RESEARCH ARTICLE

Molecular Characterization of Resistant Accessions of Cocoa (*Theobroma cocoa* L.) to Phytophtora Pod Rot Selected on-Farm in Côte-d'Ivoire

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ABSTRACT

Cocoa is (*Theobroma cacao* L.) is a significant agricultural commodity in Côte-d'Ivoire which ranks 1st in the world cocoa export. Phytophthora pod rot (Ppr) also call Black pod is the most widespread disease of cocoa. Lost due to this disease depends on the species of the pathogen and vary globally from 15% et 80% of the farm production. Due to the impact of cocoa on the economy, resistance to Phytophthora Pod rot (Ppr) is becoming increasingly important criterion of selection for new cocoa varieties in Côte-d'Ivoire. In this decade, the CNRA has selected new cocoa cultivars with involvement of farmers. Some of these cultivars have shown resistance scoring using detached pod test. Based on 12 microsatellites primers, the results of the genetic diversity and structure of these resistance plant materials are presented. Control clones from breeders collections were used basedon their relationship to the recent genetic structure of cocoa. All the farmers' accessions presented high heterozygosity value. Most of farmers' accessions were hybrids between amelonado and upper amazons groups. The neighbour joining analysis performed after 500 bootstrapping revealed 6 genetic groups respectively close to the 5 control groups of amelonado, maranon, contamana, Iquitos and Nanay. Control clones from the scavina group especially, those known be resistant to Pprat the CNRA Research station are quite different to the resistance accessions on the farmers' field. One group remained unknown based on the controls used. It is recommended that the genetic structure identified through these promising plants in farmers' fields be further exploited in breeding to obtain new hybrid or clonal cocoa varieties with low incidence of Ppr.

INTRODUCTION

Theobroma cacao L. also referred to as cocoa is a tropical tree belongs to the Malvaceae sensulato native to tropical forest of Amazonian basin (Alverson *et al*., 1999; Motamayor *et al.,* 2002). Cocoa is cultivated extensively for the unique source of cocoa butter and its derived product, chocolate. More than 70 percent of the world's cocoa is produced by small scale farmers of West African countries especially Cote d'Ivoire which supplies 40% of the world's Cocoa. Export of dried cocoa beans makes the largest agricultural commodity contribution to foreign exchange earnings, gross domestic product, and development of producing countries. Sustainability of the smallholder cocoa production systems is threatened by the

effect of pest and diseases. Globally, the most widespread disease is Phytophthora pod rot (Ppr), also called black pod, which is caused by four different species of Phytophthora. The more aggressive *P. megakarya* occurs only in West Africa. In areas invaded by *P. megakarya* in Côte-d'Ivoire, the pods losses have increased from an average of 15 %, in the presence of *P. palmivora*, to an average of 30-35% the recent past (Kébé, 1994). Chemical control of Ppr is possible but it is expensive given the fact that the average productivity achieved by the smallholder cocoa growers in West Africa is low. The majority of cocoa varieties grown worldwide are highly susceptible to the Ppr disease. Although significant variation for genetic resistance has been observed in germ plasm collections and breeding trials (Blaha and Lotodé,

1976; Despreaux *et al.,* 1989; Iwaro *et al.*, 2003), the frequency of resistant genotypes is generally low.

Since 2000, resistance to Ppr has become an increasingly important criterion for selection of new cocoa varieties in Côte d'Ivoire. The Centre National de Recherche Agronomique (CNRA) is engaged in a farmers' participatory approach in selecting new cocoa varieties. The programme started with a farm survey during which pods sample were collected from trees that were considered by the farmers as promising regarding Ppr (Pokou *et al*., 2008). It's known that farmers have largely used their own planting materials to establish new plantations in cocoa growing area. An assessment of the progenies obtained from pods collected using artificial inoculation of pathogen onto detached leaves has confirmed the resistance of 76 farmers' accessions representing 70% of sample shown by farmers as resistance to Ppr (Pokou *et al.,* 2008). However, little is known about the genetic diversity of these tolerant accessions. The objective of this study is to analyse the genetic identity of these promising resistance trees by using neutral molecular markers. Microsatellites or single Sequence repeats (SSR) and Single Nucleotide Polymorphisms (SNPs) are among the molecular markers that are used to characterize cocoa collections or study the evolutionary relationship (Motial *et al*., 2010; Zhang *et al.,* 2006; Schnell *et al.,* 2005, Motamayor *et al.,* 2002, 2003,2008).During the current study, the diversity of promising accessions was accessed by microsatellites markers.

Plant materials
Farm accessions

The plant material was composed of 76 progenies of trees preferred by farmers as they have particularly low Ppr incidence and have showed high resistance ability using detached leave test (Pokou *et al.*, 2008).These progenies hereafter call "farmers accession" were obtained from pods collected after visiting 280 farms from in the main producing regions of the country: Abengourou, Aboisso, Divo, Dalao, Gagnoa and Soubré (Table1).

Reference genotypes

Farm accessions were analyzed together with reference clones belong to six of the ten genetic groups defined by Motamayor *et al.* (2008): Amelonado, Maranon, Iquitos, Morona, contanama and nanay (Table 2). The amelonado are lower amazon origin while the other is upper amazon origin.

DNA extraction

DNA was extracted from0.5 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 withbuffer 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 13 microsatellite primers identified among the international standard set for cocoa germ plasm characterization (Sauder *et al*., 2004). These primers were localized on seven out of the ten cocoa linkage groups and no linkage disequilibrium has been reported (Pugh *et al.,* 2004, Brown *et al.,* 2008). 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 Biosystem) 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 distilled water 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 (Dst) diversity, where Ht = Hs + Dst. The genetic differentiation of 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. Alequin version 3.1, Genetix version 4.1and 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 Neighbor Joining (NJ) method using the dissimilarity matrix was performed to visualize relatedness of accession and reference clones after 500 boos traping (*Petit and Pons,* 1998). Darwin software version 5.01.158 was used to perform the FCA and NJ analysis.

RESULTS

Genetic diversity

Among the 13 microsatellite primers tested, 12 produced showed scorable alleles. The 12 loci analysed were highly polymorphic in all populations. In total, 110alleles were identified on the 12 loci. None of the geographically identified populations contained the totality of the alleles. The mean number of alleles per locus varies from 1.8 in Divo to 5.9 in Abengourou (Table 3).The mean number of allele par locus in the control population is 6.8. The frequently occurring allelesvaried from 29 in Divo to 76 in Abengourou. For all the six regional groups of accessions and the control, Ho is 0.47. The average observed heterozygosity (Ho) per population is range from 0.29 in Divo to 0.58 in Soubré. The expected heterozygosity (He) ranged from 0.38 in Divo to 0.64 in Soubré. The total diversity (Ht) was high (0.65) and average within-population diversity was also high (Hs = 0.57) while between population diversity was very low (Dst = 0.082).

Table 1: Number of promising trees selected on farmers' fields in six cocoa growing regions

Selection criteria	Regions						Total
	Abengourou	Aboisso	Divo	Gagnoa	Daloa	Soubré	
Low incidence of *Phytophthora*	24	8	3	4	19	18	76

Table 2: Reference clones and their related group used in our study: Maranon, Amelonado, Iquitos, Morona, contanama and nanay

	Genetic group					
	Amelonado	Maranon	Iquitos	National	contanama	Nanay
Reference clones	IFC1, IFC5	PA7	IMC5, IMC35, IMC67	MO9	SCA5, SCA12, SCA9	NA32

Table 3: Mean heterozygozity level of farm accessions per region

Region	He	Ho	Mean number of alleles/locus
Aboisso	0.62	0.51	5
Abengourou	0.61	0.50	5.91
Divo	0.38	0.29	1.8
Daloa	0.54	0.49	4.4
Gagnoa	0.62	0.55	2.8
Soubré	0.64	0.58	5.58

He: expected heterozygosity; Ho: observed heterozygosity

Population structure

The Fst has been determine for each pair of population. Within farmers' populations, Fst value range from 0.0011 between Aboisso and Daloato 0.06808 between Divo and Gagnoa. However, the P-value showed that only value between Soubré and Daloa with Fst of 0.04231is significant (Table 4).Population from Abengourou, Daloa and Soubré showed differences with controls clones.

By considering individuals from the whole farmers' populations, the factorial correspondence analysis showed that most of farmers' resistance accessions are hybrids between amelonado in one side and the other groups belong to upper amazons. The graphical show the genotypes from Maranon, Iquitos and Nanayare more close to framers' resistance trees while control genotypes from contamana which are all scavina clones have presented a separate group (figure1).

Neighbour joining analysis performed using the dissimilarity matrix splits farmers' accessions into six groups (Figure 2). Each of this group is composed of individuals from several regions. The largest group is close to the amelonado IFC clones, there is one group close to Iquitos (IMC) and nanay (NA) control, one group close to national (MO), one close to the maranon (PA), the smallest group is close to the contamana (Scavina clones). Based on the control used in the current study, the last group is unknown.

DISCUSSION

The main challenges in analyzing any molecular dataset for characterization study are to (a) explore whether a given population is homogenous or contains genetically distinct subgroups, and (b) identify quantitative evidence that support the presence of these groups. Using microsatellites markers, we investigated the genetic differentiation and structure of cocoa accessions resistant to Ppr from farmers' field in Côte-d'Ivoire. The allelic diversity present in these populations is lower than what have been identified in the breeding population in Côte-d'Ivoire (Pokou *et al*, 2009).The regional population

analysis showed that gene diversity was high in Soubré and Abengourou where sample size are the more numerous. Abengourou and Soubré are also the regions where *P.megakarya* have been identified (Koné, 1999, Kébé *et al.*, 2005). Farmers in these regions can easily make observation on tree behaviour for their susceptibility or tolerance to Ppr. Besides, Abengourou is located at the border area of Ghana, the second largest producer and farmers of the two countries may have exchanged plant material. Most of planting materials released in Ghana are upper amazon origin (Opoku *et al.,* 2007). Large diversity of upper amazons has already been shown before (Lanaud 1986; Laurent *et al.,* 1993; N'Goran *et al.,* 1994, Motamayor *et al*., 2008). Some Upper amazon materials are also known to be resistant to phytophthora pod rot (*Iwaro et al*, 2003).

Observed heterozygosity Hoin all populations analysed was lower than the expected He. The occurrence of a deficit in heterozygotes has been a common observation in cocoa populations (N'Goran *et al.,* 2000).A possible explanation for this could be the small sample size. The highest value of observed heterozygozity was found in Soubré region. This region is consider to be the current cocoa belt in terms of production. Survey carried out on farmers' field has also showed that the farms in these regionshave younger tree on average. In region where the disease pressure is low (Divo, Daloa and Gagnoa), the number of resistant trees is also low.

Although a small percentage of total variation, the factorial correspondence analysis (FCA) showed that most of the resistance clones are hybrids between amelonado and upper amazons groups or between upper amazons. Amelonado is known to be susceptible to most of the cocoa diseases including Ppr (lockwood, 1976).However, several works have showed the heterosis effect of hybrids made between amelonado and upper amazons for different agronomic trait (Dias *et al*., 2003, Atanda, 1973). In Côte-d'Ivoire, most of the first generation hybrids released to farmers for their superior performance including the resistance to Ppr were made with the original parental admixtures between local amelonado and different upper amazon origin of Iquitos and calabacillo (IMC), Nanay (NA), Parinari (PA) (Besse, 1977).The PA clones in the germplasm collection are known to be resistance to Ppr while the IMC are moderately resistant. The most resistance clones in the germ plasm collection is SCAVINA clones especially Sca6 which is ancestor of the current cocoa cultivar released??? Which one. Our result showed the possibility that the resistance material selected on farm had ancestor in the first generation cultivar released because none of scavina clones where identified as probable parental clone in the FCA plot. However, It not possible at this stage of the analysis to know the

Table 4: Fst value of each pair of population (P value)

	Aboisso	Abengourou	Divo	Daloa	Gagnoa	soubré
Aboisso						
Abengourou	0.00746 (0.86486)					
Divo	0.00606 (0.76577)	0.04389 (0.23423)				
Daloa	0.00114 (0.53153)	0.02759 (0.05405)	0.00671 (0.63063)			
Gagnoa	0.02348 (0.10811)	0.01931 (0.37838)	0.04231 (0.37838)	0.03725 (0.09910)		
Soubré	0.2348 (0.18018)	0.01931 (0.09910)	0.04239 (0.17117)	0.03725 (0.00901)**	0.02364 (0.28829)	
control	0.3014	0.03278 (0.04505)*	0.06430 (0.26126)	0.6037 (0.00901)**	0.2929 (0.37838)	0.02924 (0.02703)*
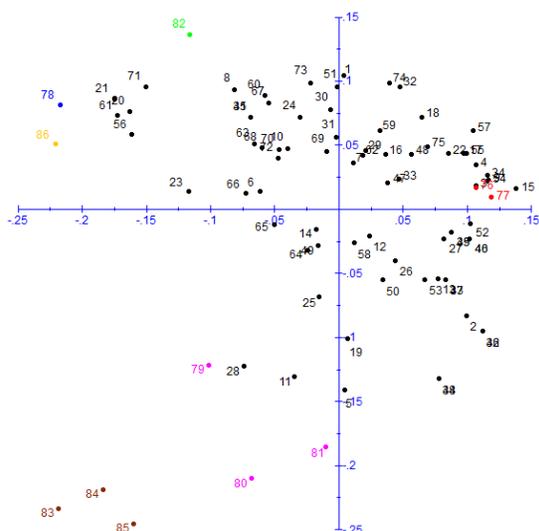
Fig. 1: Factorial correspondence analysis of farmers accessions and control clones:
Aboisso (1--9), Abengourou (10--32), Divo (33--35), Daloa (36--54), Gagnoa (55--58), Soubré (59--75), •Amelonado (76-77), •maranon (78), •Iquitos (79-81), •National (82), •contanama (83-85), •Nanay (86).
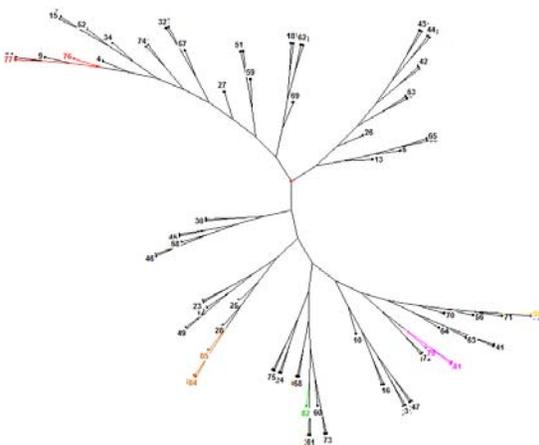
Fig. 2: Neighbor Joining Tree performed with accessions from all regions:
Aboisso (1--9), Abengourou (10--32), Divo (33--35), Daloa (36--54), Gagnoa (55--58), Soubré (59--75), •Amelonado (76-77), •maranon (78),• Iquitos (79-81), •National (82), •contanama (83-85), •Nanay (86).

generation of ancestor. The significance ofFst value between control and each of the farmers' resistance accessions population is an indicator that the selected material is not direct progenies from the control.

The NJ analysis was conducted to distinguish and separate subgroups combined within the resistance accessions and the reference clones. Except one subgroup, all other subgroups are genetically close to at least one control group. The largest group is more close to amelonado origin. Indeed, amelonado was the first cultivar to be introduced in West Africa in the 19th century (Wood, 1991).Most of old farms were established using amelonado material. Farmers' interest to amelonado is due to the density of its beans. However, amelonado is susceptible to Ppr and its production is lower than the others genetic origins of Upper amazons. In Côte-d'Ivoire, upper amazons where first introduced in 1954 from the formal West Africa Cocoa Research Institute (WACRI) (Besse, 1977). However, our analysis showed that the background of four out of five genetic groups identified are upper amazon origins. These cultivars are high yielding and are resistance to Ppr which was the only disease at that period and this may explain its spread in producing regions.

Conclusion and perspectives for utilization in varietal selection

The knowledge of the diversity of the populations and the understanding of the origins and characteristics are by themselves essential for an effective use in breeding. In Cote d'Ivoire, the cocoa breeding programme has been based on the creation of hybrids between different genetic groups. From 1990onward, a reciprocal recurrent selection programme has been set up with the purpose of improving simultaneously the characteristics of the two main genetic groups: Upper Amazon Forastero (UA) and a mixture of Lower Amazon Forastero (LA) and Trinitario (T). Inter-group crosses between some selected trees have been the first approach to select new inter-group hybrid varieties. However, it has been observed in that crosses between subgroup performed as well or even better than the inter-groupcontrol varieties (Lachenaud *et al.* 2001).The current study reports for the first time the result of the characterisation of promising materiel for resistance to Ppr. In the study, it was confirmed that four groups belong to UA and onlyone belongs to amelonado. It appears feasible to exploit the best different genetic group found in this study to improve the resistance of Ppr in the on-going breeding program.

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