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Diversity in bean Caffeine content among wild *Coffea* species: Evidence of a discontinuous distribution

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Abstract

Caffeine is a metabolite of great economic importance, especially in coffee. Previous evaluations have already focussed on wild species of coffee trees, but this assessment included six new taxa from Cameroon and Congo and involved a simplified method that generated more accurate results. Two main results were obtained: (1) Cameroon and Congo were found to be a centre of diversity, encompassing the entire range of caffeine content; (2) four groups of coffee tree species – CAF1, CAF2, CAF3 and CAF4 – were established on the basis of discontinuities in the caffeine content range. The trace levels of caffeine in CAF1 is due to an absence of accumulation in beans – a factor that is controlled by a major gene. The other classes, i.e., CAF2, CAF3 and CAF4, were characterized by the extents of their caffeine accumulation. Caffeine content was found to increase twofold from CAF2 (0.55% dmb) to CAF3 (1.1% dmb) and from CAF3 to CAF4 (2.3% dmb). This discontinuous distribution is discussed from an evolutionary standpoint.

Keywords: *Coffeae*; Caffeine; Diversity

1. Introduction

Caffeine is a purine alkaloid, i.e. 1,3,7-trimethylxanthine, derived from xanthosine. This is a secondary metabolite of great interest in coffee because of its impact on beverage quality (its accumulation in green coffee beans increases the bitterness of the drink) and affects health (insomnia). This explains current research initiatives aimed at identifying genes involved in its biosynthesis (Mizuno et al., 2003; Ogawa, Herai, Koizumi, Kusano, & Sano, 2001; Uefuji, Ogita, Yamaguchi, Koizumi, & Sano, 2003) or obtaining genetically modified varieties with lower caffeine contents (Ogita, Uefuji, Yamaguchi, Koizumi, & Sano, 2003)

The *Coffea* genus (Rubiaceae family) includes two subgenera, *Baracoffea* and *Coffea*. Coffee trees *sensus stricto* belong to the sub-genus *Coffea*. Wild species are endemic to intertropical forest zones in Africa, Madagascar, Mauritius, Comoros and Réunion (Anthony, 1992; Bridson & Vercourt, 1988; Charrier, 1978; Chevallier, 1947; Lebrun, 1941; Stoffelen, 1998). Systematics experts have described over 80 species, including two cultivated species, namely *C. arabica* L. and *C. canephora* Pierre. All taxa but *C. arabica* ($2n = 4x = 44$) are diploid with the same chromosome number ($2n = 2x = 22$).

In green coffee beans, caffeine content (CAF) varies markedly between species and within species. Species averages range from 0% dry matter basis (dmb) in *C. pseudozanguebariae* to 2.5% dmb in *C. canephora* (Anthony, Noirot, & Clifford, 1993; Charrier & Berthaud, 1975; Ky et al., 2001; Mazaffera, Silvarolla, Alves de Lima, & Medina Filho, 1997). Within *C. canephora*,

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CAF ranges from 1.5% to 3.3% dmb (Ky et al., 2001). Most of the variation (about 94%) is under genetic control (Barre et al., 1998; Charrier & Berthaud, 1975; Montagnon, Guyot, Cilas, & Leroy, 1998).

In cultivated species, CAF results from an accumulation process in green beans, which depends on the fructification time (FT), i.e., the period from flowering to ripening, and the daily amount of caffeine accumulated (Akaffou et al., 2003). In this paper, we present a biochemical evaluation of caffeine content diversity in wild *Coffea* genetic resources. The main incentives behind this study were: (1) to add six undescribed taxa from central Africa, thus increasing the number of evaluated diploid species to 21, and (2) to document the discontinuous distribution with respect to CAF. The implications for further investigations are discussed.

2. Materials and methods

2.1. Plant material

Table 1 gives the geographical origin of the coffee tree species and taxa. Each species or taxon was represented by four accessions. The 15 diploid species and the six undescribed taxa are field-maintained at the IRD coffee breeding station (Man, Côte d'Ivoire).

2.2. Sample preparation

Coffee cherries were harvested at full maturity and depulped using the wet processing method. After desiccation on silicagel, 50 green beans per tree were frozen in liquid nitrogen before crushing in a ball mill (Dangoumill) for 2 min. The fine powder was split into six samples; three were used to estimate water content and the other three underwent further analysis.

Table 1
Geographical origin of the species and taxa

Species and taxa	Geographical origin
C. brevipes	Cameroon
C. canephora	Côte-d'Ivoire
C. congensis	Congo Democratic Republic
C. eugenioides	Kenya
C. heterocalyx	Cameroon
C. humblotiana	Comores
C. humilis	Côte-d'Ivoire
C. kapakata	Angola
C. liberica dewevrei	Central African Republic
C. liberica Koto	Cameroon
C. liberica liberica	Côte-d'Ivoire
C. pseudozanguebariae	Kenya
C. racemosa	Tanzania
C. salvatrix	Tanzania
C. pocsii	Tanzania
C. stenophylla	Côte-d'Ivoire
Coffea sp. Bakossi	Cameroon
Coffea sp. Congo	Congo Democratic Republic
Coffea sp. Ngongo 2	Congo Democratic Republic
Coffea sp. Moloundou	Congo Democratic Republic
Coffea sp. N'koumbala	Cameroon

2.3. Extraction, purification and analytical HPLC

Caffeine extraction was performed as previously described by Barre et al. (1998). Analyses were carried out on a HPLC system (Waters) consisting of a 250 mm × 4 mm Merck LiChrospher 100 RP-18 column (5 μm particle size), a C_{18} guard column and a photodiode-array detector (Waters 996). The elution system (1 ml min^{-1}) consisted of two solvents that were filtered (0.2 μm pore size filter), degassed and sonicated (Ney, 300 Ultrasonik): solvent A (10 mM acetic acid/triethylamine 1000/1, pH 5.3) and solvent B (methanol). The gradient applied was: 0–15 min, 65% solvent A, isocratic; 15–20 min, 100% solvent B, linear; 20–26 min, 100% solvent B, isocratic; 26–30 min, 65% solvent A, linear. Identification and quantification were performed at room temperature (10 μl of sample) using a reference standard (Sigma Chemical Co.) at 273 nm. The calibration curve was plotted using three replicate points for a caffeine solution at 5, 10, 25 and 50 mg l^{-1}.

The processing order was fully randomised. Every 10 samples, a control was used to check the measurement stability. Caffeine content was expressed as a percentage on dry matter basis (% dmb).

2.4. Statistical analysis

All results were analysed using the Statistica software package (5.1 version, 1997 for Microsoft Windows).

Each tree was represented by its mean caffeine content. The statistical analysis only concerned between-species variations which were tested using a one-way ANOVA. A Newman and Keul's test was carried out for multiple mean comparisons.

3. Results and discussion

3.1. General

For the 21 species analysed, caffeine content in green beans ranged from trace amounts (<0.01%), in *C. pseudozanguebariae*, *C. humblotiana* and *Coffea* sp. Bakossi, to 2.64% in *C. canephora* (Table 2).

Until now, *Coffea* species have been categorised into two groups according to their green bean caffeine content, with one grouping caffeine-free species, including most Mascarocoffea and some Mozambicoffea, and the other pooling species with caffeine in their green beans (Anthony et al., 1993; Bertrand, 1901; Charrier & Berthaud, 1975; Clifford, Williams, & Bridson, 1989; Mazaf-

Table 2
Caffeine content between and within species (the within-species range is in brackets)

Species and taxa	Mean	Range
C. brevipes	2.54	2.36–2.96
C. canephora	2.64	1.51–3.33
C. congensis	1.47	1.08–1.83
C. eugenioides	0.51	0.44–0.60
C. heterocalyx	0.92	0.86–0.99
C. humblotiana	0.00	0.00–0.01
C. humilis	1.93	1.67–2.27
C. kapakata	1.20	1.04–1.39
C. liberica dewevrei	0.94	0.81–1.10
C. liberica Koto	1.31	0.91–1.70
C. liberica liberica	1.24	1.12–1.39
C. pseudozanguebariae	0.00	0.00–0.00
C. racemosa	1.06	0.86–1.25
C. salvatrix	0.03	0.01–0.06
C. pocsii	1.27	1.04–1.71
C. stenophylla	2.27	2.05–2.43
Coffea sp. Bakossi	0.00	0.00–0.03
Coffea sp. Congo	2.27	2.11–2.37
Coffea sp. Ngongo 2	2.12	1.90–2.32
Coffea sp. Moloundou	0.58	0.52–0.61
Coffea sp. N'koumbala	2.36	1.89–2.89

Contents are expressed in percent dry matter basis (% dmb).

fera et al., 1997; D'Ornano, Chassevent, & Pougneaud, 1968). Here, trace amounts of caffeine (<0.01%) were detected in green beans of species previously classified as caffeine-free (*C. pseudozanguebariae*, *C. humblotiana*). This detection was certainly due to the simplified extraction method used (Barre et al., 1998), which limits yield loss and permits detection of lower caffeine concentrations. The notion of caffeine-free species, along with the two-group species classification, should thus now be reconsidered.

3.2. Species classification according to caffeine content

As the variance was related to the mean, the data were transformed before ANOVA, using $y = \log(x + 0.03)$. Significant between species differences were noted ($F_{20,61} = 327$; $p < 0.001$), representing 94.3% of the total variance.

Four species classes were distinguished (Fig. 1). The first class (CAF1) grouped four species with trace amounts of caffeine (<0.03% dmb). The second class (CAF2) included two species with low caffeine contents (0.51–0.58% dmb). The third class (CAF3) consisted of eight species with a caffeine content ranging from 0.92% to 1.47% dmb, whereas the last class (CAF4) contained seven species with high caffeine contents (>1.93% dmb).

Evidence of four caffeine classes is reported for the first time. The first class (CAF1) corresponds to the group of caffeine-free species described previously. Classes CAF2, CAF3 and CAF4 include species whose caffeine content was formerly considered as a quantitative trait, with a continuous distribution ranging from

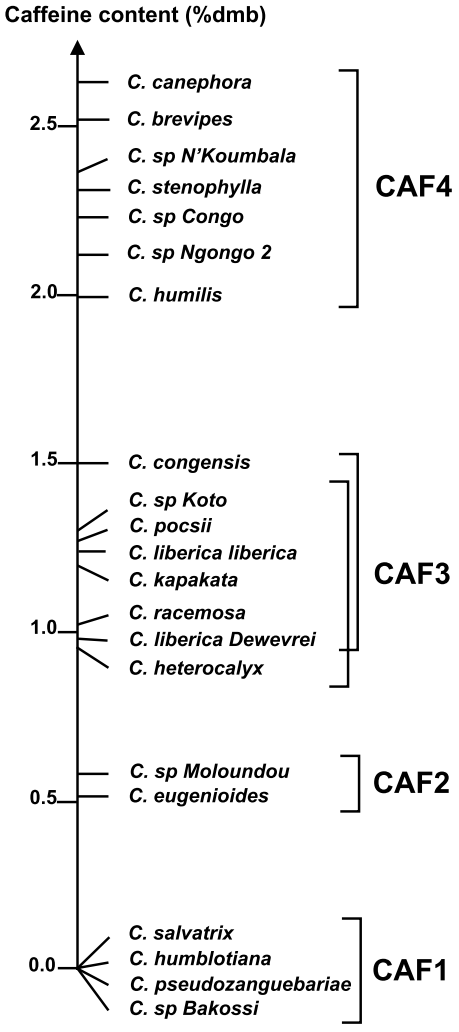

Fig. 1. *Coffea* clusters for caffeine content (expressed in % dmb). Brackets indicate Newmann and Keuls test results.

0.5% dmb in *C. eugenioides* to 2.4% dmb in *C. canephora*.

Detection of this new qualitative distribution could be due to two factors: first is, the increased number of species and the introduction of new undescribed African taxa in the analysis, thus giving rise to this new grouping. For example, the presence of *Coffea* sp. Moloundou, which showed a caffeine content similar to *C. eugenioides*, led to the formation of the CAF2 class. Similarly, *Coffea* sp. N'gongo2, *Coffea* sp. Congo, *Coffea* sp. N'Koumbala gave more weight to the CAF4 group. Secondly, the high contribution of genes in the caffeine content control led to a high discriminating power (between group/within group variance). As only 6% of the variation was explained by environmental effects, the phenotypic value provides a good estimate of the genotypic value, the within-group variance is minimised, and the discontinuity is more obvious.

Two evolutionary hypotheses could explain these discontinuities. The first one supposes the previous

existence of a continuum of species with caffeine contents ranging from trace amounts to 2.5% or more. This continuum would have been affected by a strong genetic bottleneck leading, after foundation, to only four species, each of them giving rise to CAF1, CAF2, CAF3 and CAF4, respectively. The second hypothesis is that discontinuities are due to substantial effects of major genes, e.g. the Mendelian gene controlling caffeine accumulation in beans (Barre et al., 1998), thus separating CAF1 from other classes.

3.3. Saltational evolution of caffeine content between groups

Except for CAF1 which was characterised by only trace quantities of caffeine, other groups showed saltational evolution in their caffeine content: 0.54% in CAF2, 1.07% in CAF3 and 2.31% in CAF4. Indeed, the shift from CAF2 to CAF3, or from CAF3 to CAF4, resulted in a twofold increase in CAF. The bottleneck hypothesis did not explain this kind of evolution. As the effects of genes controlling CAF are known to be multiplicative (Barre et al., 1998), as confirmed here by the relationship between variance and mean, this phenomenon could explain the doubling of CAF.

Genes frequently have multiplicative effects when the trait is the product of two independent processes. In the case of caffeine, green bean content is the result of an accumulation process over time (Clifford & Kazi, 1987). These results were confirmed in hybrids obtained by backcrossing *C. pseudozanguebariae* (PSE), with short fructification time (FT = 70 days), and *C. liberica* subsp. *Dewevrei*, with long FT (300 days). The shorter the fructification time the lower was the caffeine content (Akaffou et al., 2003). Caffeine content can be decomposed into the two following multiplicative components: the daily accumulation of caffeine (CAF/FT) and the fructification time. A quantitative trait locus was identified for both components (Akaffou et al., 2003; Ky, 2000). For example, in *C. canephora* and *C. liberica* subsp *liberica*, two species with similar fructification times (about 300 days), CAF/FT was estimated to be 0.009% d^{-1} and 0.004% d^{-1}, respectively. The fact that *C. canephora* belongs to CAF4 and *C. liberica* subsp *liberica* to CAF3 could be explained by CAF/FT. Without accumulation, these species would belong to the CAF1 class. On the other hand, fructification time in CAF1 ranges from 70 days in *C. pseudozanguebariae* (Akaffou et al., 2003) to more than 300 days in *C. humblotiana* (Charrier, 1978), indicating that trace caffeine content does not result from short FT.

This saltational evolution pattern with four classes seems to be restricted to beans. Indeed, in adult leaves of *C. salvatrix* (CAF1), *C. eugenioides* (CAF2), *C. racemosa* (CAF3), and *C. liberica* subsp. *dewevrei* (CAF4), caffeine content ranges from 0.021% to 0.073% dmb (Sivarolla, Mazzafera, Alves de Lima, Medina Filho, & Fazuoli, 1999). In leaves, caffeine content is not accumulated as in beans of species belonging to CAF1.

3.4. Central Africa as a centre of diversity for caffeine content

In East Africa, caffeine content (CAF) in beans ranges from trace amounts in *C. pseudozanguebariae* and *C. salvatrix* to 1.3% in *C. pocsii*. In West Africa, CAF ranges from 1.25% in *C. liberica* subsp. *liberica* to 2.6% in *C. canephora*. By contrast, in Cameroon and Congo, CAF ranges from trace amounts in *Coffea* sp. Bakossi to 2.6% in *C. canephora* and seems to cover the whole variation range. Even if *C. canephora* and *C. brevipes* are overlooked (both being present in West and Central African countries), the range in Central Africa is not really modified (trace contents in *Coffea* sp. Bakossi to 2.4% in *Coffea* sp. N'Koumbala). This broader range is due to the analysis of new taxa specific to this geographical zone (*Coffea* sp. Bakossi in CAF1, *Coffea* sp. Moloundou in CAF2, *Coffea* sp. Koto in CAF3, *Coffea* sp. N'gongo2, *Coffea* sp. Congo, and *Coffea* sp. Nkoumbala in CAF4). This clearly shows that Central Africa is a centre of diversity for caffeine content.

4. Conclusions and prospects

To summarize, genetic control is first involved to accumulate caffeine in green beans, and then, second, to determine the extent of caffeine accumulation.

Between-species variations within classes, and between-tree variations within species could result from the accumulation of mutations with minor effects. Conversely, these evolutionary processes cannot explain the caffeine content differences between CAF2 and CAF3 or between CAF3 and CAF4. Genes with major effects seem to be involved in the latter cases. Consequently, the two processes, i.e. the accumulation of small mutations over time and the saltational mode of evolution, seem to apply in the *Coffea* sub-genus with respect to caffeine content.

A current major research focus is to identify and characterise genes involved in between-class differentiation and in caffeine accumulation. Three genes involved in the caffeine pathway were recently identified in *C. arabica* (Mizuno et al., 2003; Ogawa et al., 2001; Uefuji et al., 2003). Their expression in leaves and fruits of species belonging to the four caffeine content classes, especially in species such as *C. pseudozanguebariae*, has to be quantified to understand whether such genes are crucial for caffeine accumulation in beans. From a practical standpoint, the decrease in caffeine content observed in leaves of genetically modified *C. arabica*, by an antisense strategy, indicates the involvement of the caffeine

synthase gene in caffeine synthesis (Ogita et al., 2003), but gives no indication concerning the level of caffeine accumulation in green beans.

Caffeine is an alkaloid known to pass through cell membranes (Pfrunder, Wanner, Frischknecht, & Baumann, 1980). Experiments on callus or protoplast cultures, as well as germination tests, have clearly shown that caffeine is released into the external medium, leading to similar contents within plant cells and medium (Mösli-Waldhauser & Baumann, 1996). In plants, as observed for *C. arabica* seedlings, caffeine content depends on the organ and growth stage, (Aerts & Baumann, 1994). It also differs between leaves and seeds in different diploid species (Sivarolla et al., 1999). Within-bean accumulation has been explained by the formation, inside the vacuole, of a caffeine and chlorogenic acid complex (Baumann & Röhrig, 1989), i.e., caffeine chlorogenate (Horman & Viani, 1972; Kappeler, Baumann, & Greutert, 1987; Payen, 1846; Sondheimer, Covitz, & Marquisee, 1961). Consequently, the relationship between caffeine and chlorogenic acid contents also should be investigated within and between classes, in both leaves and fruits.

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