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Effectiveness of the fatty acid and sterol composition of seeds for the chemotaxonomy of *Coffea* subgenus *Coffea*

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ABSTRACT

The chemotaxonomic relationships between *Coffea* (subgenus *Coffea*) species have been poorly studied to date and the compounds tested so far – chlorogenic acids, diterpenoids and purine alkaloids – did not enable the establishment of phylogenetic relationships analogous to those revealed by chloroplast and nuclear DNA studies. In the present study, the relationships between African *Coffea* species were assessed on the basis of their seed lipid composition. Fatty acids and sterols were determined in 59 genotypes belonging to 17 distinct *Coffea* species/origins. Principal Component Analysis of fatty acid and sterol data enabled easy identification of the few species for which one or several compounds could serve as a quantitative signature. Hierarchical Clustering classified the *Coffea* species in seven groups with both fatty acids and sterols. However, while groupings based on seed fatty acid composition showed remarkable ecological and geographical coherence, no phylogeographic explanation was found for the clusters retrieved from sterol data. When compared with previous phylogenetic studies, the groups deduced from seed fatty acid composition were remarkably congruent with the clades inferred from nuclear and plastid DNA sequences.

1. Introduction

The genus *Coffea* L. (Rubiaceae) contains 103 species (Davis et al., 2006), all originating from tropical Africa (41 species), Madagascar and the Comoros (59) and the Mascarenes (3). *Coffea* species occur naturally in almost all forest ecosystems of the Afrotropic ecozone: lowland evergreen forests, moist deciduous forests, freshwater swamp forests, mountain forests, and tree savannas. Most *Coffea* species are found in a rather limited geographical area, with the exception of *Coffea canephora* and *Coffea liberica,* which are widely distributed throughout the Guineo-Congolian Region (Stoffelen, 1998). Two African *Coffea* species are of major economic importance: *Coffea arabica* and *C. canephora* (robusta coffee). Since African *Coffea* species hybridize satisfactorily with one another, wild African species are considered as an important potential gene reservoir for the breeding of the two cultivated coffee species for both resistance to pathogens and bean quality traits.

Coffea have been the focus of only few molecular phylogenetic studies (Lashermes et al., 1997; Cros et al., 1998; Maurin et al., 2007) using plastid and/or nuclear ribosomal DNA sequence polymorphism. Though the number of species sampled differed (from 23 to 87), the three studies exhibit a very strong congruence with respect to the major lineages of the genus *Coffea*. The five major clades identified on the basis of plastid and nuclear DNA data also showed unambiguous geographical coherence (Lashermes et al., 1997; Cros et al., 1998; Maurin et al., 2007) and were therefore recently termed (Maurin et al., 2007) using the Afrotropic forest zone terminology: Upper Guinea, Lower Guinea/Congolian, East-Central Africa, East Africa, Madagascar/Comoros/Mascarene clades.

The chemometric discrimination of the two cultivated coffee species *C. arabica* and *C. canephora* has been an active area of research during the last ten years (Carrera et al., 1998; Casal et al., 2003; González et al., 2001; Ky et al., 2001; Martín et al., 2001; Rui Alves et al., 2003; Speer and Kölling-Speer, 2006). By contrast, very little has been done for the chemotaxonomy of the genus *Coffea* so far. The main non-parietal components of *Coffea* seeds are lipids (10–35% dry weight), proteins (8–12%), sucrose (4–11%), chlorogenic acids (1–12%), mainly caffeoylquinic and feruloylquinic acids, purine alkaloids (0–3%), mainly caffeine, and trigonelline (<2%) (Campa et al., 2004, 2005; Clifford et al., 1989; Dussert et al., 2001). As secondary metabolites, purine alkaloids, chlorogenic acids and diterpene esters are potentially valuable compounds from a phylogenetic point of view (Wink, 2003). To date, chlorogenic acid and purine alkaloid analyses have been carried out in about 30 *Coffea* species enabling three groups to be distinguished that do not strictly match the five phylogenetic clades of this genus (Clifford et al., 1989; Anthony et al., 1993). One group was formed by two East African species, *Coffea pseudozanguebariae* and *Coffea salvatrix*, whose seeds contain a distinctive glycoside, termed mozambioside (Prewo et al., 1990). The two other groups

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correspond roughly to species originating respectively from Africa and from Madagascar. Seed diterpenoids were examined in nine African coffee species by de Roos et al. (1997). Though no clear taxonomic structure was revealed, possibly because of the limited number of samples analyzed, this work provided valuable biochemical signatures for two groups of species: the occurrence of 16-*O*-methyl kahweol in seeds of the West African species *Coffea stenophylla*, and of an unidentified diterpene ester in the two East African species *C. pseudozanguebariae* and *C. salvatrix*. None of the seed secondary metabolites tested until now enabled species phylogenetic relationships to be established that were comparable with those revealed by chloroplast and nuclear DNA studies (Lashermes et al., 1997; Cros et al., 1998; Maurin et al., 2007). To our knowledge, except in the works cited above, no other compounds have been tested so far for *Coffea* chemotaxonomy.

The present study aimed to assess the value of two major seed lipid classes, fatty acids (FA) and sterols, to investigate the chemotaxonomic relationships in the genus *Coffea*. These compounds were chosen because of their great interest for the chemometric discrimination of the two cultivated coffee species (Carrera et al., 1998; Rui Alves et al., 2003) and because they have already been shown to be effective compounds for chemotaxonomy. Leaf and seed FA have been successfully used to study phylogenetic relationships at different taxonomic levels: classification of (i) families within a plant group, e.g. Angiosperms (Aitzetmüller, 1993; Mongrand et al., 1998) or Gymnosperms (Mongrand et al., 2001), (ii) tribes and genera within a family, e.g. Ranunculaceae (Aitzetmüller and Tsevegsüren, 1994), Rubiaceae (Mongrand et al., 2005); for instance, Mongrand et al. (2005) observed that the tribe Coffeae could be clearly distinguished from other tribes of the family Rubiaceae on the basis of their leaf FA composition, (iii) genera and species within a tribe, e.g. Nicotianae (Maestri and Guzman, 1995), (iv) species within a genus, e.g. *Brassica* (Barthet, 2008), *Pinus* (Wolff et al., 2000). Sterols have also been widely used for chemotaxonomic purposes, mainly in marine organisms (e.g. Dunstan et al., 2005). Though sterols are less frequently employed, a few studies have shown that they could also be a powerful tool in plant chemotaxonomy (Patterson et al., 1991; Zygadlo, 1993; Brenac and Sauvaire, 1996).

2. Results and discussion

2.1. Fatty acid composition

Seed fatty acids were analyzed by gas chromatography in 59 genotypes belonging to 14 *Coffea* species originating from Angola, Cameroon, Central African Republic, Ivory Coast, Comoro Islands, Kenya, Mozambique, Republic of Congo, and Tanzania (Table 1, Fig. 1). Eighteen peaks were identified in the chromatograms of the 59 genotypes studied. None of the peaks was exclusive to a *Coffea* species or to a group of species. Consequently, only the ten FA quantifiable accurately – i.e. when their relative percentage was higher than 0.1% – were retained for multivariate statistics. The overall FA composition obtained in the present study was in full agreement with previous reports dealing with the FA composition of seeds of the two cultivated species, *C. arabica* and *C. canephora* (Dussert et al., 2001; Rui Alves et al., 2003; Martín et al., 2001). Palmitic (16:0), stearic (18:0), oleic (18:1Δ^9) and linoleic (18:2$\Delta^{9,12}$) acids were the major FA in all species (Table 2). For each of the 10 FA retained, interspecific variability was considerable as estimated by the interspecific max/min ratio, which varied from 1.5 for 16:0 (44.7% in *Coffea humblotiana* down to 29.3% in *Coffea kapakata*) to 7 for 18:3 (3.1% in *Coffea eugenioides* down to 0.4% in *Coffea racemosa*). There was a significant effect of the species for all FA, as tested by ANOVA. In many cases, one FA could serve as a quantitative signature of one species since its mean value was significantly different from all other species means, as tested by the Newman and Keuls test. For example, the linolenic acid (18:3) content of *C. eugenioides* was significantly higher than that of all other species; the linoleic acid percentage of *Coffea charrieriana* was significantly lower than that of all other species; *Coffea congensis* seed 20:0 content was significantly higher than that of all other species.

2.2. Principal component analysis of fatty acid data

Four principal components (PC) had an eigenvalue higher than 1. These first four PC accounted for 34%, 21%, 17% and 11% of the total variance of the data set, respectively. The loading plot obtained for the first two factors (Fig. 2) shows that most FA vectors

Table 1
Distribution and ecology of the *Coffea* species studied.

Species/origin	Distribution (ecoregion)[a]	Habitat	Origin of genotypes studied
C. canephora Pierre	Guinean–Congolian forests	Semi-deciduous forest (humid areas such as valleys)	West: Ivory Coast, Central: South Central African Republic, North: North Central African Republic
C. charrieriana Stoffelen & Anthony	Lower Guinean forests	Evergreen forest (wet areas)	Cameroon
C. congensis Froehner	Congolian forests	Sand banks along rivers of Central African lowlands	Central African Republic
C. eugenioides Moore	Albertine Rift montane forests	Mountain forest	Kenya and Tanzania
C. heterocalyx Stoffelen	Lower Guinean forests	Evergreen forest	Cameroon
C. humblotiana Baill.	Comoros forests	Mountain forest	Comoro Islands
C. humilis Chevalier	Upper Guinean forests	Evergreen forest (wet areas)	Ivory Coast
C. kapakata Chevalier	Lower Guinean forests	Woodland	Angola
C. liberica Hiern	Guinean–Congolian forests	Rainforest with open canopy	West: Ivory Coast = var. *liberica*, Central: Cent. Afr. Rep. = var. *dewevrei*
C. pseudozanguebariae Bridson	East African coastal forests (north)	Coastal dry forest on coral rag	Kenya and Tanzania
C. racemosa Lour.	East African coastal forests (south)	Seasonally dry semi-deciduous-forest	Mozambique
C. salvatrix Swynnerton	East African coastal forests (south)	Seasonally dry semi-deciduous forest	Mozambique
C. sessiliflora Bridson	East African coastal forests (north)	Forest patches in woody savannah	Kenya
C. stenophylla Don	Upper Guinean forests	Semi-deciduous forest (drier areas such as top of hills)	Ivory Coast

[a] See Davis et al. (2006) for a detailed description of *Coffea* species geographical distributions.

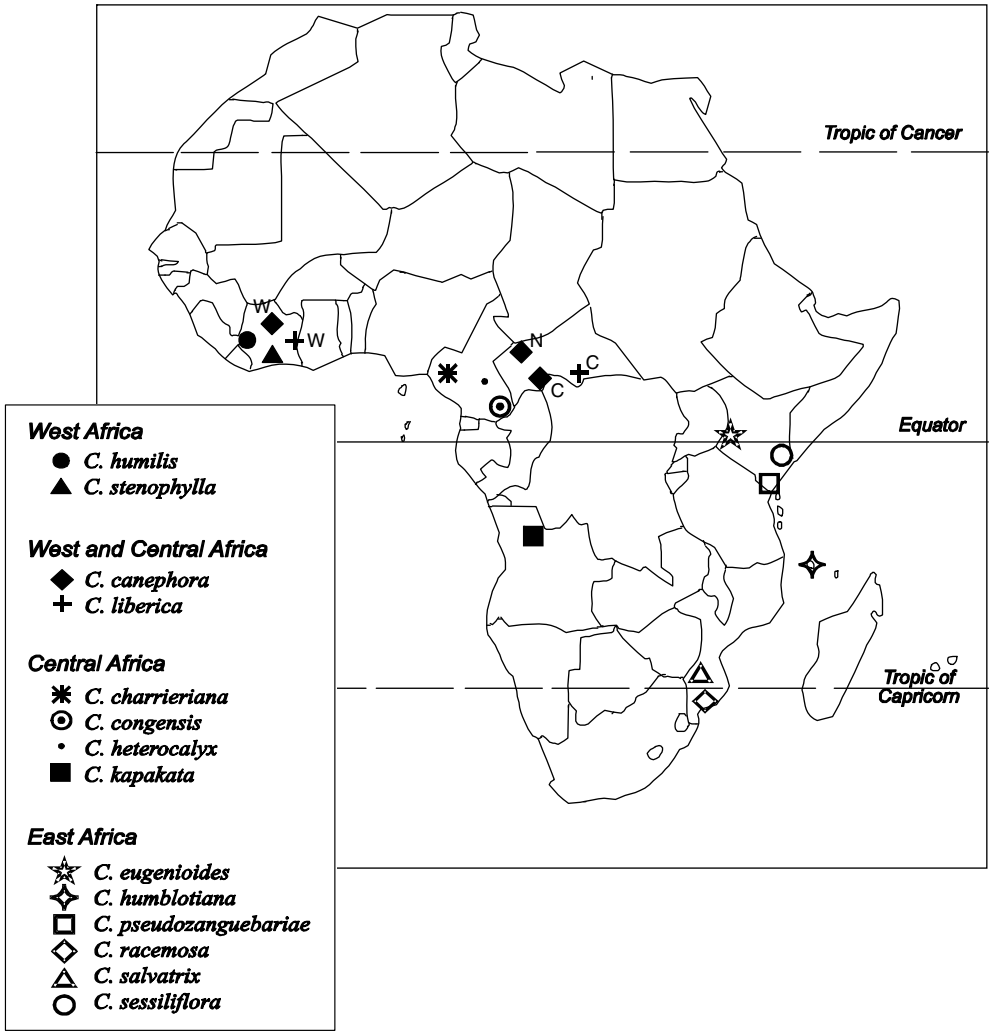

Fig. 1. Geographical origin of the plant material studied.

Table 2
Fatty acid composition of seeds. Means followed by the same letter were not significantly different at $P = 0.05$ according to the Newman and Keuls test. N = number of genotypes studied per species or origin for *C. canephora* and *C. liberica*. F and P: results of one-way ANOVAs.

Species/origin	N	16:0	18:0	18:1Δ^{9}	18:1Δ^{11}	18:2Δ^{9,12}	18:3Δ^{9,12,15}	20:0	20:1Δ11	22:0	24:0
C. canephora Central (C)	4	34.9^{cde}	6.4^{e}	11.8^{b}	0.3^{bc}	41.9^{b}	0.7^{def}	3.0^{cd}	0.4^{b}	0.5^{bcde}	0.2^{cdef}
C. canephora Nana (N)	4	35.0^{cde}	6.3^{e}	9.0^{de}	0.3^{bc}	43.7^{ab}	1.2^{cd}	3.1^{cd}	0.5^{ab}	0.7^{b}	0.3^{c}
C. canephora West (W)	4	31.2^{f}	8.8^{cd}	14.2^{a}	0.4^{bc}	40.0^{c}	1.0^{cdef}	3.1^{cd}	0.5^{a}	0.5^{bcde}	0.3^{cd}
C. charrieriana	3	44.2^{a}	16.7^{a}	4.4^{h}	0.1^{d}	28.3^{e}	0.6^{def}	3.5^{bc}	0.2^{c}	1.2^{a}	0.9^{a}
C. congensis	3	32.8^{ef}	9.2^{cd}	10.1^{cd}	0.3^{bc}	37.7^{c}	0.6^{ef}	6.9^{a}	0.4^{ab}	1.4^{a}	0.5^{b}
C. eugenioides	4	37.2^{bc}	7.0^{de}	4.9^{gh}	0.3^{bc}	44.5^{a}	3.1^{a}	2.1^{d}	0.2^{c}	0.5^{bcde}	0.2^{cdef}
C. heterocalyx	3	38.9^{b}	7.4^{de}	6.3^{fg}	0.2^{cd}	43.0^{ab}	1.0^{cdef}	2.4^{cd}	0.4^{b}	0.4^{de}	0.2^{ef}
C. humblotiana	2	44.7^{a}	6.3^{e}	5.7^{gh}	0.4^{b}	35.0^{d}	2.6^{b}	4.3^{b}	0.2^{c}	0.5^{bcde}	0.2^{def}
C. humilis	4	39.7^{b}	7.8^{de}	9.8^{cd}	0.6^{a}	38.1^{c}	0.8^{def}	2.2^{cd}	0.4^{b}	0.5^{bcde}	0.2^{cdef}
C. kapakata	1	29.3	6.9	9.8	0.4	47.8	0.9	3.5	0.4	1.0	0.3
C. liberica var. *dewevrei* (C)	4	35.1^{cde}	7.0^{de}	9.5^{cd}	0.3^{bc}	43.6^{ab}	1.2^{cde}	2.3^{cd}	0.5^{ab}	0.5^{bcde}	0.2^{ef}
C. liberica var. *liberica* (W)	4	33.2^{def}	7.9^{de}	9.4^{cd}	0.2^{bcd}	44.6^{a}	1.4^{c}	2.2^{cd}	0.4^{b}	0.4^{de}	0.2^{def}
C. pseudozanguebariae	4	31.7^{f}	16.3^{a}	8.8^{de}	0.2^{bcd}	38.5^{c}	0.7^{def}	3.1^{cd}	0.2^{c}	0.4^{cde}	0.1^{f}
C. racemosa	4	35.7^{cde}	10.1^{bc}	11.4^{bc}	0.4^{b}	38.6^{c}	0.4^{f}	2.3^{cd}	0.2^{c}	0.6^{bc}	0.2^{cde}
C. salvatrix	3	34.5^{cde}	11.0^{b}	10.6^{bcd}	0.3^{bc}	39.6^{c}	1.4^{c}	1.8^{d}	0.2^{c}	0.3^{e}	0.1^{f}
C. sessiliflora	4	33.3^{def}	16.6^{a}	7.4^{ef}	0.2^{d}	37.6^{c}	0.6^{def}	3.5^{b}	0.1^{c}	0.6^{bcd}	0.1^{ef}
C. stenophylla	4	36.0^{cd}	8.3^{de}	9.1^{de}	0.7^{a}	39.6^{c}	0.8^{def}	4.3^{b}	0.4^{b}	0.6^{bcd}	0.2^{cdef}
F		26.2	66.5	43.8	18.5	50.0	28.5	25.3	32.6	29.8	65.9
P		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

were homogeneously distributed within the correlation circle, with moderate to high loading on both factors, indicating satisfactory representation of the information contained in most FA on the PC1-PC2 score plot. PC1 clearly opposed saturated and unsaturated FA. PC2 opposed medium (16:0–18:0) to long saturated FA (20:0–24:0), and monoenic acids to linolenic acid.

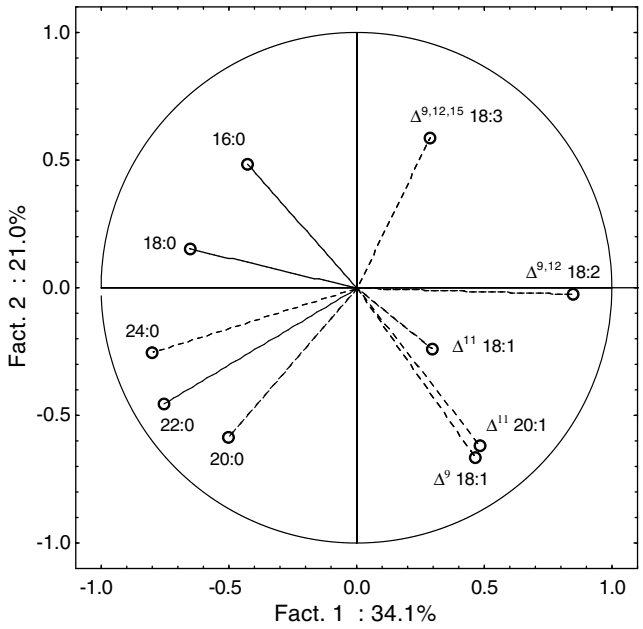

Fig. 2. Principal Component Analysis of the seed fatty acid composition of 59 *Coffea* genotypes: correlations (factor loadings) of seed FA with the first two factors (principal components).

The PC1–PC2 score plot obtained through the analysis of the FA composition first unambiguously separated four species, (*C. charrieriana*, *C. congensis*, *C. eugenioides* and *C. humblotiana)* from one another and from the 10 other *Coffea* species, which were located close together in the central part of the score plot (Fig. 3A). PCA thus appears to be an efficient tool to reveal species exhibiting a peculiar trait in their FA composition: *C. eugenioides* and *C. humblotiana* had the highest 18:3 content (>2%, Table 2), *C. charrieriana* genotypes had the lowest polyunsaturated FA content (<30%, Table 2) and *C. congensis* had the highest long (C $\geqslant$ 20) FA content (Table 2). In Fig. 3B, the enlargement of the central area of the PC1–PC2 score plot shows that the remaining 10 species could also be classified through PCA. Though PC1 and PC2 were strongly dependent on the particular FA composition of the four species cited above, it is worth mentioning that they also allowed the remaining 10 species to be distinguished by origin (Fig. 3B). *Coffea* species originating from East Africa are grouped in the left-upper part. Species originating from West and Central Africa, *C. canephora*, *C. liberica* and *C. kapakata* are all located on the right. The different origins of the two *Coffea* species that are naturally widely distributed, *C. canephora* and *C. liberica,* were well separated. The two species present in West Africa only, *Coffea humilis* and *C. stenophylla*, were grouped together close to the origin of both axes. The only significant incongruence identified in the PC1–PC2 score plot concerns the grouping of *Coffea heterocalyx* and *C. salvatrix,* which occur naturally in Central and East Africa, respectively. This incongruence was not apparent in the PC3–PC4 score plot, where *C. heterocalyx* was grouped with the other *Coffea* species originating from Central Africa (data not shown).

2.3. Hierarchical cluster analysis of fatty acid data

The first remarkable outcome of the dendrogram obtained by Hierarchical cluster analysis (HCA) of FA data is that all genotypes within a species were grouped together. In addition, seven well-supported geographical groups of species, termed FA1 to FA7, were clearly revealed (Fig. 4). The first and second clusters contained genotypes of *C. charrieriana* and *C. congensis*, respectively, both from Cameroon. Group FA3 was made up of the two species originating from Kenya and Tanzania, *C. pseudozanguebariae* and *Coffea sessiliflora*. The fourth group was constituted of the two species occurring in Mozambique, *C. racemosa* and *C. salvatrix*. Group FA5 was made up of species from Central Africa and included all genotypes of the two widespread species, *C. canephora* and *C. liberica*, whatever their origin (west, central, north). The sixth group contained the two species occurring only in West Africa, *C. humilis* and *C. stenophylla*. Finally, group FA7 was made up of *C. eugenioides* and *C. humblotiana*.

This classification of African *Coffea* inferred from seed FA composition was also remarkably congruent with the phylogeny of this genus based on nuclear and plastid DNA sequences as revealed in previous studies (Lashermes et al., 1997; Cros et al., 1998; Maurin et al., 2007). The very high proportion of well-classified species observed in the contingency table obtained (Table 3) demonstrates the outstanding value of seed FA for the chemotaxonomy of the genus *Coffea*. Only one discrepancy appeared: on the basis of their FA composition, the *C. congensis* genotypes formed a separate group, while based on DNA polymorphism studies, this species was included in the Lower Guinea/Congolian Clade, very close to *C. canephora* (Lashermes et al., 1997; Cros et al., 1998; Maurin et al., 2007). At first sight, the original seed FA composition of *C. congensis* could be interpreted as reflecting its particular ecology since it is the only rheophytic species of the genus *Coffea*. It is found on sand banks along rivers of Central African lowlands or in seasonally flooded forests. However, in a recent molecular study on the structure of the *Coffea* genus using microsatellite markers, Cubry et al. (2008) also observed that *C. congensis* genotypes formed an original group separated from other species in the Lower Guinea/Congolian Clade.

Regarding non-African *Coffea* species, *C. humblotiana* was unfortunately the only species available in the CNRA-Ivory Coast field collections. Given that more than half of *Coffea* species are endemic to Madagascar, a new study is needed to assess whether non-African species have different seed FA composition from African species. A previous study by Chassevent et al. (1974) reported that Madagascan species exhibited higher palmitic acid content (40–80%) and lower linoleic acid content (6–37%) than African *Coffea*. This hypothesis is in agreement with the data obtained here for *C. humblotiana* whose seeds contained the highest palmitic acid content out of all the *Coffea* we analyzed (Table 2). In the present study, *C. humblotiana* was grouped with *C. eugenioides* in the FA phenogram obtained by HCA. This grouping was certainly favoured by the too low representation of Madagascan species in the present study.

C. charrieriana is a poorly known species, which was only very recently described (Stoffelen et al., 2008), and has never previously been included in a phylogenetic study. In the present work, *C. charrieriana* did not join any of the four major African phylogenetic clades. Its original seed FA composition, similar to that described for Madagascan species (Chassevent et al., 1974), is not the only peculiar biochemical characteristic of this species: it may be the only naturally caffeine-free species in West Africa (Davis et al., 2006). The absence of caffeine in seeds is also a common feature of *Coffea* species from Madagascar (Clifford et al., 1989). The taxonomic position of *C. charrieriana* undoubtedly requires further investigations using molecular tools.

Two African *Coffea* species are very widely distributed throughout the whole Guineo–Congolian Region: *C. canephora* and *C. liberica* (Stoffelen, 1998). For *C. canephora*, we investigated three origins and for *C. liberica* two, to assess possible infraspecific geographical differentiation. Significant differences were observed for all major fatty acids between the three origins of *C. canephora* tested (Table 2), leading to their clear separation in the corresponding phenogram (Fig. 4). These differences are congruent

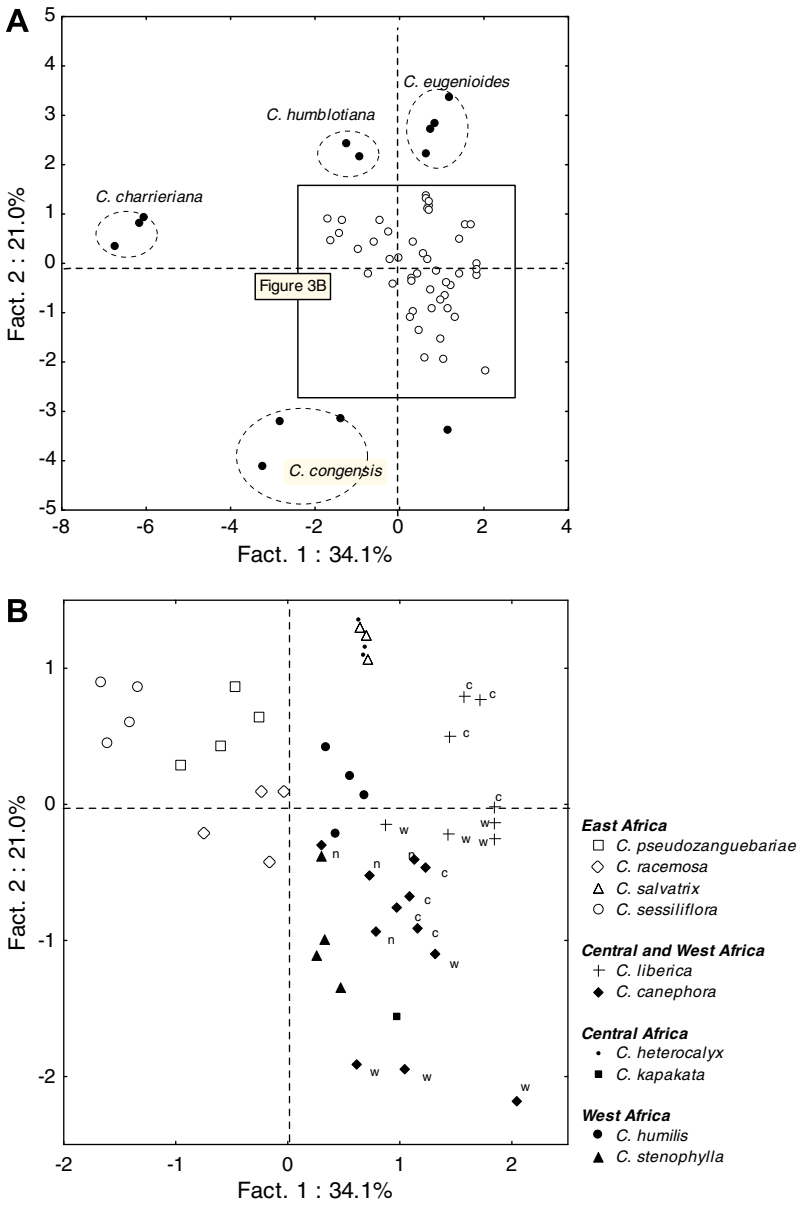

Fig. 3. (A) Principal Component Analysis of the seed fatty acid composition: PC1–PC2 score plot of the 59 genotypes of *Coffea* studied. (B) Enlargement of the central area of the PC1–PC2 score plot.

with the infraspecific genetic structure of this species as revealed using RFLP markers (Dussert et al., 2003). In contrast, the two origins of *C. liberica* did not show any significant variation in their FA composition, even though they are classically distinguished as two botanical varieties, *C. liberica* var. *liberica* (West Africa) and *C. liberica* var. *dewevrei* (Central Africa), whose taxonomic differentiation was recently supported by molecular data (N'Diaye et al., 2005). This discrepancy reveals that seed FA is less efficient at the infraspecific level than at the infrageneric level for chemotaxonomic purposes.

2.4. Sterol composition

Thirteen sterols were identified in seeds of the 50 genotypes studied (Table 4). None of them was limited to one *Coffea* species or to a group of species. The overall sterol composition obtained in the present study was in full agreement with previous studies on the seed sterol composition of the two cultivated species, *C. arabica* and *C. canephora* (Carrera et al., 1998). The major sterols were also the same in all *Coffea* species studied: β-sitosterol (37–57%), stigmasterol (12–36%), campesterol (11–22%) and Δ^5 avenasterol (1.2–15.2%). These four sterols are the predominant sterols in most oilseed species (Phillips et al., 2002). For all sterols, infrageneric variability as assessed by the max/min ratio was considerable. As with seed FA, ANOVA and post-hoc multiple comparisons of means enabled identification of sterols that could serve as a quantitative signature for the discrimination of one *Coffea* species: campestanol, stigmasterol and Δ^7 campesterol for *C. humblotiana*; Δ^7 stigmastenol, $\Delta^{5,23}$ stigmastadienol and Δ^7 avenasterol for *C. congensis*.

2.5. Principal component analysis of sterol data

Four principal components (PC) had an eigenvalue of more than 1. They accounted for 39%, 19%, 15% and 9% of the total variance, respectively. In contrast to FA, the PC1–PC2 loading plot (Fig. 5)

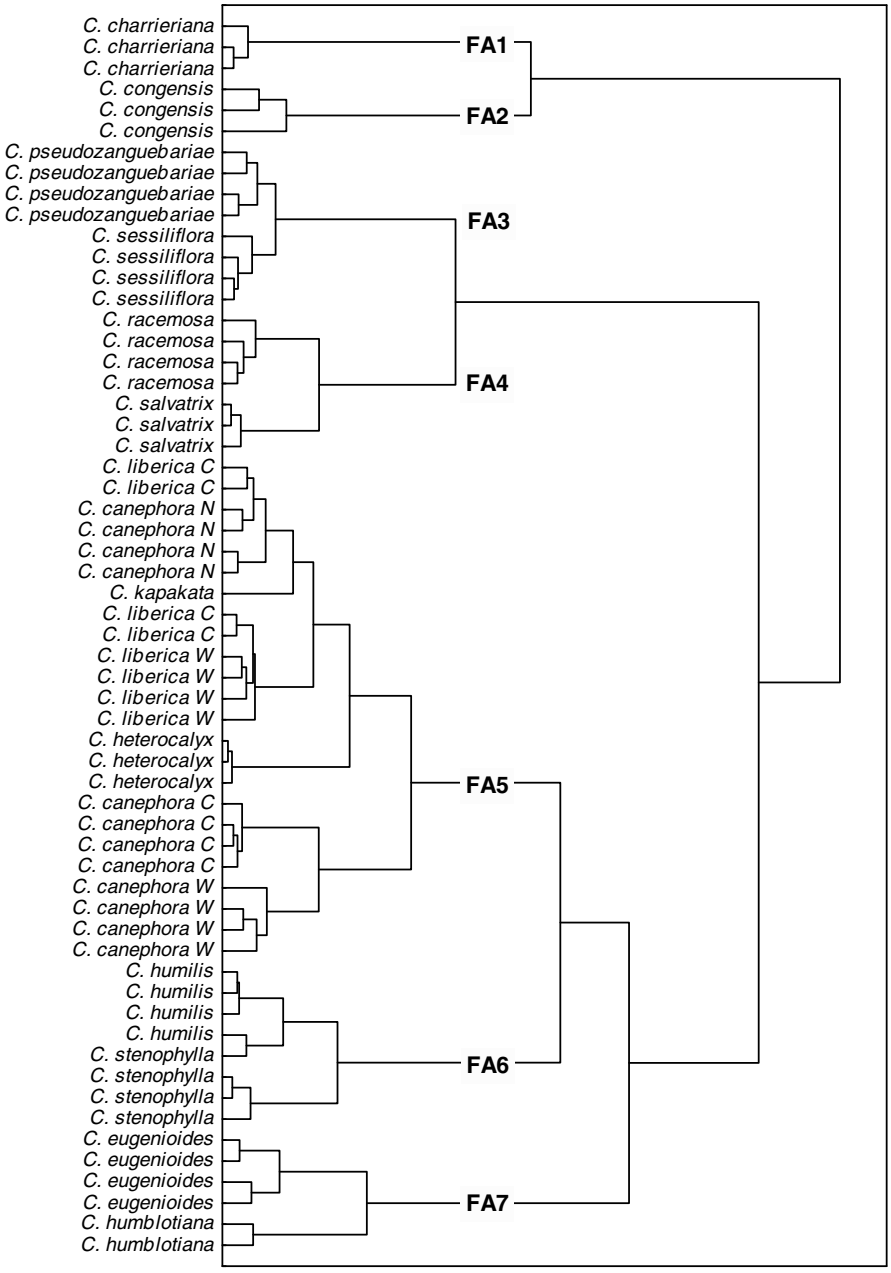

Fig. 4. Hierarchical Clustering Analysis of the seed fatty acid composition of 59 *Coffea* genotypes.

showed high colinearity within three groups of sterols: (i) sitostanol, campestanol and Δ^7 campesterol, (ii) Δ^7 avenasterol, $\Delta^{5,23}$ stigmastadienol and Δ^7 stigmastenol, (iii) campesterol, clerosterol, Δ^5 avenasterol and $\Delta^{5,24}$ stigmastadienol. The PC1–PC2 score plot obtained by the analysis of sterols clearly separated the two species *C. congensis* and *C. humblotiana*, from one another and from the remaining *Coffea* species (Fig. 6A). These two species were already identified above by ANOVA and post-hoc tests for their original sterol composition. As with seed FA, PCA thus appears to be a valuable tool to identify species with clear quantitative biochemical signatures: for instance, the possibility to discriminate *C. congensis* from all other *Coffea* species using its Δ^7 avenasterol, $\Delta^{5,23}$ stigmastadienol and Δ^7 stigmastenol contents. However, it appears that the first two groups of collinear vectors described above were mostly imposed by the original sterol composition of only two species. We thus expected that PC1 and PC2 would not be not effective for the classification of the remaining 15 species/origins. Enlargement of the central part of the PC1–PC2 score plot in Fig. 6B shows that two species, *C. canephora* and *C. racemosa*, were correctly separated from other species by PCA. Nonetheless, no well-supported geographical groups of species were identified on the PC1–PC2 score plot.

2.6. Hierarchical cluster analysis of sterol data

As with FA data, seven groups, termed S1–S7, could be distinguished through the HCA of sterol data (Fig. 7). However, in contrast to the FA phenogram, all genotypes within a species were not systematically grouped together: four of the 50 genotypes studied were not located in the correct cluster according to the species criterion (one genotype of *C. pseudozanguebariae*, one genotype of *C. humilis* and two genotypes of *C. liberica*). Moreover, the remarkable geographical coherence of FA-based clusters could not be recovered using sterols. For instance, the four species from

Table 3
Correspondence between groups of species obtained through Hierarchical Clustering Analysis of seed FA data and clades inferred from DNA sequences.

Chemotaxonomy (groups) based on FA composition	Phylogeny (clades) based on nuclear and chloroplastic DNA sequences (Cros et al., 1998; Maurin et al., 2007)			
	Upper Guinea	Lower Guinea/Congolian	East Africa	East-Central Africa
FA6	*C. humilis* *C. stenophylla*			
FA5		*C. canephora* *C. heterocalyx* *C. kapakata* *C. liberica* var. *liberica* *C. liberica* var. *dewevrei*		
FA2		*C. congensis*		
FA3–FA4			*C. pseudozanguebariae* *C. racemosa* *C. salvatrix* *C. sessiliflora*	
FA7				*C. eugenioides*

Table 4
Sterol composition of seeds. Means followed by the same letter were not significantly different at $P = 0.05$ according to the Newman and Keuls test. N = number of genotypes studied per species or origin for *C. canephora* and *C. liberica*. F and P: results of one-way ANOVAs. COL = cholesterol, CPR = campesterol, CPN = campestanol, STR = stigmasterol, Δ^7CPR = Δ^7 campesterol, $\Delta^{5,23}$S = $\Delta^{5,23}$ stigmastadienol, CLE = clerosterol, β-SIT = β-sitosterol, SIN = sitostanol, $\Delta^{5,24}$S = $\Delta^{5,24}$ stigmastadienol, Δ^7S = Δ^7 stigmastenol, Δ^5A = Δ^5 avenasterol, Δ^7A = Δ^7 avenasterol.

Species/origin	*N*	COL	CPR	CPN	STR	Δ^7CPR	$\Delta^{5,23}$S	CLE	β-SIT	SIN	$\Delta^{5,24}$S	Δ^7S	Δ^5A	Δ^7A
C. canephora Central (C)	4	0.2^{e}	19.2^{ab}	0.5^{d}	17.4^{de}	0.3^{de}	0.2^{b}	1.0^{a}	44.7^{bcd}	0.4^{c}	0.9^{a}	0.1^{c}	14.8^{a}	0.2^{cd}
C. canephora Nana (N)	3	0.2^{de}	20.1^{ab}	0.5^{d}	18.0^{de}	0.3^{de}	0.2^{b}	0.9^{abc}	43.0^{bcd}	0.5^{c}	0.7^{ab}	0.1^{c}	15.2^{a}	0.2^{cd}
C. canephora West (W)	1	0.2	19.1	0.6	15.1	0.2	0.2	1.0	49.5	1.3	0.9	0.4	11.5	0.2
C. charrieriana	3	0.3^{cde}	20.7^{ab}	0.5^{d}	26.5^{bc}	0.3^{de}	0.3^{b}	0.7^{c}	46.4^{abcd}	0.6^{c}	0.2^{ab}	0.2^{c}	2.9^{de}	0.3^{cd}
C. congensis	3	0.2^{de}	17.6^{abc}	1.1^{b}	19.2^{d}	0.8^{b}	1.4^{a}	0.8^{bc}	44.0^{bcd}	2.5^{abc}	0.4^{ab}	3.5^{a}	6.4^{bcd}	2.4^{a}
C. eugenioides	4	0.4^{cde}	13.5^{cd}	1.0^{bc}	26.2^{bc}	0.7^{bc}	0.3^{b}	0.5^{d}	50.4^{abc}	3.3^{ab}	0.3^{ab}	0.4^{c}	2.8^{de}	0.3^{cd}
C. heterocalyx	2	0.3^{cde}	15.9^{abc}	1.1^{b}	28.1^{b}	0.8^{b}	0.4^{b}	0.9^{ab}	39.9^{cd}	3.1^{ab}	0.7^{ab}	0.4^{c}	7.6^{bc}	0.8^{b}
C. humblotiana	2	0.7^{ab}	16.0^{abc}	1.9^{a}	36.6^{a}	1.5^{a}	0.3^{b}	0.4^{d}	36.7^{d}	3.3^{ab}	0.1^{ab}	1.1^{bc}	1.2^{e}	0.2^{cd}
C. humilis	3	0.3^{cde}	17.4^{abc}	1.0^{bc}	17.4^{de}	0.5^{bcd}	0.3^{b}	0.9^{abc}	49.8^{abc}	2.6^{abc}	0.7^{ab}	0.2^{c}	8.8^{bc}	0.2^{cd}
C. kapakata	1	0.3	18.5	0.4	22.6	0.3	0.2	1.0	48.2	0.6	0.3	0.1	7.4	0.2
C. liberica var. *dewevrei* (C)	4	0.3^{cde}	18.0^{abc}	0.7^{cd}	17.6^{de}	0.3^{cde}	0.2^{b}	0.8^{abc}	51.2^{abc}	1.3^{bc}	0.6^{ab}	0.1^{c}	8.8^{bc}	0.1^{d}
C. liberica var. *liberica* (W)	4	0.2^{e}	16.4^{abc}	0.7^{cd}	12.3^{e}	0.3^{de}	0.2^{b}	0.7^{bc}	57.0^{a}	1.1^{bc}	0.7^{ab}	0.4^{c}	9.5^{b}	0.6^{bc}
C. pseudozanguebariae	3	0.5^{bcd}	22.3^{a}	0.4^{d}	20.8^{cd}	0.1^{e}	0.2^{b}	0.8^{bc}	47.0^{abcd}	0.4^{c}	0.4^{ab}	0.2^{c}	6.8^{bcd}	0.1^{d}
C. racemosa	3	0.4^{cde}	20.1^{ab}	0.7^{cd}	17.5^{de}	0.4^{cde}	0.3^{b}	0.8^{abc}	40.7^{cd}	1.5^{bc}	1.0^{a}	0.5^{c}	15.2^{a}	0.9^{b}
C. salvatrix	3	0.3^{cde}	17.8^{abc}	0.4^{d}	17.7^{de}	0.2^{de}	0.2^{b}	0.8^{abc}	53.4^{ab}	0.4^{c}	0.3^{ab}	0.1^{c}	8.1^{bc}	0.1^{d}
C. sessiliflora	3	0.7^{a}	17.3^{abc}	1.1^{b}	22.4^{bcd}	0.6^{bcd}	0.3^{b}	0.8^{abc}	45.1^{bcd}	4.2^{a}	0.6^{ab}	0.3^{c}	6.4^{bcd}	0.4^{cd}
C. stenophylla	4	0.5^{abc}	11.7^{d}	0.5^{d}	21.4^{cd}	0.6^{bcd}	0.2^{b}	0.8^{abc}	56.4^{a}	1.4^{bc}	0.4^{ab}	1.5^{b}	3.8^{cd}	0.7^{b}
F		7.90	9.07	15.02	11.56	13.33	10.17	9.37	6.12	5.23	2.82	19.79	16.36	37.96
P		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

East Africa were split into the groups S1, S2 and S3. The two species occurring only in West Africa, *C. humilis* and *C. stenophylla*, were also separated into two different groups, S3 and S6, respectively. *C. canephora* genotypes, which formed an independent cluster (S4), were not grouped with *C. liberica* individuals. Because of these discrepancies, sterols did not prove to be particularly useful for a better understanding of the taxonomic relationships within the genus *Coffea*. Consequently, the classification of African *Coffea* as inferred from seed sterol composition was not compared with the phylogeny of this genus based on nuclear and plastid DNA data.

2.7. Comparison of the effectiveness of fatty acids and sterols for Coffea chemotaxonomy

The reasons for the higher value of seed FA for *Coffea* chemotaxonomy in comparison with sterols remain to be investigated. However, differences in the metabolism of storage lipids and sterols may partly explain this result. FA are accumulated in significant amounts (10–35% dry weight) in *Coffea* seeds in the form of triacylglycerols, which are synthesized over a long period during seed development and, once assembled, are deposited in specialized organelles termed oil bodies, lowering the possible impact of rapid environmental variations on the final FA composition. By contrast, sterols represent only 0.05–0.16% of the seed dry weight (data not shown) and are restricted to membranes. Considering the numerous physiological and biophysical roles played by sterols in membranes (Hartmann, 1998; Lindsey et al., 2003), one can assume that seed sterol composition is significantly influenced by micro-environmental conditions and can undergo rapid changes. This would partly explain the non-negligible variation observed between genotypes of the same species in their final seed sterol composition and the limited usefulness of this chemical class for the chemotaxonomy of the genus *Coffea*.

The apparent high value of seed FA for the chemotaxonomy of *Coffea* should however be considered with caution. Indeed, in various oil crops such as rape, sunflower and soybean, numerous studies demonstrated the existence of significant environmental effects on the FA composition of seeds (e.g. Baux et al., 2008; Cherry et al., 1985; Hou et al., 2006; Izquierdo et al., 2002; Oliva et al., 2006; Richards et al., 2008). Among them, year and location are factors that are frequently cited for their impact on seed FA composition. Variations in fatty acid composition are mostly due to differences in temperature and precipitation from year to year and from location to location. Therefore, one cannot ascertain that a similar congruence with previous phylogenetic studies would have be found if the coffee species studied have been grown in another environment or analyzed a different year.

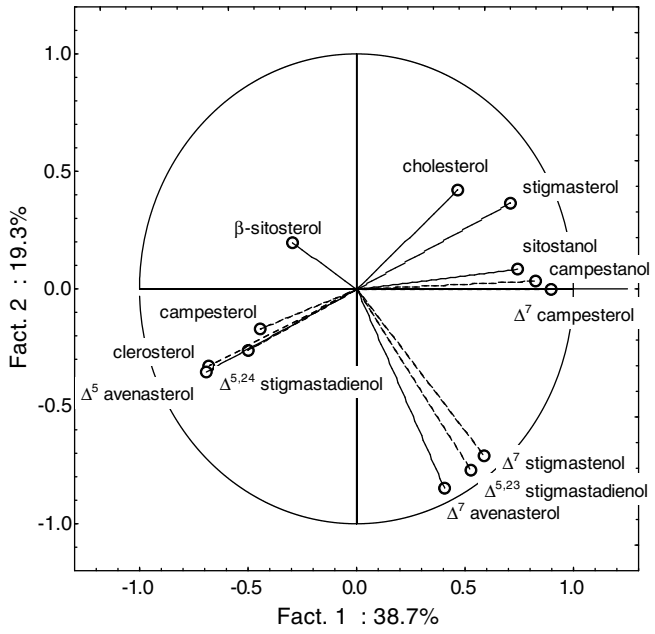

Fig. 5. Principal Component Analysis of the seed sterol composition of 50 *Coffea* genotypes: correlations (factor loadings) of seed sterols with the first two factors (principal components).

Among possible undesirable effects, the genotype × environment interaction is the most unfavourable one regarding the chemotaxonomic usefulness of seed FA since it is expected to alter considerably genotype classification. In a recent study based on an experimental design involving various locations in Colombia and several *C. arabica* genotypes, it has been shown that the genotype × environment interaction had a very low impact on seed FA composition (Bertrand et al., 2008). This finding thus constitutes an encouraging result towards the effectiveness of seed FA for *Coffea* chemotaxonomy.

Besides, phylogenetic *Coffea* groups are remarkably consistent with geographical regions, which also correspond to distinct ecosystems. One could therefore logically consider that the value of seed FA for the chemotaxonomy of *Coffea* species is to a large extent due to the adaptative feature of these compounds. However, to our knowledge, no hypothesis has yet been proposed regarding the adaptive role of storage lipids (as opposed to membrane lipids). We rather suggest that the FA composition of total lipids reflects genetic drift during speciation. This hypothesis is supported by the observation that two species of the same clade/FA group may be adapted to contrasting ecological niches. For instance, the two species in group FA6 (Upper Guinea clade), *C. humilis* and *C. stenophylla*, are found in very different microhabitats. Berthaud (1986) emphasized that trees of *C. humilis* grow only on thalwegs (valley floors) or on the banks of back-waters and pools, which are the most humid micro-areas of the rainforest in Ivory Coast, while *C. stenophylla* was found only in the drier areas of semi-deciduous forests of Ivory Coast, such as on the top of the hills. Despite these differences, *C. stenophylla* and *C. humilis* have similar seed FA composition. The hypothesis that differences in seed FA composition between clades are mostly governed by random evolutionary processes is also substantiated by the observation that these differences are quantitatively rather limited, challenging the hypothesis of selection through adaptative success. For example, it is difficult to believe that a higher stearic acid content of only a few percent in species in East Africa would increase fitness for conditions found in woody savannah.

2.8. Conclusions

The present work establishes the very high value of seed FA for the chemotaxonomy of African *Coffea* species. Groups obtained through HCA of FA data exhibited remarkable eco-geographical coherence and were congruent with previous phylogenetic studies. None of the other compounds tested so far, sterols (present work), chlorogenic acids and purine alkaloids (Clifford et al., 1989; Anthony et al., 1993), and diterpenoids (de Roos et al., 1997) revealed such congruence. Our results also suggest extending the present approach to other *Coffea* species, originating from an increased variety of ecosystems, including those of Madagascar. They finally indicate that the genus *Coffea* could provide a valuable model to investigate the reasons for the differences observed among different biochemical families with respect to their chemotaxonomic value.

3. Experimental

3.1. Plant material

Fresh mature seeds of the *Coffea* species studied (Table 1) were provided from the field collections of the *Centre National de Recherche Agronomique*, Ivory Coast, which is certainly the richest collections of wild African *Coffea* species in the world. This germplasm was collected from the wild during several survey missions organized by IRD in Ivory Coast, Cameroon, Central African Republic, Guinea, Kenya, Republic of the Congo, and Tanzania (Fig. 1), with the exception of *C. heterocalyx*, *C. racemosa* and *C. salvatrix* materials which were donated to CNRA from other collections (Anthony et al., 2007). For the two species with wide natural distribution, *C. canephora* and *C. liberica*, three and two infraspecific groups, respectively, were built on the basis of geographical origin. For *C. liberica*, the two groups also coincided with distinctive taxonomic varieties: *C. liberica* var. *liberica* for the material originating from Ivory Coast, *C. liberica* var. *dewevrei* for that collected in Central African Republic. For each species/group studied, one to four distinct wild genotypes were randomly chosen and sampled separately in the fields. For each genotype studied, seeds were freeze-dried upon receipt at Montpellier and placed over silica-gel in a hermetic plastic box and stored at −80 °C until use. Prior to lipid extraction, seeds were reduced to a fine powder using an analytical grinder (IKA© A15, Germany). Powders were placed over silica-gel in a hermetic plastic box and stored at 4 °C.

3.2. Lipid extraction

Total lipids were extracted from 2 g samples of dried powder using a modified Folch method (Folch et al., 1957) with methylene chloride replacing chloroform. Roughly, the bean material was homogenized for 30 s in 10 ml of methylene chloride/methanol (2/1) using an IKA (Staufen, Germany) T25 Ultra-turrax prior to filtration onto a glass filter cup (pore size 4). The residue was extracted again with 10 ml of methylene chloride/methanol (2/1) in the same conditions. The filtrate was transferred in a 60 ml glass separatory funnel and washed with 4 ml of 0.73% NaCl solution by vigorous shaking by hand. After the resulting mixture separated into two phases, the lower phase was recovered. All steps were performed at room temperature. Extracted lipids were quantified gravimetrically after evaporation to dryness under nitrogen at 40 °C. The dry residue was then dissolved in 1 ml of methylene chloride/methanol (2/1) and stored at -20 °C until further analysis.

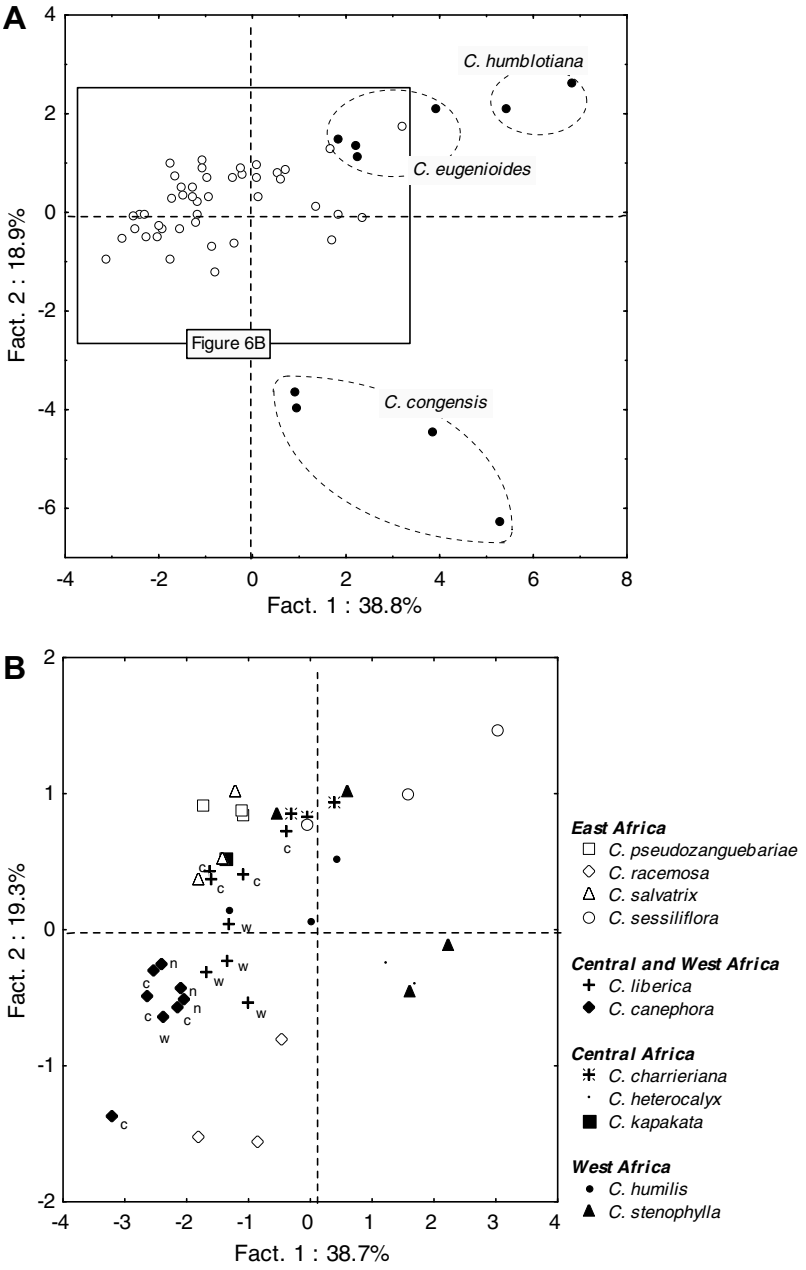

Fig. 6. (A) Principal Component Analysis of seed sterol composition: PC1–PC2 score plot of the 50 genotypes of *Coffea* studied. (B) Enlargement of the central area of the PC1–PC2 score plot.

3.3. Fatty acid analysis

The FA composition of total lipids was determined as described in Laffargue et al. (2007). FA methyl esters (FAME) were prepared according to the ISO-5509 standard. Lipid extracts were first saponified with 4 ml of a 0.5 M methanolic solution of sodium hydroxyde at 90 °C for 10 min and then methylated with 5 ml of 14% BF_3 methanolic solution at 90 °C for 3 min. GC analyses were performed using an HP 6890 system with flame ionisation detection (FID). A Famewax capillary column (RESTEK, France), 30 m × 0.25 mm × 0.25 μm was used. The analyses were carried out in program temperature mode from 185 °C to 225 °C at 4 °C/min and then in the isothermal mode for 10 min at 225 °C. Helium was used as carrier gas at 40 cm s^{-1}. Both injector and detector were at 230 °C. FAMEs were identified by comparing their retention times with those of the FAME standards (Supelco) and were quantified as percentages of total FA (w/w). For each genotype, the FA composition was analyzed in triplicate, i.e. from three different lipid extracts.

3.4. Sterol analysis

Total sterols were first purified by successive saponification of total lipids and thin-layer chromatography (TLC) of the unsaponifiable lipids. Briefly, 50 mg of lipids combined with 0.2 mg of dihydrocholesterol (internal standard) were dissolved in 1.5 ml of 2 M solution of potassium hydroxide in ethanol–water (100:5, v/v) and heated in a 90 °C water-bath with constant stirring for 60 min. After cooling, 3 ml of water and 3 ml of diethyl ether were added. The mixture was vigorously mixed and after it separated into two phases, the upper phase was recovered. Extraction was repeated two times by adding 3 ml of diethyl ether, gently shaken by hand,

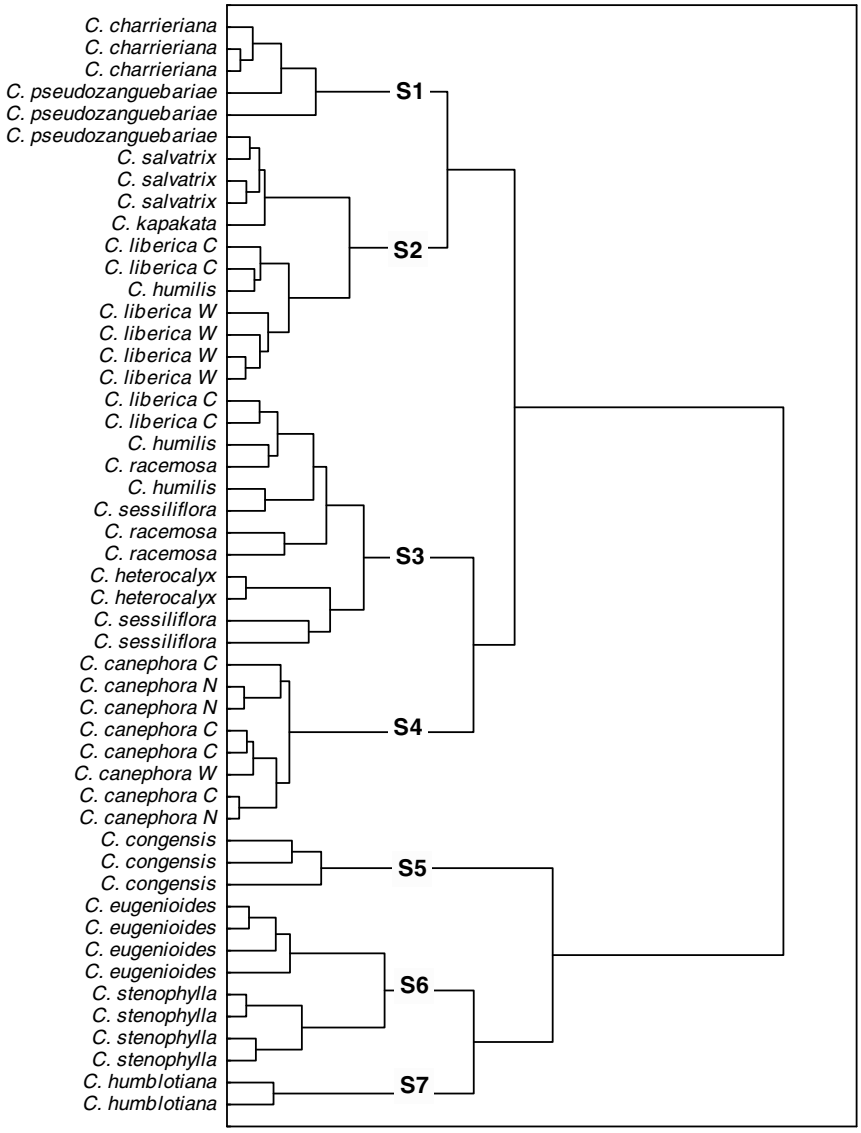

Fig. 7. Hierarchical Clustering Analysis of the seed sterol composition of 50 *Coffea* genotypes.

and after separation of the two phases, the upper phase was recovered. The three upper phases were combined, dried under a nitrogen stream and the resulting unsaponifiable fraction was dissolved in 300 µl diethyl ether and dried using anhydrous sodium sulphate. The unsaponifiable fraction was spotted on pre-coated TLC plates (silica G 60, 20 × 20 cm, 0.25 mm thickness) from Merck (Darmstadt, Germany). Unsaponifiable lipids were separated using the developing solvent system hexane/diethyl ether (1/1, v/v). A 0.1% 2′,7′ dichlorofluorescein ethanolic solution was sprayed on the plates and the sterol band was identified under UV (365 nm) by comparing the retention factors (Rf) with that of dihydrocholesterol, scraped off the plate and suspended in 4 ml of diethyl ether. The mixture was heated for 15 min at 60 °C and filtered through a filter paper. The residue was extracted again two times and the filtrates were combined prior to evaporation to dryness under nitrogen at 40 °C. The dry residue was then dissolved in 15 µl of methylene chloride. GC analyses were then performed using a SAC-5 capillary column (Supelco, France), 30 m × 0.25 mm × 0.25 µm. The analyses were carried out at 285 °C using helium as carrier gas at 33 cm s^{-1}. Both injector and detector were at 300 °C. Sterols were identified by comparing their retention times with those of standards (Supelco) and were quantified as percentages of total sterols (w/w). For each genotype, the sterol composition was analyzed in triplicate, i.e. from three different lipid extracts.

3.5. Statistics

Principal Component Analysis and Hierarchical Clustering Analysis (Ward's pair-wise grouping method, Euclidian distance) were carried out using Statistica (©Statsoft, Tulsa, USA). Data were standardized prior to performing HCA.

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