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ORIGINAL ARTICLE

Adaptive radiation in *Coffea* subgenus *Coffea* L. (Rubiaceae) in Africa and Madagascar

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Abstract Phylogeographic analysis of the *Coffea* subgenus *Coffea* was performed using data on plastid DNA sequences and interpreted in relation to biogeographic data on African rain forest flora. Parsimony and Bayesian analyses of *trn*L-F, *trn*T-L and *atp*B-*rbc*L intergenic spacers from 24 African species revealed two main clades in the *Coffea* subgenus *Coffea* whose distribution overlaps in west equatorial Africa. Comparison of *trn*L-F sequences obtained from GenBank for 45 *Coffea* species from Cameroon, Madagascar, Grande Comore and the Mascarenes revealed low divergence between African and Madagascan species, suggesting a rapid and radial mode of speciation. A chronological history of the dispersal of the *Coffea* subgenus *Coffea* from its centre of origin in Lower Guinea is proposed. No relation was found between phylogenetic topology and the age of emergence of the volcanic islands that *Coffea* species have colonised in the Indian Ocean, suggesting dispersal from mainland Africa after the emergence of the youngest island, Grande Comore, 500,000 years ago. Additional sequences were obtained from GenBank for 24 species of other Rubiaceae genera, including the *Rubia* genus whose origin has been dated from the Upper Miocene. Estimates of substitution rates suggested that diversification in *Coffea* subgenus *Coffea* occurred about 460,000 years ago or as recently as the last 100,000 years, depending on the cpDNA region considered and calibration. The phylogenetic relationships based on plastid sequences confirmed biogeographic differentiation of coffee species, but they were not congruent with morphological and biochemical classifications, or with the capacity to grow in specific environments. Examples of convergent evolution in the main clades are given using characters of leaf size, caffeine content and reproductive mode.

Keywords Africa · Biogeography · *Coffea* · Evolution · Phylogeny · Plastid sequences · Rubiaceae

Introduction

Coffeeae tribe belongs to the Ixoroideae monophyletic subfamily of Rubiaceae family and is close to the tribes Gardenieae and Pavetteae (Bremer and Jansen 1991; Davis et al. 2007). The coffee species share the typical coffee bean morphology, i.e. a groove on the flat side of the seed. They have been described in two genera, *Coffea* L. and *Psilanthus* Hook. f., which differ in their flower morphology (Leroy 1980; Bridson 1987; Davis et al. 2005). Each genus has been divided into two subgenera: *Coffea* subgenus *Coffea* (95 species), *Coffea* subgenus *Baracoffea* (J.-F. Leroy) J.-F. Leroy (nine species), *Psilanthus* subgenus *Psilanthus* (two species) and *Psilanthus* subgenus *Afrocoffea* (Moens) (20 species) (Bridson 1988; Davis et al. 2005, 2006; Davis and Rakotonasolo 2008). Both genera occur naturally in tropical Africa; *Coffea* also occurs in Madagascar, Grande Comore and the Mascarenes, and *Psilanthus* in south-east Asia, Oceania and northern Australia. Research has mainly focused on the *Coffea* subgenus *Coffea*, which comprises the majority of coffee species, including those of economic importance, *C. arabica* L. (65% of world production) and *C. canephora* Pierre ex A. Froehner (35%) (more details at www.ico.org).

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Coffea subgenus *Coffea* is represented by 41 species in Africa, 58 in Madagascar, one in Grande Comore and three in the Mascarenes, each area having 100% endemicity for its species (Davis et al. 2006). All species are perennial woody bushes or trees that differ greatly in morphology, size and ecological adaptation. They can constitute valuable markers of evolution in the African rain forest since coffee trees have colonised various types of forest, including humid evergreen forest, evergreen forest, mixed evergreen-deciduous forest sometimes seasonally dry, deciduous forest, savannah woodland, gallery forest, coastal forest and temporarily flooded riparian forest (Davis et al. 2006). Except for *C. canephora* and *C. liberica* Bull. ex Hiern from West and Central Africa and *C. eugenioides* S. Moore from East Africa, coffee species have a rather restricted distribution, sometimes only a few square kilometres. Three centres of species diversity have been identified in Madagascar (mainly in the evergreen humid forests of eastern Madagascar), Cameroon (14 species) and Tanzania (16 species, mainly in the eastern Arc Mountain) (Davis et al. 2006). However, many characters considered in taxonomy are weak and variable, and many species have not been fully characterized, so it is hard to draw valid conclusions about their relationships (Bridson 1982; Stoffelen 1998; Davis et al. 2005). All species are diploid ($2n = 2x = 22$), except *C. arabica*, which is tetraploid ($2n = 4x = 44$) (Charrier and Berthaud 1985). They are self-incompatible except the tetraploid species *C. arabica* and the diploid species *C. heterocalyx* Stoff. (Coulibaly et al. 2002) and *C. anthonyi* Stoff. & F. Anthony (Stoffelen et al. 2009), which are self-compatible.

Molecular phylogenies of coffee species have been established based on variations in intergenic spacer sequences (Lashermes et al. 1996; Cros et al. 1998; Tesfaye et al. 2007) and introns (Tesfaye et al. 2007) of plastid DNA, internal transcribed spacer (ITS) sequences of rDNA (Lashermes et al. 1997) and a combination of four plastid regions and ITS (Maurin et al. 2007). Low sequence divergence was found between *Coffea* and *Psilanthus*, indicating that molecular data do not support the recognition of two genera (Lashermes et al. 1997; Cros et al. 1998). Enlarging the number of *Coffea* species and *Psilanthus* species did not resolve the relationship between the two genera (Maurin et al. 2007). At species level, a small number of parsimony-informative characters were found in molecular studies and the primary clades were weakly supported in the trees. This was attributed to the recent origin of the genus *Coffea* and a radial mode of speciation (Lashermes et al. 1997; Cros et al. 1998). All the studies pointed to a correspondence between the main groups of species and their geographical origin. In Africa, groups of species were identified in West Africa, West and Central Africa, East-Central Africa and East Africa (Lashermes et al. 1997; Cros et al. 1998; Maurin et al. 2007). A lack of sequence divergence was found in the Madagascan species, and consequently their position has remained unresolved (Maurin et al. 2007).

The major objectives of the present study were to (1) reconstruct the phylogenetic relationships within *Coffea* subgenus *Coffea* using sequence data from non-coding regions of plastid DNA, (2) determine the relationships of new species from Central Africa, (3) investigate divergence times within *Coffea* subgenus *Coffea* and (4) propose a chronological history of coffee radiation using biogeographic data of African flora.

Materials and methods

Species sampling and outgroup selection

Sequences of the intergenic spacers *trn*L-F, *trn*T-L and *atp*B-*rbc*L were produced for 24 *Coffea* subgenus *Coffea* species, 2 *Psilanthus* species and 1 outgroup plant (Table 1). The sampling scheme covered the biogeographic diversity of the *Coffea* subgenus *Coffea* in Africa as shown by previous studies (Cros et al. 1998; Maurin et al. 2007). All coffee accessions were collected during IRD (formerly ORSTOM) missions in Africa (Anthony et al. 2007) and maintained in greenhouses at the IRD centre in Montpellier. One species from Cameroon (Anthony et al. 1985) and four species from Congo (de Namur et al. 1987) could not be identified and provisional names were used. *Gardenia jasminoides* J. Ellis was chosen as the outgroup based on previous molecular studies in the Rubiaceae family (Cros et al. 1998; Bremer et al. 1999; Andreasen and Bremer 2000).

Biogeographic groupings

The African coffee species were grouped according to their biogeographic origin using the terminology of Maurin et al. (2007): Upper Guinea (UG), Lower Guinea/Congolian region (LG/C), East-Central Africa (E-CA) and East Africa (EA). Based on a chorological analysis, Upper Guinea, Lower Guinea and Congolia were recognised as subcentres of endemism in the Guineo-Congolian Regional Centre of Endemism (G-C) (White 1979, 1983).

Sequence generation

Total DNA was obtained from fresh leaves using the method of Lashermes et al. (1993), modified by Paillard et al. (1996). DNA samples were purified using QIAquick columns (QIAGEN). Target regions were amplified in 25 µl reactions with approximately 20–25 ng of total DNA, 1× colorless GoTaq Flexi Buffer [50 mM KCl,

Table 1 Accessions used in cpDNA analysis and their geographical distribution according to Davis et al. (2006)

Species	Geographical distribution	GenBank accession number		
		*atp*B-*rbc*L	*trn*L-*trn*F	*trn*T-*trn*L
Coffea accessions				
C. anthonyi Stoff. & F. Anthony	Cameroon, Congo	FJ493346	FJ493323	FJ493373
C. arabica L.	Ethiopia	FJ493347	FJ493319	FJ493374
C. brevipes Hiern	Cameroon, Gabon, Congo, DRC	FJ493348	FJ493320	FJ493375
C. canephora Pierre ex A. Froehner	West, Central and East-Central Africa	FJ493349	FJ493321	FJ493376
C. charrieriana Stoff. & F. Anthony	Cameroon	FJ493350	FJ493322	FJ493377
C. congensis A. Froehner	Cameroon, CAR, Gabon, Congo, DRC	FJ493351	FJ493324	FJ493378
C. costatifructa Bridson	Tanzania	FJ493352	FJ493325	FJ493379
C. eugenioides S. Moore	DRC, Burundi, Rwanda, Uganda, Sudan, Kenya, Tanzania	FJ493353	FJ493326	FJ493380
C. heterocalyx Stoff.	Cameroon	FJ493354	FJ493327	FJ493381
C. humilis A. Chev.	Liberia, Ivory Coast	FJ493355	FJ493328	FJ493382
C. kapakata (A. Chev.) Bridson	Angola	FJ493356	FJ493329	FJ493383
C. liberica var. *dewevrei* (De Wild. & T. Durand) Lebrun	Central Africa, Uganda, Sudan	FJ493357	FJ493330	FJ493384
C. liberica var. *liberica* Bull. ex Hiern	West Africa	FJ493358	FJ493331	FJ493385
C. pocsii Bridson	Tanzania	FJ493359	FJ493332	FJ493386
C. pseudozanguebariae Bridson	Kenya, Tanzania	FJ493360	FJ493333	FJ493387
C. racemosa Lour.	Mozambique, Zimbabwe, South Africa	FJ493361	FJ493334	FJ493388
C. salvatrix Swynn. & Phillipson	Malawi, Mozambique, Zimbabwe	FJ493362	FJ493335	FJ493389
C. sessiliflora ssp. *sessiliflora* Bridson	Kenya	FJ493363	FJ493336	FJ493390
C. stenophylla G. Don	Guinea, Sierra Leone, Ivory Coast	FJ493364	FJ493337	FJ493391
Coffea sp. 'Congo'	Congo	FJ493365	FJ493338	FJ493392
Coffea sp. 'Mayombe'	Congo	FJ493366	FJ493339	FJ493393
Coffea sp. 'Ngongo2'	Congo	FJ493367	FJ493340	FJ493394
Coffea sp. 'Ngongo3'	Congo	FJ493368	FJ493341	FJ493395
Coffea sp. 'Nkoumbala'	Cameroon	FJ493369	FJ493342	FJ493396
Related genus *Psilanthus*				
P. ebracteolatus Hiern	West and Central Africa	FJ493370	FJ493343	FJ493397
P. mannii Hook. f.	West and Central Africa	FJ493371	FJ493344	FJ493398
Outgroup				
Gardenia jasminoides J. Ellis		FJ493372	FJ493345	FJ493399

DRC Democratic Republic of Congo, *CAR* Central African Republic

10 mM Tris-HCl (pH 9.0 at 25°C) and 0.1% Triton X100], 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.25 μM of each primer and 0.75 U of GoTaq DNA polymerase (Promega). The PCR program consisted of 5 min at 95°C followed by 34 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C, and a final extension of 72°C for 8 min. The primers used are listed in Table 2. Amplified products were cleaned using the GFX PCR kit (GE Healthcare). Sequencing reactions were performed by Cogenics using Sanger technology, separately for each strand to obtain independent forward and reverse sequences. Forward and reverse fragments were assembled and, in the case of differences, new reactions were performed. All sequences were deposited in GenBank (Table 1).

Sequence comparisons

Sequences were obtained from GenBank for 45 species of *Coffea* subgenus *Coffea* and 24 species belonging to other Rubiaceae genera (Table 3). The *Coffea* species originated from Cameroon (*C. bakossi* Cheek & Bridson, *C. mayombensis* A. Chev., *C. montekupensis* Stoff.), Grande Comore (*C. humblotiana* Baill.), the Mascarenes (*C. macrocarpa* A. Rich., *C. mauritiana* Lam., *C. myrtifolia* (A. Rich. ex DC.) J.-F. Leroy) and Madagascar (38 species). The other Rubiaceae accessions were considered as representative of the subfamilies Ixoroideae, Cinchonoideae and Rubioideae, according to results of previous studies (Natali et al. 1995; Bremer et al. 1999; Andreasen

Table 2 Amplification primers for *trn*L-F, *trn*T-L and *atp*B-*rbc*L

Intergenic spacer	Primer	Sequence	Reference
*trn*L-F	Forward (11L)	5′-GGTTCAAGTCCCTCTATCCC-3′	Taberlet et al. (1991)
	Reverse (14R)	5′-ATTTGAACTGGTGACACGAG-3′	
*trn*T-L	Forward (9L)	5′-CATTACAAATGCGATGCTCT-3′	Taberlet et al. (1991)
	Reverse (12R)	5′-TCTACCGATTTCGCCATATC-3′	
*atp*B-*rbc*L	Forward (4L)	5′-GAAGTAGTAGGATTGATTCTC-3′	Savolainen et al. (1994)
	Reverse (4R)	5′-TACAGTTGTCCATGTACCAG-3′	

and Bremer 2000). These subfamilies are generally recognised as being the three major lineages within Rubiaceae (Rydin et al. 2008).

Phylogenetic analyses

Sequence alignments were initially performed with CLUSTAL W (Thompson et al. 1994) and manually adjusted using the MegAlign program of the DNASTAR package (Lasergene v7.2) without difficulty due to low levels of nucleotide variation. Sequence divergence (distance) between accessions was calculated by the DNA-DIST program in PHYLIP (Felsenstein 1995) using the Kimura-2-parameter model. The data on the intergenic spacers *trn*L-F, *trn*T-L and *atp*B-*rbc*L were not analysed separately because they all exhibited low levels of sequence divergence. Nucleotide diversity of combined sequences was estimated for biogeographic regions using the Arlequin v3.1 software package (Excoffier et al. 2005).

Phylogenetic analyses were conducted using maximum parsimony (MP) and maximum likelihood (ML) methods implemented in PAUP* 4.0b10 (Swofford 2001). Parsimony analyses (Swofford et al. 1996) were performed using the heuristic search method with a random addition sequence of ten replicates, tree-bisection-reconnection (TBR) branch swapping, and the MULTREES option. All nucleotide substitutions were weighted equally. Branch support was examined in the maximally parsimonious trees (MPTs) with the bootstrap method (Felsenstein 1985) using PAUP* 4.0b10. Bootstrap values were calculated from 10,000 replicates with the random addition and heuristic search option. Gaps were treated either as missing data or as additional characters. As MP analysis ignores information on branch lengths, we also used maximum likelihood (ML), which includes an estimation of branch length and assumes that changes are more likely along long branches than short ones. The ML heuristic analysis was run for 10 random-addition-sequence replicates with TBR branch swapping and the HKY85 sequence evolution model. Bootstraps were calculated using 10,000 replicates.

Bayesian inference of phylogeny was implemented using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). MrBayes was run with two simultaneous analyses with four parallel chains in each, starting with a random tree and sampling one tree for each 100 generations. The temperature of the chains and other parameters were left at default value. The program was run up to 1–3 $\times$ 10^6 Markov Chain Monte Carlo (MCMC) generations to arrive at the stationary phase (average standard deviation of split frequencies <0.01).

Divergence time estimation

Divergence time was estimated using two calibration dates: (1) Colonisation of volcanic islands by *Coffea* species is assumed to have followed their emergence from the Indian Ocean, 8 mya ago for Mauritius (MacDougal and Chauman 1969), 2 mya for Reunion Island (Emerick and Duncan 1982) and 0.5 mya for the Grande Comore (Nougier et al. 1986; Rocha et al. 2005). (2) The origin of the genus *Rubia* is assumed to be coincident with the first occurrence of fossil pollen records, dated from the Upper Miocene (Muller 1981). Numbers of substitutions per site were calculated using branch lengths for *Rubia* in the ML analysis and the estimated age of this species, and compared to those found in the *Coffea* clades.

Results

Coffee sequence characteristics

The *trn*L-F sequences generated for 26 coffee species ranged in length from 331 to 356 bp. Aligned sequences contained two deletions, one 8 bp and one 11 bp in length. Aligned with the outgroup sequence, the coffee sequences presented an insertion of 10 bp and a deletion of 1 bp. The *trn*L-F matrix was composed of 357 aligned positions, 21 (5.9%) of which were variable, with 7 (2.0%) parsimony-informative (Table 4). The maximum divergence was 2.4% between coffee sequences [i.e. *C. liberica* var. *dewevrei* (De Wild & T. Durand) Lebrun-*C. stenophylla* G. Don] and 3.6% between the outgroup and coffee (i.e. *C. stenophylla*) sequences.

Table 3 Sequences of the intergenic spacers *trn*L-F, *trn*T-L and *atp*B-*rbc*L obtained from GenBank for 45 species of *Coffea* subgenus *Coffea* and 24 species belonging to other Rubiaceae genera

Species	Origin	*trn*L-*trn*F	*trn*T-*trn*L	*atp*B-*rbc*L
Coffea subgenus *Coffea*				
C. abbayesii J.-F. Leroy	Madagascar	DQ153805		
C. andrambovatensis J.-F. Leroy	Madagascar	DQ153879		
C. ankaranensis J.-F. Leroy ex A.P. Davis & Rakotonas.	Madagascar	DQ153774		
C. arenesiana J.-F. Leroy	Madagascar	DQ153807		
C. augagneuri Dubard	Madagascar	DQ153800		
C. bakossi Cheek & Bridson	Cameroon	DQ153835		
C. bertrandii A. Chev.	Madagascar	DQ153791		
C. betamponensis Portères & J.-F. Leroy	Madagascar	DQ153788		
C. boiviniana (Baill.) Drake	Madagascar	DQ153793		
C. buxifolia A. Chev.	Madagascar	DQ153809		
C. coursiana J.-F. Leroy	Madagascar	DQ153784		
C. dubardii Jum.	Madagascar	DQ153802		
C. farafanganensis J.-F. Leroy	Madagascar	DQ153772		
C. heimii J.-F. Leroy	Madagascar	DQ153798		
C. homollei J.-F. Leroy	Madagascar	DQ153769		
C. humblotiana Baill.	Grande Comore	DQ153778		
C. kianjavatensis J.-F. Leroy	Madagascar	DQ153849		
C. lancifolia A. Chev.	Madagascar	DQ153770		
C. leroyi A.P. Davis	Madagascar	DQ153771		
C. liaudii J.-F. Leroy	Madagascar	DQ153801		
C. littoralis A.P. Davis	Madagascar	DQ153808		
C. macrocarpa A. Rich.	Mauritius	DQ153838		
C. mangoroensis Portères	Madagascar	DQ153870		
C. manombensis A.P. Davis	Madagascar	DQ153812		
C. mauritiana Lam.	Mauritius & Reunion	DQ153836		
C. mayombensis A. Chev.	Cameroon	DQ153828		
C. mcphersonii A.P. Davis	Madagascar	DQ153790		
C. millotii J.-F. Leroy	Madagascar	DQ153776		
C. montekupensis Stoff.	Cameroon	DQ153826		
C. montis-sacri A.P. Davis	Madagascar	DQ153797		
C. moratii J.-F. Leroy	Madagascar	DQ153869		
C. myrtifolia (A. Rich. ex DC.) J.-F. Leroy	Mauritius	DQ153844		
C. perrieri Drake ex Jum. & H. Perrier	Madagascar	DQ153794		
C. pervilleana (Baill.) Drake	Madagascar	DQ153779		
C. rakotonasoloi A. Davis	Madagascar	DQ153783		
C. ratsimamangae J.-F. Leroy ex A.P. Davis & Rakotonas.	Madagascar	DQ153811		
C. resinosa (Hook. f) Radlk.	Madagascar	DQ153795		
C. richardii J.-F. Leroy	Madagascar	DQ153782		
C. sahafaryensis J.-F. Leroy	Madagascar	DQ153780		
C. sakarahae J.-F. Leroy	Madagascar	DQ153806		
C. sambavensis J.-F. Leroy ex A.P. Davis & Rakotonas.	Madagascar	DQ153785		
C. tetragona Jum. & H. Perrier	Madagascar	DQ153773		
C. tsirananae J.-F. Leroy	Madagascar	DQ153810		
C. vatovavyensis J.-F. Leroy	Madagascar	DQ153777		
C. vianneyi J.-F. Leroy	Madagascar	DQ153803		
Other Rubiaceae				
Asperula cynanchica L.		DQ662135	DQ662135	

Table 3 continued

Species	Origin	*trn*L-*trn*F	*trn*T-*trn*L	*atp*B-*rbc*L
Asperula tinctoria L.		DQ662136		
Chione venosa (Sw.) Urban		AF152687		
Cinchona pitayensis (Wedd.) Wedd.		AF152684		
Cinchona pubescens Vahl			AJ346963	AJ233990
Cosmibuena grandiflora (Ruíz Lopez & Pav.) Rusby		AF152686		
Diplospora dubia (Lindl.) Masam.				DQ131725
Exostema caribaeum (Jacq.) Schult.				AJ233991
Exostema purpureum Grisebach		AF152696		
Galium baillonii Brandza				X81671
Gardenia volkensii K. Schum.		AF201044		
Guettarda speciosa L.		AF152725		
Hillia valerii Standl.				X81683
Ixora coccinea L.		AJ620117	AJ620117	
Ixora finlaysoniana Wall. ex G. Don.				DQ131744
Ixora parviflora Lam.				X76477
Pavetta abyssinica Fresen.			FM207133	
Pavetta barbertonensis Bremek.		AF152668		
Rubia cordifolia Hochst. ex A. Rich.				DQ131777
Rubia horrida Thunb. Puff.		DQ662167	DQ662167	
Rubia peregrina L.				X76474
Rubia tinctorum L.			FJ695421	X76465
Tricalysia cryptocalyx Baker		AF152669		
Tricalysia elliotii (K. Schum.) Hutchinson & Dalziel				DQ131791

The *trn*T-L sequences ranged from 348 to 511 bp in length across the coffee species. Two ambiguous regions containing variable numbers of A and T repeats were removed because of possible sequencing errors caused by *Taq* polymerase stuttering. Five indels ranging from 1 to 172 bp were required to align the coffee sequences. Their alignment with the outgroup sequence required six additional indels of 1–6 bp. *G. jasminoides* presented a 32-bp region with a 7-bp inverted repeat at each end. The *trn*T-L matrix was composed of 524 aligned positions, 17 (3.2%) of which were variable, with three (0.6%) parsimony-informative (Table 4). The maximum divergence was 0.9% between coffee sequences [i.e. *C. kapakata* (A. Chev.) Bridson-*C. pocsii* Bridson] and 2.1% between the outgroup and coffee (three species) sequences.

The length of *atp*B-*rbc*L sequences ranged from 684 to 723 bp. A variable region of 2 bp randomly containing A, T, C or G was found between a 7-bp inverted repeat sequence. This variable region was not used for subsequent analyses. Eight indels ranging from 1 to 32 bp were required to align the coffee sequences. Two additional indels of 1 bp were included in the alignment of the outgroup sequence. The *atp*B-*rbc*L matrix was composed of 757 aligned positions, 27 (3.6%) of which were variable, with 11 (1.5%) parsimony-informative (Table 4). The maximum divergence was 0.7% between coffee sequences (three pairs of species) and 2.4% between the outgroup and coffee (i.e. *P. mannii*) sequences.

Analysis of African species

The combined plastid data comprised 1,638 bp of aligned sequence of 26 coffee species and *G. jasminoides* as outgroup (Table 4). With gaps treated as missing data, parsimony analysis produced 61 MPTs, with a consistency index (CI) of 0.942 (0.840 excluding uninformative characters), a retention index (RI) of 0.941, and a rescaled consistency index (RC) of 0.887. The topology of the MP analysis of *Coffea* species supported two sister clades and several subclades that were consistent with biogeographic regions (Fig. 1). Clade I comprised four subclades corresponding to one species from East Africa (subclade Ia), the remaining East African species (Ib), *C. arabica* and two species from East-Central Africa and Lower Guinea (Ic), and two species from Upper Guinea (Id). Clade II comprised species exclusively native to the Guineo-Congolian region. Three subclades were supported corresponding to three species from the Lower Guinea/Congolia region, closely related to *C. canephora* (subclade IIa), three species from the Lower Guinea/Congolia region (IIb), and the unidentified species

Table 4 Characteristics of the cpDNA regions used in the phylogenetic analyses: number of aligned, variable and parsimony-informative positions, consistency index (CI), retention index (RI) and rescaled consistency index (RC) of MP analyses

Region	Material	Aligned positions (bp)	Variable positions (bp)	Parsimony-informative positions (bp)	CI	RI	RC
*trn*L-F	24 *Coffea* and 2 *Psilanthus* species	357	21 (5.9%)	7 (2.0%)	Not analysed separately		
	69 *Coffea* species	299	24 (8.0%)	8 (2.7%)	1.000	1.000	1.000
	69 *Coffea* and 13 Rubiaceae species	323	103 (31.9%)	62 (19.2%)	0.869	0.931	0.810
*trn*T-L	24 *Coffea* and 2 *Psilanthus* species	524	17 (3.2%)	3 (0.6%)	Not analysed separately		
	24 *Coffea* and 7 Rubiaceae species	681	305 (44.8%)	249 (36.6%)	0.940	0.967	0.908
*atp*B-*rbc*L	24 *Coffea* and 2 *Psilanthus* species	757	27 (3.6%)	11 (1.5%)	Not analysed separately		
	24 *Coffea* and 12 Rubiaceae species	654	228 (34.9%)	187 (28.6%)	0.910	0.958	0.872
All combined	24 *Coffea* and 2 *Psilanthus* species	1,638	65 (4.0%)	21 (1.3%)	0.948	0.905	0.858

from Congo (IIc). *C. charrieriana* Stoff. & F. Anthony and *C. liberica* var. *liberica* Bull. ex Hiern were included in clade II, but they were weakly supported as subclades IId and IIe respectively. The *Psilanthus* species formed two sister clades but their position was weakly supported by bootstrap values. Conflicts between MPTs lay in the position of *C. liberica* var. *liberica* and the *Psilanthus* species, which were placed either in clade II or as sisters to clades I and II. The topology of the Bayesian majority rule consensus tree (Fig. 1) was identical to that of the MP analysis, except for the position of the *Psilanthus* species, which were grouped as sister clades of clades I and II in the MP analysis, and of clade II in the Bayesian analysis. As phylogeny of the *Psilanthus* species remained uncertain, *P. ebracteolatus* and *P. mannii* were not included in subsequent analyses. Except for subclade IId, all clades and subclades were supported by at least one synapomorphy (Fig. 1). A maximum of four synapomorphies was observed for subclade Id. Ten *Coffea* species were characterised by at least one autapomorphy. By contrast, the outgroup presented many more specific characters (25). Few indels were present in subclades, but there were more many in the outgroup.

With gaps treated as new characters, MP analysis yielded 54 MPTs (CI = 0.948, RI = 0.905, RC = 0.858), the consensus of which resembled the tree found with gaps treated as missing data (data not shown). Bootstrap values increased in general, except for *C. liberica* var. *dewevrei*, *C. heterocalyx* and *C. kapakata*.

Considering the species grouped in biogeographic regions, estimates of nucleotide diversity (Nei 1987) were 0.026 ± 0.013 in Lower Guinea and Congolia (13 species), 0.021 ± 0.012 in East-Central Africa (6 species), 0.019 ± 0.012 in East-Central Africa (4 species) and 0.008 ± 0.005 in Upper Guinea (4 species).

Analysis of African and Madagascan species

The *trn*L-F sequences of African species and of the outgroup were aligned with those of three species from Central Africa and 42 species from the Madagascar region, available in GenBank (Table 3). One 1-bp deletion was required to align the sequences of three Madagascan species [i.e. *C. augagneurii* Dubard, *C. pervilleana* (Baill.) Drake, *C. ratsimamangae* J.-F. Leroy ex A.P. Davis & Rakotonas.] and one species from Grande Comore (i.e. *C. humblotiana*) with those of the remaining species. The matrix was composed of 299 aligned positions, 24 (8.0%) of which were variable, with 8 (2.7%) parsimony-informative. Maximum divergence was 2.5% between coffee sequences (*C. leroyi* A.P. Davis-*C. liberica* var. *dewevrei*) and 3.6% between the outgroup and coffee (i.e. *C. leroyi*, *C. stenophylla*) sequences.

With gaps treated as missing data, MP analysis produced a single MPT (CI = 1.0, RI = 1.0, RC = 1.0), the topology of which was identical to that of the Bayesian analysis (Fig. 2). All species from the Madagascar region fitted in clade I. No difference was detected among 10 species from Madagascar, 2 species from the Mascarenes (i.e. *C. macrocarpa*, *C. myrtifolia*) and those from East Africa. Twenty-seven species formed a subclade, in which three species from northern Madagascar (i.e. *C. augagneurii*, *C. pervilleana*, *C. ratsimamangae*) and one from Grande Comore (i.e. *C. humblotiana*) grouped together. The species from Cameroon (i.e. *C. bakossi*, *C. mayombensis*, *C. montekupensis*) were placed in clade II. Referring to their distribution, clades I and II were consequently named the clades A-IO (Africa-Indian Ocean) and G-C (Guinea-Congolian) respectively.

Analysis of Rubiaceae species

The *trn*L-F sequences of African and Madagascan species and of the outgroup were aligned with those of 12 Rubiaceae species belonging to other genera than *Coffea*, and available in GenBank (Table 3). Several indels (1–19 bp) were required to align the new sequences with those of coffee. The matrix was composed of 323 aligned positions, 103 (31.9%) of which were variable, with 62 (19.2%)

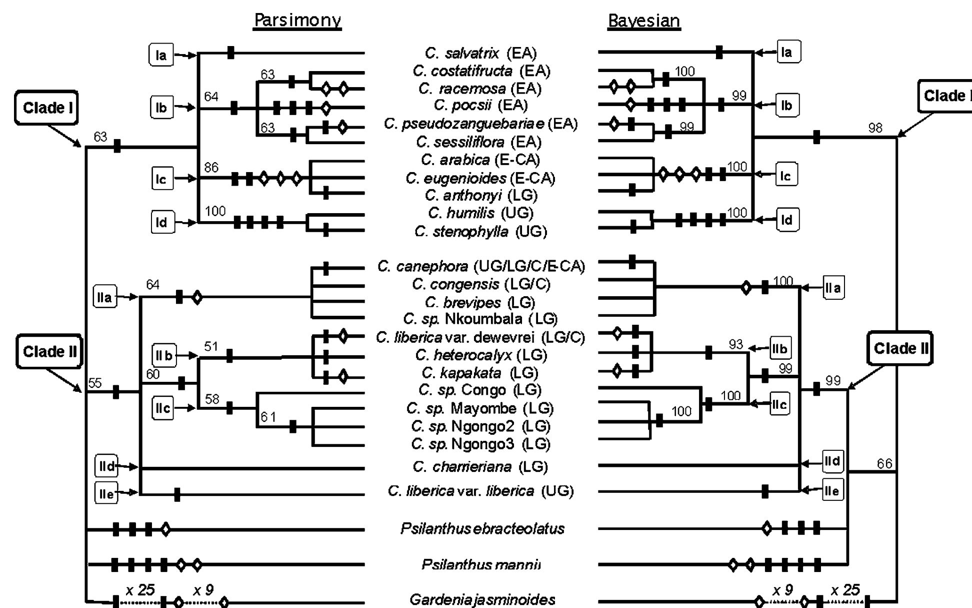

Fig. 1 Phylogenies of 24 *Coffea* subgenus *Coffea* species and two *Psilanthus* species from Africa, using sequences of intergenic spacers *trn*L-F, *trn* T-L and *atp*B-*rbc*L, with gaps coded as missing data. *G. jasminoides* was used as the outgroup. Substitutions and indels appearing once are represented by *solid boxes* and *open rhombi* respectively. For geographical groupings of species, see White (1979) and Maurin et al. (2007). *UG* Upper Guinea, *LG* Lower Guinea, *C* Congolia, *E-CA* East-Central Africa, *EA* East Africa. *Left* Strict consensus tree generated by MP analysis (CI = 0.942, RI = 0.941, RC = 0.887), with bootstrap values (>50%) listed above branches. *Right* Bayesian majority rule phylogeny with posterior probabilities (>50%) listed above branches

parsimony-informative. Divergence was found to be high between *Rubia* and *Coffea* (24.5–26.1%) and other Ixoroideae species (23.8–24.6%). By contrast, divergence was only 1.1–4.3% within the Ixoroideae subfamily.

The HKY85+G model (Hasegawa et al. 1985) was identified by Modeltest as the best nucleotide substitution model. Base frequencies were A = 0.341, C = 0.195, G = 0.108 and T = 0.356, the ti/tv ratio was 0.767, and the estimated value of the gamma shape parameter was 0.826. ML and NJ analyses produced the same phylogenetic relationships supporting the monophyly of Rubiaceae (Fig. 3). The tree of the *trn*L-F sequences showed three main strongly supported lineages, corresponding to the subfamilies Rubioideae, Cinchonoideae and Ixoroideae. The distribution of branch lengths was variable among branches. Long branches were observed for subfamily branching while short branches were observed within subfamilies. The clades A-IO and G-C were closely grouped with the other Ixoroideae species.

Divergence time

The molecular phylogenetic trees generated here and in previous studies did not show any relation between phylogenetic topology and the age of emergence of the volcanic islands that *Coffea* species have colonised in the Indian Ocean. On one hand, no difference was detected in the *trn*L-F region among 2 species from Mauritius (i.e. *C. macrocarpa* and *C. myrtifolia*), 10 species from Madagascar and the East African species of our study. On the other hand, the species from Grande Comore (i.e. *C. humblotiana*) presented a sequence identical to that of three species from Madagascar. Such similarities among species from East Africa, Madagascar, Mascarenes and Grande Comore indicate that dispersal of the *Coffea* subgenus *Coffea* in the Indian Ocean occurred after the emergence of volcanic islands. Given the age of the youngest island (i.e. Grande Comore), dispersal of *Coffea* subgenus *Coffea* species from mainland Africa probably occurred during the last 500,000 years.

Based on the origin of the *Rubia* genus, substitution rates estimated in the ML analyses varied from 15.5×10^{-9} subst. per site per year to 99.6×10^{-9} subst. per site per year (Table 5). The *Coffea* subgenus *Coffea* could thus have diverged about 460,000 years BP or as recently as the last 100,000 years, depending on the cpDNA region considered and calibration.

Discussion

General findings

The present study provided new plastid sequences from *Coffea* subgenus *Coffea* species. The intergenic spacer *trn*T-L was sequenced for the first time and new species

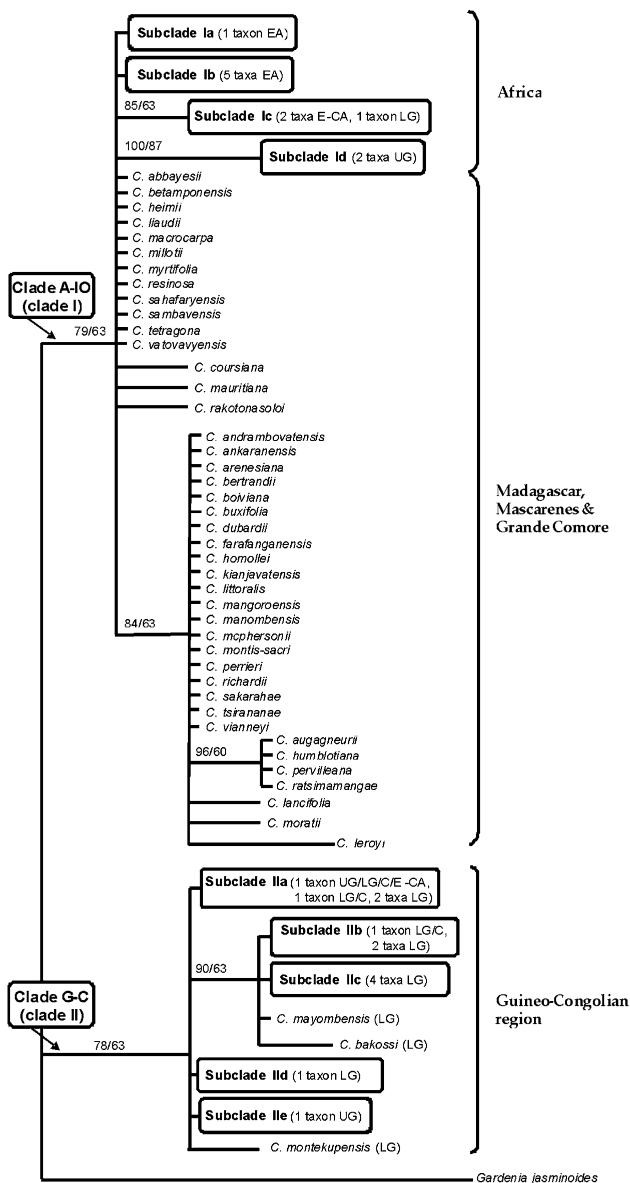

Fig. 2 Single MPT resulting from analysis of *trn*L-F sequences of 69 *Coffea* subgenus *Coffea* species from Africa (27), Madagascar (38), Grande Comore (1) and the Mascarenes (3), with gaps coded as missing data (CI = 1.0, RI = 1.0, RC = 1.0). *G. jasminoides* was used as the outgroup. Bayesian posterior probabilities are listed above branches, parsimony bootstrap values below. Clades and subclades are identified in Fig. 1

were included in the phylogenetic analysis. Non-coding regions were chosen rather than coding regions because they are under lower selection pressure and reveal more divergence among related species (Dixon and Hillis 1993; Gielly and Taberlet 1994). However, non-coding cpDNA regions present variable evolutionary rates and bring variable numbers of potentially informative characters (Shaw et al. 2005; 2007). In coffee, sequences of the intergenic spacers *trn*L-F and *atp*B-*rbc*L were successfully used in previous phylogenetic studies (Cros et al. 1998; Maurin

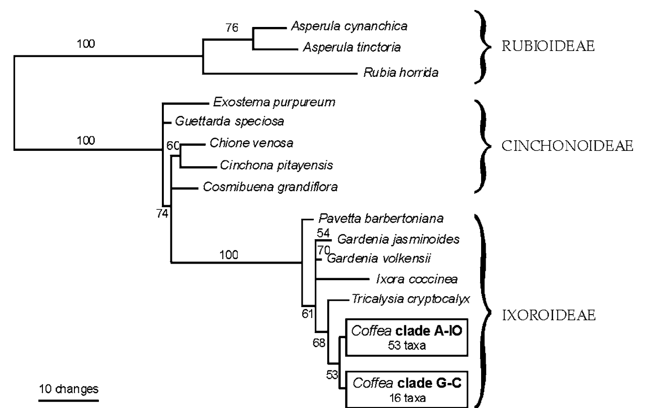

Fig. 3 Maximum likelihood tree of 69 *Coffea* subgenus *Coffea* species and 13 species belonging to other Rubiaceae genera based on analysis of *trn*L-F sequences with gaps coded as missing data. *Numbers* indicate bootstrap support values above 50% in 10,000 replicates. Clades A-IO and G-C are identified in Fig. 2

et al. 2007; Tesfaye et al. 2007). The substitutions identified in this study were confirmed by separating forward and reverse sequencing reactions. Our sequences showed a nucleotide composition and a transition/transversion rate similar to those observed for angiosperms in the intergenic regions *trn*L-F (Bakker et al. 2000) and *atp*B-*rbc*L (Manen and Natali 1995; Morton and Clegg 1995; Hoot and Douglas 1998). Sequence divergence was low in the *Coffea* subgenus *Coffea* ($\leq$2.4%), as shown in previous studies of the *trn*L-F region (Cros et al. 1998), other plastid regions (*trn*L-F intron, *rpl*16 intron and *acc*D-*psa*1) and the internal transcribed spacer (ITS 1/5·8S/ITS 2) of nuclear rDNA (Maurin et al. 2007). Divergence was, however, higher in this study than in the intergenic spacers (*atp*B-*rbc*L, *trn*S-G, *rpl*2-*rps*19 and *rps*19-*rpl*22), introns (*atp*F, *trn*G and *trn*K) and genes (*mat*K, *rpl*2, *rps*19 and *rpl*22) of chloroplast genomes sequenced by Tesfaye et al. (2007). Few parsimony-informative characters were found, only 21 on a 1,638 bp length (1.3%), which explained why the main branches of phylogenetic trees were supported by a low number of characters.

Phylogenetic relationships

The results of our analysis of African species are congruent with those previously published based on plastid and ITS sequences (Lashermes et al. 1997; Cros et al. 1998; Maurin et al. 2007; Tesfaye et al. 2007). Phylogenetic analyses of our dataset revealed two lineages in *Coffea* subgenus *Coffea*. Clade A-IO spans the entire geographical range of *Coffea* subgenus *Coffea* while clade G-C is restricted to the Guineo-Congolian region. Within clades, species were classified in subclades according to their biogeographic origin (i.e. EA, E-CA, C, LG, UG). Similar groupings were found by Maurin et al. (2007) who included 83% of *Coffea*

Table 5 Substitution rate estimated for the *Rubia* species in the ML analyses, using Upper Miocene start (11.6 mya) and end (5.3 mya) for calibration, and corresponding divergence time estimated for *Coffea* subgenus *Coffea*

Region	Calibration 11.6 mya (subs per site per year)	Calibration 5.3 mya (subs per site per year)	Divergence time estimates (years)
*trn*L-F	15.5×10^{-9}	34.0×10^{-9}	158,000–345,000
*trn*T-L	45.5×10^{-9}	99.6×10^{-9}	211,000–461,000
*atp*B-*rbc*L	20.0×10^{-9}	43.8×10^{-9}	48,000–104,000

species in their study, but the main clades were erroneously named EA-IO (East Africa-Indian Ocean) and LG/C (Lower Guinea/Congolia). These names did not reflect the biogeographical origin of studied material since clade EA-IO included a subclade from Upper Guinea and a species (i.e. *C. anthonyi*) from Lower Guinea/Congolia. Similarly clade LG/C included *C. canephora* and *C. liberica* var. *liberica* which can be found in Upper Guinea.

Our molecular analysis resolved the species from Cameroon (i.e. *C. charrieriana*) and Congo (i.e. *Coffea* sp. 'Congo', *Coffea* sp. 'Ngongo 3'), studied here for the first time, to two distinct subclades of clade G-C, thus increasing known diversity in Lower Guinea. High levels of similarity were observed in the *trn*L-F sequences of *Coffea* sp. 'Mayombe', *Coffea* sp. 'Ngongo 2' and *Coffea* sp. 'Ngongo 3', all from the south-west of the Mayombe Mountains in Congo. Moreover, their sequences were identical to that of *C. mayombensis* whose distribution covers west equatorial Africa, from southern Nigeria to Cabinda, including the Mayombe Mountains (Stoffelen 1998). Such grouping resembled that observed around *C. canephora*, a widely distributed species, grouped with species with limited distribution (i.e. *C. congensis* A. Froehner, *C. brevipes* Hiern, *Coffea* sp. 'Nkoumbala'). This confirmed previous observations on the high level of endemicity in the Mayombe Mountains (Cusset 1981, 1989).

Centre of origin

Nucleotide diversity was higher in Lower Guinea and Congolia than in any other biogeographic region, as a consequence of overlap of clades A-IO and G-C in west equatorial Africa. This suggests that Lower Guinea could be the centre of origin of *Coffea* subgenus *Coffea*. The origin may thus not be in Kenya as suggested by a biogeographic analysis (Leroy 1982), but in West-Central Africa. According to floristic records, Lower Guinea is the richest sub-centre of endemism of the Guineo-Congolian Region (White 1979). Diversity in *Coffea* subgenus *Coffea* has, however, been underestimated for a long time as shown by the case of Cameroon. In the early 1990s, only 5 species were known whereas now 15 species are recognised (Anthony et al. 2006), not including the new species of this study. Sequence diversity appeared maximal in west equatorial Africa, suggesting that Lower Guinea constitutes a major centre of speciation for *Coffea* subgenus *Coffea*. This region likely played the role of refuge for coffee trees during the last arid maximum (18,000 years BP) and previous arid phases. In Central Africa, a chain of small refuges has been located near the Atlantic Ocean: in west and south Cameroon, in the Crystal and Chaillu Mountains in Gabon and in the Mayombe Mountains in Congo (Maley 1987, 1996). These areas rich in coffee species are known to be hotspots of biodiversity (Küper et al. 2004). Forest patches could also have survived between refuges and formed forest islands in a grassy sea (Leal 2004).

Radiation in *Coffea* subgenus *Coffea*

The low rate of homoplasy and the low number of characters supporting the main branches confirmed the hypothesis of a rapid and radial mode of speciation in *Coffea* subgenus *Coffea* (Lashermes et al. 1997; Cros et al. 1998). Judging from genetic distances, the origin of *Coffea* subgenus *Coffea* is recent. For example, *trn*L-F uncorrected pairwise sequence divergence was only 0–2.4% within *Coffea* species while that between *Coffea* and *Rubia* was 24.5–26.1%. Another fact in favour of a recent origin of *Coffea* subgenus *Coffea* is the low number of insertions and deletions that were required for plastid sequence alignment. To align the *trn*L-F sequences of 42 Madagascar species with those of 26 African species, only one short deletion (1 bp) was required. Few indels were also reported for sequence alignment of cpDNA intergenic spacers (Cros et al. 1998) and introns (Tesfaye et al. 2007), and none in coding regions (Tesfaye et al. 2007). Moreover, the sequences of Madagascar species showed high similarities with those of species from the surrounding islands and from East Africa, suggesting a common origin. Biodiversity is, however, considerable in Madagascar (Myers et al. 2000), in particular for coffee trees since the region contains 60% of *Coffea* subgenus *Coffea* species (Davis et al. 2006). The majority of Madagascan species have rather limited distribution (Davis et al. 2006), which corresponds to radial and rapid speciation.

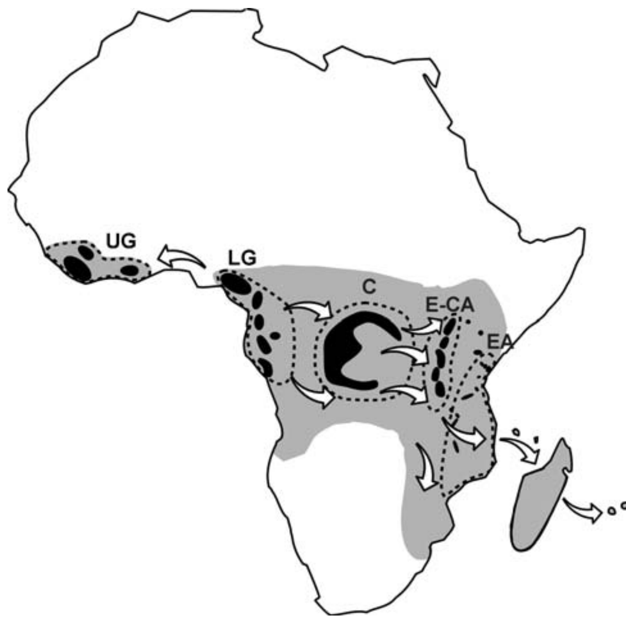

Fig. 4 Reconstruction of the dispersal of *Coffea* subgenus *Coffea* from its centre of origin in Lower Guinea. Actual distribution of *Coffea* subgenus *Coffea* and putative forest refuges during the last major arid phase (18,000 years BP) (Maley 1996; Roy 1997) are in *grey* and *black* respectively. Biogeographic regions in Africa are outlined by marks according to White (1979) and Maurin et al. (2007). *UG* Upper Guinea, *LG* Lower Guinea, *C* Congolia, *E-CA* East-Central Africa, *EA* East Africa

Coffee would have spread radially from the centre of origin located in Lower Guinea, westwards up to Upper Guinea and eastwards through Central Africa (Fig. 4). Dispersal could have benefitted from several putative refuges in the Congo-Zaire Basin (Maley 1996; Colyn et al. 1991), in East Central Africa (Lovett 1993) and in East Africa (Fjeldså and Lovett 1997; Roy 1997) where montane regions offered a great range of habitats. Colonisation of Madagascar was doubtless the result of a single dispersal event from the African mainland, followed by insular speciation. Such a scenario has been already proposed to explain speciation in the genera *Begonia* (Plana et al. 2004) and *Gaertnera* (Malcomer 2002) in Madagascar. High similarity between *C. humblotiana* from Grande Comore and three species from north Madagascar (i.e. *C. augagneurii*, *C. pervilleana*, *C. ratsimamangae*) indicate that Grande Comore was colonised by coffee trees from north Madagascar (Maurin et al. 2007) or, more likely according to the geographic position of Grande Comore, in one step when coffee trees crossed the Mozambique channel. Lastly, the species from the Mascarenes showed a common origin with the East African species in our study and with ten Madagascar species, suggesting rapid colonisation of Mauritius and Reunion Island from Madagascar.

The Dahomey Gap has recently (ca. 4,000 years BP) fragmented rain forest in the Guineo-Congolian region, isolating Upper Guinea from Lower Guinea over a distance of some 200 km in Togo and Benin (Salzmann and Hoelzmann 2005). This savannah barrier is believed to have occupied a far larger area during previous drier phases and to have separated the forest refuges of Ivory Coast-Ghana and west Cameroon by at least 1,200 km. In *C. canephora*, the existence of genetic groups distributed in Upper Guinea and Central Africa (Berthaud 1986; Dussert et al. 2003), which are easily distinguishable and hardly differentiated (Leroy et al. 1993), suggests that colonisation of Upper Guinea by coffee trees occurred before formation of the Dahomey Gap, similarly to what happened for the shea tree (Fontaine et al. 2004).

Divergence time

Radiation observed in Madagascar and the surrounding islands demonstrated that coffee trees are not remnants of a putative Cretaceous Gondwana flora (Guillaumet and Mangenot 1975; Leroy 1978). Their origin is much more recent than the Gondwana dislocation mentioned by Leroy (1982) and even more recent than the appearance of the volcanic islands around Madagascar. Given the age of the youngest island (i.e. Grande Comore), coffee dispersal occurred within the last 500,000 years. Sequence comparison between *Coffea* subgenus *Coffea* species and *Rubia*, whose origin was dated from the Upper Miocene (Muller 1981), enabled us to estimate the divergence time at about 100,000–450,000 years BP. Even though these ages should be considered as preliminary estimates, radiation in *Coffea* subgenus *Coffea* occurred probably in the second half of the Middle Pleistocene (780,000–126,000 years BP). Coffee dispersal could have benefitted from humid conditions during interglacials of the past 200,000 years (Dupont et al. 2001). The rapidity of colonisation points to the effective dispersal of coffee seeds, likely by monkeys in Africa and lemurs in Madagascar. The role of primates in seed dispersal has already been put forward to explain the rapid radiation of *Aframomum* the genus in Africa (Harris et al. 2000).

Adaptation and speciation

The phylogenetic relationships based on nucleotide sequences were not congruent with either morphological and biochemical classifications (Stoffelen 1998; Dussert et al. 2008) or with the adaptive capacity to grow in specific environments. For example, only three small-leaved species are known in Central Africa: *C. anthonyi* (Stoffelen et al. 2009), *C. charrieriana* (Stoffelen et al. 2008) and *C. kapakata* (Chevalier 1947; Bridson 1994). In our study, the first species was placed in the clade A-IO while the two others were placed in the clade G-C.

Another example is the absence of caffeine in coffee seeds. Two caffeine-free species have been reported in Africa up to now, *C. pseudozanguebariae* Bridson (Hamon et al. 1984) and *C. charrieriana* (Stoffelen et al. 2008). They were classified in clades A-IO and G-C respectively. However, these species occupy very different habitats, the coastal dry forest on a coral reef substrate close to the Indian Ocean in the case of *C. pseudozanguebariae* (Anthony et al. 1987) and rain forest in west Cameroon in the case of *C. charrieriana* (Stoffelen et al. 2008). On the other hand, all caffeine-free species from Madagascar (e.g. *C. homollei* J.-F. Leroy) (Anthony et al. 1993) were grouped in the clade A-IO together with Madagascan species containing caffeine (e.g. *C. lancifolia* A. Chev.) (Rakotomalala et al. 1992). The absence of caffeine in seeds and leaves where the component is synthesised does not appear to be associated with one or more particular lineages in *Coffea* subgenus *Coffea*. Similarly, autofertility of *C. anthonyi* (Stoffelen et al. 2009) and *C. heterocalyx* (Coulibaly et al. 2002) is a character that appeared independently in clades A-IO and G-C. These characters are examples of convergent evolution on the scale of equatorial Africa and the islands in the Indian Ocean. Finally, the high adaptive capacity of *Coffea* subgenus *Coffea* probably originates in variations in gene expression mechanisms rather than in the nucleotide composition of the genes themselves.

Accelerated rates of regulatory gene evolution could accompany rapid morphological diversification in adaptive radiation (Barrier et al. 2001). Phenotypic plasticity has been shown to affect plant morphology, anatomy and physiology (Walbot 1996; Sultan 2000) as well as the ecological organisation of populations (Miner et al. 2005). Plasticity of coffee trees could be the key to rapid colonisation of African forests from Guinea to Mozambique and, farther away, of islands in the Indian Ocean. This would explain the restricted distribution of the majority of coffee species and the number of species described up to now using morphological criteria. Further studies on the evolution of *Coffea* subgenus *Coffea* should include regulatory genes whose divergence could correlate better with phenotypic evolution than molecular evolution did.

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