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Phylogenetic Analysis of Chloroplast DNA Variation in *Coffea* L.

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The *trn*L–*trn*F intergenic spacer of cpDNA has been sequenced from 38 tree samples representing 23 *Coffea* taxa and the related genus *Psilanthus*. These sequences were used for phylogenetic reconstruction using parsimony analyses. The results suggest a radial mode of speciation and a recent origin in Africa for the genus *Coffea*. Phylogenetic relationships inferred from the cpDNA analysis suggest several major clades, which present a strong geographical correspondence (i.e., west Africa, central Africa, east Africa, and Madagascar). The overall results agree well with the phylogeny previously inferred from nuclear genome data. However, several inconsistencies are observed among taxa endemic to west Africa, suggesting the occurrence of introgressive hybridization. Evidence is also obtained for the genetic origin of the allotetraploid species *C. arabica*.

INTRODUCTION

The low frequency of structural changes in the chloroplast DNA molecule (cpDNA) together with a conservative rate of sequence evolution make it an ideal target for plant phylogenetic study (Clegg and Zurawski, 1992; Olmstead and Palmer, 1994). Since the chloroplast genome of most angiosperms is inherited unidirectionally through maternal lines (Reboud and Zeyl, 1994), numerous conservative markers can be generated that are easily interpreted and utilized in reconstructing phylogenies. In particular, analysis of cpDNA variation has proven to be valuable for revealing possible cases of introgression (Doebley, 1992; Rieseberg and Brunsfeld, 1992). Interspecific hybridization may result in a nonconcordance between chloroplast-based and nuclear-based phylogenies. Combined use of cytoplasmic and nuclear markers allows introgression to be detected and distinguished from other phenomena, such as joint retention of the ancestral condition, clinal variation, and convergence (Doebley, 1992; Rieseberg and Soltis, 1991; Rieseberg and Wendel, 1993).

Coffee trees (family Rubiaceae) are classified in two genera, *Coffea* and *Psilanthus*, each genus being divided into two subgenera (Bridson and Verdcourt, 1988). All *Coffea* species are native to the intertropical forest of Africa, Madagascar, and islands of the Indian Ocean (Mascareign and Comoro Islands), while species belonging to the genus *Psilanthus* originate from either Asia or Africa. The subgenus *Coffea* encompasses more than 80 taxa so far identified, including the two species of economic importance: *Coffea arabica* L. and *Coffea canephora* Pierre (Charrier and Berthaud, 1985; Berthaud and Charrier, 1988). *Coffea* species are diploid ($2n = 2x = 22$), except *C. arabica* ($2n = 4x = 44$) which is self-fertile and considered a segmental allotetraploid (Carvalho, 1952; Grassias and Kammacher, 1975). Although showing considerable variation in morphology, size, and ecological adaptation, *Coffea* species hybridize readily with one another and produce relatively fertile hybrids (Charrier, 1978; Louarn, 1993). Success in intergeneric hybridization has even been reported (Couturon, personal communication). The internal transcribed spacer (ITS 2) region of the nuclear ribosomal DNA has been successfully used to establish a molecular phylogeny of *Coffea* species (Lashermes *et al.*, 1997). Nevertheless, comparisons between phylogenies inferred from both chloroplast and nuclear genomes would provide a better basis for assessing species relationships.

Exclusively maternal inheritance of cpDNA was observed in interspecific hybrids between *C. arabica* and *C. canephora*, and in an intraspecific progeny of *C. canephora*, suggesting that the mode of plastid inheritance in *Coffea* is strictly maternal (Lashermes *et al.*, 1996). CpDNA variation present in the subgenus *Coffea* was assessed by restriction fragment length polymorphism (RFLP) on both the total chloroplast genome and the atpB–rbcL intergenic region (Lashermes *et al.*, 1996). Only 12 variable characters were evidenced, indicating low cpDNA variation. To increase the number of polymorphic markers, comparative DNA sequencing seems particularly relevant. The technique is relatively fast, convenient, and offers a large data set of discrete characters. The sequence of a chloroplast gene such as *rbc*L, which has been widely used for inferring

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phylogeny in plants (Clegg and Zurawski, 1992), is likely to be changing too slowly to provide enough characters for a phylogenetic analysis between congeneric species. Recent results indicate that chloroplastic noncoding regions such as the intergenic spacer between the *trn*L (UAA)3′ exon and the *trn*F (GAA) gene can be used to address questions concerning relationships among closely related species or genera (Van Ham *et al.*, 1994; Gielly and Taberlet, 1994).

In the present study we have sequenced the *trn*L–*trn*F intergenic spacer of 38 tree samples representing 23 *Coffea* taxa and the related genus *Psilanthus*. By studying the cpDNA variation, we sought to gain insights into *Coffea* evolution. Phylogenetic relationships of *Coffea* species inferred from the chloroplast DNA variation are compared with estimates based on data from the nuclear genome.

MATERIALS AND METHODS

Plant Material

Plant material was obtained from the ORSTOM collection, which resulted from several expeditions in Africa and Madagascar (Anthony, 1992). The accessions surveyed, with their origins, are indicated in Table 1. Thirty-six accessions belonged to 23 *Coffea* taxa. 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). For outgroup comparison, we analyzed an accession of *Gardenia grandiflora*.

Total DNA was isolated from lyophilized leaves as previously reported (Lashermes *et al.*, 1993), except

TABLE 1

List and Origin of Accessions Analyzed for cpDNA Variation

Taxa	Accession code	Population name	Country of origin
Coffea arabica L.	ET 12-5		Ethiopia
	Caturra		Brazil (cultivar)
C. bertrandi Chev.	Bertrandi		Madagascar
C. brevipes Hiern		Mt. Cameroon	Cameroon
		Mungo	Cameroon
C. canephora Pierre	A25		Côte-d'Ivoire (cultivar)
	IF200		Côte-d'Ivoire (cultivar)
	IF444		Côte-d'Ivoire (cultivar)
C. congensis Froehner	03 255	Louma	Central African Republic
	03 429	Anginga	Central African Republic
	03 1650	Brazzaville	Congo
C. costatifructa Bridson	08 111	Utete	Tanzania
C. eugenioides Moore	04 0005	Kimilili	Kenya
	04 1485	Cheptuyet	Kenya
C. humblotiana Baillon	Humblotiana		Comoro Islands
C. humilis Chev.	07 141	Sakré	Côte-d'Ivoire
C. kapakata Chev.	Intro. Brazil		Angola
C. liberica var. *dewevrei* Lebrun	05 797	N'Dongue	Central African Republic
	05 559	Balifondo	Central African Republic
C. liberica var. *liberica* (Hiern)	EA1		Côte-d'Ivoire
Lebrun	05 242	Taï	Côte-d'Ivoire
C. millotii Leroy	Millotii		Madagascar
C. pseudozanguebariae Bridson	08 021	Shimba	Kenya
	08 228	Uzigua	Tanzania
C. racemosa Lour.	Intro. Portugal		Mozambique
C. salvatrix Swynn. & Phil.	Intro. Brazil		Mozambique
	Intro. Tanzania		Botswana
C. sessiliflora Bridson	PA4	Shimba	Kenya
	08 161	Kitulangalo	Tanzania
C. sp. Mayombe		Mayombe	Congo
C. sp. Moloundou	OC 210	Souanké	Congo
C. sp. N'gongo II		N'gongo II	Congo
C. sp. N'koumbala	OC 105	N'koumbala	Cameroon
C. sp. X			Unknown
C. stenophylla Don	FB 1	Ira	Côte-d'Ivoire
	FA 21	Assabli	Côte-d'Ivoire
Psilanthus ebracteolatus Hiern	OA 153	Bafingdala	Côte-d'Ivoire
P. mannii Hook. f.	OA 009	Divo	Côte-d'Ivoire
Gardenia grandiflora			

that CTAB was replaced by MATAB (mixed alkyl trimethylammonium bromide) in the extraction buffer.

PCR Amplification and DNA Sequencing

Primers designed by Taberlet *et al.* (1991) were used for PCR amplification of the *trn*L–*trn*F region (5′-GGTTCAAGTCCCTCTATCCC-3′ and 5′-ATTTGAACTGGTGACACGAG-3′). The primers are constructed against distal regions of the highly conserved tRNA genes and thus are suitable for amplifying this noncoding region from a broad spectrum of higher plants. Amplifications were performed in a volume of 50 μl containing 10 mM Tris–HCl, pH 9.0, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 50 mM KCl, 150 μM each dATP, dCTP, dGTP, dTTP, 0.5 μM of each primer, 50 ng of total DNA, and 1 U of *Taq* polymerase (Promega). Reactions were performed in a PTC-100 thermal cycler (MJ Research). After 5 min heating at 95°C, 35 cycles were run. Each cycle consisted of 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C. This was followed by 4 min at 72°C. The amplification products were purified by agarose gel electrophoresis, and the concentrated DNAs were recovered using fiberglass (Appligene, France).

Direct sequencing was performed in both directions from the double-stranded DNA fragment using alternatively one of the two amplification primers. The Taq Dye Dideoxy Terminator Cycle Sequencing kit (Applied Biosystem) was used as recommended by the manufacturer. The PCR products were analyzed on an ABI373A autosequencer.

Sequence Analysis

Nucleotide sequences obtained from the 39 accessions (GenBank database under the Accession numbers U93387 to U93404) were aligned by hand. These varied in length due to the presence of several insertions and deletions (indels). Wagner parsimony phylogenetic trees (Farris, 1970) were constructed with the phylogenetic inference package (PHYLIP, version 3.4) written by Felsenstein (1989). Indels were included as single characters in the input data. An analysis was also conducted with a combined data set using the *trn*L–*trn*F sequences and 11 restriction site changes (identified by the codes r1 to r11) detected by RFLP analysis of the chloroplast genome in a previous study (Lashermes *et al.*, 1996). The DNAPARS program was used to find the most parsimonious trees. The shortest parsimonious trees were used to construct a strict consensus tree using the CONSENSE program. The bootstrap method (Felsentein, 1985) was employed to evaluate the reliability of tree topologies.

RESULTS

Sequence Analysis

The 39 sequences determined allowed the identification of only 20 distinct sequences of the *trn*L–*trn*F intergenic spacer (Fig. 1). Alignment of coffee tree sequences required the introduction of six gaps, two of which were 1 bp in length and one each of 8, 11, 20, and 30 bp. Alignment including the outgroup species, *G. grandiflora,* results in two additional gaps of 1 and 10 bp, respectively. The nature of these gaps (insertion or deletion) was hypothesized on the basis of outgroup comparison. All inferred gaps in coffee tree sequences were found to be deletions except for a gap 1 bp in length which could correspond to a putative insertion. The length of the *trn*L–*trn*F spacer varies between 314 (accession 14) and 343 bp in individual accessions, the multiple alignment being 344 bp in length.

Identical sequences were found for several accessions belonging to the same or different *Coffea* species. Sequence types were shared by up to 10 accessions. Among all coffee tree species, the multiple alignment resulted in 32 variable characters (26 nucleotide substitutions and 6 indels), and 254 aligned nucleotide sites (74%) were invariant. The transition/transversion ratio is 1.8. Pairwise nucleotide differences of aligned positions excluding gaps were calculated. A maximum inter-*Coffea* sequence divergence of 3.4% was observed between accessions of *C. kapakata* (S5) and *C. stenophylla* (S13). Intergenic sequence divergence between *Coffea* and *Psilanthus* accessions ranged from 0.3 to 3.5% of nucleotides. Sequence comparisons with the outgroup accession *G. grandiflora* gave divergence values ranging from 2.1 to 4.2% nucleotides.

Phylogenetic Analysis

Parsimony analysis of *trn*L–*trn*F sequence data involving 38 coffee tree accessions and the outgroup species *G. grandiflora* was performed. Maximally parsimonious tree topologies (data not shown) required only 42 steps and have a consistency index of 0.95, indicating a low level of homoplasy (parallel or convergent evolution). However, for most of the branches, the number of supporting character states is rather low.

One of the 97 most parsimonious trees obtained when both *trn*L–*trn*F sequence characters and restriction site changes of cpDNA were included in the parsimony analysis was arbitrarily chosen and is presented in Fig. 2. The topologies require 47 steps and have a consistency index of 0.91. The trees have almost the same topology as those generated when only the sequence data are considered. Among coffee tree species, *Psilanthus manni* and *C. kapakata* have the most autapomorphies. The two representatives of *Psilanthus* (*P. manni* and *P. ebracteolatus*) do not appear associated. Bootstrap values (BV), i.e., the frequencies of occurrence of each monophyletic group among 100 bootstrap replicates, ranged from 26 to 100%. Among the *Coffea* taxa, five major groupings can be distinguished, which represent distinct cpDNA lineages.

A strong geographical correspondence is observed (Fig. 2). Taxa originating from west and central Africa

```
                                             1                                               49
S1  (acces. 1, 2, 13, 31)                    TTTGATCCCCCAACTATTTATCCTATCCCCCTTTCGTTAGCGGTTCAAA
S2  (acces. 28)                              .............-..............T....................
S3  (acces. 29)                              .............-...................................
S4  (acces. 14)                              .................................................
S5  (acces. 17)                              ...........--------------------..................
S6  (acces. 23, 24, 25, 26, 27)              .................................................
S7  (acces. 12)                              .................................................
S8  (acces. 4, 5, 6, 7, 8, 9, 10, 11, 20, 33) .................................................
S9  (acces. 21, 34)                          .................................................
S10 (acces. 30)                              .................................................
S11 (acces. 32)                              .................................................
S12 (acces. 18, 19)                          ..........................................-------
S13 (acces. 35, 36)                          .........T.......................................
S14 (acces. 16)                              .........T.......................................
S15 (acces. 22)                              .................................................
S16 (acces. 3)                               .................................................
S17 (acces. 15)                              .................................................
S18 (acces. 38 i.e. P. mannii)               .................................................
S19 (acces. 37 i.e. P. ebracteolatus)        .................................................
S20 (acces. 39 i.e. G. grandiflora)          .............-................T..................

     50                                                                          127
S1   AAACCTTATTCATTTACTCTATTCTCTTAGAAATCGATCTGGACGGAAAAGCCCTTTTCTTATCACAAATCTTGTGTT
S2   ..............................................................................
S3   ..............................................................................
S4   ..............................................................................
S5   ....................................................T..GG.....................
S6   ..............................................................................
S7   ..............................................................................
S8   ....................................................T.........................
S9   ....................................................T.........................
S10  ....................................................T.........................
S11  ..............................................................................
S12  ----................................................T.........................
S13  ................T............T................................................
S14  .............................T................................................
S15  ..............................................................................
S16  ..............................................................................
S17  ..............................................................................
S18  ...............................--------.......................................
S19  ..............................................................................
S20  .T.................................A..........................................

     128                                                                         205
S1   ATTTATGATATACATATAAATGAACATCTTTGAGCAAGAAATACCCATTTGAATGGTTTACAATCGATATAACTATTC
S2   ....................................................C.........................
S3   ....................................................C.........................
S4   ..............................................................................
S5   .......................................CT.....................................
S6   ..............................................................................
S7   ...........................................T..................................
S8   ..............................................................................
S9   ..............................................................................
S10  ..............................................................................
S11  ..............................................................................
S12  ..............................................................................
S13  ..............................................................................
S14  ..............................................................................
S15  ....................................................G.........................
S16  ..............................................................................
S17  ....................................................G.........................
S18  .................................T..........................................C.
S19  ..............................................................................
S20  ....................----------.............................................C..
```

FIG. 1. Aligned nucleotide sequences of the intergenic spacer between the *trn*L (UAA) 3′exon and the *trn*F (GAA) gene (see Table 1 for accession code). Dashes denote alignment gaps and dots denotes bases in common with the first sequence. The arrow indicates the beginning of *trn*F (GAA). The −35 and −10 promoter elements are overligned.

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     206                                                                        283
S1   ATACTGAAACTTACAAAGTACTCTTTTTTAAGATACAAGAAATTCTAGTACCTAGATAAAATTTTGTAATCCCCTTTC
S2   .............................................................C................
S3   .............................................................C................
S4   ..........................................................................----
S5   ........................C.........C..........................C................
S6   .............................................................C................
S7   .............................................................C................
S8   ..................................C..........................C................
S9   ........................C.........C..........................C................
S10  ........................C.........C..................G.......C................
S11  ........................C.........C........C.........G.......C................
S12  ........................C.........C..........................C................
S13  ...........C.................................................C................
S14  ...........C.................................................C................
S15  .............................................................C................
S16  .............................................................C................
S17  .............................................................C....A...........
S18  ..................................C..........................C................
S19  ..................................C..........................C................
S20  ..................................C..........................C............C...

     284          -35              -10                      → trnF gene         361
S1   CTTCTTTTAATTGACATAG-CCCCCCTTTTCTCATAAAATGAGGATGCTACATTGGGACTGGTCGGGATAGCTCAGAT
S2   ...................-..........................................................
S3   ...................-..........................................................
S4   --------------------------....................................................
S5   ...................-..........................................................
S6   ...................-..........................................................
S7   ...................-..........................................................
S8   ...................-..........................................................
S9   ...................-..........................................................
S10  ...................-..........................................................
S11  ...................-..........................................................
S12  ...................-..........................................................
S13  ...................-..........................................................
S14  ...................-..........................................................
S15  .............G.....-..........................................................
S16  .............G.....-.........C................................................
S17  .............G.....-.........-................................................
S18  ..GT...............-..T.......................................................
S19  ...................-..........................................................
S20  ...................A.....T..................................................C.
```

FIG. 1—*Continued*

are distributed in three clades. A first clade comprises *C. humilis* and *C. stenophylla*. This clade is distinct, as indicated by a BV of 100%. A second strongly supported clade comprises the species endemic to the uplands of the ridge region, *C. eugenioides* and *C. arabica*, together with *C. sp.* Moloundou collected in Congo. Other west and central African taxa are grouped in a third clade which is only weakly supported at its base. In addition, three of the four homoplastic characters required by the most parsimonious trees are distributed among this group of taxa. On the other hand, sublineages appear relatively well supported. The taxa *C. sp.* N'gongo and *C. sp.* Mayombe show a close relationship as well as *C. sp.* N'koumbala and the members of the canephoroid group (*C. brevipes*, *C. canephora*, *C. congensis*). The taxon *C. sp* X is associated with an accession of *C. liberica*.

Species native to the region between the Kivu ridge and the Mozambique channel represent a fourth clade, with the exception of *C. costatifructa*. However, this grouping is supported by only one character.

The fifth clade comprises the two analyzed species originating from Madagascar and *C. humblotiana*, native to the Comoro Islands. Divergence between these species and the species endemic to Africa appears relatively low.

DISCUSSION

Phylogenetic analysis of chloroplast DNA in addition to biparentally inherited nuclear genes provides a means of evaluating phylogenetic hypothesis as well as a test of whether cytoplasmic gene flow has occurred.

Sequence analysis of the intergenic spacer between the *trn*L (UAA)3' exon and the *trn*F (GAA) gene provides valuable additional data on the cpDNA variation among coffee tree species. As expected from a noncoding region, the divergence values calculated between taxa

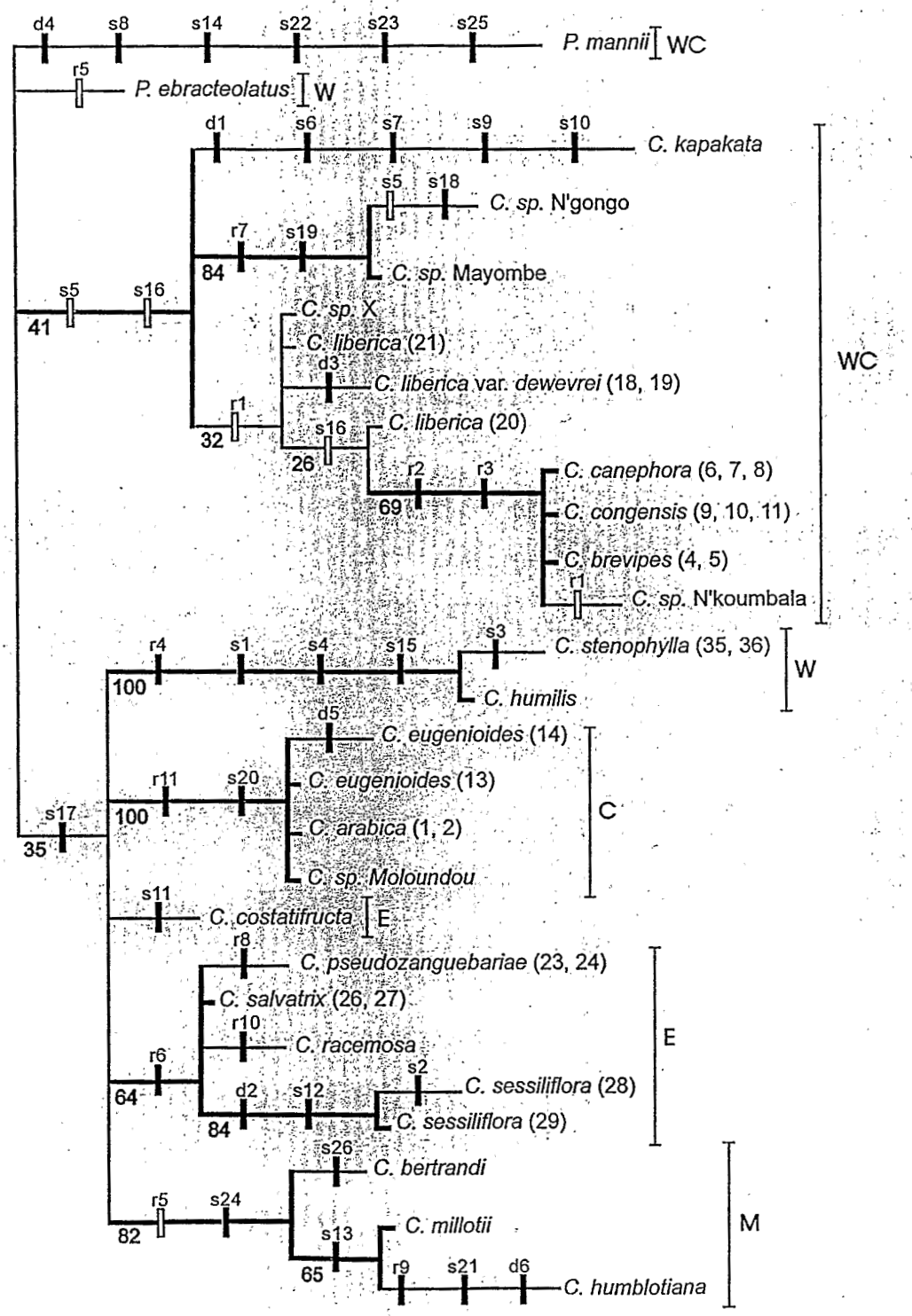

FIG. 2. One of the most parsimonious trees (97 found) derived from parsimony analysis of cpDNA polymorphism. Characters appearing only once on the tree are solid boxes; parallel and reversal changes are designated with open boxes. The characters coded r1 to r11, d1 to d6, and s1 to s25 correspond to restriction site changes of cpDNA, indels, and nucleotide substitutions on the *trn*L–*trn*F intergenic spacer, respectively. Numbers below branches are bootstrap values (100 replicates). Clades present in the strict consensus topology are indicated by grey lines. The letters following each clade indicate the geographical distribution of the relevant species: W (west Africa), WC (west and central Africa), C (central Africa), E (east Africa), and M (Madagascar and the Comoro Islands).

(0 to 3.4%) are much higher than the maximum sequence divergence value of 0.6% previously estimated by RFLP on the overall chloroplast genome (Lashermes *et al.*, 1996). The mutations, nucleotide substitutions, as well as short insertion/deletion events appeared randomly distributed along the *trnL–trnF* sequence. The observed transition/transversion ratio is equivalent to those previously reported in different genera for intergenic regions of chloroplast genome (Zurawski and Clegg, 1987; Gielly and Taberlet, 1994).

Evolutionary rates of nucleotide substitution differ between the different genomes (i.e., chloroplast, mitochondrial, nuclear) and vary depending upon the specific portion of DNA analyzed (Wolfe *et al.*, 1987; Clegg and Zurawski, 1992). Therefore, the divergence distance estimated from the cpDNA may not directly reflect that of the species. However, the low level of cpDNA variation exhibited by coffee tree species is probably related to a recent origin of the genus *Coffea*, as previously suggested (Lashermes *et al.*, 1996). Furthermore, both the rather low number of characters supporting the respective branchings and the low rate of homoplasy revealed by parsimony analyses of cpDNA variation suggest a rapid and radial mode of speciation for the primary clades. As a consequence, most of the identified clades are weakly supported, and additional DNA sequence data are necessary in order to increase resolution among the basal nodes of *Coffea* phylogeny.

The overall relationships of coffee tree species derived from parsimony analysis of either the *trnL–trnF* sequences or the combined data set showed good agreement with the phylogeny derived from ITS sequence analysis (Lashermes *et al.*, 1997). Congruence between the cpDNA- and ITS-derived phylogenies includes the arrangement of *Coffea* taxa into several major groups corresponding to geographic regions (i.e., Madagascar, east Africa, central Africa, west and central Africa). However, detailed comparisons between ITS- and cpDNA-based phylogenies revealed several discrepancies.

One discrepancy concerns the tetraploid species *C. arabica*. CpDNA from *C. arabica* appeared similar to cpDNA from *C. eugenioides* and *C.sp.* Moloundou, while the ITS2 region of *C. arabica* diverged markedly from the sequences of those taxa and appeared almost identical to the sequences of canephoroid species. The allotetraploid origin of *C. arabica* has recently been investigated using DNA-based markers (Lashermes *et al.*, 1995). The present results strongly support the hypothesis that a species related to *C. eugenioides* and *C.sp.* Moloundou was the maternal progenitor species. In addition, the lack of cpDNA divergence observed between those putative cytoplasmic donors and *C. arabica* could argue for a relatively recent origin of *C. arabica*.

Other inconsistencies with the ITS-derived phylogenies involve taxa from west Africa. CpDNA of *C. stenophylla* appears closely related to that of *C. humilis*, while the nuclear ribosomal DNA sequence analysis as well as a preliminary RFLP analysis using nuclear single-copy probes (Lashermes *et al.*, 1995) associated *C. stenophylla* with *C. sp.* Ngongo and *C. sp.* Mayombe. The taxa *C. sp.* X, which could correspond to the recently documented species *Coffea heterocalix* from Cameroon (Stoffelen *et al.*, 1996), has a cpDNA identical to that of an accession of *C. liberica*, while nuclear genome analyses indicated a close relationship among *C. sp.* X, *C. eugenioides*, and *C. sp.* Moloundou. These discrepancies could be interpreted as the result of interspecies transfer of cpDNA mediated by hybridization. CpDNA "capture" attributable to introgressive hybridization between related species has already been reported in various genera including *Brassica* (Palmer *et al.*, 1983), *Helianthus* (Reiseberg *et al.*, 1990), *Gossypium* (Wendel and Albert, 1992), *Populus* (Smith and Sytsma, 1990), and *Zea* (Doebley, 1989). Since *Coffea* species hybridize readily with one another under artificial conditions (Charrier, 1978; Louarn, 1993), introgressive hybridizations are likely to have occurred during species differentiation. Reticulate evolution, which is a widespread phenomenon (Grant, 1971; Rieseberg and Soltis, 1991; Rieseberg and Wendel, 1993), could have played an significant role in coffee trees. Evidence for such events among west African taxa complicates phylogeny reconstruction.

The results presented here are consistent with an African origin of the genus *Coffea* (Charrier, 1978; Leroy, 1982). The low divergence observed when comparing taxa from Madagascar to the species endemic to Africa suggests a recent colonization of Madagascar. The hypothesis that lineage separation stemmed from the Mesozoic breakup of Gondwana (Leroy, 1982) is contradicted by the low levels of sequence divergence observed. Long-distance dispersals (Raven and Axelrod, 1974) are also likely to have occurred in the colonization of the volcanic islands of the Indian Ocean, such as the Comoro Islands. Narrowing of the Mozambique channel in relation to episodic glacial advances during the Quaternary period (Hamilton, 1976) could have facilitated such dissemination.

In addition, cpDNA variation analysis does not support the present classification of coffee trees into two genera, namely, *Coffea* and *Psilanthus*. A similar observation was reported in a study of nuclear ribosomal DNA sequences (Lashermes *et al.*, 1997). Both molecular analyses indicate a close relationship of species belonging to the two genera and prompt modifications in the adopted classification based on flowering and flower characteristics (Leroy, 1980; Bridson, 1987).

A general conclusion emerging from the present study is that no single source of information should be used unequivocally to determine phylogenetic relation-

ships among the closely related but highly diversified taxa associated with the genus *Coffea*. The combined use of chloroplast and nuclear molecular markers is invaluable for assessing the occurrence of genetic exchange among related forms. Nevertheless, further analysis using additional multiple nuclear and cytoplasmic markers (Rieseberg and Brunsfeld, 1992) would be required to determine the actual extent and evolutionary significance of introgression between coffee tree species.

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