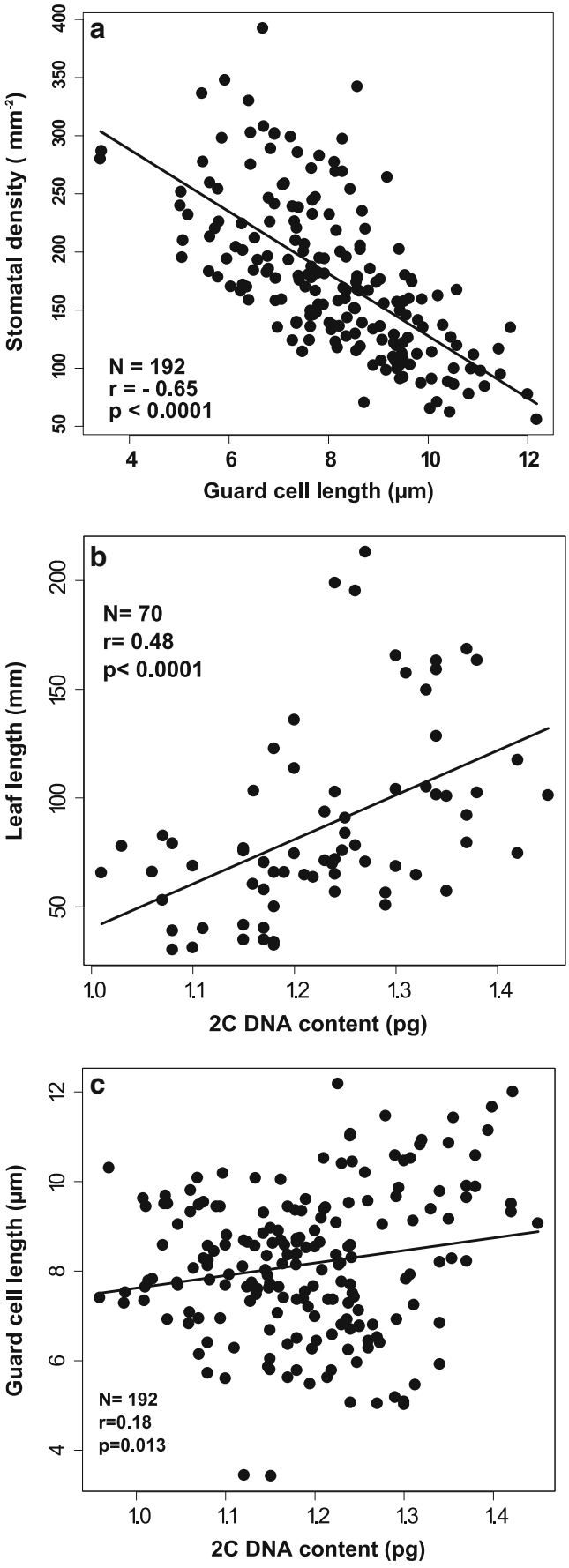


Fig. 5 Linear regression showing: **a** A negative correlation between stomata density and guard cell length; **b** Medium positive correlation between leaf length and 2 C DNA content; and **c** weak positive correlation between guard cell length and 2 C DNA content

Propidium iodide was used as an intercalary dye, as it is not sensitive to base composition. Taking 400 mg leaves (corresponding to 4 cm^2 for Noirot et al. 2003a), Barre et al. (1996) concluded that PI saturation is obtained with 330 µg/mL, but that this tends to destabilize the DNA. We used only 1 cm^2 leaf tissue and the typical concentration of 50 µg/mL PI, six-fold less than Barre et al. (1996). This gave reproducible relative peak positions for *Coffea* to standard (see the column CV in Table 2).

Noirot et al. (2005) demonstrated that temperature modifies chromatin-condensation and consequently PI accessibility, but the effects (increase/decrease PI accessibility) are not similar for *Coffea* and the standard. So, the practice for decades has been to work over ice with cold buffers, as done here. The level of Triton was raised from the typical 0.1 % (*w*/*v*) to 0.5 %. Cytosolic compounds (such as caffeine or phenolics, Noirot et al. 2003b) can also cause stoichiometric errors in the estimation of 2 C DNA content, possibly giving artefactual intraspecific variation (Noirot et al. 2000). Our buffer contained 1 % PVP to absorb polyphenols, and fresh 20 mM metabisulfite as antioxidant. The African species used to test the relationship between lyophilized and fresh material (Fig. 1) cover a large range of chlorogenic acids and caffeine content (Campa et al. 2005), while Malagasy are caffeine-free or display a low caffeine content (Rakotomalala 1992). They also have low levels of total chlorogenic acids (<2.5 % dry matter basis, Anthony et al. 1993). Yet the values of these biochemically diverse species apparently give the same relationship whether they are analyzed on lyophilized or fresh material, which is reassuring. Finally, as well demonstrated by Noirot et al. (2003 caffeine, 2005), there is no perfect solution to suppress all effects (negative and positive) having an impact on the genome size measurement. However, these authors clearly recommend that this "should not prompt scientists to drop genome size investigations using flow cytometry". When estimating genome size for new species, as it is the case here, "it is still better to have a slightly biased estimation than no estimation at all". We therefore used a linear relationship to convert the ratio of fluorescent signals to the genome size applying necessary precautions (Triton, PVP, metabisulfite, cold, an internal standard, and high replication).

Coffea genome size variation and leaf parameters

Regarding leaf parameters, for *Coffea* species, despite the absence of or poor correlation with genome size, it generally appeared that the smaller genome species from dry

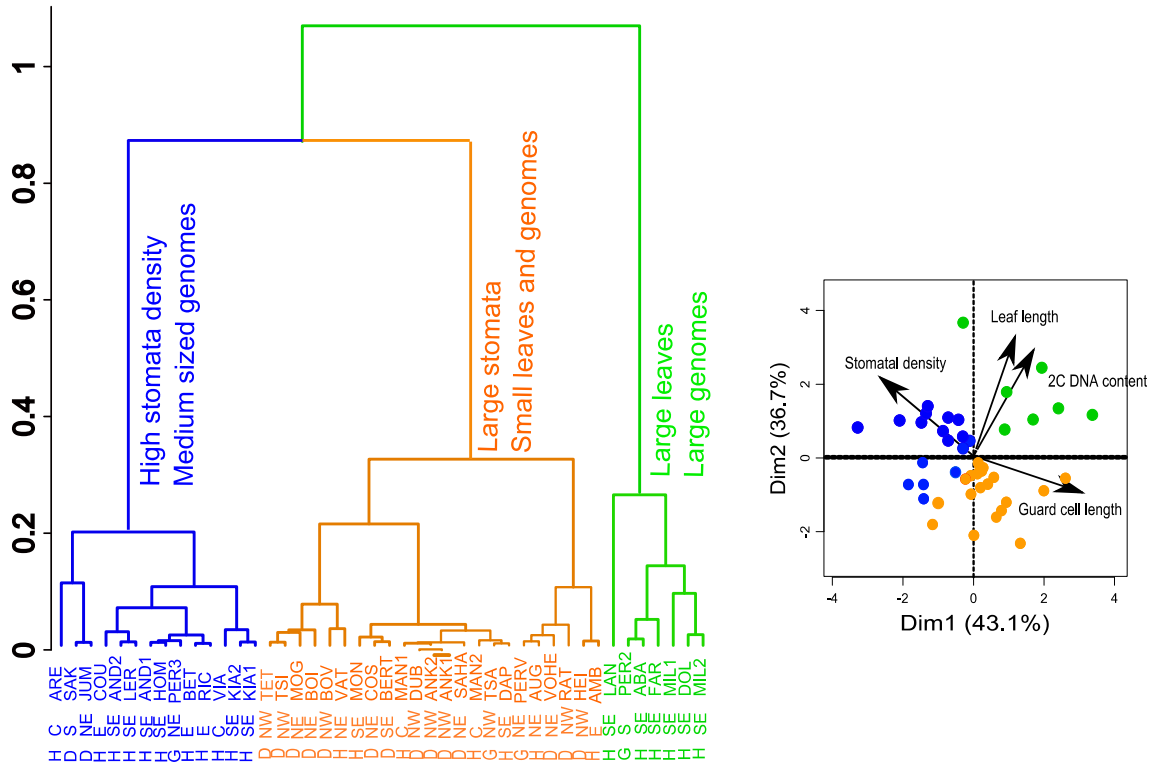


Fig. 6 Hierarchical clustering on factorial coordinates of a principal component analysis and WARD aggregation method. The four variables used are stomata density, guard cell length, leaf length, and genome size. The geographic origin (*NE* northeast, *NW* northwest, *C* center, *S* south, *E* east, and *SE* southeast as noted in Fig. 4a) and the habitat (*D* dry, *H* humid, and *G* gallery forest) of each population are indicated. Population codes are those used for Fig. 3 and Table 1

environments had smaller leaves with larger stomata, while larger genome species from humid habitats had longer leaves with a higher stomata density (Fig. 6). The positive correlations between genome and stomata sizes documented within eudicots, or between genome size and leaf shape and structure, reported across angiosperms, are seldom noted in trees (Hodgson et al. 2010). However, trees tend to have a small genome size and small and dense stomata, even though within tree samples there is no significant relationship between genome size and any of these physiological traits (study on 101 angiosperms, Beaulieu et al. 2008). One possible explanation could be that trees have a genome size range that is generally too small to detect such correlation. If genome size sets the minimum size of both guard cells and epidermal cells, the resulting change in stomatal density may predispose a species to a particular ecological and life history strategy. For *Coffea*, if the genome sizes were larger, there would probably be a more significant correlation between genome size and stomata size and density. This would probably also indicate that the species are well adapted to their growing environment and may not be or may be less able to colonize new niches. This last point would disagree with the assumption of recent speciation in Madagascar (Maurin et al. 2007).

Moreover, GCL is a complex trait that also depends on other factors such as hormonal regulation of plant growth and development, drought stress, and light quality. Moreover, *Coffea* genome size appeared also related to adaptive traits such as rainfall and water deficit (Cros et al. 1995; Noirot et al. 2003a; this study). Hence, for *Coffea* as for all other trees, the interaction between all these factors may result in an absence of clear relationship between each of these traits and the genome size.

Finally, for *Coffea* species, the global trend is that species with the smaller genomes and smaller and thicker leaves generally grow in dry areas of east Africa or northern Madagascar, while species from humid forests of west and central Africa or eastern and southeastern Madagascar have bigger genomes and larger and thinner leaves.

Geographical distribution of *Coffea* genome size

Genome size variation was observed at different levels throughout the genus *Coffea*, i.e., the main geographic regions (Mascarocoffea, west plus west-central Africa, and east Africa) and the series among Mascarocoffea. Although the *Coffea* phylogeny is not well resolved, three main clades corresponding to the main geographic regions were identified in all studies (Lashermes et al. 1997; Cros et al. 1998; Maurin et al. 2007). All African species and all Mascarocoffea were differentiated by their mean genome size (1.43

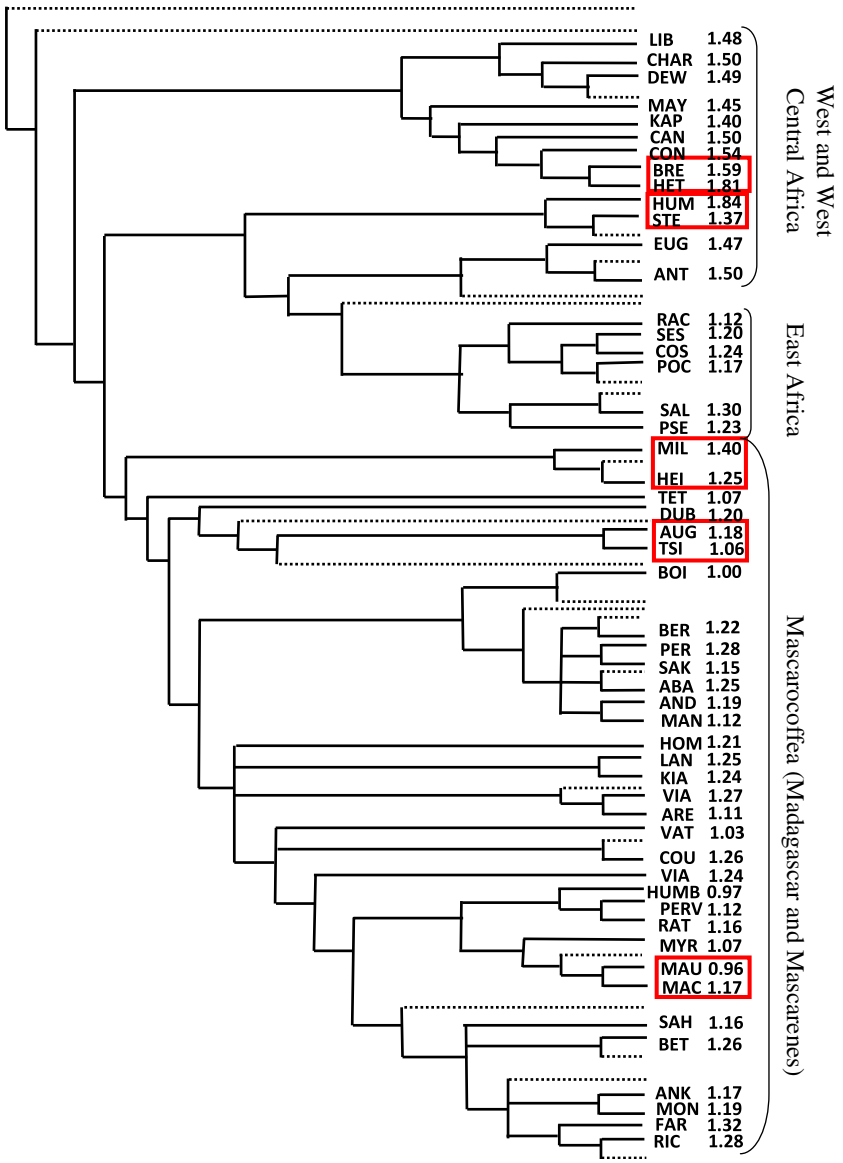


Fig. 7 Genome sizes plotted on the *Coffea* phylogeny tree adapted from Maurin et al. (2007). Only branches for species studied here were kept, while the others are dotted lines. *Red boxes* underline examples of closely related species differing by their genome size (see text). 2 C DNA values are in pg for lyophilized material

and 1.19 pg, respectively). Interestingly, within each landmass (Africa and Madagascar), a genome size gradient was identified: east to west for Africa and north to southeast for Madagascar. Furthermore, despite some exceptions, notably with respect to the two smallest genomes found in humid habitat of Mauritius and Comoros, the genome size distribution according to habitat in Madagascar as well as in Africa was in line with the general trend, i.e., the smaller genomes in dry forests or dry environments and the bigger genomes in humid forests (Figs. 4 and 6). Previous gradients were emphasized in Africa (Cros et al. 1995; Noirot et al. 2003a), the first with rainfall and the second with water deficit. Large-genome species appear to be less frequent in environments characterized by low precipitation and high temperatures (Knight and Ackerly 2002). Similarly, the genome size variation for diploid *Lasiocephalus* species (Asteraceae), was closely correlated with environmental and life traits (elevation, habitat, and growth form, Dušková et al. 2010).

At the geographical level, the genome size ranges for Mascarocoffea (0.96–1.41 pg) and for east African species (1.05–1.47 pg) were similar and lower than for west plus west-Central Africa (1.33–1.84 pg). However, the differences between minimum and maximum values are similar, indicating dynamic genome size variation in the three regions.

Given that the genome sizes are similar between east Africa and Madagascar (1.18 and 1.20 pg/2C respectively), it could be argued that the Malagasy species likely came from east Africa as previously postulated by Charrier (1978) and Leroy (1980). Under this scenario, it would be more likely that migrants from east Africa have been reached first the western coast or the north coast of Madagascar corresponding to rather dry environments. The founding species may have been adapted to dry environments in a first place, and only recently

radiated into more humid environments of Madagascar. This could be congruent with the fact that dry forests in Madagascar would be older than humid ones (Wells 2003). Moreover, climate was rather stable in Madagascar until 2–3 My ago (Yoder and Nowak 2006), contrary to what was observed in tropical Africa during Miocene (Morley 2000). This may partly explain a more pronounced speciation process in west plus west-central Africa and finally the significant differences in genome size between humid forest species found in Africa compared to Madagascar.

Finally, dispersal event(s) could more likely explain the origin of Malagasy *Coffea* species, while environmental factors and recent dispersal radiation would be responsible for the total endemism of Mascarocoffea. However, given the range of genome size variation, two questions could be addressed: has there been an increase or a decrease in DNA content, or both? Can genome size variation be correlated with genetic relationships among species?

Coffea genome size variation and species relationships

Coffea genome size and species relationships either from the molecular phylogenic tree or from their genetic divergence were not associated. Indeed, plotting genome sizes on Maurin's tree (the one including the wider Mascarocoffea sampling) did not reveal any clear relationship between genome size and phylogeny neither in Africa nor Madagascar. Indeed, some unexpected relationships appeared. For instance, *Coffea stenophylla* and *Coffea humilis* (two west African species) proved to be genetically close despite their very different 2 C DNA contents (1.33 vs. 1.84 pg), likewise for the two west-Central African species *Coffea brevipes* (1.57 pg) and *Coffea heterocalyx* (1.81 pg). In the same way, in Madagascar, *C. millotii* and *Coffea heimii* appeared to be genetically close despite their distinctive genome sizes (1.40 vs. 1.25 pg), as did *Coffea augagneuri* (1.18 pg) and *Coffea tsirananae* (1.06 pg) (red boxes; Fig. 7).

Relationships among species can also be estimated through the cross-hybridization success rate or through the F1 hybrid pollen fertility rate. For instance in Madagascar, two pairs of species, i.e., *Coffea resinosa* and *C. millotii*, with a 31.4 % cross-hybridization success rate and *Coffea vatovavyensis* and *Coffea bertrandii* with a 73 %F1 hybrid pollen fertility rate (Charrier 1978), should be considered as relatively close while they differed significantly by their 2 C DNA content (1.27 vs. 1.40 pg for the former and 1.03 vs. 1.22 pg for the latter). Regarding African species, *C. humilis* × *Coffea liberica* var *liberica* inter-specific F1 hybrids have good fertility (54–67 % pollen viability, Louarn 1993), while the two species differ significantly by their genome size (1.84 and 1.44 pg, respectively) and belong to separate clades. Altogether, these observations lead to the same conclusion that there is no relationship between genome size and genetic divergence or between genome size and phylogeny. This conclusion supports previous reports on *Hordeum* (Jakob et al. 2004), *Aegilops* (Eilam et al. 2007), and *Lasiocephalus* (Dušková et al. 2010).

Finally, like the cotton tribe (Wendel and Cronn 2002) or the genus *Agropyron* (Caetano-Anollès 2005), our results suggest that during *Coffea* genome evolution, both DNA content increase and/or decrease occurred independently in Africa and in the Indian Ocean Islands.

Acknowledgments Razafinarivo NJ receives a PhD fellowship from IRD and benefits from the RubiComp IRD funds. The Ueshima Coffee Company (UCC) contributes to maintaining the field collection at Kianjavato, Madagascar. We thank FOFIFA authorities and all people who facilitated access and field work at the Kianjavato research station, Madagascar.

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