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Inheritance and restriction fragment length polymorphism of chloroplast DNA in the genus *Coffea* L.

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Abstract CpDNA variation among 52 tree samples belonging to 25 different taxa of *Coffea* and two species of *Psilanthus* was assessed by RFLP analysis on both the total chloroplast genome and the *atpB-rbcL* intergenic region. Twelve variable characters were distinguished allowing the identification of 12 different plastomes. The low sequence divergence observed might suggest that *Coffea* is a young genus. The results were in contradiction with the present classification into two genera. Additionally, cpDNA inheritance was studied in interspecific hybrids between *C. arabica* and *C. canephora*, and in an intraspecific progeny of *C. canephora*, using PCR-based markers. Both studies showed exclusively maternal inheritance of

tetraploid and is self-fertile, while other species are diploid and generally self-incompatible (Charrier and Berthaud 1985).

Analysis of chloroplast DNA (cpDNA) variation has proven to be immensely valuable for plant phylogenetic reconstruction (Clegg and Zurawski 1992; Olmstead and Palmer 1994). Before using cpDNA as an evolutionary marker in the genus *Coffea*, different features such as the mode of cpDNA inheritance and the importance of intraspecific variation (Harris and Ingram 1991) need to be considered. In addition, the value of cpDNA for studying genetic relationships between *Coffea* species is related to the type and extent of the observed variation

Materials and methods

Plant material

The plant material included in the cpDNA variation analysis was obtained from a field-based collection resulting from several expeditions in Africa and Madagascar (Anthony 1992). The sampling strategy was to maximise the likelihood of detecting variation among accessions. Therefore, *Coffea* species were represented by several samples when more than one genetic group had been revealed by previous agro-morphological studies (Hamon et al. 1995). In total, 50 samples belonging to 25 taxa were analysed. The closely related genus *Psilanthus* was also represented by two species, *P. ebracteolatus* and *P. mannii*, which belong to the subgenera *Afrocoffea* and *Psilanthus*, respectively (Bridson and Verdcourt 1988). The accessions surveyed and their origins are indicated in Table 1.

For the determination of cpDNA inheritance, the plant material studied included two interspecific hybrids (Et30×IF181T and Caturra×IF181T) between *C. arabica* (female parent) and *C. canephora*, 15 individual trees resulting from an intraspecific cross in *C. canephora* between DH160-02 (female parent) and IF200, and

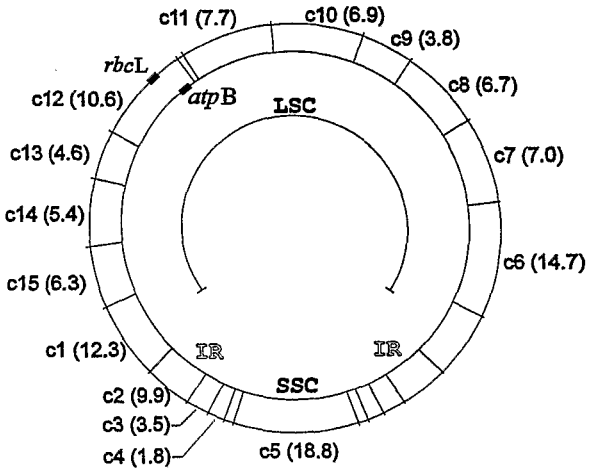

Fig. 1 Location of *Lactuta sativa* cpDNA *Sac*I fragments (Jansen and Palmer 1987) used as probes in this study. The size (kb) of each

Table 1 Origin of the accessions analysed for cpDNA variation

Taxa		Accession code	Origin
1	*Coffea arabica* L	ET 12-5	Ethiopia
2		Catura amarillo	Brazil (cultivar)
3		Marsabit/3099	Kenya
4		Marsabit/3058	Kenya
5		hibrido de Timor	Timor island
6	*C. bertrandi* Chev.	Bertrandi	Madagascar
7	*C. brevipes* Hiern	Mount Cameroon	Cameroon
8		Mungo	Cameroon
9	*C. canephora* Pierre	IF 444	Côte-d'Ivoire (cultivar)
10		IF 200	Côte-d'Ivoire (cultivar)
11		IF 155	Côte-d'Ivoire (cultivar)
12		IF A25	Côte-d'Ivoire (cultivar)
13		IF 410	Côte-d'Ivoire (cultivar)
14		IF 160	Côte-d'Ivoire (cultivar)
15	*C. congensis* Froehner	03 255	Central African Rep.
16		03 1650	Congo
17		03 429	Central African Rep.
18		03 103	Central African Rep.
19	*C. costatifructa* Bridson	08 111	Tanzania
20	*C. eugenioides* Moore	04 1485	Kenya
21		04 010	Kenya
22		04 005	Kenya
23	*C. farafanganensis* Leroy	Farafanganensis	Madagascar
24	*C. humblotiana* Baillon	OB 080	Comoro islands
25	*C. humilis* Chev.	07 141	Côte-d'Ivoire
26	*C. kapakata* Chev.	intro. Brazil	Angola
27		intro. Tanzania	Angola
28	*C. liberica* Hiern	pop. Koto/EC 05	Cameroon
29	*C. liberica* var. *dewevrei*	pop. N'Dongue/05 797	Central African Rep.
30	(De Wild. & Th. Dur) Lebrun	pop. Balifondo/05 559	Central African Rep.
31	*C. liberica* var. *liberica*	EA1	Côte-d'Ivoire
32	(Hiern) Lebrun	pop. Taï/05 242	Côte-d'Ivoire
33	*C. millotii* Leroy	Millotii	Madagascar
34	*C. perrieri* Drake	Perrieri	Madagascar
35	*C. pervilleana* Drake	Pervilleana	Madagascar
36	*C. pseudozanguebariae* Bridson	08 228	Tanzania
37		08 021	Kenya
38	*C. racemosa* Lour.	intro. Tanzania	Mozambica
39		intro. Portugal	Mozambica
40	*C. salvatrix* Swynn. & Phil.	intro. Tanzania	Botswana
41		intro. Brazil	Mozambica
42	*C. sessiliflora* Bridson	PA 4	Kenya
43		08 161	Tanzania
44	*C. sp.* Mayombe	Mayombe	Congo
45	*C. sp.* Moloundou	OC 210	Congo
46	*C. sp.* N'gongo II	OC 282	Congo
47	*C. sp.* N'koumbala	OC 105	Cameroon
48	*C. sp. X*	Sp X	unknown
49	*C. stenophylla* Don	FB 1	Côte-d'Ivoire
50		FA 21	Côte-d'Ivoire
51	*Psilanthus ebracteolatus* Hiern	OA 153	Côte-d'Ivoire
52	*P. mannii* Hook. f.	OA 009	Côte-d'Ivoire

restriction sites had been assayed. One-hundred-and-four restriction sites were shared by all 52 accessions while eight sites varied among taxa (Table 2). All variable sites are in the large single copy of the chloroplast genome. Digestion with *Eco*RI revealed significantly more polymorphism (six sites) than the *Eco*RV or *Dra*I digests (one site each).

A heterogeneous profile was detected for coffee species originating in Madagascar using the c6 probe on the *Eco*RI digest (Fig. 2). Incomplete digestion was ruled out since repeated assays produced consistent results for all samples. Differences in intensity of hybridisation signals between profiles were constant across experiments and genotypes.

Table 2 Chloroplast DNA restriction fragment length polymorphisms observed among 52 accessions of coffee species

Character	Enzyme		Probe (position[a])	Nature
Total cpDNA	1	*Eco*RI	c6	Restriction-site mutation
	2	*Eco*RI	c6	Restriction-site mutation
	3	*Eco*RI	c6	Restriction-site mutation
	4	*Eco*RI	c7, c8	Restriction-site mutation
	5	*Eco*RI	c11	Restriction-site mutation
	6	*Eco*RI	c13, c14	Restriction-site mutation
	7	*Eco*RI	c6	Restriction-site mutation
	8	*Dra*I	c13	Restriction-site mutation
atpB-rbcL region	9	*Hae*III	(380)	Restriction-site mutation
	10	–	(418–692)	Insertion/deletion (30 bp)
	11	–	(370–418)	Insertion/deletion (30 bp)
	12	–	(130–370)	Insertion/deletion (10 bp)

[a] Position sites referring to the *atpB-rbcL* sequence of *C. arabica* (CHCARBCLA, EMBL database)

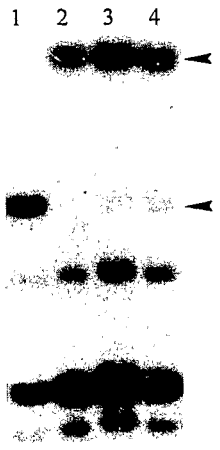

Fig. 2 Autoradiograph showing a heterogeneous profile detected for coffee species originating in Madagascar using a c6 cpDNA probe on an *Eco*RI digest. *Lane 1* is *C. sessiliflora* (42), *lane 2 C. racemosa* (38), *lane 3 C. farafanganensis* (23) and *lane 4 C. millotii* (33). *Arrows* indicate variant fragments

For different accessions, the *Eco*RI single digest was used to sequentially align the various fragments in order to estimate the total length of the coffee cpDNA genome. The length calculated by the summation of the sizes of the different restriction fragments reached 170 kb. Since the set of probes used represent only 95% of the lettuce cpDNA, and as small restriction fragments could be undetected, the size of the total coffee chloroplast genome was estimated as roughly 175 kb. The 112 restriction sites surveyed therefore represent approximately 0.4% of the coffee cpDNA.

RFLP analysis of the *atpB-rbcL* region

PCR was used to amplify *atpB-rbcL* intergenic spacers from the 52 accessions studied. A single band was obtained which varied in length from approximately 930 to 960 bp, depending on the accession. Restriction fragments were analysed following digestion with each of the four restric-

Chloroplast DNA variation in the genus *Coffea*

Combinations of the eight restriction-site changes revealed by RFLP analysis on the chloroplast genome, and the four variable characters detected on the *atpB-rbcL* intergenic fragments, produced 12 different cpDNA plastome types among the 52 coffee-tree samples analyzed (Table 3). No intraspecific variation was detected within any of the 12 species for which more than one individual tree was studied. The distinct plastome types identified are shared by accessions from either one, two, three or four different species. *Coffea* species belonging to the different plastomes always originate from the same area (West Africa, Central Africa, East Africa or Madagascar), except for the type-B plastome including *C. costatifructa* and *C. kapakata* which are found in East and Central Africa, respectively. Maximum divergence was observed between plastome D, which encompasses species originating from West Africa (*C. brevipes*, *C. canephora*, *C. congensis*) and plastome K, which includes *C. humblotiana* found in the Mascarenes. Plastomes D and K diverged for 5 of the 12 polymorphic characters (four of the eight restriction-site changes on total cpDNA). The two species belonging to the genus *Psilanthus*, *P. ebracteolatus* and *P. mannii*, did not differ from *Coffea* species, and were included in two relatively divergent plastomes.

Inheritance of cpDNA

The amplified *atpB-rbcL* fragments of two interspecific hybrids between *C. arabica*, used as female parent, and *C. canephora* (4x) were analysed (Fig. 3). Only the cpDNA patterns of arabica parents, either Et30 or Caturra, were observed, indicating strictly maternal inheritance. The *ndhC-trnV* fragment phenotypes were determined for 15 individual trees derived from the cross between DH160-02 and IF200 (Fig. 4). In all trees examined, a 1100-bp frag-

Table 3 Distribution of the species analysed among the different plastomes identified. For each plastome, the state of the 12 polymorphic characters encoded in the coffee chloroplast DNA is indicated

Plastome type	Character states												Taxa
	1	2	3	4	5	6	7	8	9	10	11	12	
A	0	0	0	1	0	0	1	0	0	0	0	0	*C. arabica, C. eugenioides, C. sp.* Moloundou
B	0	0	0	1	0	0	1	0	0	0	0	1	*P. mannii, C. costatifructa, C. kapakata*
C	1	0	0	1	0	0	1	0	0	0	0	1	*C. liberica, C. sp. X*
D	1	0	1	0	0	0	1	0	0	0	0	1	*C. canephora, C. congensis, C. brevipes*
E	0	0	0	1	1	0	1	0	0	0	0	1	*C. stenophylla, C. humilis*
F	0	0	0	1	0	0	1	1	0	0	0	1	*C. sp.* Mayombe, *C. sp.* N'gongo II
G	0	0	1	0	0	0	1	0	0	0	0	1	*C. sp.* N'koumbala
H	0	0	0	1	0	0	0	0	1	0	0	1	*C. pseudozanguebariae*
I	0	1	0	1	0	0	0	0	0	0	0	1	*C. salvatrix, C. sessiliflora*
J	0	0	0	1	0	0	0	0	0	0	1	1	*C. racemosa*
K	0	0[a]	0	1	0	1	1	0	0	1	0	1	*C. humblotiana*
L	0	0[a]	0	1	0	1	1	0	0	0	0	1	*C. millotti, C. farafanganensis, C. pervilleana, C. bertrandi, P. ebracteolatus*

[a] Heterogeneous profile is observed; only the predominant character is taken in consideration

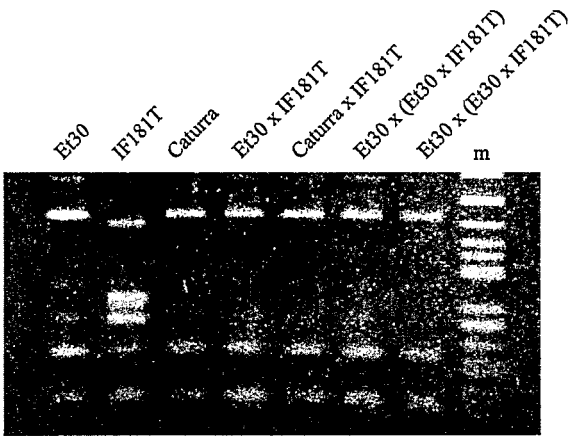

Fig. 3 Electrophoretic pattern of the 960-bp PCR-amplified *atpB-rbcL* fragment from two interspecific hybrids between *C. arabica* (accessions Et30 and Caturra) and *C. canephora* (IF181T) and the parental genotypes, after double digestion with *Alu*I and *Sau*3A

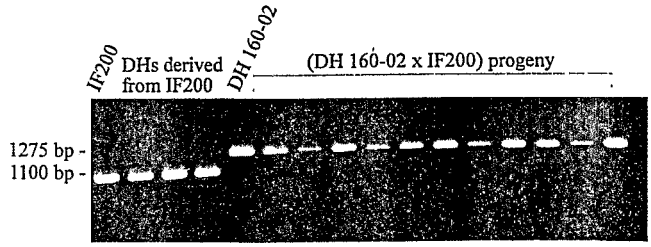

Fig. 4 Electrophoretic pattern of length variation of the PCR-amplified *ndhC-trnV* fragment generated from the clone IF200, doubled haploids derived from IF200, DH160-02, and individual trees resulting from the cross DH160-02×IF200

(Fig. 4), the IF200-specific fragment (1275 bp) was observed as expected.

Discussion

The size of the chloroplast genome of *Coffea* is roughly 175 kb, which is larger than the previous estimate (157 kb) for another species belonging to the Rubiaceae, *Psychotria bacteriophila* (Bremer and Jansen 1991), and the cpDNA size of most land plants (Palmer 1985). An RFLP analysis involving double enzyme digests would be required to construct a physical map of the *Coffea* chloroplast genome. However, single-digest fragments analysis did not show any major structural rearrangements from the other Rubiaceae species analysed using the same set of heterologous probes (Bremer and Jansen 1991). *Coffea* species have the chloroplast genome arrangement typical of most angiosperms examined (Palmer 1985), with one large and one small single copy region (LSC and SSC respectively) separated by an inverted repeat (IR). The large size estimated for coffee seems to be mainly due to the presence of a wide inverted repeat. However, a more accurate estimation of coffee cpDNA size is necessary.

Fifty individual trees representing 25 taxa were assayed to determine the extent and type of cpDNA variation present in the subgenus *Coffea*. Despite a relatively large number of taxa analysed, no intraspecific variation was detected by RFLP analysis on either the total chloroplast genome or the *atpB-rbcL* region, and cpDNA divergence between several species was either reduced or absent. Additional cpDNA analysis involving digestion with numerous restriction enzymes should be used to reveal and study intraspecific variation in coffee species. Another strategy would be to focus cpDNA variation analysis on several

non-coding regions, known to display higher mutational rates than coding regions (Palmer et al. 1988). In this regard, preliminary results obtained with the *ndhC-trnV* fragment in *C. canephora* are promising.

The four site changes observed in the chloroplast genome between the most divergent plastomes (D and K) relative to the 112 restriction sites, representing approximately 672 nucleotides, led to an estimate of maximum overall sequence divergence of 0.6%. Although published studies vary with regard to genome coverage, choice and number of restriction enzymes, sample sizes, and method of evaluation, the amount of cpDNA diversity in *Coffea* can be compared to that in other genera of flowering plants. Genera for which cpDNA sequence diversity has been reported include: *Coreopsis* (0.2%, Crawford et al. 1990), *Tripsacum* (0.36%, Larson and Doebley 1994), *Mitella* (0.5%, Soltis et al. 1990), *Glycine* subg. *Glycine* (1.3%, Doyle et al. 1990), *Gossypium* (2.1%, Wendel and Albert 1992), *Brassica* (2.6%, Palmer et al. 1983), and *Plantago* (3.4%, Wolff and Schaal 1992). Thus, as compared to these genera, *Coffea* exhibits relatively little cpDNA variation.

The few differences observed in this group of *Coffea* chloroplast genomes may be due in part to the relatively long life cycles of coffee-trees, whose generation times have been estimated to be between 20 and 30 years (Berthaud 1986). The result of long life cycles could be a slow accumulation of mutations because of the long "generation time", or reduced nucleotide substitution rates, as has been observed in palms (Wilson et al. 1990) and in *Viguiera* (Schilling and Jansen 1989). In addition, if one assumes

1994). In the present study, a unique heterogeneous pattern was observed across different species. Other explanations, such as cross homologies between cpDNA and both nuclear and mitochondrial genomes (Timmis and Scott 1983; Stern and Palmer 1984; Sederoff et al. 1986), could be advanced. However, further studies involving isolated cpDNA are required before it can be asserted that such phenomena occur in coffee.

In conclusion, the present results reaffirm the utility of cpDNA variation in systematic studies at the intraspecific level (reviewed in Soltis et al. 1992). However, the low cpDNA variation in *Coffea* requires a study of the most variable regions, such as the intron and intergenic spacer (Gielly and Taberlet 1994), in order to extend the level of resolution of cpDNA. The determination of chloroplast DNA inheritance in coffee-trees was a pre-requisite for use of the chloroplast molecule in tracing the evolutionary history of *Coffea* species. Restriction analysis of PCR-amplified intergenic fragments of chloroplast DNA appeared to be very powerful in examining cpDNA transmission. The use of PCR guarantees high sensitivity and allows the analysis of large sample sets. Such markers could be very useful in further studies on cpDNA gene flow and interspecific hybridisation in natural populations.

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