

Genetic Diversity in Cocoa (*Theobroma cacao* L.) Germplasm Collection from Ghana

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ABSTRACT. *Theobroma cacao* L. with its center of diversity in Central and South America was first introduced to West Africa in the

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mid-19th century and today the region produces 70% of the world's cocoa. Several distinct cocoa types have been introduced, cultivated, and intercrossed across the region. Also, bi-parental crosses involving selections from various introductions have been planted on a large scale. Consequently, a wide range of genetic diversity that could be exploited for crop improvement is expected. The present study has been, therefore, undertaken to assess the degree and distribution of genetic diversity present in cocoa germplasm collections from the Cocoa Research Institute (CRIG), seed gardens and materials from farmers' plantations in Ghana, using molecular markers. Two hundred and thirty-five trees representing all the cocoa-growing regions of Ghana were sampled *in situ* from farmers' fields and grouped as *farmers' collection*. Another set of 104 trees was collected from breeders' seed gardens, called *breeders' collection*. Thirty-eight parental clones from the CRIG's collection, used in producing the bi-parental crosses, comprised the third category, called *parental clones*. The collections were screened with the set of 17 mapped microsatellite markers. Average gene diversity was high in all populations, with mean observed heterozygosity of 0.738. Although the highest was recorded in accessions from breeders' and parental collections, genetic diversity in the farmers' collection was comparable with them. Despite the low level of differentiation [$F_{st} = 0.076$] found across all the three groups, sufficient genetic differences existed between them, separating breeders' collection from farmers' collection. The study also revealed the pattern of adoption of available planting materials by farmers on their fields. doi:10.1300/J411v20n01_04 [Article copies available for a fee from The Haworth Document Delivery Service: 1-800-HAWORTH. E-mail address: <docdelivery@haworthpress.com> Website: <<http://www.HaworthPress.com>> © 2007 by The Haworth Press, Inc. All rights reserved.]

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INTRODUCTION

Since the mid-19th century, cocoa germplasm has been brought to West Africa and specifically to Ghana through diverse means. Notable among them were the introduction from Fernando Po in 1878 by a Ghanaian, Tetteh Quarshie; Governor Griffiths' introductions in 1887; introductions by missionaries as well as introductions made to Aburi Botanic Garden (farm, established as an Agricultural Station in the 19th century) in the early 1900s (Lockwood and Gyamfi, 1979). This intro-

duced germplasm was originally Lower Amazon Forastero variety, now known as West African Amelonado and Trinitarios. Posnette (1943) collected Amelonados and Trinitarios from Aburi Botanic Garden, experimental stations and farmers' fields in Ghana and established them at what became the West African Cocoa Research Institute (WACRI) at Tafo, Ghana. Some of these selections and their first generation selfings were used as parents of the first cocoa bi-parental crosses developed at WACRI (Rogers and Knight, 1953; McKelvie, 1956) that were subsequently called 'Series II' hybrids. The most significant introduction after that was Posnette's introduction from Trinidad that included Upper Amazon cocoa (Pound, 1938; Posnette, 1948). This introduction became a basis of the development of modern varieties, not only in Ghana but also in West Africa and to some extent in other cocoa-growing areas of the world. The impact was such that currently the four main cocoa-producing countries in West Africa, namely, Côte d'Ivoire, Ghana, Nigeria and Cameroon, contribute more than 70% of the world cocoa beans production, with a distinctive so-called West African Amelonado taste. Despite quite well documented history of germplasm introductions into the West African region, the real situation regarding existing range of germplasm and their distribution across cocoa-growing regions is not well known among the producing countries. This fact raises the question of efficiency and level of adoption by farmers of materials released by breeders as well as how much and what type of the available germplasm was utilized in breeding programmes.

In Ghana, for example, so-called F3 Amazon material was distributed from the mid-fifties (Hammond, 1957). Centrally operated bi-clonal seed gardens first started production in about 1967, producing Series II hybrids by natural pollination (Glendinning and Edwards, 1962). From about 1971 they produced modified Series II hybrids by mass manual pollination of freshly opened flowers (Edwards, 1973), using different pollen parents from time to time. From the early seventies, seed gardens were planted as mono-clone blocks of self-incompatible clones that were manually pollinated with different suites of pollen parents, depending on the clone. Despite the availability of improved seed, surveys on farms during material collection indicated that there is still a significant number of farmers who select planting materials from their own or other neighbouring farms. The ongoing study (data not shown) reveals that only 40.7% of farmers obtain planting materials from government sources or research institute. The impact of this practice on cocoa production is not very clear, but it certainly has impact on

the genetic structure of the national stock of cocoa trees. This case study was thus undertaken to use molecular markers to assess the genetic diversity in cocoa materials collected from different sources, such as, CRIG's germplasm collection, seed gardens and farmers' fields.

Variation in allele frequency at many unlinked loci is the preferred way to estimate genetic diversity and differentiation. Generally, molecular markers have been superior to morphological and biochemical markers for this purpose (Melchinger et al., 1994). Simple sequence repeat (SSR) markers are particularly attractive as they are abundant in plant and animal genomes with high levels of polymorphism, locus specificity, reproducibility, and most importantly, their co-dominant mode of inheritance (Donini et al., 1998). Recently, the SSRs/microsatellites have been increasingly used in DNA fingerprinting of cacao germplasm. These markers have been used for individual clone identification (Saunders et al., 2004), parentage analysis (Schnell et al., 2005), diversity assessment (Lanaud et al., 1999; 2001), and determination of the origin and domestication of cocoa (Motamayor and Lanaud, 2002).

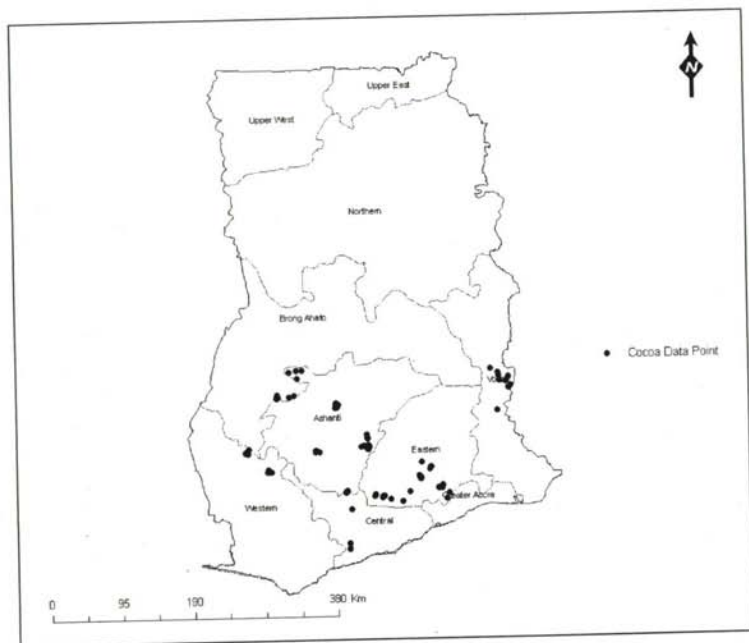
In the present study, the set of 17 SSRs was utilized to fingerprint the 377 cocoa germplasm accessions collected from farmers' plantings, seed gardens and Cocoa research Institute of Ghana. The objective was to determine the genetic diversity and population structure in the cocoa collection from Ghana, specifically to address the issue of impact of breeding efforts on planting materials being used by farmers. This study is a part of an international collaborative project on DNA fingerprinting of cocoa germplasm in West Africa and the resulting information should reveal the pattern of genetic diversity present in one of the major cocoa-producing country in the region—Ghana.

MATERIALS AND METHODS

Plant Material

Leaf samples were collected from three hundred and seventy seven accessions of field-grown cocoa trees representing the major cocoa growing regions of Ghana and core of germplasm collection of the Cocoa Research Institute of Ghana (CRIG). GPS (Geographical Positioning Survey) data were obtained for all accessions and their collection sites are presented on Figure 1. Each selected tree was labeled permanently for future identification and the list is presented in Table 1. The accessions were categorized as *parental clones*, *breeders' collection*

FIGURE 1. Map of Ghana with indication of sites where materials for this study were collected



and *farmers' collection*. The *parental clones*, collected from CRIG, consisted of two groups: (1) the Upper Amazon material first introduced to Ghana by Posnette, often referred to as the Trinidad introductions (T), and a representation of the source population (IMC, Nanay, Parinari and Scavina) and (2) locally selected Amelonado and Trinitarios that Posnette assembled at Tafo. The accessions belonging to F3 Amazon progenies and Series II hybrids planted in different cocoa research stations of CRIG and seed gardens were grouped as *breeders' collection*. The *farmers' collection* consisted of accessions collected from different farmers' farms, mainly representing the six cocoa-growing regions of Ghana (Table 1). The farmers' collections were named after the regions from where they have been collected considering each collection from each region separately (Table 1). The accessions collected from Tetteh Quarshie farm and Aburi Botanic Garden were grouped separately. Seven trees were selected from Tetteh Quarshie farm that were noted as being among the first introduced trees on this

TABLE 1. Cocoa accessions collected from gene banks of CRIG and farmers' farms representing all the cocoa growing regions in Ghana

Germplasm Collection	H_{nb} (Over all loci)	H_o (Over all loci)	$P_{0.95}$	A	B
Parental Collection					
Upper Amazon (81) ^a	0.764	0.760	1.000	7.1	4.7
Local Amelonado & Trinitario (23)	0.756	0.754	1.000	6.3	3.6
Breeder's Collection					
Series II hybrids & F3 Amazon Progenies (38)	0.757	0.747	1.000	6.7	4.8
Total	0.759	0.754	1.000	6.7	4.4
Farmers' Collection					
Tetteh Quarshie Farm (7)	0.589	0.526	0.880	3.2	2.4
Aburi Gardens (10)	0.718	0.687	1.000	5.2	4.3
Volta Region (16)	0.753	0.727	1.000	6.7	3.6
Central Region (9)	0.770	0.718	1.000	4.9	3.8
Eastern Region (28)	0.758	0.742	1.000	6.5	3.6
Ashanti Region (51)	0.771	0.764	1.000	10.8	4.4
Brong Ahafo Region (18)	0.743	0.722	1.000	5.9	4.2
Western Region (96)	0.741	0.741	1.000	6.9	4.6
Total	0.730	0.703	0.985	6.3	3.9
Overall Mean	0.738	0.717	0.990	6.4	4.0

H_{nb} = unbiased gene diversity (Nei, 1978)

H_o = observed heterozygosity; $P_{0.95}$ = proportion of polymorphic loci when the most frequent allele does not exceed 95%

A = mean number of alleles per locus

B = effective number of alleles per locus

^a Number of individual per group are indicated in brackets

farm and in Ghana. These included those 2 trees that are believed to be from the original Amelonado introduction in Ghana. A total of 11 populations were, therefore, considered for the present study.

Farms were randomly selected in each of the six cocoa growing regions of Ghana. The selection of trees in farmers' plantations was mainly based on *Farmers' Participatory Approach* wherein trees were selected according to the individual farmers' perception of 'best performing' and 'worst performing' trees, as well as other factors like incidence of pod rot, medium tree vigor and absence of Cocoa Swollen Shoot Virus. Random sampling was also followed during selection.

DNA Extraction and PCR Amplification

Genomic DNA was extracted from fresh mature leaves, following a CTAB-based protocol (Russell et al., 1992) and diluted to a working concentration of approximately 2.5 ng/ μ l. Collected accessions were screened with the set of 17 SSR markers (Table 2) previously described (Lanaud et al., 1999; Risterucci et al., 2000; Saunders et al., 2004). All PCRs were performed in a total volume of 5 μ l in a gradient cyler PTC 200 (MJ Research, USA) using the following cycling parameters: 94°C for 4 min. (initial denaturation) followed by 35 cycles of denaturing at 94°C for 30 sec, annealing for 1 min at 51°C or 46°C (depending on the primers) and elongation at 72°C for 1 min. This was followed by final elongation at 72°C for 7 min. The amplified PCR products were separated on a 6% denaturing polyacrylamide gel (PAGE). The HyperladderV (Cat. No BIO-33032, Bionline, UK) with fragment sizes ranging between 25 and 500 bp was used as a molecular weight standard for estimation of amplified products sizes.

TABLE 2. Seventeen microsatellite markers used in the study with mean number of alleles, estimates for genetic variation for the whole collection per marker (H_o , H_s , F_{st}) and Hardy-Weinberg equilibrium test (D_{st})

Marker name	EMBL accession number	No. of alleles per locus	H_o	H_s	Gene diversity per locus	F_{st}
mTeCIR1	Y16883	8	0.921	0.768	0.766	0.073
mTeCIR6	Y16980	6	0.806	0.697	0.695	0.075
mTeCIR7	Y16981	6	0.697	0.676	0.676	0.159
mTeCIR8	Y16982	6	0.839	0.713	0.712	0.043
mTeCIR11	Y16985	8	0.762	0.751	0.746	0.044
mTeCIR12	Y16986	10	0.617	0.851	0.860	0.049
mTeCIR15	Y16988	7	0.684	0.757	0.759	0.089
mTeCIR17	Y16990	5	0.615	0.665	0.668	0.078
mTeCIR18	Y16991	6	0.577	0.644	0.638	0.112
mTeCIR22	Y16995	5	0.644	0.666	0.666	0.131
mTeCIR24	Y16996	7	0.612	0.585	0.583	0.081
mTeCIR25	Y16997	10	0.766	0.765	0.760	0.087
mTeCIR26	Y16998	6	0.554	0.754	0.757	0.044
mTeCIR33	AJ271826	9	0.791	0.829	0.829	0.064
mTeCIR37	AJ271942	11	0.810	0.856	0.858	0.045
mTeCIR40	AJ271943	9	0.896	0.795	0.794	0.052
mTeCIR60	AJ271958	8	0.608	0.812	0.813	0.057
Mean		7.5	0.718	0.740	0.718	0.076

H_o = observed heterozygosity

H_s = expected heterozygosity

For each SSR marker, the raw gel data were manually scored following presence-absence format, in which the presence of a band was scored as 1 and its absence as 0. Microsatellites being co-dominant in nature scored two bands for an individual genotype if it was heterozygous for that marker, or a single band if it was homozygous.

Data Analysis

To estimate the genetic diversity present in the cocoa germplasm collections, the mean number of alleles per polymorphic locus, effective number of alleles per locus, average observed heterozygosity (H_o) and mean gene diversity (H_{nb}) (Nei 1978) was calculated using Genetix v. 4.04 and TFPGA v. 1.3. The pairwise genetic distances between individual germplasm accessions were calculated from the raw data based on Nei's unbiased genetic distance (Nei, 1978). The matrix was then subjected to cluster analysis using the unweighted pair-group method with arithmetic average (UPGMA) with TFPGA v. 1.3. Bootstrap values were calculated for each node of the cluster following 1,000 permutations across loci.

Genetic differentiation was quantified using F-statistics (F_{st}) (Wright 1969), as described by Weir and Cockerham (1984), using FSTAT v. 2.9 (Goudet 1995). F_{st} values were estimated per allele per locus and overall. Bootstrap values were calculated following 1,000 permutations across loci. FSTAT performs bootstrapping across loci (Efron 1982) and provides rigorous testing of hypotheses of genetic differentiation. The Exact Hardy-Weinberg test was used to assess the deviation from HW equilibrium and was performed by GENEPOP v. 3 (Raymond and Rousset, 1995).

RESULTS

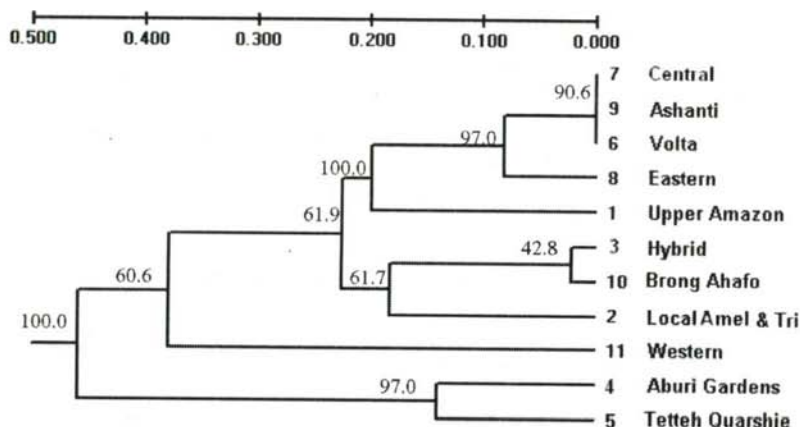
Genetic variation was assessed using the set of 17 SSR markers with 377 cocoa accessions under study, which were grouped into three "genetic" categories: *parental collection* with two populations (104 accessions), *breeders' collection* with one population (38 accessions) and *farmers' collection* with 8 populations (235 accessions) (Table 1). All the microsatellite markers showed polymorphism across each of these populations. A total of 127 alleles were recorded across 11 populations (categorized based on genetic and geographical grouping). Allelic richness differed substantially among the populations (see column A of Ta-

ble 1). The unbiased mean gene diversity H_{nb} (Nei, 1978), over all the groups was 0.738. However, the overall mean gene diversity over parental and breeders' collections (0.759) was significantly ($P < 0.034$) higher than that of the farmers' populations (0.730). Similarly, the mean number of alleles was high for accessions belonging to parental and breeders' collections (6.7), which also recorded the highest observed heterozygosity (0.754) (Table 1), although there was limited sampling of accessions from parental and breeders' collections. The lowest gene diversity (0.589) and observed heterozygosity (0.526) was recorded in the Tetteh Quarshie population, which also recorded the lowest number of mean effective number of alleles (2.4). The mean effective number of alleles was determined by considering those alleles with frequencies equal to or higher than 0.05. It was found that the overall mean effective number of alleles across different genetic groups was low (4.0). However, breeders' collections had the maximum number of effective alleles (4.8) followed by Upper Amazon accessions (4.7).

The number of alleles at each locus varied from 5 (mTcCIR17 and mTcCIR22) to 11 (mTcCIR37) with a mean of 7.5 alleles per locus (Table 2). Tests for departures from Hardy-Weinberg Equilibrium (HWE) revealed deviations from HWE in all the populations, and the heterozygote deficiency was highly significant ($P < 0.001$) across populations and loci. The F_{st} value, averaged over all loci, was 0.076 as estimated through bootstrapping, with a confidence interval of 99% (Table 2). This shows a low level (< 0.5) of differentiation among the three genetic populations described in the study. Nei's estimation of heterozygosity (H_s) was 0.740, which is 7.8% higher than the observed heterozygosity (H_o) of 0.718.

The genetic relationship among 11 cocoa populations was further analyzed using UPGMA cluster analysis (Figure 2). Two major clusters were formed, one grouping together the accessions from Tetteh Quarshie farm and Aburi Botanic Garden, and the other with accessions from parental, breeders' and farmers' collections. Despite the fact that the accessions from Upper Amazon, local Amelonado and Trinitarios as well as F3 Amazon progenies and Series II hybrids clustered into two separate groups, the recorded genetic distance between them was very low (data not shown). Among populations from farmers' plantings, the accessions from Volta, Eastern, Central and Ashanti regions clustered together whereas those from Western and Brong Ahafo regions formed two separate clusters.

FIGURE 2. UPGMA dendrogram of 11 cocoa populations based on Nei (1978) unbiased minimum genetic distances, bootstrap values indicated as percentages above each branch were obtained from 1,000 replications by re-sampling loci



DISCUSSION

The set of 17 SSR markers used in this case study enabled an approximate estimation of genetic diversity and population differentiation in cocoa germplasm collected from CRIG's research stations and farmers' fields located in the cocoa-growing regions of Ghana. The reliability of estimates for genetic variation, such as H_s , H_o , F_{st} and genetic distances, depends more on the number of loci than on the number of individuals sampled (Baverstock and Mortiz, 1996). The estimates of F_{st} varied widely from marker to marker, which may be due to the small set of SSR markers used in this study for assessing genetic diversity. The low level of genetic divergence ($F_{st} = 0.076$) recorded indicated a high level of similarity among the 11 populations studied. This confirms that most of these populations originated from a common source, i.e., breeders' seed gardens (Opoku et al., 2006). However, the high mean gene diversity of breeders' over the farmers' collections provides sufficient evidence that there were genetic differences between both populations to separate breeders' germplasm from that of farmers. The results revealed that there is relatively less preference for breeders' germplasm among the farmers, and this has also been evident from the socio-economical data recorded during collection missions (Opoku et al., 2006). The re-

sults also indicate that there is limited usage of available parental germplasm by the breeders in their breeding programmes to improve the existing cocoa varieties.

Nei's estimation of gene diversity recorded in the analysis showed a high genetic diversity within the cocoa accessions ($H_{nb} = 0.738$). However, the high level of similarity among the 11 populations showed that the high genetic diversity recorded in this study existed within the individual populations than between them.

A high level of gene diversity (0.756) in accessions from 'Local Amelonado and Trinitario' population (Table 1) was an unexpected observation as the studies of other researchers have shown that lower Amazon Forastero or Amelonado accessions were mostly homozygous with low genetic diversity (N'Goran et al., 1994; Motamayor and Lanaud, 2002; Motamayor et al., 2003; Schnell *personal communication*). The high heterozygosity observed in these accessions might be due to the presence of introgressions of Upper Amazon genes from the released improved varieties. This may also be attributed to the grouping of Amelonado accessions with Trinitarios, which are highly heterozygous in nature in comparison to Amelonados.

The deviation of the loci from Hardy-Weinberg equilibrium ($D_{st} = 0.060$) observed in the present study could have a number of explanations. It might be mainly because of the way the cocoa germplasm collection has been grouped in the present study or may be due to the possession and nature of self-incompatibility phenomenon in cocoa. It might also be due to the evolutionary pathway of original cacao populations, where genetic drift may have caused the fixation of alleles in specific populations (Motamayor et al., 2003). However, the accessions from the Tetteh Quarshie farm and local Amelonado are mainly self-compatible, though this theory is still being discussed.

Tetteh Quarshie's introduction was one of the successful establishments of cocoa in Ghana during the mid-19th century and from then onwards it spread across the country with farmers purchasing pods from this farm. The cocoa produced at the Tetteh Quarshie farm is Amelonado type, which originated from Brazil. The Amelonado accessions from Brazil have shown a reduced genetic diversity similar to other traditional cultivars, such as Criollo or Nacional (Motamayor et al., 2003). The accessions belonging to this group were generally uniform in several attributes, including tree-to-tree uniformity and susceptibility to Cocoa Swollen Shoot Virus (CSSV).

The separate clustering of accessions from Tetteh Quarshie farm and Aburi garden compared to rest of cocoa populations (9) under study is

quite interesting. These accessions were among the first introductions into the country and then spread across the country; however, the results indicated that presently these accessions have little or no influence on the current plantings in farmers' fields. It appears that they are no longer widely distributed or utilized in the country. Subsequent hybridization with later introductions and adoption of farmers' own or newly released improved germplasm may be the reason for this discrepancy.

The accessions from Western region clustered separately from those of breeders' collection and populations of other regions indicating that the breeders' germplasm had less impact on planting materials in the West of the country in comparison with other regions. It proves to be in agreement with the historical records that cocoa cultivation in Ghana had spread from other adjacent regions to the West. Additionally, the seed gardens from which farmers could obtain improved planting materials developed by breeders are fewer in the region and are inaccessible due to poor road network. Interviews with farmers indicated that they plant materials collected from any source including their own old farms. The germplasm in the West region also constitute the most recently developed improved planting materials. On the other hand, farmers from other regions mostly collect seeds from the improved seed gardens. This explains why the farmers' collections from these regions clustered with the breeders' collections.

Another interesting observation is that the germplasm from Central, Ashanti, Volta, and Eastern regions, which constitute the earliest cocoa-growing regions of Ghana, clustered together and separately from the parental clones and breeders' collection, whereas the accessions from Brong Ahafo region clustered closer to the breeders' collections. It is not clear, but some historical records show that most of Brong Ahafo plantings were done at the time when Series II hybrids had been developed and were popular in the country. Thus, most farmers in this region might have used those varieties as planting material. However, in the case of the other regions, a substantial number of farmers might have, in addition to breeders' varieties, collected materials from their own farms or other neighboring sources.

The results have revealed that breeders' collections had varied impact on the cocoa cultivation in the country, which could affect cocoa production. Most of the hybrids developed by breeders have parents with self-incompatibility genes, but the selection is such that the progenies going to farmers are cross-compatible. Thus, use of planting material from farmers' fields year after year could have deleterious impact

on yield and other desirable traits as a result of increased incompatibility in the subsequent progenies.

The findings of this case study, therefore, indicate that there is a scope for better utilization of available parental germplasm in breeding programs for developing varieties with better yielding abilities and resistance to diseases with increased number and better accessibility of seed gardens to the farmers. There could be free distribution of seeds to the farmers during field days and many other measures to spread the utilization of improved varieties by the farmers. These measures would further help in improving the adoption rate of new varieties by farmers and in combination with competent farming practices would create the profitable cocoa farming system that will attract a young generation of farmers.

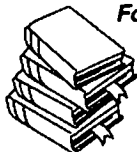
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