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Genome Size Variations in Diploid African *Coffea* Species

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Flow cytometry was conducted to evaluate genome size diversity among African diploid species of the *Coffea* genus. The study included 15 species and six new taxa from Congolese and Cameroonian forest regions which have yet to be botanically characterized. Between-population differences were also recorded in some cases. These evaluations using an internal standard were highly correlated with previous results obtained with an external standard, but differences of up to 18 % existed for some species, involving stoichiometric errors. Consequently, genome size variation between species and within species are discussed as true genome size differences or stoichiometric errors. Environmental and phenotypic correlations with genome size are also discussed.

Key words: *Coffea*, genome size, flow cytometry, interspecific diversity, intraspecific diversity.

INTRODUCTION

The *Coffea* genus (Rubiaceae family) includes two subgenera, *Baracoffea* and *Coffea*. Coffee trees *sensu stricto* belong to the subgenus *Coffea.* In the wild, they are endemic to intertropical forest zones in Africa, Madagascar, Mauritius, Comoros and Réunion (Chevallier, 1929, 1938, 1942, 1947; Leroy, 1963; Charrier, 1978; Bridson and Vercourt, 1988; Anthony, 1992; Stoffelen, 1998). Systematists have described over 80 species, including two cultivated species, *C. arabica* L. and *C. canephora* Pierre. All taxa are diploid with the same chromosome number ($2n = 2x = 22$), except *C. arabica* ($2n = 4x = 44$).

A first flow cytometry evaluation of genome size, using propidium iodide (PI) as dye and *Oryza sativa* ssp. *japonica* as external standard, was carried out on 12 diploid species of the subgenus *Coffea* (Cros *et al*., 1995). This first evaluation revealed a broad genome size range, from 0·95 pg in *C. racemosa* Lour., an East African species, up to 1·78 pg in *C. humilis* Chev., a West African species (Cros *et al*., 1995). However, several African *Coffea* species and taxa have not yet been evaluated, in particular new taxa collected in the Congo and Cameroon (Anthony *et al*., 1985; De Namur *et al*., 1987).

Since then, an accurate flow cytometry method has been developed to determine small genome size differences (0·025 pg) in coffee trees (Barre *et al*., 1996). The method differs from the former by: (*a*) the use of an internal standard, *Petunia hybrida* (2·85 pg; Marie and Brown, 1993); (*b*) a statistically defined sample size; and (*c*) a full randomization or Fisher's block experimental design. We used this new method to assess genome size diversity from a more complete set of *Coffea* species. Fifteen African species, some of them including several populations (*C. canephora* Pierre, *C. congensis* Froehner, *C. brevipes* Hiern) or subspecies (*C. liberica* ssp. *liberica* Hiern., *C. l.* ssp. *Dewevrei* De Wild. et Dur.) were studied. Six new taxa from Congolese and Cameroonian forest regions which have yet to be botanically characterized were also evaluated.

Some genome size differences are artefactual due to the presence of cytosolic compounds (Noirot *et al*., 2000, 2002; Price *et al*., 2000). In coffee trees, caffeine increases dye accessibility, while chlorogenic acids (CGA) decrease it (Noirot *et al*., 2003). As caffeine and CGA contents vary between trees, this leads to pseudo-variations in genome size within and between species. This also induces genome size differences when using an internal or external standard. In the first case, leaves of standard and target are chopped together, thus releasing cytosolic compounds of both species in the buffer; while in the second case, leaves are chopped separately and cytosolic compounds of the target cannot act on standard nuclei and vice versa. Such induced differences between internal and external standardization are described within a back-cross progeny (Noirot *et al*., 2003).

Consequently, in the present study, the genome sizes of new undescribed taxa along with those of botanically characterized species were compared with those obtained using external standard (Cros *et al*., 1995). Variations in genome size between species and within species are then discussed while taking the stoichiometric error into account.

MATERIALS AND METHODS

Plant material

Table 1 gives the geographical origin of all species and populations. All trees were maintained in tropical conditions in a glasshouse in Montpellier. Five plants were evaluated for most species or populations. Each plant was represented by four extracts obtained from two leaves. The internal standard was *Petunia hybrida* (2·85 pg; Marie and Brown, 1993).

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TABLE 1. *Geographical origin of species, taxa and populations*

Species, taxa and populations	Geographical origin
C. brevipes	Cameroon (Mt Cameroon)
C. brevipes	Cameroon (Kumba-Loum)
C. canephora	Cameroon
C. canephora	Côte d'Ivoire
C. canephora	Central African Republic
C. canephora	Congo
C. congensis	Cameroon
C. congensis	Central African Republic
C. congensis	Congo
C. costatifructa	Tanzania
C. eugenioides	Kenya
C. heterocalyx	Cameroon
C. humilis	Côte d'Ivoire
C. kapakata	Angola
C. liberica ssp. *liberica*	Côte d'Ivoire
C. liberica ssp. *Dewevrei*	Central African Republic
C. liberica Koto	Cameroon
C. pocsii	Tanzania
C. pseudozanguebariae	Kenya
C. racemosa	Tanzania
C. salvatrix	Tanzania
C. sessiliflora	Kenya
C. stenophylla	Côte d'Ivoire
C. sp. Bakossi	Congo (Bakossi)
C. sp. Congo	Congo
C. sp. Mayombe	Congo
C. sp. Ngongo 2	Congo (Ngongo)
C. sp. Ngongo 3	Congo (Ngongo)
C. sp. Moloundou	Congo (Souanké)
C. sp. Moloundou	Cameroon (Moloundou)
C. sp. Nkoumbala	Cameroon (Nkoumbala)

Underlined species were evaluated by Cros *et al.* (1995).

Sample preparation and cytometric measurements

Nuclei were extracted by chopping the leaves (Galbraith *et al*., 1983) in a slightly modified (0·5 % Triton X-100, pH 9) lysis buffer (LB) described by Dolezel (1989). The addition of mercaptoethanol in the buffer avoided polyphenol oxidation. Leaf samples (4 cm^2) were chopped for about 30 s at constant rate of chopping in a Petri dish containing 2 ml of lysis buffer. The solution was filtered through nylon cloth (50 µm mesh size) and then kept on ice for at least 2 h (this incubation time is very important in *Coffea* species). Several preparations were pooled when a large volume of extract was required. This extraction method and buffer gave high nuclear fluorescence stability, i.e. less than 2 % variation after 6 h incubation.

Nuclei were stained with PI (95–98 % Sigma P 4170). In all experiments, including those involving dilution, a saturating final concentration of 330 µg ml^{-1} (Barre *et al*., 1996) was used.

A FACScan cytometer (Becton Dickinson, Franklin lakes, NJ, USA) with an argon laser (15 mW) at 488 nm and an emission pulse area of 585 nm ± 22 nm was used. FL2 area and FL2 width were measured for 1024 channels. A window was defined for each sample so that only G1 nuclei were monitored. Five hundred nuclei were counted for each measurement. The zero offset of the analogue-to-digital converter was checked with *Petunia* nuclei (Barre *et al*., 1996). There were no gain fluctuations in the amplifier system. The voltage was maintained at a constant high level throughout each experiment. The processing order was fully randomized within each experiment day.

Statistical analysis

A nested ANOVA model was used to compare populations and trees within populations in species represented by more than one population. For other species, a one-way ANOVA was applied, whereby each tree was represented by a mean of four evaluations. Modes of standardization were compared using the linear regression model. All analyses were carried out using the Statistica Software package (5.1 version, 1997 for Microsoft Windows).

RESULTS

Genome size comparison between populations within species

Three species, *C. canephora*, *C. congensis* and *C. brevipes*, and a botanically uncharacterized taxon, *C. sp.* Moloundou, were represented by several populations (2–4). Another species, *C. liberica*, included two subspecies, *C. l.* ssp. *liberica* and *C. l.* ssp. *Dewevrei*, and one population, *C. sp.* Koto.

A nested-ANOVA was carried out within each species to test the between-population factor and the between-tree (within population) factor. Three types of results were recorded: (1) in *C. canephora* and *C. sp.* Moloundou, there were no differences between populations nor any differences between trees within populations; (2) in *C. brevipes*, there were no differences between populations, but there were differences between trees within populations; and (3) for *C. congensis* and *C. liberica*, significant differences were noted for both factors (Table 2).

C. congensis, a Cameroonian population, had a slighly larger mean genome size (2C = 1·51 pg) than other populations (2C = 1·48 pg) from the Central African Republic and the Congo, but this difference represented only 2 %.

In *C. liberica*, the two subspecies *C. l.* ssp. *liberica* and *C. l.* ssp. *Dewevrei,* had the same genome size (2C = 1·40 pg). These two subspecies differed from the Koto population collected in Cameroon (2C = 1·51 pg). In this case, the difference in genome size was about 8 %.

Genome size of new botanically uncharacterized Coffea *taxa*

All of these new taxa are native to Cameroon and the Congo (Brazzaville). As the genome sizes of the two *C. sp*. Moloundou populations did not differ, that of Souanké was retained for the ANOVA (Table 3). The genome size of these new taxa varied significantly ($F_{5,24}$ = 20·2; $P < 0{\cdot}0001$) from 2C = 1·31 pg for *C. sp.* Ngongo 2 to 1·44 pg in *C. sp*. Moloundou. Three groups could be defined according to the genome size: group A, including only *C. sp*. Ngongo 2; group B, with three taxa (*C. sp.* Congo, *C. sp.*

TABLE 2. *Genome size comparisons between populations and trees within populations in five diploid species of the* Coffea *genus*

Species	Population or subspecies	DNA content	Pop *F* test	Tree *F* test
C. canephora	Cameroon	1·429[a] (1·425–1·438)	$F_{1,8}$ = 0·80 *P* = 0.51	$F_{16,20}$ = 1·37 *P* = 0.24
	Côte d'Ivoire	1·431[a] (1·325–1·521)		
	Central African Republic	1·440[a] (1·423–1·455)		
	Congo	1·448[a] (1·432–1·469)		
C. congensis	Central African Republic	1·477[a] (1·455–1·510)	$F_{2,12}$ = 6·90 *P* = 0·010	$F_{12,15}$ = 4·86 *P* = 0·0026
	Congo	1·478[a] (1·460–1·495)		
	Cameroon	1·509[b] (1·499–1·521)		
C. liberica	Côte d'Ivoire (subsp. *liberica*)	1·396[a] (1·358–1·425)	$F_{1,12}$ = 17·9 *P* = 0·0003	$F_{12,15}$ = 8·25 *P* = 0·0001
	Central African Republic (subsp. *Dewevrei*)	1·406[a] (1·395–1·414)		
	Cameroon (Koto)	1·511[b] (1·419–1·547)		
C. sp. Moloundou	Cameroon (Moloundou)	1·432[c] (1·404–1·453)	$F_{1,8}$ = 0·87 *P* = 0·38	$F_{8,10}$ = 1·18 *P* = 0·40
	Congo (Souanké)	1·444[c] (1·423–1·461)		
C. brevipes	Cameroon (Kumba-Loum)	1·519[b] (1·502–1·545)	$F_{1,8}$ = 0·16 *P* = 0·60	$F_{8,10}$ = 7·49 *P* = 0·002
	Cameroon (Mt Cameroon)	1·523[b] (1·496–1·544)		

The DNA content column includes mean and between-tree range in parenthesis (below).
The results of the Newmann–Keuls multiple mean comparison test within species are indiced by superscript letters.
The Pop *F* test column gives ANOVA results for the between-population factor; the subscript numbers following of the *F* are degrees of freedom. The Tree *F* test column gives ANOVA results for the between-tree factor.

TABLE 3. *Nuclear DNA content in new uncharacterized taxa*

Taxons	DNA content	Range
C. sp. Ngongo 2	1·308[a]	1·295–1·320
C. sp. Congo	1·358[b]	1·309–1·417
C. sp. Nkoumbala	1·366[b]	1·346–1·400
C. sp. Mayombe	1·369[b]	1·350–1·399
C. sp. Ngongo 3	1·417[c]	1·383–1·445
C. sp. Bakossi*	1·430	–
C. sp. Moloundou Souanké	1·444[c]	1·423–1·461

Letters indicate multiple mean comparison results using the Newman–Keuls test.
The *F* test (df1 = 4, df2 = 5) concern between-tree differences within populations.
* Represented by only one tree

Nkoumbala and *C. sp.* Mayoumbe) and 2C = 1·36–1·37 pg; group C, also with three taxa, *C. sp. Ngongo 3, C. sp.* Bakossi and *C. sp.* Moloundou, and 2C = 1·42 – 1·44 pg.

Genome size in botanically characterized species

A population choice was applied in the analysis to obtain a balanced design when species were represented by more than one population. The selected population generally had a mean genome size close to that of the whole species. A random choice was made for species represented by two populations. Consequently, *C. brevipes* was represented by the population from Mount Cameroon, *C. canephora* by the population from the Central African Republic, *C. congensis* by the population from the Congo. *C. liberica* was an exception since it was represented by the two subspecies *C. l.* ssp. *Dewevrei* and *C.* sp. Koto population. Lastly, as the within species variation increased along with the genome size, a logarithmic transformation was applied to the data before performing the one-way ANOVA.

Genome size ranged from 2C = 1·04 pg in *C. racemosa* to 1·76 pg in *C. humilis*. Between-species variations were highly significant ($F_{14,60}$ = 518; $P < 0·0001$) and represented 99 % of the total variance. In these conditions, it was not surprising that each species differed from the others when the genome size difference was above 0·027 pg (Table 4).

Most species generally had a specific genome size. Exceptions concerned: (*a*) the group of three species—*C. sessiliflora* (2C = 1·11 pg), *C. pseudozanguebariae* (2C = 1·13 pg) and *C. costatifructa* (2C = 1·15 pg), where the nuclear DNA content increased over a gradient by 0·02 pg step^{-1} (the smallest genome size of this set, *C. sessiliflora*, did not differ significantly from that of *C. pseudozanguebariae*, and the latter did not differ from

TABLE 4. *Nuclear DNA content in African coffee species*

Species	DNA content	Range
C. racemosa	1·035[a]	1·030–1·039
C. pocsii	1·083[b]	1·059–1·141
C. sessiliflora	1·109[c]	1·080–1·128
C. pseudozanguebariae	1·131[cd]	1·115–1·133
C. costatifructa	1·150[d]	1·129–1·162
C. salvatrix	1·221[e]	1·200–1·248
C. stenophylla	1·286[f]	1·267–1·314
*C. kapakata**	1·323	–
C. eugenioides	1·364[g]	1·344–1·384
C. liberica ssp. *Dewevrei*	1·406[h]	1·395–1·414
C. canephora RCA	1·440[i]	1·423–1·455
C. congensis CR	1·478[j]	1·460–1·495
C. liberica Koto	1·511[k]	1·419–1·547
C. brevipes Mt Cameroon	1·523[k]	1·496–1·544
C. heterocalyx	1·737[l]	1·718–1·751
C. humilis	1·764[l]	1·732–1·792

Species are arranged in increasing DNA amounts.

Letters indicate multiple mean comparison results using the Newman–Keuls test.

The F test (df1 = 4, df2 = 5) concern between-tree differences within populations.

* Represented by only one tree

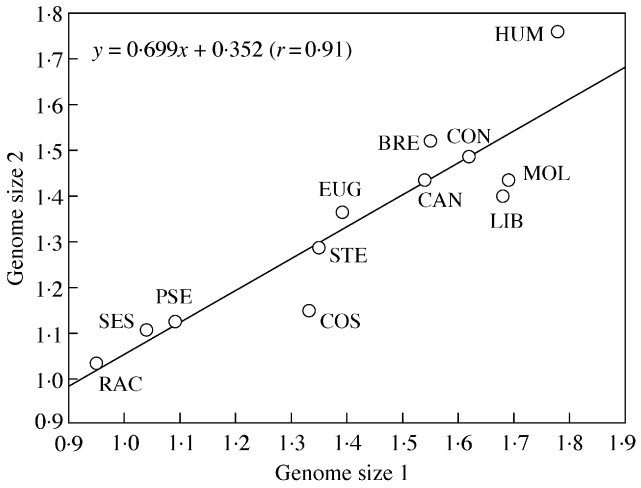

FIG. 1. Relationship between evaluations by Cros *et al.* (1995) (genome size 1) and current evaluations (genome size 2).

that of *C. costatifructa,* but the *C. sessiliflora* and *C. costatifructa* genome sizes differed significantly); (*b*) *C. brevipes* and *C. l.* Koto; and (*c*) *C. humilis* and *C. heterocalyx*.

C. kapakata, represented by only one accession from Angola, was not processed in the ANOVA. The genome size of this accession was 2C = 1·32 pg, i.e. midway between *C. stenophylla* and *C. liberica*.

Correlation with previous evaluations

The genome size range in the subgenus *Coffea* was similar to that observed by Cros *et al*. (1995) using external standardization. For the 12 diploid species assessed in both studies, there was a high and significant correlation (r = 0·91; P < 0·0001) between evaluations (Fig. 1). Nevertheless, the slope (b = 0·7) and intersect (a = 0·35) differed from the expected b = 1 and a = 0, respectively. Some species, i.e. *C. pseudozanguebariae*, *C. brevipes*, *C. eugenioides* and *C. humilis*, were close to the theoretical regression line $y = x$. In contrast, the difference between standardization methods was greater for *C. liberica* (18 %), *C. sp.* Moloundou (16 %), and *C. costatifructa* (14·5 %). For these species, internal standardization led to a smaller genome size.

DISCUSSION

Various impacts of stoichiometric error on genome size evaluations

The genome size range in the subgenus *Coffea* was similar to that observed by Cros *et al*. (1995) using external standardization and a strong correlation between the two evaluations was recorded. Nevertheless, the slope and intersect differed from the expected results. The standardization mode (internal in the present case) could be responsible for these differences through nucleus–cytoplasm interactions (Noirot *et al*., 2000, 2002). Indeed, basic assumptions concerning genome size estimation using flow cytometry do not apply when dye accessibility to DNA is not identical for standard and target nuclei (Noirot *et al*., 2003).

External standardization became unreliable when a marked difference was noted for *C. liberica* (18 %), *C. sp.* Moloundou (16 %), and *C. costatifructa* (14·5 %). For these species, internal standardization led to a smaller genome size. This could correspond to higher dye accessibility of *Petunia* nuclei DNA due to caffeine release in the buffer during chopping. In all cases, internal standardization should have led to lower bias (lowering the bias does not mean eliminating it), since the standard and target nuclei conditions were the same with regards to the antagonistic effects of caffeine and phenols. Consequently, they should be quite similar with respect to the extent of dye accessibility to DNA. Note that both standardization modes could be considered reliable when internal–external differences are not significant, as for *C. pseudozanguebariae*, *C. eugenioides*, *C. brevipes* and *C. humilis.*

Internal standardization lowers the bias but does not eliminate it completely. The stoichiometric error obtained when using internal standardization is about 5 % in coffee trees (Noirot *et al*., 2002). This is, however, much lower than differences between standardization mode. This also means that a difference of less than 5 % between all modes would not reflect a true DNA content variation. This state of uncertainty concerns between-tree variations determined within *C. brevipes, C. congensis* and *C. liberica.* The latter case confirms previous results, where between-tree variations were due to stoichiometric error (Noirot *et al*., 2002).

Caffeine and chlorogenic acid, two compounds abundant in coffee leaves and fruits, could explain intraspecific variations in genome size (Noirot *et al*., 2003), and also interspecific variations. Indeed, the caffeine content ranges

from 0 in *C. pseudozanguebariae* to 2·54 % dry matter basis in *C. canephora*. Similarily, the chlorogenic acid content is 1·1 and 11·3 % in these two species, respectively (Charrier and Berthaud, 1975; Anthony *et al*., 1993; Ky *et al*., 2001). Such variations could lead to pseudo-differences in genome size between species. Nonetheless, stoichiometric error cannot explain the entire genome size range observed within the genus.

Are between-species and between-population genome size variations valid speciation criteria in coffee trees?

The accuracy of the method permitted us to separate most species by their genome size when there was a difference of at least 0·03 pg. Genome size could be a species-specific feature and exceptions seemed to be due to a lack of power of either the experiment (replicate number) or to the Newmann–Keuls test (this test is less powerful than the *F* test). In both cases, this problem could be overcome by using a slightly higher replicate number.

An absence of difference between populations was recorded within *C. canephora*, *C. brevipes* and *C. sp.* Moloundou. The higher genome size determined for the *C. liberica* Koto population could suggest speciation. This hypothesis is supported by specific morphological traits: the Koto trees have long-shaped leaves with spatuled acumen and large, black fruits (fruits are red and smaller in the two other *C. liberica* subspecies), with thick, fibrous pulp (Anthony, 1992). Similarly, based on the species-specific genome size hypothesis, the Congolese *C. congensis* population could have been undergoing a speciation process, but stoichiometric error might also have been a key factor.

The species-specific genome size hypothesis would confirm the pre-classification—on the basis of morphological traits—of the collected accessions into new taxa. This was the case for *C. sp.* Ngongo 2, whose genome size differed from that of all other uncharacterized taxa.

Conversely, an absence of genome size differences did not imply that taxa could be considered as belonging to only one species. For example, the genome size did not differ between *C. sp.* Ngongo 3 and *C. sp.* Moloundou, but they differed markedly with respect to their morphological traits and mating system (allogamous *vs*. autogamous). In summary, the genome size differences noted here indicate a potential change in species status, while nevertheless keeping in mind that minor differences could reflect a stoichiometric error. In contrast, a similarity in genome size is not sufficient evidence of a lack of speciation between taxa in coffee trees.

Is genome size variation correlated with adaptive traits in coffee trees?

Correlations between genome size and abiotic factors are well known, but are often reversed. For example, genome size increases with elevation in *Dasypyrum villosum* (Caceres *et al*., 1998) but not in *Artemisia* genus (Torrell and Vallès, 2001).

In the *Coffea* subgenus, genome size generally increases from East to West Africa (Cros *et al*., 1995). Geographical gradients have already been observed in the UK, i.e. southern plant species generally have a larger genome size than northern species (Grime and Mowforth, 1982). In coffee trees, the east–west gradient was confirmed by our study on a larger sample. Cros *et al.* have shown that the gradiant is related to rainfall, opposing xerophytic species from East Africa, like *C. racemosa*, *C. sessiliflora, C. salvatrix* and *C. pseudozanguebariae* (Chevallier, 1947), to West African species such as *C. humilis* that are adapted to the sempervirens forest (Chevallier, 1947). This hypothesis is further supported by two other examples focused within a country.

In the Central African Republic, three species, *C. congensis* (2C = 1·48 pg), *C. canephora* (2C = 1·45 pg) and *C. l.* ssp. *Dewevrei* (2C = 1·41 pg) differ in adaptative terms (Berthaud and Guillaumet, 1978). *C. congensis* is strictly limited to sempervirens forests that are periodically flooded by the Oubangui River. *C. canephora* is found from the humid sempervirens forest to the semi-deciduous forest, but always in well drained soils. In the first type of forest, *C. canephora* could be close to *C. congensis* on the edges of flooded zones. *C. l.* ssp. *Dewevrei* grows in forest galleries from the semi-decidious forest to the savannah zone and is sympatric to *C. canephora* in semi-deciduous forests. In this example, the genome size, despite low between-species variations, seems to be positively correlated with the soil water deficit. The low genome size variation could also explain the ecological overlapping of these three species, i.e. the sympatry encountered for *C. congensis* and *C. canephora*, and also for *C. canephora* and *C. l. Dewevrei*. Note the absence of sympatry between the two extremes, i.e. *C. congensis* and *C. l.* ssp. *Dewevrei* (Berthaud and Guillaumet, 1978).

Another example of a relationship between water deficit and genome size was observed in Côte d'Ivoire, where four species, i.e. *C. humilis*, *C. canephora*, *C. l.* ssp. *liberica* and *C. stenophylla*, can be collected in the wild. *C. humilis*, the species with the largest genome size (2C = 1·76 pg), grows in the most humid forest of Côte d'Ivoire (Taï forest, along the Liberian border), whereas *C. stenophylla* with the lowest genome size (2C = 1·29 pg) is particularily well adapted to long cold dry seasons (up to 6 months) (Portères, 1962)—the two other species grow under intermediate water deficit conditions.

A correlation does not reflect causality, and other contrasts should be taken into account to differentiate East and West African species. In West African species, fructification time is longer (10 months *vs*. 2 months in East African species), red fruits are more frequent, caffeine content is higher (Anthony *et al*., 1993) and flower numbers per node are higher. This set of gradients is highly colinear. Consequently, it is difficult to attribute causality between genome size and each of these traits. In addition, there are always some exceptions: *C. stenophylla* with a low genome size has a long fructification time (10 months), *C. sp.* Bakossi produces black fruits, but has a genome size (1·43 pg) typical of a red fruit species, and *C. heterocalyx*, a species with large genome size, has few flowers per node.

Consequently, it appears important to undestand the role of genome size variation on fitness during speciation before attibuting a causality relationship between any adaptative traits and genome size variation.

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