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Full Length Research

Genetic diversity of outstanding Cacao accessions (*Theobroma cacao* L.) from farmers' field in Côte-d'Ivoire using SSR markers

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Cocoa tree (*Theobroma cacao* L.) is an important commodity of Côte-d'Ivoire which lead the world for cocoa export. In six producing regions of the country (Abengourou, Aboisso, Divo, Gagnoa, Daloa), 489 best trees have been selected by a breeder-farmer participatory manner based on the knowledge of farmers about their planting material. Seedlings from each of these selected trees were used to study the genetic diversity and the genetic structure using 12 microsatellites markers. Parental clones from seed gardens were used as control populations. The microsatellites markers amplified 143 alleles in farmers' accessions and 78 alleles in control populations. The farm accessions revealed high within region and low between region diversity. Most of the farm accessions appeared to belong to hybrids intermediate between Upper Amazon (UA) and Lower Amazon (LA) Amelonado parental genotypes. However, certain of accessions appeared to be rather pure UA or LA types. An important finding of this study is the allelic richness which is higher in farmers' accession than seed gardens parental clones suggesting the possibility of enlarging the breeder's collection by introducing farm accessions into working collection.

Key words: *Theobroma cacao*, farmer accession, microsatellites markers, diversity.

INTRODUCTION

Theobroma cacao L. also referred to as cocoa is a tree crop native to tropical forests of American continent (Motamayor *et al*., 2002). The species belongs to the Malvaceae native (Alverson *et al*, 1999). Cocoa is cultivated extensively as the unique source of cocoa butter and its derived product, chocolate. About 70 percent of the world's cocoa is supply by West African countries of Cote d'Ivoire, Ghana, Nigeria and Cameroon. Export of dried cocoa beans makes the largest agricultural commodity contribution to foreign exchange earnings, gross domestic product, and development of producing countries. Cultivation of cocoa is one of the predominant income producing enterprises in rural area of Côte-d'Ivoire where more than 95% of cacao is produced by smallholder farmers with low yield varying between 0.4 to 0.6 Kg/ha. Cultivation of cocoa is largely extensive and the use of unimproved planting materials, the effect of pest and diseases, the ageing of cocoa farms and the poor adherence to the suite of good agricultural practices for cocoa production largely accounts for the low productivity (Freud *et al*, 2000). Although selected material has been developed with a yield potential of 2 tons per hectare, the ability to resist against diseases such as black pod are yet to be improved (Lachenaud *et al.,* 2001). Breeders are working to collect different sources of the partial resistance genotypes to enlarge the genetic base of working collection.

In 2000, the Centre National de Recherche Agronomique

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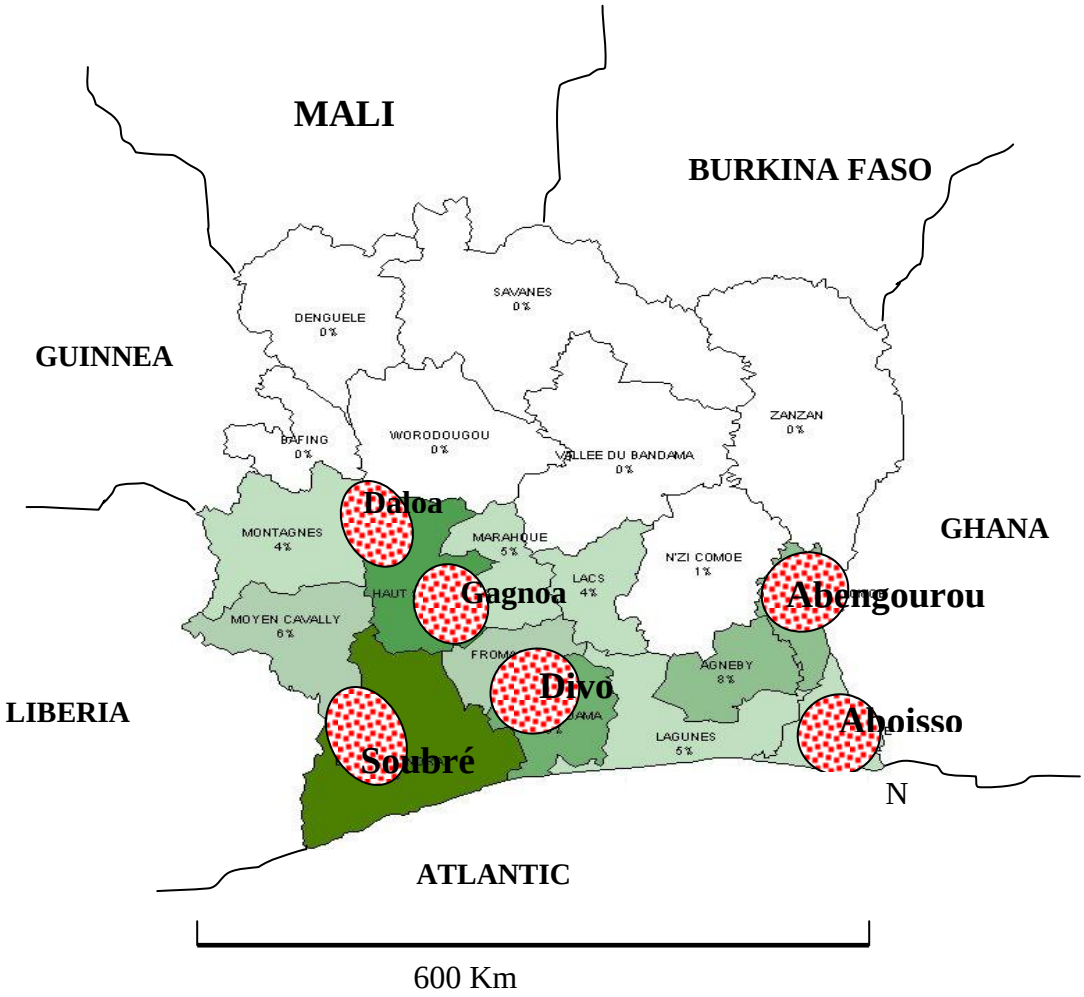

Figure1. Cocoa producing regions in Côte d'Ivoire where cocoa farm accessions were collected Sample collecting areas.

(CNRA) included a farmers' participatory approach in the cocoa breeding programme. The objective is to make use of farmers' knowledge to select promising accessions in farmers' fields and to compare the best varieties selected by breeders with farmers' selections. The programme started with a farm survey carried out on harvest season during which pods were collected from promising trees (Pokou *et al.,* 2005). Results are presented here on the level of genetic diversity, evaluated with SSR markers. Single Sequence Repeat (SSR) or micro satellite markers are useful as they are highly informative, co dominant and are simple to maintain and exchange between laboratories. SSRs are among the molecular markers being used to characterize cocoa collections or study the evolutionary relationship (Motial *et al*, 2010; Pokou *et al*., 2009; Motamayor *et al*, 2008, 2003,2002; Zhang *et al*, 2006; Schnell *et al,* 2005; Bhattacharjee *et al*., 2004).

PLANT MATERIALS

Farm Accessions

During the 2001-2003 main harvesting periods, i.e. October to January, visits were carried out to 280 farms in main cocoa producing regions in Côte d'Ivoire (Figure1): Abengourou, Aboisso , Divo, Dalao, Gagnoa and Soubré . Farmers were asked if they knew of trees in their farms with particularly high yield, with low *Phytophthora* pod rot (Ppr) incidence or with low insect infestation (infestation of mirids on pods). Trees preferred by farmers for the above mentioned criteria were observed during the visits for their growing conditions to assess if a particular tree had possibly been favoured by the environment. In total 489 trees were selected in a breeder-farmer participatory manner. The number varied from 144 in Abengourou to 55 in Daloa (Table1).

A sample of five seeds was taken at random from pods obtained on each of the selected mother trees and sowed in a nursery. One plant of each progeny (called hereafter "farm outstanding accession") was used as representative of the preferred mother tree of the farm in this study.

Reference Genotypes

Farm accessions were analysed together with reference clones composed of parental clones used in seedgardens

Table1. Number of accessions collected in farmers' field per growing area.

	Collecting areas					
	Abengourou	**Aboisso**	**Divo**	**Daloa**	**Gagnoa**	**Soubré**
Number of accessions	144	89	57	55	86	58

Table 2.Reference parental clones of released varieties.

	Genetic Group		
	Lower amazon	**Trinitario**	**Upper Amazon**
Clones name	IFC1, IFC2, IFC5, IFC15, IFC412	ISC46, IFC306, IFC307, IFC312, IFC316, UF11, UF676, POR	PA35, PA7, SCA6, IMC67, NA32, T85/799, UPA412, UPA409, UPA603, UPA608, IMC47

in Côte d'Ivoire (Table 2). These genotypes were split into Upper Amazon (UA), Lower Amazon (LA) and Trinitario (TR) origin according to Cheesman 1944 (Table 2).

DNA Extraction

DNA was extracted from 0.5 g of fresh leaves collected from each of the selected accession and references clones. Leaves were cleaned, frozen in liquid nitrogen and ground. DNA was isolate with buffer containing 100 mM TRIS-HCL pH 8.0, 2% MATAB, 20mM EDTA, 1% PEG6000 and 0.5% sodium sulphite and then purified using the phenol-chloroform method (Karakousis and Langridge, 2003).

Molecular Analysis

In vitro amplification was performed by PCR (Polymerase Chain Reaction) with 12 microsatellite primers identified as an international standard set for cocoa germplasm characterization (Sauder *et al*, 2004; Lanaud *et al*., 1999). These primers were localized on seven out of the ten cocoa linkage groups (Brown *et al,* 2008, Pugh *et al.* 2004). Each primer was used to amplify 2ng of DNA in 10µl of reaction mixture using the PTC 200 instrument (MJ Research, Waters town, MA). The electrophoresis of all products was conducted on an ABI 3100 Genetic Analyzer (manufactured by Applied Bio system) using performed optimized polymer as described by Schnell *et al* (2005). Briefly, after PCR reactions, each sample was prepared by combining 1.0 µl of PCR product with 20µl of dH_2O and 0.1 µl of Gene Scanrox, denatured at 95°C for 5 min and placed immediately in ice. Electrophoresis was carried out using run module for fragment analysis. Alleles of the microsatellite loci were scored according to their size using Gene Mapper software.

Genetic Diversity Parameters

The data obtained were used to estimate the following genetic diversity parameters: average number of alleles per locus, heterozygosity and gene diversity (Nei, 1978). The estimated value of total gene diversity (Ht) was subdivided into within-population (Hs) and between-population (Gst) diversity, where Ht = Hs +Gst. The genetic differentiation is based on geographic origin is given by the Fst value and estimate of the proportion of the diversity present between the populations in relation to the total diversity. F-stat version 2.9.3.2 software packages were used to calculate the genetic parameters. A Factorial Correspondence Analysis (FCA) was performed to visualize the structure of the farmers' outstanding accessions. The Neighbour Joining (NJ) method using the dissimilarity matrix was performed to visualize relatedness of accessions and reference clones after 500 bootstrapping (*Petit and Pons,* 1998). Darwin software version 5.01.158 was used to perform the FCA and NJ analysis.

RESULTS

Genetic Diversity

The 12 loci analysed were highly polymorphic in all populations. In total, 143 alleles were identified in farmers'

Table 3. Genetic parameters of farmers accessions for each region, where He = expected heterozygosity, Ho = observed heterozygosity, MN-AL = Mean number of alleles par locus.

Regions	He	Ho	MN-AL
Aboisso	0.5906	0.4978	8.0
Abengourou	0.6131	0.4963	8.5
Divo	0.5667	0.4607	7.0
Daloa	0.59	0.4978	7.083
Gagnao	0.6036	0.4978	7.16
Soubré	0.6258	0.5442	7.5833

Table 4. Genetic diversity parameters of farmers accessions for each SSR locus, where Ho = observed heterozygosity, Ht = total gene diversity, Hs = within-population diversity and Dst = between population diversity.

Locus	N	Ht	Hs	Dst
mTcCir 3	20	0.786	0.789	0.004
mTcCir 6	11	0.660	0.664	0.004
mTcCir12	14	0.810	0.817	0.007
mTcCir 15	13	0.212	0.213	0.001
mTcCir 19	12	0.656	0.658	0.002
mTcCir 21	23	0.622	0.625	0.003
mTcCir 24	6	0.207	0.209	0.002
mTcCir 25	13	0.709	0.712	0.003
mTcCir 26	5	0.679	0.686	0.007
mTcCir 9	9	0.458	0.458	0.000
mTcCir 17	6	0.212	0.213	0.001
mTcCir 18	11	0.666	0.671	0.005
Total	**143**	**0.605**	**0.609**	**0.004**

Table 5. Genetic parameters of reference populations, where N = number of alleles, He = expected heterozygosity, Ho = observed heterozygosity, MN-AL = Mean number of alleles par locus.

Group	N	He	Ho	MN-AL
Upper Amazon (UA)	62	0.5937	0.6424	5.0833
Lower amazon (LA)	26	0.3342	0.3542	2.3333
Trinitario	49	0.5937	0.5222	4.5000

accessions on the 12 SSR loci. None of the geographically identified populations contained the totality of the alleles. The mean number of alleles per locus varies from 7.0 in Divo to 8.5 in Abengourou (Table3). The frequently occurring allelesvaried from 5 at locus Cir26 to 20at locus Cir3. The average observed heterozygosity (Ho) per population is range from 0.46 in Divo to 0.54 in Soubré while the expected heterozygosity (He) ranged from 0.59 in Daloa to 0.625 in Soubré. For all the six populations of accessions, Ho is 0.505 (Table 4). The total diversity (Ht) ranged from 0.207 at locus mTcCIR24 to 0.786 at locus mTcCIR3. In the overall population, Ht was high (0.609) and average within-population diversity was also high (Hs = 0.605) while between population diversity was very low (Dst = 0.004).

As regard the reference populations, the total number of alleles was 78 among which 62 are from Upper amazons (UA) population, 49 from Trinitario (TR) and 26from lower Amazons (LA). The mean number of alleles per locus varied from 2.16 in lower amazon to 5.5 in trinitario (Table5). The average observed heterozygosity (Ho) per population is range from 0.45 in lower amazon to

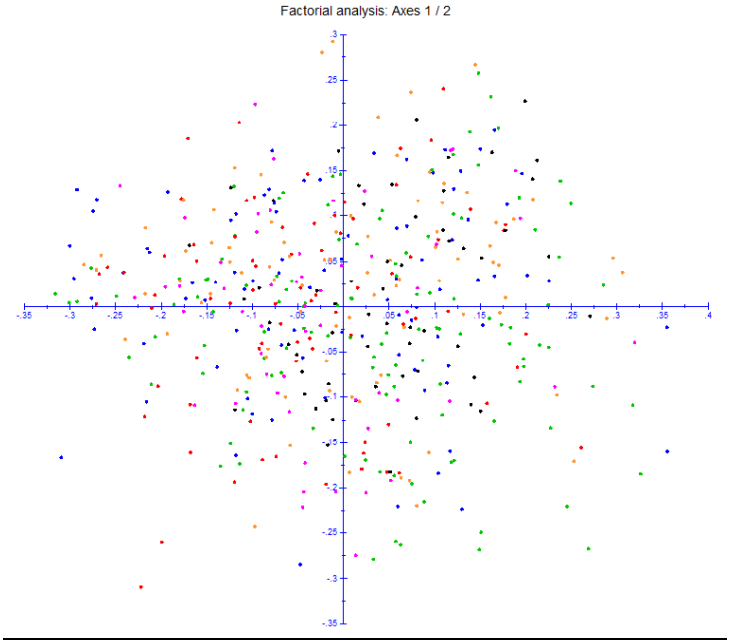

Figure 2. FCA performed with farmers accessions from the six regions
● Aboisso, ● Abengourou, ● Divo, ● Daloa, ● Gagnoa, ● Soubré

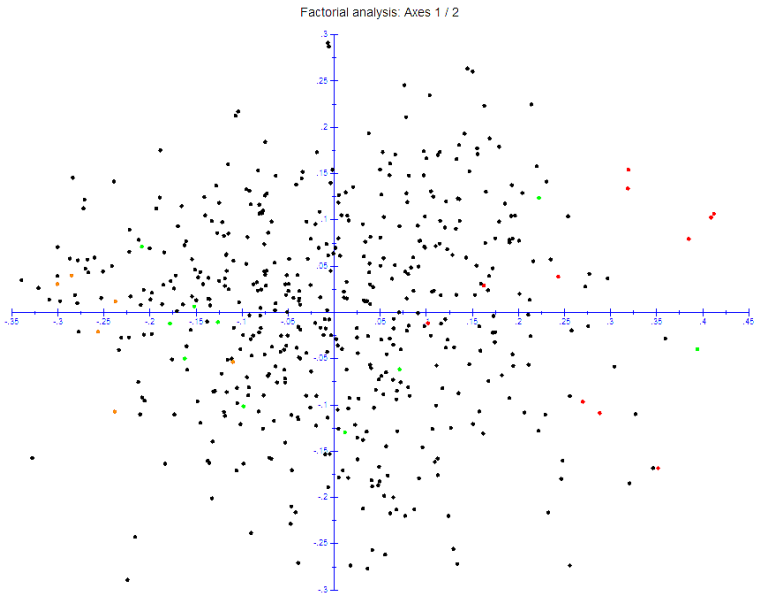

Figure 3.Scatter plot of accessions with farmers' accessions and reference clones:● lower amazon (amelonado),● Trinitario, ● Upper Amazon

0.70 in trinitario while the expected heterozygosity (He) ranged from 0.45 in LA to 0.68 in HA population. For all the three reference populations, the observed heterozygosity is 0.598. The total diversity (Ht) was high (0.728) and average within-population diversity was also high (Hs = 0.600) while between population diversity was relatively low (Dst = 0.129).

Population Structure

The diversity of on farm accessions was visualized with regards to the reference genotypes. Firstly farm accessions from the regions were compared by FCA without the reference genotypes. It appears a large diversity of farmers' accessions. Beside, these populations

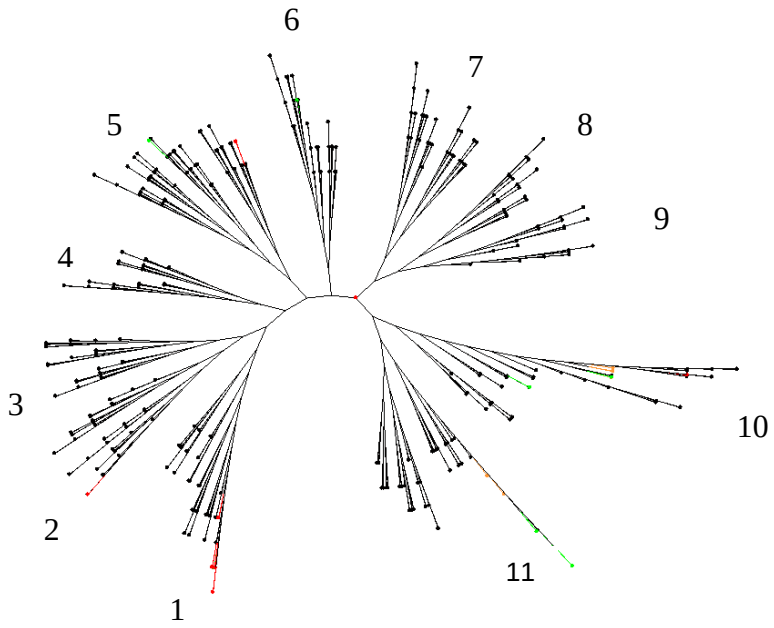

Figure 4. Neighbor Joining Tree with farmers' accessions and reference clones: ● Farm accessions,● lower amazon (amelonado),● Trinitario, ● Upper Amazon

based only on the geographic areas are largely overlapping, suggesting low between region diversity. No separate group has been found on the scatter plot. Secondly, in other FCA, all the farms' accessions were compared to the reference genotypes of UA, LA and Trinitario origins (Figure2). In the scatter plot representing 30% of the total variation, the reference clones are separate according to their genetic origin: LA, Trinitario and UA. However, it appears that the variability of farmers' accessions is slightly larger than that of seed gardens parental clones. Most of accessions appeared as hybrids between the UA and LA(Figure3). However, some accessions are very close to pure LA and UA genotypes. Then, the Neighbour Joining Tree performed with farmers' material and reference clones showed 11 groups of individuals among which, 4 are not similar to none of the reference clones clusters(Figure 4).

DISCUSSION

The present study is the first that show the genetic diversity and genetic differentiation amongst large populations from
farmers' field in Côte d'Ivoire. All the microsatellites analysed showed a high level of polymorphism within and between populations. The set of SSR markers utilized in this study has been widely used for assessing genetic diversity of cocoa populations in West Africa. Such was the case of the reciprocal recurrent populations in Côte-d'Ivoire, germplasm from Nigeria and collections from Cameroon and Ghana (Pokou *et al,* 2009; Aikpokpodion *et al,* 2009; Efombagn *et al*, 2008; Opoku *et al,* 2007).The total alleles found in farmers' best material is higher than the number of alleles in seed gardens parental population suggesting the introduction of cocoa from several origins. Important allelic diversity in farmers' accession have been showed in Ghanaian cocoa collection (7.5 alleles per locus) which contain accessions collected locally and international origin (Opoku *et al*., 2007) and collection also in Cameroon with mean number of alleles of 9.41 (Efombagn *et al*., 2008). In the present study, the greatest number of alleles has been found in Abengourou region while the heterozygosity was higher in Soubré region. Abengourou is located close to the border of Ghana, the second larger producing country. Indeed, according to the history, large scale cultivation of cocoa in West Africa started in Ghana with seed from amelonado(LA) origin previously introduced from Principe and Sao-tome island (Wood, 1991). Then, the most diverse varieties (upper amazons)were introduced in the West Africa cocoa Research institute (WACRI) in Ghana (Freud, 2000). The higher number of alleles comparingto seed gardens parental clones might be the result of introduction from Ghana where upper amazon varieties have been widely released earlier (Opoku *et al.*, 2007). The high gene diversity indicates substantial levels of admixture in the gene pool for farmers' germplasm. Important level of heterozygosity found is consistence to the farmers' practices. In Côte d'Ivoire, amelonado progenies were the first varieties introduced by the French missionary, later on in 1975, the first improved varieties composed of hybrids between upper amazon and lower amazon or trinitario origin have been released

(Besse,1977). Natural hybridisation may have been occurred in farmers' field between locally existing populations Amelonado and upper amazon introduced from Ghana.

(Freud *et al.,* 2000). Indeed, a survey made in farmers' field showed that farmers widely used their own best planting material to produce the seeds for establishing new plantations in addition to improved varieties released (Pokou *et al*., 2005).

In all regions, observed heterozygosity is near to 0.5 or higher, except in Divo region (0.46). These high values for heterozygosity are likely to be partly related to the origin of accessions analysed which are composed of seedlings obtained from open pollination in farmers' field. Consequently, these accessions may have resulted from selfing or recombination with neighbouring trees. However, in all accessions, observed heterozygosity (Ho) was lower than the expected heterozygosity (He). The occurrence of a deficit in heterozygotes, indicating some degree of inbreeding, has been a common observation in cocoa populations (N'Goran *et al.* 2000). The allelic richness in this study is higher than reported in recurrent selection populations in Côte-d'Ivoire suggesting the possibility of using farmers' accession to enlarge the breeders collection (Pokou *et al*, 2009).

In addition to estimating its genetic diversity, the current study provides a comprehensive examination of population structure. The Factorial Correspondence Analysis (FCA) was conducted to distinguish and separate subgroup within germplasm. Although a small percentage of total variation was captured in this study, the expected grouping of reference populations was observed. Globally, upper amazon individuals are separated from lower amazons and trinitario. However, no separate group was observed within farmers' populations according to the growing region suggesting that the same genetic background were found in all producing regions. Indeed, the same hybrids varieties from the seed gardens were distributed in different parts of the countries. This may explain why the diversity of the best planting materials in the six regions is quite similar. An important finding from our data was that most of farmers' planting materials are genetically intermediate between the UA and LA (Amelonado) reference genotypes. In addition, a certain amount of farm accessions are very near to the UA and LA reference genotypes.

These may represent rather pure UA and Amelonado origins. A significant part of the hybrid genotypes observed in our study may therefore have derived from natural out-crossing between improved hybrids with remaining populations of LA, UA or African Trinitario types growing in farmers' fields. The Neighbour joining tree confirms the suggestion of new introduction of genotypes different from the released material from the seed gardens. This study shows that the breeders' collection might be used to enlarge the working collection by introducing some farmers' material.

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