UNIVERSITY OF CAPE COAST

FINGERPRINTING OF BLACK POD DISEASE-RESISTANT *THEOBROMA CACAO* L. ACCESSIONS USING MICROSATELLITE MOLECULAR MARKERS

EDWARD KOFI ABBAN

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BY

EDWARD KOFI ABBAN

A THESIS SUBMITTED TO THE DEPARTMENT OF MOLECULAR BIOLOGY AND BIOTECHNOLOGY OF THE SCHOOL OF BIOLOGICAL SCIENCES OF THE UNIVERSITY OF CAPE COAST IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF MASTER OF PHILOSOPHY DEGREE IN BOTANY

MAY 2009

DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original work and that no part of it has been presented for another degree in this university or elsewhere.

Candidate's Signature: …… Date: ……

Supervisors' Declaration

We declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

Principal Supervisor's Signature: …… Date: ……

DR. ISAAC GALYUON

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DR. J. F. TAKRAMA

ABSTRACT

Black pod is a disease of *Theobroma cacao* L. and can cause up to 100% losses in crop yield. Breeding for resistance can reduce or prevent the incidence of the disease. This study was therefore designed to assess black pod disease resistance in 205 accessions of five populations; 'T' clones, progenies of 'T' clones, Amazonia, Series II hybrids and a control population of susceptible accessions by the leaf disc method and genetic fingerprinting using 13 mTcCIR microsatellite molecular markers.

Scores from the leaf disc test were 1.24, 1.50, 1.68, 1.97 and 3.33 for the 'T' clones, progenies of 'T' clones, Amazonia, Series II hybrids and the controls respectively. This indicates that the 'T' clones were the most resistant. A total of 127 alleles were detected with the mTcCIR microsatellite primers. Out of these, 23 were found unique to the 'T' clones. The observed (H_O) and expected (H_E) mean heterozygosity over all the populations were 0.625 and 0.656 indicating a heterozygote deficit among these populations despite the high genetic variability. Cluster analysis also indicated low genetic diversity among populations but a high genetic difference between individuals within a population.

Therefore, the 'T' clones could be employed in breeding for resistance to the black pod disease. The unique and rare alleles found in the 'T' clones could have accounted for their high resistance. The low genetic base observed indicates the need for introduction of exotic varieties in breeding programmes to broaden the genetic base of the cocoa germplasm in Ghana.

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CHAPTER ONE

INTRODUCTION

This chapter covers the justification to this study and its objectives. It starts with cocoa introductions into Ghana, cocoa diseases, cocoa resistant varieties developed in Ghana followed by genetic diversity studies, genetic diversity in cocoa, and the use of molecular markers as a tool for fingerprinting, and linkage map construction in cocoa trees.

Introduction of cocoa into Ghana

Theobroma cacao L. (cocoa) is a crop with major economic importance, since cocoa is grown by more than two million growers in more than 50 countries. Cocoa fat-rich seeds are the unique source of cocoa solids and cocoa butter, fundamental raw materials for the chocolate and cosmetic industries (Pires *et al*., 1998).

Cocoa growing in Ghana was first reported in 1815 in the *De Goudkust*, which was published in Amsterdam (Dickson, 1963). Dickson, however, surmises that the crop was growing before 1800, probably around the Dutch settlements near Elmina, but development was prevented by tribal wars, which caused farmers to concentrate on food farming.

The Basel Mission in 1859 introduced cocoa to Akropong Akwapim from Cape Palmas, Liberia. By November, 10 seedlings were growing but only one plant survived to commence cropping in 1865 (Wanner, 1962). Pods and seedlings were distributed to Mission stations at Aburi, Akwapim Mampong and Krobo Odumase as well as to farmers, but it seems unlikely that many plants survived to produce pods, partly because cocoa proved difficult to grow and the local farmers were then not very interested (Lockwood and Gyamfi, 1979).

Tetteh Quarshie, who worked as a labourer on a cocoa plantation in Fernando Po, returned to Ghana in 1878 and brought with him a number of pods, and established a farm at Akwapim Mampong. Tetteh Quarshie's farm was successful and in 1883 he began to sell all his pods to other locals or use them to raise seedlings, so it was until late 1885 that he prepared the first cocoa for export. The introduction from Fernando Po was mainly of the West African Amelonado type, but it also included *Calabacillo* material (Auchinleck and Knowles, 1962).

In 1887, the Governor of the Gold Coast, Sir William Branford Griffiths, brought Amelonado seedlings from Sao Tome to establish a plot at Aburi for distribution of cocoa seedlings to local chiefs and the Basel Missions. Dickson (1963) argues that Governor Griffiths' introduction was the most important source of seeds in the development of Ghana's cocoa industry, but most authorities agree that Tetteh Quarshie's great contribution was to popularize cocoa growing.

In 1889, the Basel Mission at Begoro again imported pods from Cameroon and were successful in raising a number of seedlings (Wanner, 1962). In 1900 the Aburi gardens received 12 plants of the 'Pentagona' type in October and two plants each of 'Red Forastero' and 'Criollo' from the Botany Department, Jamaica, from Kew (Johnson, 1901). The Pentagona cocoa was not officially released from Aburi because of low yields and high losses due to pests and diseases. However, many pods were stolen from the Pentagona trees and were the source of the Pentagona types later found on farms (Lockwood and Gyamfi, 1979). There were subsequent introductions from Venezuela and Trinidad to the Aburi botanic gardens through to 1909 (Lockwood and Gyamfi, 1979).

In 1943, Posnette made an extensive collection of most of the important Amelonados and local Trinitarios from farmers' fields in Ghana and established them at Tafo to serve as a genebank for cocoa breeders and researchers. Some of these selections formed parents of the first hybrid cocoa developed at Cocoa Research Institute of Ghana (CRIG), the Series II hybrids (Posnette, 1951). The most significant introduction after these was Posnette's introduction of Upper Amazon cocoa from Trinidad in 1944. This introduction was meant to broaden the genetic variability in cocoa germplasm used in breeding (Adomako and Adu-Ampomah, 2000). Lockwood and Gyamfi (1979) report that Posnette used the letter 'T' as a code for the materials he introduced from Trinidad followed by a stand number in the field. Most of the seedlings were established at Tafo and 121 accessions were identified (T1 – T121).

This introduction has made significant contribution to the development of modern day varieties not only in Ghana but also in West Africa and to some extent in other cocoa growing areas of the world. In recent years, through a British research team and efforts at CRIG, some introductions have further been made, the bulk of which is also Upper Amazon in origin. Most of these introductions have been established at CRIG and have contributed to the planting material currently in some farmers' fields in Ghana.

Cocoa diseases

Diseases are among the most important limitations to cocoa production in Ghana. When left uncontrolled, cocoa diseases alone lead to extremely high crop losses (Domfeh *et al.*, 2008). It is thought that not the best varieties of *T. cacao* have been planted worldwide; and quality may have taken second place to quantity in the choice of tree, and the monocultures have laid themselves open to devastating fungal pathogens (Wood and Lass, 1985).

The three most important and damaging cocoa diseases are black pod rot (caused by *Phytophthora* sp.), which occurs worldwide and has the largest impact (global yield loss of 20 to 30% and tree deaths of 10% annually), and frosty pod rot (*Moniliophtora roreri*) and witches broom (*M. perniciosa*, formerly *Crinipellis perniciosa*) that are restricted to tropical America (Franzen and Mulder, 2007), which account for about 5% and 30% of total annual losses, respectively. Other diseases of cocoa include cocoa swollen shoot virus, Dieback and *Ceratocystis* wilt – *Ceratocystis fimbriata* (Dand, 1997).

Pests of cocoa include insects, small mammals and birds. There are 65,000 kinds of insect pests, with relatively small number causing economic effect on cocoa. Damage to cocoa through insect falls into two main categories: those that directly attack the tree and those that transmit or through which pathogens are established. Those that cause direct or primary damage to cocoa are the *Distantiella theobroma* and *Salbergella singularis* (marids) or *Helopeltis* sp. and *Pseudodoniella* sp. (capsids) and *Conopomorpha cramerella*. Mealy-bugs and *Xyleborus* beetle cause secondary infections (Dand, 1997). Small mammals and birds can also do considerable damage by attacking cocoa pods. Of the mammals, rats, monkeys and civets probably account for most of the destruction. Parrots and woodpeckers also feed on the pods and can damage the crop. Losses from vertebrate pests may account for up to 5-10% of the world crop (Entwistle, 1972) making them one of the major causes of damage to cocoa.

Cocoa resistant varieties developed in Ghana

Cocoa breeding in Ghana began in 1937 with the selection of locally available materials, which were made up of the uniform variety known as 'West African Amelonado' originating from the lower Amazon basin, and non-Amelonado introductions described as local-Trinitario's (Glendinning, 1967). These had poor establishment capabilities, long generation time, and low yields (Adu-Ampomah, 1994) and were susceptible to either Cocoa Swollen Shoot Virus Disease (CSSVD) or black pod disease.

In recent years, CRIG, through its breeding programmes, has developed cocoa hybrids for enhancement of production. Six different groups of hybrids coded 'Series I-VI' have been developed, some with general purposes and others with certain specific purposes. Series I, II and III were developed for general purposes. Among these, the Series II hybrids (Plate 1a) were found to be the most promising in terms of establishment ability and precocity (Glendinning, 1957) with satisfactory bean size and flavour. Subsequently, the series II hybrid was approved for commercial use (Lockwood and Gyamfi, 1979).

Series IV was developed specifically to curb the problem of cocoa swollen shoot virus infection as most of the plants used as parents were observed to show some level of tolerance to infection with swollen shoot virus. Series V and VI were developed purposely for black pod and drought tolerant, respectively. Thus, all the plants used as parents in the series V development were observed to have some resistance to black pod, while the plants used as parents for Series VI were drought tolerant.

Between 1971 and 1991, a series of progeny trials were established in Ghana, using selected Upper Amazon materials as parents. This led to the selection and release to farmers of Inter-Upper Amazon hybrids, which are more resistant to CSSV than the Series II hybrids. The selected Inter-Upper Amazon hybrids also had, at least, the same level of other desirable agronomic characteristics as the Series II hybrids (Thresh *et al*., 1988; Adomako *et al.*, 1999). Since 1986, Inter-Upper Amazon hybrid seeds have also been produced in seed gardens and supplied to farmers as planting materials in Ghana

alongside the Series II hybrids. The Series II hybrids are gradually being replaced with the Inter-Upper Amazon hybrids (Plate 1b).

Plate 1a: Pods of Series II Hybrid cacao variety.

Plate 1b: Inter-Upper Amazon cacao variety.

A breeding programme was again initiated in 1982 to identify better parents for hybrid seed production (Adomako *et al*., 1999). Ten Upper Amazon cocoa selections were evaluated for their general combining ability for yield, pod and bean characteristics, black pod disease incidence and vegetative characteristics. Some outstanding female parents were identified, which included Parinary (Pa) 150, Pa 7 and Pound (P) 7. They were found to be better than the two widely used seed garden female parents T85/799 and T60/887, especially in terms of net yield (Adomako *et al*., 1999b).

All the new hybrids of Upper Amazon origin are characterised by exceptional vigour, with early bearing and high yields. These improvements are largely due to hybrid vigour, which results when crosses are made between plants from two genetically distinct populations (Allen and Lass, 1983). The traditional West African Amelonado variety bears fruit about five years after planting, and attains a maximum yield of 500-1000 kg/ha under good field management. Under similar conditions, the Upper Amazon-derived hybrids bear fruit about two years earlier than the West African Amelonado variety and attains a maximum yield of about 3000 kg/ha (Toxopeus, 1964).

Genetic diversity studies

Study of genetic diversity is the process by which variations among individuals or groups of individuals or a population is analyzed by a specific method or a combination of methods (Mohammadi and Prasanna, 2003). The data often involve numerous measurements and in many cases, combinations of different types of variables. Diverse data sets have been used by researchers

to analyze genetic diversity in crop plants; most important among such data sets are pedigree data (Bernardo, 1993; Messmer *et al.*, 1993; Van Hintum and Haalman, 1994), passport data, (morphological data) (Smith and Smith, 1992; Bar-Hen *et al.*, 1995), biochemical data obtained by analysis of isozymes (Hamrick and Godt, 1997) and storage proteins (Smith *et al*., 1987), and recently, DNA based marker data that allow more reliable differentiation of genotypes (Mohammadi and Prasanna, 2003).

Since each of these data sets provide different types of information, the choice of analytical method depends on the objective of the experiment, the level of resolution required, the resources and technological infrastructure available, as well as the operational and time constraints (Karp *et al.*, 1997). Analysis of genetic diversity in crop is important for crop improvement and provides essential information to enable more efficient use of available genetic resources (Mohammadi and Prasanna, 2003). Additionally, it is a platform for stratified sampling of breeding populations (Mohammadi and Prasanna, 2003) by grouping populations into subgroups with similar genetic characteristics.

Accurate assessment of the levels and patterns of genetic diversity can be invaluable in crop breeding for diverse applications including analysis of genetic variability in cultivars (Smith, 1984; Cox *et al*., 1986), identifying diverse parental combinations to create segregating progenies with maximum genetic variability for further selection (Barrett and Kidwell, 1998), and introgressing desirable genes from diverse germplasm into the available genetic base (Thompson *et al*., 1998). An understanding of genetic relationships among inbred lines or pure lines can be particularly useful in

planning crosses, in assigning lines to specific heterotic groups, and for precise identification with respect to plant varietal protection (Hallauer and Miranda, 1988).

Study of genetic diversity in germplasm collections can facilitate reliable classification of accessions and identification of subsets of core accessions with possible utility for specific breeding purposes (Mohammadi and Prasanna, 2003). Significant emphasis has being paid to comprehensive analysis of genetic diversity in numerous crops including major field crops, such as wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), maize (*Zea mays* L.), barley (*Hordeum vulgare* L.), and soybean (*Glycine max* (L) Merr.). However, cocoa has received very little attention in terms of its genetic diversity.

Cocoa genetic diversity

Genetic diversity refers to the variations at the level of individual genes (polymorphism). It represents all of the genetically determined differences that occur between individuals of a species in the expression of a particular trait or set of traits. Knowledge in genetic diversity in germplasm collection is a prerequisite for successful breeding programmes. Information on the genetic diversity of germplasm collections helps in deciding what sources to cross for making new genetic combinations; it also helps in selecting parents for maximising heterotic responses and identifying populations that should be maintained to preserve maximum genetic diversity in the germplasm (Adu-Ampomah, 1994).

Knowledge in genetic structure of cocoa can help in understanding how the species evolved, and to establish relationship between the great variability of pods and seeds encountered in natural populations. It can also help in determining gene flow; refine breeding strategies involving different populations and direct germplasm collection strategies.

Genetic diversity of cocoa has been studied on samples belonging to different morphogeographic groups. Such studies have distinguished between Criollo and Forastero with Trinitario widespread between them. Thorough investigation into the origin and diversity of the Criollo and Trinitario groups has been done Motamayor *et al*. (2000a and b). The results showed that several morphological Criollo types could be encountered. Studies by Lanaud *et al*. (2000) have indicated a very small proportion of polymorphic loci among the individuals of the Criollo varieties; hardly any molecular differences have been observed, despite the morphotypes observed. However, significant genetic variability has been identified among the Forastero populations originating from South America; from Ecuador to Guyanas (Pound, 1938, 1945; Allen and Lass, 1983). The analysis also showed that the diversity of current Criollo overlapped with that of the Trinitario clones studied.

The search for additional genetic diversity is concentrated on the upper Amazonian basin because the region is considered the center of origin for the species (Cheesman, 1944) and has the highest level of diversity (Laurent *et al*., 1993a, 1994; Figueira *et al*., 1994; N'Goran *et al*., 1994). The efficiency of molecular markers in assessing the organization of genetic variability and

phylogenetic relationships in plant complex-species has already been demonstrated. On the basis of isozymes data, upper Amazonia is considered to be the primary centre of diversity (Lanaud, 1987; Warren, 1994).

Molecular markers

Morphological and molecular markers are the most commonly used genetic markers in genetic diversity studies and linkage map construction in plants (Lalitha, 1999). Morphological markers that have been identified in cocoa include the axil spot gene, the albino seed gene and the crinky leaf dwarf gene (Harland and Frenchville, 1927). However, studies have shown that most morphological markers mask the effects of linked minor genes, making it impossible to identify desirable linked characters (Tanskley *et al*., 1989).

Biochemical and molecular markers employed in genetic diversity studies include protein-based markers (isozymes or allozymes) and DNA-based markers. The DNA-based markers include Restriction Fragment Length Polymorphisms (RFLP), Random Amplified Polymorphic DNA (RAPDs), Amplified Fragment Length Polymorphisms (AFLPs) and microsatellites (Simple Sequence Repeats or SSRs) (Gupta *et al.,* 1999). Environmental effects on isozyme markers make them unsuitable for extensive genetic diversity study.

The RFLP technique is laborious, time consuming, expensive, requires relatively large amounts of uncontaminated DNA and usually involves the use of isotopes (Couch and Fritz, 1990). Moreover, the isotopic labelling of probes

for use in RFLP studies can also be a limiting factor in the utilization of RFLP in developing countries. The main advantage of RAPD marker is the simplicity of the technique and its low cost (Powell *et al*., 1996); but it lacks good reproducibility, which hampers comparison between individual studies. AFLP, though highly effective in detecting DNA polymorphisms, is technically demanding.

Microsatellites are co-dominant in nature, allowing all possible genotypes to be distinguished in any segregating generation and easy to detect via Polymerase Chain Reaction (PCR) microsatellite analysis. They require less DNA template than RFLP and RAPD markers and are, therefore, convenient for genetic analysis on plants. Compared to RFLP and RAPD, microsatellite detects more alleles and a high level of polymorphism and is equally a powerful tool for estimation of heterozygosity (Brown *et al*., 1996). Microsatellites are preferentially used for genetic studies (Jarne and Lagoda, 1996) due to their numerous advantages over the other molecular markers.

In view of these numerous advantages and high efficiency in estimation of genetic variability, mTcCIR microsatellite markers were used in this work.

Justification

Cocoa is the third produce on the world market of raw materials after sugar and coffee. Cocoa is mainly produced on smallholdings, and according to the World Cocoa Foundation (http://www.worldcocoafoundation.org/), 40 to 50 million people depend upon cocoa for their livelihood, worldwide. About three million tons of cocoa are produced annually, from which 70% is contributed by Africa and the demand for cocoa is increasing. This production corresponds to a global market value of $5.1 billion (Lanaud *et al.*, 2008). Black pod is the most serious disease constraint to cocoa production worldwide, especially in West and Central Africa, where some 65% of the world's cocoa is largely cultivated by subsistence farmers (Assoumou, 1997; ICCO, 1998). *P. megakarya* is the most aggressive compared to *P. palmivora* and can cause between 60 and 100% crop loss (Djiekpor *et al*., 1981; Dakwa, 1988).

The continuous progress of *P. megakarya* through the cocoa-producing countries of West Africa has the potential to significantly reduce the world's cocoa production and to impact negatively on the resource-poor farmers, leading to socioeconomic, and possibly political as well as ecological, instability (Rice and Greenberg, 2000).

Chemical control can be effective against fungal diseases but is polluting and often too expensive. Integrated pest management centered on the use of resistant material, coupled with other methods of control (cultural, biological) is probably the best way of combating this pathogen over the long term to ensure sustainable control of the pathogen.

Consequently, disease resistance is the primary trait targeted by cocoa breeders. Sources of resistance have been identified for black pod (Iwaro *et al*., 2006), however, the molecular basis of cocoa resistance genes remain unknown (Lanaud *et al*., 2008). Decoding the cocoa genome will contribute significantly to our understanding of the functional aspect of cocoa resistance.

Identification and propagation of black pod disease genetically resistant trees will reduce the incidence of the disease and cost of fungicides used in controlling the disease. Knowledge of the genetic diversity of these resistant tree crops will indicate sources from which planting materials were obtained by farmers as well as their adaptivity to their environment and diseases. Fingerprints of black pod disease resistant trees will serve as the basis for genetic verification of any resistant varieties identified by farmers and breeders. This diversity result will be incorporated into the improved breeding programs at CRIG, Tafo to obtain seedlings with greater genetic resistance.

Objectives

This study was therefore designed to:

1. determine the level of resistance of farmer-selected and breeder-selected cocoa accessions resistant to black pod disease by leaf disc test;
2. identify and fingerprint black pod disease resistant cocoa trees from CRIG genebank and farmer's farms using microsatellite markers; and
3. determine the genetic diversity among black pod disease-resistant cocoa from the CRIG genebank and black pod-tolerant trees from farmer's farms using microsatellites.

CHAPTER TWO

LITERATURE REVIEW

In this chapter the botany, genetic map and diseases of cocoa have been reviewed. Microsatellites, their production and design as well as nomenclature has also been reviewed with emphasis on their use in cocoa. Detection of resistance to black pod disease by leaf disc test, breeding resistant trees and detection of black pod resistant gene by quantitative trait locus and its incorporation into the marker assisted selection programs has been reviewed.

Botany of cocoa

Theobroma cacao L. is a diploid fruit tree specie (2n=2x=20) with a small genome (380 Mb) (Lanaud *et al*., 1992; Figueira *et al*., 1992), similar to that of rice. *T. cacao* originated from the tropical rainforest of South America and is one of the major cash crops for many tropical countries. The fruits of *T. cacao* (or pods) contain 20 to 40 beans which are used to produce chocolate and cocoa butter after a post harvest treatment including fermentation, drying and torrefaction steps. The cocoa tree (Plate 2a) belongs to the genus *Theobroma* (family Sterculiaceae, order Malvales), a group of small trees that grew in the wild in the Amazon Basin and other tropical areas of South and Central-America, from 15º South to 18º North. There are twenty-two species

in the genus with some economic values, of which four species are cultivated: *T. cacao, T. grandiflorum* (willd. ex Spreng) Schum., *T. bicolour* Humb. and Bonpl., and *T. augustifolium* Mociño and Sessé. Only *T. cacao* is used for chocolate production (Cuatrecasas, 1964). *Theombroma grandiflorum,* known as *cupussu* (or cupuacu), is cultivated for production of sweet beverages, ice creams, confections and *cupulate* obtained from fermented seeds (Alves and Figueria, 2002). *Theobroma* literally means *food of the gods*, referring to the cultural and religious status of cocoa.

Plate 2a: Cocoa tree showing growth habit and pods.

The natural habitat of *Theobroma* is the lower canopy of the evergreen rain forest, hence it is cultivated as shade crop (shade canopies). In this habitat rainfall is heavy (1500 to 2000 mm per year is required for the cocoa tree), the temperature is relatively uniform throughout the year (the mean minimum and maximum temperatures are 18 to 21ºC and 30 to 32ºC, respectively), with a constant high humidity (100% during the day, falling to 70 to 80% during the night) during the wet and dry seasons (the dry season should preferably be shorter than three months and not totally dry), and the shade is dense. The cocoa tree can grow in a wide range of soil types, with good physical structure and reasonable quantity of nutrients and organic matter in the top layer, good water retention properties and drainage, and a pH of 5.0 to 7.5 (Wood and Lass, 1985; Fowler *et al*., 1998).

The cocoa fruit, commonly known as a pod, contains seeds which are embedded in mucilaginous pulp. This mucilage contains a germination inhibitor which delays germination inside the pod (Wood and Lass, 1989). However, once the pod is opened the mucilage decomposes rapidly and germination begins since the seed has no dormant period. The flowers are borne on the trunk (Plate 2b) and branches, a habit referred to as cauliflory or truncate. The flowers are only produced on wood of a certain minimum physiological age, usually two or three years old under favourable growing conditions. Flowers are borne on long pedicels and have five free sepals, five free petals, ten stamens and ovary of five united carpels. The ten stamens which form the androecium of the flower are in two whorls, the outer whorl consists of five long non-fertile staminodes, while the inner whorl has five

fertile stamens. The stamens bear two anthers which lie in the pouch of the corresponding petal. The ovary has five parts containing many ovules arranged around a central axis. The flowers are generally pink with darker tissue in the staminodes and petals, but there is a considerable variation between cultivars in the size and colour of the flowers (Wood and Lass, 1989).

Plate 2b: Cocoa trunk with flowers.

Cocoa trees produce large numbers of flowers at certain times of the year depending on local conditions and genotype. However, only 1-5 percent of the flowers are successfully pollinated to produce pods, although higher proportions have been recorded for the Amelonado type (Posnette and Entwistle, 1957). Pollination is effected by various small insects; the most important of these pollinating insects are midges belonging to several genera

of the family Ceratopogonidae (Wood and Lass, 1989).

Three main genetic types of cocoa have been traditionally recognised: Criollo (Plate 3a), Forastero (Plate 3b), and Trinitario (Plate 3c). The Criollo type is known for the high-quality, nutty-flavored chocolate that it produces. Contrary to Criollo, Forastero cacao generally has high vigour and prolificacy, and is traditionally sub-classified into Upper and Lower Amazonian groups. Forastero cacao constitutes an important genetic type for commercial production; its presence as an ancestor was recognised in aproximatyely 80% of the world plantations (Cheesman, 1944). The Trinitario genetic group is generally considered as a hybrid of Criollo cacaos and lower Amazonian Forastero *Amelonado* obtained by repeated introductions of the latter to the island of Trinidad over the larger part of the century (Pound, 1938; Motamayor *et al*., 2003).

Plate 3a: Pod of Criollo cocoa variety.

Plate 3b: Pod of Forastero cocoa variety

Plate 3c: Pod of Trinitario cocoa variety.

Genetic map of cocoa

Mapping involves putting markers (and genes or QTL) in order, indicating the relative distances among them, and assigning them to their linkage groups on the basis of their recombination values from all pairwise combinations (Toure *et al.*, 2000). Genetic mapping is therefore a basic tool of genomic research. Several linkage maps have already been published for *Theobroma cacao* L. based on codominant markers, such as restriction fragment length polymorphisms (RFLPs), isoenzymes and a small number of simple sequence repeats (SSRs) and, in some of the maps, associated with dominant amplified fragment length polymorphisms (AFLP) and random amplified polymorphic DNA (RAPD) markers (Lanaud *et al*., 1995; Crouzillat *et al*., 1996; Risterucci *et al*., 2000). These markers have been used to locate quantitative trait loci (QTLs), such as disease resistance and yield factors (Crouzillat *et al*., 2000; Flament *et al*., 2001; Clement *et al*., 2003; Queiroz *et al*., 2003; Risterucci *et al*., 2003).

Nearly 15 different mapping populations of different sizes have been produced and mapped during the last 10 years, mainly with the objective to map QTLs (Lanaud *et al.*, 2008). Among them, two mapping populations, with a largest number of individuals have been used to constitute high density maps: A progeny of 250 trees, belonging to the cross UPA 402 x UF 676 have been planted in Côte d'Ivoire. UPA 402 is a Forastero genotype which originated from Upper Amazonia in Peru, UF 676 is a Trinitario corresponding to a hybrid between a Criollo and Forastero genotype from Lower Amazonia in Brazil. Part of this population has been used until now to

establish the Centre de Cooperation Internationale en Recherches Agronomique pour le Developpement (CIRAD) cocoa reference map on which all new markers have been successively mapped including AFLP, RFLP, and SSR. This map (783 cM, with an average interval distance between markers of 1.7 cM) included 465 codominant markers, and among them 268 SSR markers (Pugh *et al*., 2004).

The new microsatellite markers are distributed throughout all linkage groups of the map, but their distribution is not random. The length of the map established with only SSRs is 769.6 cM, representing 94.8% of the total map. The current level of genome coverage is approximately one microsatellite every 3 cM. This new reference map provides a set of useful markers that is transferable across different mapping populations and will allow the identification and comparison of the most important regions involved in the variation of the traits of interest and the development of marker-assisted selection strategies (Pugh *et al*., 2004).

A progeny of 1500 trees, planted in Brazil, and corresponding to an F2 population has been established from an F1 hybrid between ICS1 and Scavina 6. ICS 1 is a Trinitario genotype (hybrid between a Criollo genotype and a Forastero genotype from Lower Amazonia in Brazil) and Scavina 6 is a Forastero genotype which originated from Upper Amazonia of Peru. A part of this population (250 individuals) is currently used to map markers defined in the expressed sequence tags collection. A project, currently developed by CIRAD and Centre National de Génotypage (CNG) aims to map a set of about 800 new markers defined in genes having a high similarity with known

function genes (115 SSR and about 700 single nucleotide polymorphism) on the 2 mapping populations mentioned above (Lanaud *et al.,* 2008). A large number of these SNP, identified from an EST collection recently produced correspond to a polymorphism between Criollo and Lower Amazon Forastero. These gene maps will constitute a substantial resource to help the whole genome sequence assembly. If needed, a densification of these maps will be possible by adding other SNP markers defined in the EST collection (Lanaud *et al.,* 2008).

Black pod infection

Black pod or *Phytophthora* pod rot has been the primary fungal disease affecting cacao production worldwide since the 1920's. Overall losses are estimated at approximately 30% of the world production (Renard, 1999), that is, equal to or greater than 900,000 metric tons of cacao beans. Control of this disease has been based on the use of genetic resistance and chemical treatment (Blaha, 1999; Efron *et al.,* 1999; Lawrence, 1978; McGregor, 1982).

Although complete resistance has not been detected, differences in susceptibility among clones or hybrids derived from crosses have been observed in various countries, including Brazil (Rocha and Vello, 1971), Cameroon (Blaha and Lotodé, 1977), and Papua New Guinea (Saul, 1993). Thus, genetic improvement of resistance is possible for this trait, which could be transmitted additively (Ndoumbé *et al.,* 2001; Tan and Tan, 1990), with narrow-sense heritability values equivalent to broad-sense heritability values up to 0.7 (Cilas *et al.,* 1998).

Four main species of *Phytophthora* are known to cause black pod disease, and these vary in both their aggressiveness and the level of crop loss. *Phytophthora megakarya* is the most aggressive. In contrast, *P. palmivora* is less aggressive and can cause crop losses of 4·9–19% (Blencowe and Wharton, 1961; Dakwa, 1984). However, it is also more aggressive than *P. capsici* (Lawrence *et al*., 1982). *Phytophthora citrophthora* is more aggressive than *P. palmivora* or *P. capsici*, and requires less time for zoospore germination and penetration on unwounded, detached pods (Campêlo *et al.*, 1982).

P. palmivora has a world-wide distribution on cocoa; *P. megakarya* is confined to several countries of West Africa, while *P.capsici* is confined to South America, Central America and the West Indies. Another species, *P. citrophthora*, has been identified from cocoa in Bahia, Brazil. *P. citrophthora* is more virulent on unwounded, detached pods than *P. palmivora* or *P. capsici*, but is relatively rare (Wood and Lass, 1985).

Phytophthora species have historically been delimited by their morphology, cytology and biochemistry (Sansome *et al.*, 1975; Brasier and Griffin, 1979; Kaosiri and Zentmyer, 1980; Hardham *et al.*, 1986; Appiah *et al.*, 2003). There are, however, difficulties in using phenotypic taxonomic characters, in that some characters overlap between species, and significant variation exists among isolates of the same species (Brasier and Griffin, 1979; Erwin and Ribeiro, 1996; Appiah, 2001; Appiah *et al.*, 2003). Molecular approaches can provide reliable methods for pathogen identification and disease diagnosis, and the technology available has sufficient sensitivity to

enable detection of variation between organisms at the level of a single base change (Schlick *et al.,* 1994).

There are differences in the symptoms caused by the four *Phytophthora* species: the initial symptom is the appearance of a small translucent spot on the pod surface and is the same for all species (Opoku *et al*., 2007). This spot soon turns to a chocolate brown colour, then darkens and expands rapidly with a slightly irregular margin. This margin can advance at an average of 12 mm in 24 hours. *P. palmivora* does not usually produce a bloom of sporangia. Since *P.megakarya* is mainly a soil borne pathogen, most of the soil particles carried by ants, tend to be contaminated with the propagules of the fungus. It is therefore very common to see black pod infections associated with ants tents (Plate 4) (Opoku, 2004). Chemical control of black pod by spraying with copper fungicide is a well-established control method. Cultural techniques, such as shade reduction, regular harvesting and frequent weed control may reduce infection, but some losses from black pod are almost inevitable if the area is subject to long periods of high humidity (Opoku *et al*., 2007).

Plate 4: *P. megakarya* black pod associated with ant tent (proximal infection)

Infection of other tissues can also occur but they do not normally cause severe economic loss. However, damage depends not only on the species of *Phytophthora* but also on the environmental conditions under which the trees are growing as well as the climatic conditions during the fruit-producing period. The major economic loss is from infection of the pod. Pods can be infected at any age, but most significant economic loss arises from infection during the two months prior to ripening. Pods infected (Plate 5) at this stage can be a total loss because the fungus can easily pass from the pod husk to the seed-coat of the bean in a developing green pod.

Plate 5: Black pod infested cocoa pods on a tree.

Studies on cocoa tissue susceptibility have shown that the physiological stage of the fruit has an effect on the success of infection (Blaha and Lotodé, 1976). The duration of pod ripening may also be important in disease expression in the field, since the time pods are exposed to the pathogen is variable (Berry and Cilas, 1994a). The field incidence of the disease is influenced by environmental factors. Rainfall provides favourable conditions for successful infection by the pathogen, since it results in high humidity and low temperature. Rainfall has been reported to cause increase in black pod attacks in farmers plots in different cocoa growing areas of Cameroon (Ndoumbé, 2002).

It has also been shown that the more pods a tree bears, the more susceptible it is to black pod disease (Berry and Cilas, 1994b). Rain splash (primary inoculum) has been shown to be responsible for infection on pods that grow near the soil surface (Meideros, 1976). Trajectory splash, for example, can disperse the spores up to a distance of 1.5–2 m, whereas wind-blown droplets can disperse the spores up to 12 m, but at lower frequencies (Gregory and Maddison, 1981).

Two types of resistance in pods have been reported; one to the penetration of the pod by the pathogen and the other to the growth of the pathogen after penetration (Risterucci *et al.,* 2003). Resistance to *Phytophthora* exhibits a continuum of phenotypic variations in the species *Theobroma cacao* suggesting that several genes are involved (Risterucci *et al.,* 2003). However, among all the germplasm analysed, no tree has ever been found to be completely resistant in the field. A polygenic control of resistance has already been suggested (Blaha and Lotodé, 1976; Enriquez and Soria 1996) and field resistance seems to be additive (Tan and Tan 1990; Cilas *et al*., 1998). An artificial inoculation tests by Blaha and Lotodé (1976) classified one hundred clones according to their level of resistance.

A method based on inoculation made on leaves was developed by Nyassé (1997) and a significant positive correlation between leaf-test data and pod-rot rate in the field was observed. The resistance trait heritability and the validity of leaf inoculation test for *P. megakarya* studied in a diallel crossing scheme in Cameroon (Ndoumbé *et al*., 2001; Nyassé *et al*., 2002), suggested that the leaf test could be used as an early predictor of resistance in cacao in

the field. Leaf tests have also been used for quantitative traits analyses for resistance on cacao (Flament *et al*., 2001) and sugar beet (Setiawan *et al*., 2000).

Black pod resistance

In Ghana, heritability of single plant yield and incidence of black pod disease in cocoa has been studied in three long-term clone trials (Lockwood *et al.*, 2007). The low heritability of single plant yield, which has been known for 80 years, has been widely overlooked in cocoa research and extension, compromising the success of clone selection programmes (Lockwood *et al.*, 2007). Broad sense heritabilities of 20, 18 and 15 entries were estimated. They were 0.15, 0.05 and 0.15 for yield in pods per plant, and 0.26, 0.19 and 0.40 for incidence of 'bad' pods, mostly due to black pod disease, caused by *Phytophthora* sp. The heritability of the incidence of black pod disease is high enough to justify mass selection where family level data are not available. This finding currently is being applied on a large-scale programme in Ghana to select clones that are high-yielding in the presence of *P. megakarya* (Lockwood *et al.*, 2007).

During the 1990s, resistance to *Phytophthora* pod rot (Ppr) received considerable attention (Efombagn, 2005). An early screening test based on leaf disc inoculations was developed (Nyassé *et al*., 1995) that proved to be correlated with field level of infection (Tahi *et al*., 2000). Potential sources of resistance to *P. megakarya* were detected in accessions selected in farmers' field in Cameroon, based on farmers' information and on the leaf disc test.

The results showed that farmers' knowledge is a useful input in cocoa breeding programmes. Therefore, further exchange of Ppr resistant genotypes among cocoa collections in different countries should be beneficial for cocoa breeding and should be encouraged (Efombagn, 2005).

Opoku *et al.* (2004) in the development of resistant varieties and search for biochemical agent(s) for the control of *Phytophthora megakarya* determined the resistance of some apparently black pod resistant trees identified on farmers farms using T60 as a control with resistant mean score of 1.15. The result of this study suggested most of the clones from farmers farms were potential sources of black pod resistant materials. They indicated that crosses between the selected trees and further screening will be done on field plantings and clones and progenies with greater resistance to black pod will be selected for the seed gardens and/or distributed to farmers.

Microsatellites (SSRs)

Microsatellites (SSRs) are present in the majority of eukaryotic genomes and consist of simple, short tandemly repeated di- to penta-nucleotide sequence motifs (Beckman and Soller, 1990). The allelic variation in microsatellite loci can easily be detected by PCR using specific flanking primers. Polymorphism based on variation in the number of repeated motifs is probably due to slippage during DNA replication or unequal crossing-over (Levinson and Gutman, 1987). Microsatellites have been widely used in many crop species due to their abundance, high degree of polymorphism, locus specificity, reproducibility, low amount of DNA required, suitability for

multiplexing on automated systems and, above all, their codominant mode of inheritance (Pugh *et al.,* 2004). These characteristics make SSRs an attractive option for increasing the density of the cacao linkage map.

Microsatellite loci are fairly evenly distributed along the linkage groups and no report on any significant clustering of these markers is available (Pugh *et al.,* 2004). Data on physical mapping of microsatellites on group chromosomes using deletion stocks also confirm that the microsatellites are not physically clustered in specific regions of the chromosomes (Roder *et al*., 1998a), so that the microsatellite markers should prove useful for complete coverage of the genome (Roder *et al*., 1998a). The availability of extensive molecular maps of microsatellites open new avenues for tagging genes of economic importance, not only for marker-assisted selection, but also for cloning genes leading to the development of transgenic plants for crop improvement (Pugh *et al.,* 2004).

Production of microsatellite markers and primer design

Several procedures have been developed to produce SSR-enriched gDNA libraries. Billote *et al*. (1999) described an easy method for developing microsatellite markers in tropical crops that has subsequently been used with success (Billote *et al*., 2001; Aranzana *et al*., 2002; Dirlewanger *et al*., 2002). A genomic library enriched for (GA)n and (CA)n has been constructed from the cacao (*Theobroma cacao* L.) clone Catongo based on the procedure described (Billote *et al*., 1999). The main criteria for primer design were to produce well-matched primers that were 16–24 nucleotides long, had an

average GC content ranging between 40% and 50% and an annealing temperature between 45^{o}C and 55^{o}C and were preferably G- or C- rich at the 3' end.

Marker nomenclature

All loci are designated according to the nomenclature guidelines presented by Risterucci *et al*. (2000) and Lanaud *et al*. (2004). SSRs were denoted as mTcCIRX where m corresponds to microsatellite, Tc to *Theobroma cacao*, CIR to CIRAD and X to the microsatellite number.

Cocoa and microsatellites

Microsatellites have been applied in studies of DNA fingerprinting, genetic diversity, variety characterization and genetic mapping in cocoa (Charters and Wilkinson, 2000; Faleiro *et al*., 2004; Pugh *et al*., 2004; Saunders *et al*., 2004). Lanaud *et al*. (1999) developed the first group of simple sequence repeat (SSR) markers for *T. cacao*. More recently, Pugh *et al*. (2004) developed 387 new SSR markers for this species. However, all these SSR loci were isolated using dinucleotide probes during the screening of the genomic library. Consequently, SSR loci consisting of repeats of tri- and tetra-nucleotides remain to be searched in the *T. cacao* genome.

The genetic diversity and natural population structure of *Theobroma cacao* from the Brazilian Amazon has also been evaluated by microsatellite markers (Maria *et al.*, 2006). From 19 microsatellite loci tested, 11 amplified scorable products were observed, revealing a total of 49 alleles, including two

monomorphic loci. The Brazilian Upper Amazon population contained the largest genetic diversity, with the most polymorphic loci, the highest observed heterozygosity; and the majority of rare alleles, indicating that this region might be considered part of the centre of diversity of the species. The observed heterozygosity for all the Brazilian populations (H_o = 0.347) was comparable with values reported for other similar Upper Amazon Forastero cacao populations (Maria *et al.,* 2006). The Lower Amazon populations, traditionally defined as highly homozygous, had an unexpectedly high observed heterozygosity (H_o = 0.372), revealing rare and distinct alleles, with large identity with the Upper Amazon population (Maria *et al.,* 2006).

Thus, it was argued that part of the Lower Amazon population might derive from successive natural or intentional introductions of planting material from other provenances, mainly Upper Amazon. Maria *et al.* (2006) reported most of the loci exhibited a lower observed heterozygosity than expected, suggesting that self-pollination might be more common than usually assumed in cacao, but excess of homozygotes might also derive from sub-grouping (Wahlund effect) or from sampling related individuals. Most of the gene diversity was found to occur within groups, with small differentiation between the four Brazilian Amazon populations, typical of species with high gene flow.

Genetic diversity in cocoa germplasm of Southern Cameroon has been revealed by SSR markers (Efombagn *et al*., 2006). The range of polymorphism of 194 cocoa accessions collected in farms in Southern Cameroon during field surveys and 71 Trinitario and Upper Amazon clones available in genebanks on-station was assessed using 13 SSR markers.

Gene diversity, genetic differentiation and genetic similarities were analysed for the different populations. In total, 282 alleles were detected within all the populations studied. The farm accessions were highly differentiated based on their geographical origin, with accessions coming from the east province clustering together with local Trinitario accessions from the genebank.

Accessions from the Centre-South provinces clustered with Amazon and hybrid accessions, suggesting more uptake of seed garden materials in farms in these provinces. Genetic diversity parameters indicated that the farmers' planting material was not highly diverse, and was genetically close to parental genotypes available in genebanks. However, some promising Upper Amazon clones ('T' clones) used as parents of released hybrid varieties were genetically distant from the accessions. This result suggests that the progenies of these parents have so far been poorly used in the cocoa farms surveyed.

A great deal of research effort has been expended to the development of new genetically modified cocoa plants with improved productivity, and resistance, and beans of good industrial quality. Ioná *et al*. (2007) have also reported the development and characterization of novel tetra-, tri- and di-nucleotide microsatellite markers in cocoa. The availability of suitable genetic markers is important for the efficient selection and breeding of perennial species, such as cocoa.

Ioná *et al*. (2007) have described the development of 123 microsatellite loci of cocoa, and used an optimized protocol to construct and screen a microsatellite-enriched genomic library from which they isolated 64 di-nucleotide, 45 trinucleotide and 14 tetra-nucleotide microsatellite loci.

The primers were tested on samples from five different *T. cacao* accessions, one accession each from *T. grandiflorum* and *Herranea* sp. Among the 123 loci, 54 were polymorphic, 61 were monomorphic and eight had no amplification products. Such markers will be useful in future studies since they would increase the accuracy of genotypic assessments in diverse cocoa tree populations as well as in other species of the *Theobroma* genus.

Genetic diversity in cocoa germplasm from Ghana, using microsatellite markers, has also been reported (Opoku *et al.*, 2007). Two hundred and thirty five trees representing all cocoa growing regions in Ghana were sampled from farmers' fields, 104 trees from breeders' seed gardens and 38 parental clones (CRIG's collections) used in producing the bi-parental crosses were screened with a set of 17 mapped microsatellite markers. Average gene diversity was high in all populations, with mean heterozygosity of 0.738. The highest genetic diversity was recorded for the accessions from breeders' and parental collections. However, genetic diversity in the farmers' collections was comparable. Despite the low level of differentiation (Fst = 0.076) found across all three populations, sufficient genetic differences existed between them, separating breeders' collection from farmers' collection (Opoku *et al.*, 2007).

Microsatellite markers were used as a diagnostic tool to detect and label off-types from cocoa breeding evaluation at Tropical Agricultural Research and Higher Education Center (CATIE), Costa Rica (Takrama *et al.*, 2005). Using 24 microsatellites, the genetic identity of parental trees and progeny was determined by capillary electrophoresis. The microsatellites that detected differences between multiple trees of a parental clone were then used

to fingerprint progeny made with off-type parental trees. The analysis resulted in the identification of two type of UF 273 parental clones involved in nine crosses. Among 285 offsprings, 149 plants or 52.3% were identified as the offsprings of type II (off-type) of UF 273. This finding shows that genotyping parental stock before proceeding on large-scale breeding programme is an essential step.

Resistance breeding

Breeding for resistant genotype appears to be a plausible alternative to the control of the menace pest and diseases pose to cocoa cultivation in Ghana. To be able to breed for resistance to any disease in plants, there is the need for the understanding of the genetics of the host plant resistance to the disease, presence of populations which have variation in the trait concerned (germplasm) and development of reliable screening techniques. Recent breeding approaches at CRIG include mutation breeding, reciprocal recurrent selection (RRS) programme, breeding for resistance to vectors and marker-assisted selection (Adu-Ampomah *et al.,* 2002).

Cocoa germplasm in Ghana has been screened for resistance; and resistant varieties developed to withstand the cocoa swollen shoot virus disease (Adu-Ampomah *et al.,* 2002), whiles resistant trees to blackpod disease have also been identified and currently undergoing field test at CRIG (data unpublished). However, conventional or formal plant breeding programmes conducted in developing countries have been criticized for ignoring indigenous germplasm and failing to breed for adaptation to

conditions faced by small-scale farmers (Atlin *et al*., 2001).

Black pod quantitative trait locus

A QTL is the location of a gene that affects a trait that is measured on a quantitative (linear) scale. QTLs are identified via statistical procedures that integrate genotypic and phenotypic data (Toure *et al.,* 2000). QTLs are assigned to chromosome locations based on the positions of markers on a linkage map (Risterucci *et al.,* 2003).

Regions of the genome involved in yield, vigor, and resistance to *Phytophthora palmivora* in *Theobroma cacao* have been identified for QTL mapping (Clement *et al.,* 2003). Three heterozygous clones, one Upper Amazon Forastero (IMC 78) and two Trinitario (DR 1 and S 52), were crossed with the same male parent, a Lower Amazon Forastero (Catongo), known to be highly homozygous. Observations were made on progeny over nine consecutive years. One to three QTL related to yield were detected in each of the three populations, located on chromosomes 1, 2, 4, 5, 9, and 10.

They explained between 8.1 and 19.3% of the phenotypic variation and showed various levels of repeatability. In IMC 78, the QTL detected on chromosome 5 was the most repeatable over years. The QTL for the average individual pod weight on chromosome 4 was the most significant with a logarithm of the odds ratio (LOD) of 17.3 and an R 2 of 43.7. QTL related to these traits have been identified in the same region of the genome in clones of different genetic groups.

Marker-assisted selection

Molecular tags, a prerequisite for marker-assisted selection (MAS), have been developed for many agronomic traits in several crop plants using different kinds of molecular markers. The essential requirements for MAS in a plant breeding programme are: (1) marker(s) should co-segregate or be closely linked (1 cM or less) with the desired trait; (2) an efficient means of screening large populations for the molecular markers should be available; and (3) the screening technique should have high reproducibility across laboratories, be economical to use and should be user friendly. Molecular markers are especially advantageous for agronomic traits that are otherwise difficult to score. Molecular marker studies using near isogenic lines (NILs) (Muehlbauer *et al*., 1988; Young *et al*., 1988; Martin *et al*., 1991), recombinant inbred lines (RILs) (Mohan *et al*., 1994) or bulked segregant analyses (BSA) (Michelmore *et al*., 1991) have accelerated the mapping of many genes in different plant species.

Comparing the advantages and disadvantages associated with the use of different marker systems one may choose a suitable marker technology, for a specific purpose, such as plant breeding, DNA fingerprinting, genetic diversity analysis and comparative mapping. It should be recognized that different marker systems may be suitable for different purposes. However, the very high cost of their development restricts their use in many laboratories. In addition, the locus specificity and high level of polymorphism associated with microsatellites make them the marker system of choice for molecular marker-aided MAS election in practical plant breeding (Pugh *et al.,* 2004).

CHAPTER THREE

MATERIALS AND METHODS

The methods used to collect physiological and molecular data for this work are described in this chapter.

Plant material

A total of 205 cocoa accessions were used in this work. These comprised 117 trees from selected cocoa farms in the Jukwa District of the Central Region and 88 from the CRIG genebank at Bechem in the Brong Ahafo Region (Fig.1). These were regrouped into five populations (Table 1). Leaf samples were collected for all accessions from healthy branches located between the green and semi-green parts for resistance test and DNA analysis. The cocoa trees selected from farmers' farms were identified by farmers as their best trees (Plate 6) based on productivity, low incidence of black pod, medium tree vigour and absence of CSSVD. A few susceptible trees were also selected and used as controls.

Table 1. Cocoa accessions collected from breeders' and farmers fields.

Germplasm collections	Population	Number of Accessions
Farmers field Collection		
Control (susceptible)	1	20
Series II hybrids	2	47
Amazonia	3	50
Breeder's collections		
'T' Clones	4	48
Progenies	5	40
Total		205

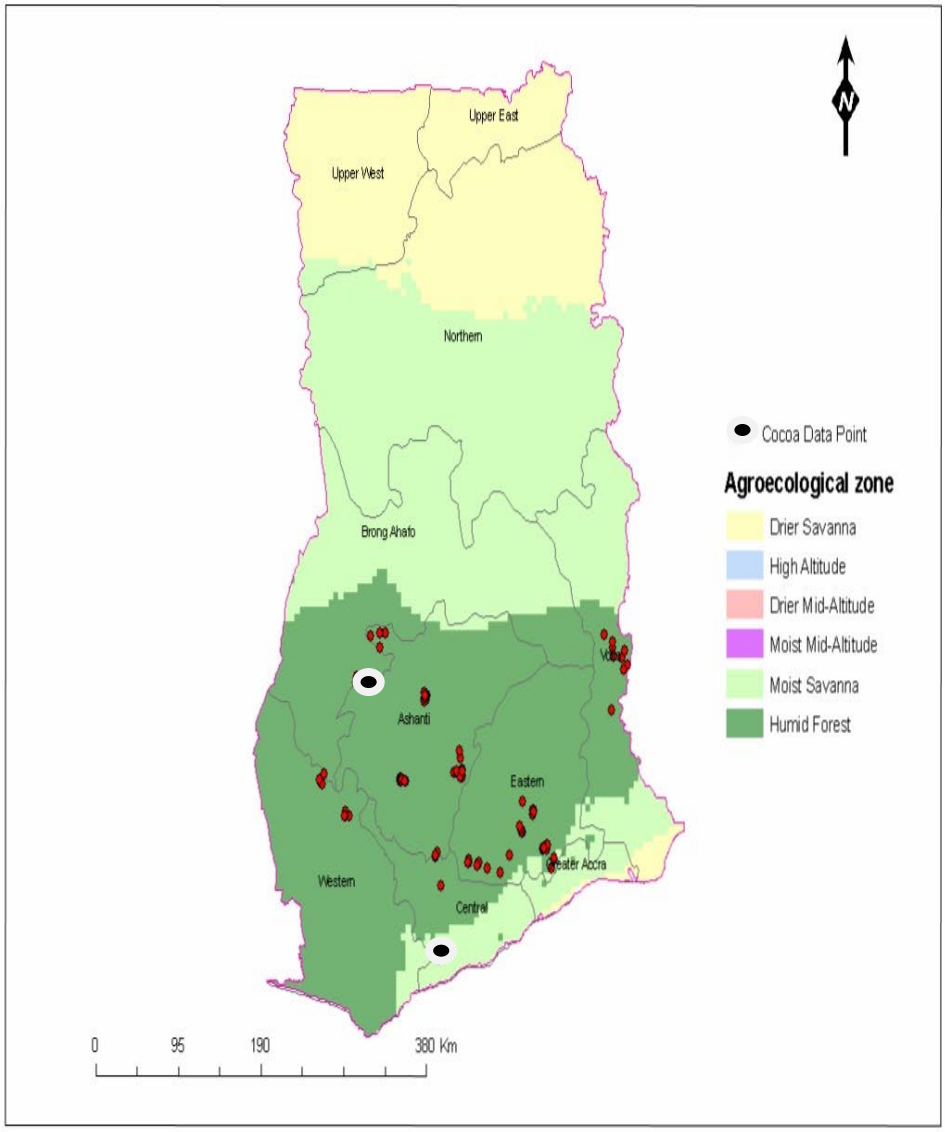

Fig. 1: Map of Ghana showing collection sites of cocoa accessions used for the study .

Plate 6: A farmer's best tree.

Evaluation of resistance

The level of resistance among clonal materials and progenies (from Bechem) and from farmers' farms (Jukwa) was evaluated by leaf-disc inoculation with zoospores of *Phytophthora megakarya*. There were five replica experiments for each leaf sample. Leaf samples (8-10 leaves) from plagiotropic branches located between green and semi-green parts of the stem or about 2-months-old leaf, of similar age and exposition to sunlight, free of disease and insect damage were harvested.

All leaf samples from selected trees were labelled individually and kept in small transparent perforated polythene bags (without water) and then in bigger transparent polythene bags with 20 ml of tap water to keep the micro-environment humid. The leaves were kept overnight. Eight to 10 leaf discs were cut with a cork borer of 0.75 cm diameter, kept on filter paper in Petri dishes lined with moistened cotton wool. The leaf discs are then arranged with the underside of the leaf disc upwards in inoculating trays lined with humidified foam. There were 80 discs randomly placed in each of the five trays. Ten microliters of a suspension containing $2x10^5$ zoospores/ml of *P. megakarya* was used to inoculate each leaf disc using a micro-pipette. The inoculation trays are covered and stored at room temperature for five days. The lesions which developed on the leaf discs were scored on the 5^{th} day of incubation on a scale of 0-5.

This scale was based on the increasing size of the necrotic/chlorotic area as follows: 0 - no symptom, 1 - penetration points, 2 - network of points; 3 - weblike patch; 4 - mottled patch; and 5 - true patch or necrosis. A digital

picture (JPEG) of the disc was taken and using Microsoft Office picture manager software, the sizes of the lesions were determined in mega pixels (MP) (Plate 7a and b).

Plate 7a: Leaf disc with inoculants of zoospores of *P .megakarya*

Plate 7b: Leaf disc showing developed lesions

DNA extraction

Genomic DNA was extracted from fresh mature leaves of the selected trees using a modified CTAB-based protocol by Sappal *et al*., (1994) and Russell *et al*., (1992). Five leaf discs each of 1 cm diameter frozen in liquid nitrogen for 15 seconds were ground to fine powder in 1.5 µl eppendof tube.

The powdered tissue was mixed with 500 µl of CTAB extraction buffer [100 mM Tris-HCl (pH 8), 1.4 M NaCl, 20 mM EDTA (pH 9), 2% (w/v) CTAB, 2% (w/v) PVP] and 0.5 µl (0.1% v/v) β-mercaptoethanol, and homogenized by vortexing. The homogenate was incubated at 65^{o}C for 30 min with intermittent vortexing. After cooling to room temperature, equal volume of chloroform–isoamyl alcohol (24:1 v/v) was added followed by several inversions of the tube to ensure complete emulsification. It was then centrifuged at 14000 rpm for 15 min.

The aqueous phase was recovered and the chloroform-isoamyl alcohol (24:1) extraction was repeated. The aqueous supernatant was frozen at –20^{o}C overnight after addition of two-thirds volume of ice-cold isopropanol to precipitated DNA. The DNA was pelleted by centrifuging at 14000 rpm for 5 min followed by washing in 1ml washing buffer (76% ethanol, 10 mM ammonium acetate) and 1 ml 80% ethanol. The pellet was then dried at room temperature and re-suspended in 100 µl of low salt TE buffer [10 mM Tris-HCl (pH 8), 1 mM EDTA].

DNA quantification

Five microliters of the re-suspended DNA is mix with a loading dye (bromophenol blue) in an eppendorf tube. This is loaded into wells of a 0.8% (w/v) DNA agarose gel and electropherised in 1X TAE buffer (40 mM Tris-acetate, 0.5 M EDTA (pH 9) at 100 V for 45 min. The agarose gel is then stained in an ethedium bromide solution for 10min and view on a UV transilluminator. DNA samples were quantified by comparing intensities of bands with 0.1 µg/µl lambda III DNA marker on 0.8% (w/v) DNA grade agarose gel using a UV transilluminator and camera interfaced with Mistubishi Video copy processor (Plate 8). This was followed by DNA dilution to a working concentration of 2.5 ng/µl.

Plate 8: UV transilluminator and camera interfaced with Mistubishi video copy processor.

Polymerase chain reaction (PCR)

Applied Biosystems 2720 Thermal Cycler was used to perform PCR amplification in a 10 µl reaction mixture containing 0.1 µl of 5U *Taq polymerase* (Bioline, UK), 2 µl of 2.5 ng DNA, 0.2 µl of 10 mM dNTPs, 1 µl of 25 mM $MgCl_2$, 1 µl of 10X NH_4 buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8 at 25^oC), 0.1% Tween-20], and 0.5 µl of both forward and reverse 10 µM mTcCIR SSR primers with 4.7 µl of sterile distilled water per reaction mixture. Thirteen mTcCIR SSR primers were used to genotypically characterize the cocoa accessions. The PCR profile included an initial denaturation step at 94^oC for 4 min, 35 repeats of the following amplification cycles: 30 sec at 94^oC, 1 min at 46/51^oC, and 1 min at 72^oC; and a final extension step at 72^oC for 7 min and then put on hold at 4^oC at infinity. The amplified products were stored at -20ºC until they were needed to run gels.

Running of polyacrylamide gel (PAGE)

Ten microliters denaturing buffer [96% (v/v) formamide, 5% bromophenol blue, 5% xylene cyanole 0.5 M EDTA] was added to each PCR reaction product in the 96 well plate. An equal amount of the denaturing buffer was also added to a 10 ul DNA ladder (25 bp Hyperladder V) (Bioline, UK). The samples were denatured at 95^oC for 5 minutes, then kept on ice and 5µl of each sample (DNA ladder in the first well) loaded in each sample well of a 4 mm thick sequencing gel (6% polyacrylamide, 7 M Urea). Gels were run at 100 W for 1.5 hours using a BIO-RAD Sequi-Gen® GT Nucleic Acid Electrophoresis Cell and a power pack (Bio-Rad Pac 3000) and 1x TBE as

running buffer (Plate 10). The products were visualised by Silver staining (Promega, UK) using the method described by Bassam *et al*. (1991).

The gel (attached to the back glass plate using bind-silane) was fixed in 10% glacial acetic acid solution for 30 min, rinsed with deionised water 3 times (2 min for each rinse) prior to staining in silver nitrate solution [1 g/l $Ag(NO_3)$, 3 ml 37% formaldehyde (1.5 ml/l)] also for 30 min. After this, the gel was rinsed in deionsed water for 5 seconds and put in a pre-chilled developing solution containing sodium carbonate (30 g/l), 1.5 ml 37% formaldehyde per litre, sodium thiosulfate (2 mg/l) and observed carefully till the stained bands were visible. The gel was then quickly transferred into a stop solution (10% glacial acetic acid), rinsed in distilled water and dried at room temperature overnight. All steps of the staining procedure were performed on orbital shaker at 50 revolutions per min.

Gel scoring

The PAGE bands were scored by visual inspection on a transilluminator (Plate 11) based on the situation of co-dominance or dominance. The distance travelled by the molecular marker and that of the individual sample bands were measured using a ruler and their relative front (Rf) values calculated using the formula:

$$\mathrm{Rf} = \frac{\text{Distance moved by band}}{\text{Total Distance moved by the dye}}$$

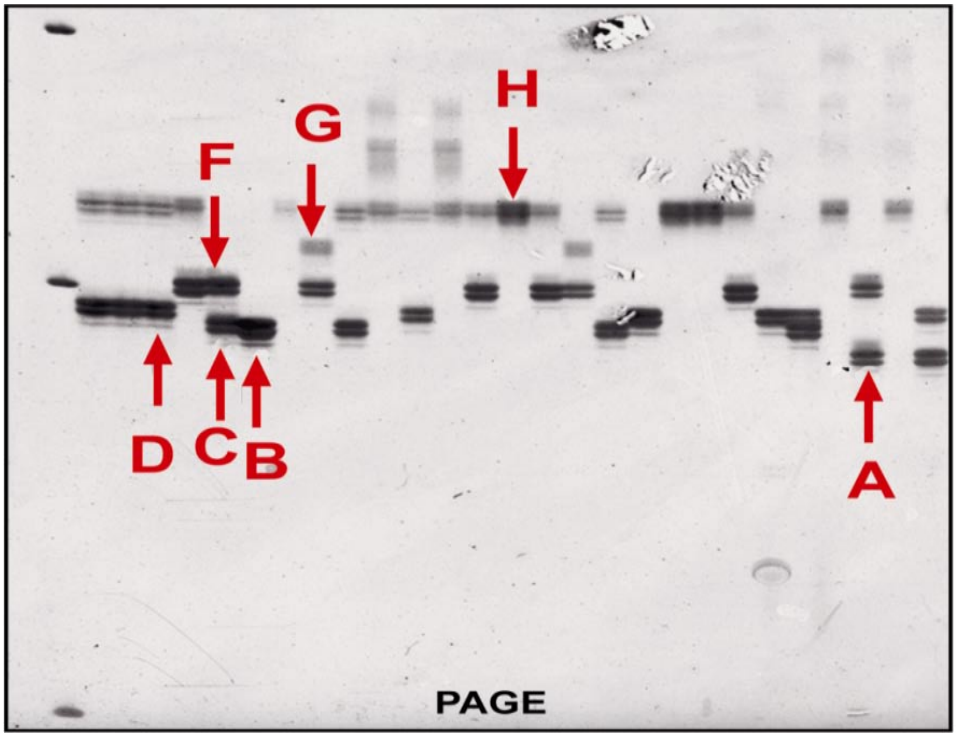

Plate 9: DNA bands showing on 6% acrylamide gel after silver staining

Statistical data analysis

Evaluation of resistance

The data for the five populations were analysed using the MINTAB statistical software 13.32 (Appendix B).

Microsatellite analysis

Genetic diversity

The genetic diversity of the cocoa accessions was studied by using a computer program, FSTAT version 2.9.3.2 developed by Goudet (2002). The following genetic parameters were calculated: Nei's estimation of observed heterozygosity (H_O), the expected heterozygosity (H_E) and total heterozygosity

(H_T). Gene diversity per locus and population (Nei, 1973), genetic distance, allele frequency, and test for Hardy-Weinberg Equilibrium were calculated using Tools for Population Genetic Analysis (TFPGA) version 1.3. Frequency of cross-pollination (C) was also calculated manually using Wrights (1952) frequency of cross pollination formula below:

$$C = \frac{1 - F_{IS}}{1 + F_{IS}}$$

Where, F_{IS} is a measure of heterozygote deficiency of individuals within each population.

Population structure

Wrights fixation indices

If there is no random mating throughout a population, this could be due to the population having structured subpopulations. Wright (1965) developed Fixation Indices to describe the amount of structure within populations. These are F_{IS}, F_{ST} and F_{IT}. The genetic structure within and among populations studied in this work was assessed with Wright's Fixation Indices F_{IS} and F_{ST} (G_{ST}) as described by Weir and Cockerham (1984), using FSTAT version 2.9.3.2 (Goudet, 2002). F_{IS} a measure of heterozygote deficit of individual members within each population is given by:

$$F_{IS} = \frac{H_E - H_O}{H_E}$$

Genetic differentiation

G_{ST} is Wright's coefficient of genetic differentiation among the individual populations making up the entire dataset, which is given by:

$$G_{ST} = \frac{H_T - H_E}{H_T}$$

G_{ST} has been the most commonly used measure of genetic differentiation between populations. It can be thought of as the probability of drawing two alleles that are identical by descent from the same subpopulation. It varies between 0 (no genetic divergence between subpopulations) and 1 (subpopulations fixed for different alleles).

Gene flow

Other parameters calculated were Wrights (1952) estimation of gene flow (N_m) and interpopulation gene diversity (D_{ST}). Wrights estimation of gene flow was calculated using the formula below:

$$N_m = \frac{1 - G_{ST}}{4G_{ST}}$$

Cluster analysis

TFPGA version 1.3 was also employed perform cluster analysis using Unweighted Pair Group Method with Arithmetic Averages (UPGMA) (Nei, 1978). Cluster analysis is used to arrange objects of interest into a branching hierarchy of groups (a tree, or dendrogram) based on how similar or dissimilar the objects are in terms of a number of attributes that are known for each object. Thus, the UPGMA cluster analysis was carried out to reveal similarities between the five populations.

In addition, data was analysed using the PowerMarker version 3.25 and Mega 4 to obtained UPGMA cluster (tree) (Nei and Takezaki, 1983) with all the variables. This revealed the similarities between the individual accessions.

CHAPTER FOUR

RESULTS

In this chapter results from the evaluation of resistance of sample trees are presented. Microsatellite analysis of data, some unique allele sizes and genetic diversity parameters for the molecular data has also been stated.

Analysis of resistance values

Mean lesion size and score for 'T' clones

The result of the leaf disc tests of 'T' clones are presented in Table 2. The lesion scores were generally low and ranged from 0.846 to 1.525 on a scale of 0-5. The results suggested that most of the trees selected have high levels of black pod resistance. Six clones had mean scores of less than 1 (below 0.0380 mega pixels) and no clones had mean score of more than 2 (above 0.1660 mega pixels). Tree with code number T50 had the least lesion score of 0.846 (0.0182 mega pixels) and tree T34 had the highest lesion score of 1.525 (0.1054 mega pixels). The results clearly suggest that the test trees have varying degrees of tolerance.

Mean lesion size and score for progenies

The results of the leaf disc tests on the progenies are presented in Table 3. The lesion scores were similar ranging from 0.900 to 2.430 (0.0252 to

0.2210 mega pixels). The progeny A1/211 x A1/197 had the least lesion score while Na121 x IMC57 had the highest lesion score.

Table 2: Mean lesion size and score of leaf disc of 'T' clones to *P. megakarya*

Clone Code	Mean lesion size (MP)	Mean lesion score	Ranking
T50	0.0182	0.846	1st
T72	0.0209	0.867	2nd
T62	0.0337	0.967	3rd
T26	0.0358	0.983	4th
T54	0.0396	1.013	5th
T57	0.0438	1.046	6th
T59	0.0438	1.046	6th
T40	0.0497	1.092	8th
T6	0.0497	1.092	8th
T55	0.0502	1.096	10th
T17	0.0502	1.096	10th
T14	0.0513	1.104	12th
T69	0.0524	1.113	13th
T4	0.0545	1.129	14th
T37	0.0545	1.129	14th
T46	0.0561	1.142	16th
T30	0.0566	1.146	17th

Table 2: Continued.

Clone Code	Mean lesion Size (MP)	Mean lesion score	Ranking
T20	0.0604	1.175	18th
T14A	0.0630	1.196	19th
T1	0.0636	1.200	20th
T41	0.0646	1.208	21st
T23	0.0668	1.225	22nd
T22	0.0673	1.229	23rd
T19	0.0689	1.242	24th
T29	0.0737	1.279	25th
T12	0.0742	1.283	26th
T3	0.0743	1.284	27th
T28	0.0748	1.288	28th
T48	0.0753	1.292	29th
T15	0.0753	1.292	29th
T18	0.0755	1.296	31st
T27	0.0758	1.304	32nd
T24	0.0769	1.309	33rd
T33	0.0854	1.371	34th
T52	0.0860	1.375	35th
T21	0.0860	1.375	35th
T45	0.0881	1.392	37th
T68	0.0908	1.413	38th

Table 2: Continued.

Clone Code	Mean lesion size (MP)	Mean lesion score	Ranking
T32	0.0918	1.421	39th
T36	0.0924	1.425	40th
T25	0.0940	1.438	41st
T2	0.0961	1.454	42nd
T43	0.0961	1.454	42nd
T14B	0.0962	1.455	44th
T49	0.0972	1.463	45th
T31	0.0982	1.471	46th
T34	0.1041	1.517	47th
T42	0.1054	1.525	48th

Table 3: Mean lesion size and score of leaf disc of progenies to *P. megakarya.*

Code	Mean lesion size (MP)	Mean lesion score	Ranking
A1/211xA1/197	0.0252	0.90	1st
A1/34xA1/154	0.0277	0.92	2nd
Na121xT60/887	0.0316	0.95	3rd
P29xPa150	0.0346	0.97	4th
Na58xT60/887	0.0431	1.04	5th
Na710xT60/887	0.0495	1.09	6th
A1/200xA1/154	0.0610	1.18	7th
A1/154xA1/198	0.0623	1.19	8th
IMC25xIMC57	0.0636	1.20	9th
P2/1BxT60/887	0.0648	1.21	10th
A1/125xA1/154	0.0648	1.21	10th
NJa58xPa150	0.0712	1.26	12th
P2/1BxPa150	0.0725	1.27	13th
A1/197xA1/154	0.0776	1.31	14th
A1/183xA1/198	0.0853	1.37	15th
A1/154xA1/16	0.0866	1.38	16th
A1/197xA1/2	0.0879	1.39	17th
A1/154xA2/34	0.0904	1.41	18th
Pa20xIMC57	0.0994	1.48	19th
A1/197xA1/154	0.1032	1.51	20th

Table 3: Continued.

Code	Mean lesion size (MP)	Mean lesion score	Ranking
T79/501xT85/799	0.1071	1.54	21st
A1/154xA1/2	0.1096	1.56	22nd
A1/4xA1/2	0.1096	1.56	22nd
A1/149xA1/4	0.1109	1.57	24th
A1/154xA1/215	0.1160	1.61	25th
A1/183xA1/154	0.1173	1.62	26th
P1xIMC57	0.1186	1.63	27th
A1/149xA1/154	0.1212	1.65	28th
A1/213xA1/154	0.1212	1.65	28th
A1/2xA1/198	0.1224	1.66	30th
IMC25xPa150	0.1250	1.68	31st
A1/154xA1/4	0.1468	1.85	32nd
A2/34xA1/2	0.1506	1.88	33rd
A1/4xA1/154	01570	1.93	34th
A1/200xA1/2	0.1647	1.99	35th
A1/154xT85/185	0.1685	2.02	36th
RB41xIMC47	0.1736	2.06	37th
Na758xT60/887	0.1826	2.13	38th
Na758xIMC57	0.1864	2.16	39th
Na121xIMC57	0.2210	2.43	40th

Mean lesion size and score for Amazonia

The results of the leaf disc tests of the Amazonia accessions are presented in Table 4. The lesion scores were generally low and ranged from 1.09 to 2.85 (0.0495 to 0.2748 mega pixels) on a scale of 0 to 5. The results suggested that most of the trees selected based on farmers identifications have high levels of black pod resistance. Forty trees had mean scores of less than 2, with 10 trees having mean scores of more than 2. Tree with code number A19 had the least lesion score and tree code A33 had the highest lesion score. The results clearly suggest that the test trees have varying degree of tolerance.

Mean lesion size and score for Series II hybrids

The results of the leaf disc tests on the Series II hybrids are presented in Table 5. The lesion scores ranged from 1.18 to 2.97 (0.0610 to 0.2901 mega pixels). The tree coded H15 had the least lesion score while tree coded H37 had the highest lesion score. These results also suggest that trees selected had varying resistances to black pod infestation. Twenty-six trees had mean scores above 1 with the mean scores of twenty-one above 2.

Mean lesion size and score for control

The results of the leaf disc tests on the susceptible trees (hybrids and Amazonia) used as control are presented in Table 6. The lesion scores ranged from 3.00 to 3.76 (0.2940 to 0.3912 mega pixels). The tree coded C4 had the least lesion score while tree coded C7 had the highest lesion score. These results also suggest that the selected trees had varying resistances to black pod infestation, although identified by farmers as susceptible to the black pod disease.

Table 4: Mean lesion size and score of leaf disc of Amazonia to *P. megakarya.*

Code	Mean lesion Size (MP)	Mean lesion score	Ranking
A19	0.0495	1.09	1st
A21	0.0495	1.09	2nd
A37	0.0597	1.17	3rd
A17	0.0674	1.23	4th
A22	0.0712	1.26	5th
A27	0.0712	1.26	5th
A30	0.0712	1.26	5th
A29	0.0725	1.27	8th
A16	0.0764	1.30	9th
A20	0.0776	1.31	10th
A23	0.0815	1.34	11th
A25	0.0828	1.35	12th
A26	0.0866	1.38	13th
A28	0.0879	1.39	14th
A18	0.0904	1.41	15th
A2	0.0917	1.42	16th
A41	0.0994	1.48	17th
A35	0.1007	1.49	18th
A36	0.1020	1.50	19th
A8	0.1032	1.51	20th

Table 4: Continued.

Clone	Mean lesion size (MP)	Mean lesion score	Ranking
A24	0.1071	1.54	21st
A39	0.1084	1.55	22nd
A13	0.1109	1.57	23rd
A3	0.1148	1.60	24th
A6	0.1186	1.63	25th
A9	0.1250	1.68	26th
A5	0.1263	1.69	27th
A48	0.1276	1.70	28th
A43	0.1314	1.73	29th
A34	0.1327	1.74	30th
A10	0.1352	1.76	31st
A12	0.1365	1.77	32nd
A31	0.1378	1.78	33rd
A44	0.1391	1.79	34th
A14	0.1404	1.80	35th
A11	0.1416	1.81	36th
A32	0.1544	1.91	37th
A7	0.1557	1.92	38th
A42	0.1570	1.93	39th
A1	0.1596	1.95	40th
A45	0.1775	2.09	41st

Table 4: continued.

Clone	Mean lesion size (MP)	Mean lesion score	Ranking
A40	0.1813	2.12	42nd
A38	0.1826	2.13	43rd
A49	0.1826	2.13	43rd
A50	0.1877	2.17	45th
A15	0.2031	2.29	46th
A47	0.2069	2.32	47th
A4	0.2095	2.34	48th
A46	0.2415	2.59	49th
A33	0.2748	2.85	50th

Table 5: Mean lesion size and score of leaf disc of Series II hybrids to *P. megakarya.*

Code	Mean lesion size (MP)	Mean lesion score	Ranking
H15	0.0610	1.18	1st
H8	0.0687	1.24	2nd
H11	0.0751	1.29	3rd
H50	0.0764	1.30	4th
H19	0.0789	1.32	5th
H14	0.0802	1.33	6th
H18	0.0802	1.33	6th
H13	0.0828	1.35	8th
H17	0.0840	1.36	9th
H3	0.0930	1.43	10th
H16	0.0956	1.45	11th
H12	0.0968	1.46	12th
H48	0.1007	1.49	13th
H9	0.1186	1.63	14th
H47	0.1237	1.67	15th
H1	0.1250	1.68	16th
H27	0.1288	1.71	17th
H5	0.1314	1.73	18th
H6	0.1314	1.73	18th
H20	0.1314	1.73	18th

Table 5: Continued.

Code	Mean lesion size (MP)	Mean lesion score	Ranking
H7	0.1352	1.76	21st
H39	0.1491	1.79	22nd
H45	0.1429	1.82	23rd
H2	0.1468	1.85	24th
H49	0.1468	1.85	24th
H25	0.1544	1.91	26th
H21	0.1672	2.01	27th
H30	0.1826	2.13	28th
H4	0.1852	2.15	29th
H33	0.1864	2.16	30th
H36	0.1864	2.16	30th
H40	0.2005	2.27	32nd
H46	0.2133	2.37	33rd
H42	0.2129	2.39	34th
H24	0.2210	2.43	35th
H26	0.2210	2.43	35th
H35	0.2236	2.45	37th
H10	0.2364	2.55	38th
H41	0.2428	2.60	39th
H32	0.2466	2.63	40th
H34	0.2466	2.63	40th

Table 5: Continued.

Code	Mean lesion size (MP)	Mean lesion score	Ranking
H29	0.2517	2.67	42nd
H38	0.2568	2.71	43rd
H31	0.2607	2.74	44th
H43	0.2707	2.82	45th
H44	0.2888	2.96	46th
H37	0.2901	2.97	47th

Table 6: Mean lesion size and score of leaf disc of control (susceptible) to *P. megakarya.*

Code	Mean lesion size (MP)	Mean lesion score	Ranking
C4	0.2940	3.00	1st
C3	0.2978	3.03	2nd
C8	0.3016	3.06	3rd
C1	0.3029	3.07	4th
C9	0.3042	3.08	5th
C10	0.3042	3.08	5th
C13	0.3042	3.08	5th
C2	0.3106	3.13	8th
C12	0.3157	3.17	9th
C19	0.3311	3.29	10th
C20	0.3349	3.32	11th
C11	0.3477	3.42	12th
C17	0.3528	3.46	13th
C14	0.3580	3.50	14th
C16	0.3631	3.54	15th
C15	0.3708	3.60	16th
C6	0.3746	3.63	17th
C18	0.3772	3.65	18th
C7	0.3900	3.75	19th
C5	0.3912	3.76	20th

Microsatellite analysis

All thirteen SSR markers used produced polymorphic bands in all accession with a total of 124 alleles (Table 7). The average number of alleles per locus for the 13 markers was 9.54. The highest number of alleles (13) was detected at the mTcCIR 1 and mTcCIR 3 loci and the lowest number (7) at the mTcCIR 6, mTcCIR 17, mTcCIR 24 and mTcCIR 26 loci. Percentage polymorphism recorded for all SSR was 100%. Plate 12 represents a profile of the microsatellite mTcCIR 15 amplification of cocoa accessions on 6% denaturing polyacrylamide gel showing polymorphic bands.

Table 7: Microsatellite sequences with repeat motifs, number of alleles and percentage polymorphic loci (% PL).

Primer Name	Sequence (5'-3')	Repeat Motif	Number of Alleles	% PL
mTcCIR1	gca ggg cag gct cag tga tgg gca acc aga aaa cga t	(ct)14	13	100
mTcCIR3	cat ccc agt atc tca tcc att ca ctg ctc att tct ttc ata tca	(ct)20(ta)21	13	100
mTcCIR6	ttc cct cta aac tac cct aaa t tta agc aaa gca atc taa cat a	(tg)7(ga)13	7	100
mTcCIR9	acc atg ctt cct cct tca aca ttt ata ccc caa cca	(ct)8n15(ct)5	8	100
mTcCIR12	tct gac ccc aaa cct gta att cca gtt aaa gca cat	(cata)4n18(tg)16	12	100
mTcCIR15	cag ccg cct ctt ggt tag tat ttg gga ttc ttg atg	(tc)19	9	100
mTcCIR 17	aag gat gaa gga tgt aag aga g ccc ata cga gct gtg agt	(gt)7n4(ga)12	7	100
mTcCIR 18	gat agc taa ggg gat tga gga ggt aat tca atc att tga gga ta	(ga)12	10	100
mTcCIR 19	cac aac ccg tgc tga tta gtt gtt gag gtt gtt agg ag	(ct)28	11	100

Table 7: Continued.

Primer Name	Sequence (5'-3')	Repeat Motif	Number of Alleles	% PL
mTcCIR 21	gtc gtt gtt gat gtc ggt ggt gag tgt gtg tgt ttg tct	(tc)11n5(ca)12	8	100
mTcCIR 24	ttt ggg gtg att tct tct ga tct gtc tcg tct ttt ggt ga	(ag)13	7	100
mTcCIR 25	ctt cgt agt gaa tgt agg ag tta ggt agg tag ggt tat ct	(ct)21	12	100
mTcCIR 26	gca ttc atc aat aca ttc gca ctc aaa gtt cat act ac	(tc)9c(ct)4tt	7	100

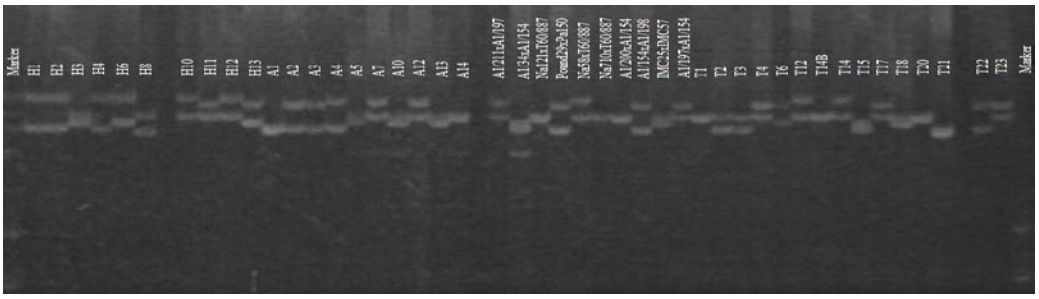

Plate 10: Representative profile of mTcCIR 15 amplification products of cocoa accessions on 6% denaturing polyacrylamide gel showing polymorphic bands.

Fingerprinted alleles revealed across all the 13 SSR markers for the best twenty resistant trees was compared with the control, (susceptible ones), to identify the presence of any unique and rare alleles based on the allele frequency (Table 8). These unique alleles are found only in the best resistant trees and absent in the susceptible ones. Except for markers mTcCIR 9 and mTcCIR 26, which had alleles common to both best trees and susceptible ones, all the other 11 markers recorded some unique alleles in varying numbers. Markers mTcCIR 1, mTcCIR 3, mTcCIR 12, mTcCIR 15 and mTcCIR 18 recorded 3 unique alleles each, mTcCIR 17 and mTcCIR 25 recorded 2 unique alleles each and mTcCIR 6, mTcCIR 19, mTcCIR 21 and mTcCIR 24 recorded an allele each. A total of 23 unique alleles were recorded.

Table 8: Alleles unique to 20 best resistant accessions compared to control.

Primer	Number of unique alleles	allele size (bp)
mTcCIR1	3	123, 142, 144
mTcCIR3	3	229, 242, 247
mTcCIR6	1	246
mTcCIR12	3	187, 199, 211
mTcCIR15	3	238, 246, 252,
mTcCIR17	2	281, 285
mTcCIR18	3	331, 335, 339
mTcCIR19	1	360
mTcCIR21	1	146
mTcCIR24	1	186
mTcCIR25	2	136, 152

Genetic variations among the cocoa populations

Heterozygosity was computed as described by Nei (1978) as shown in Table 9. The observed mean heterozygosity (H_O) and the mean expected heterozygosity (H_E) were 0.625 and 0.656, respectively, whilst the total mean heterozygosity (H_T) was 0.669. A mean value of 0.189 was estimated as the Hardy-Weinberg probability (Haldane, 1954) for five populations across each locus.

The level of genetic diversity within and among the five populations over all the loci was determined by calculating the Nei (1978) gene diversity and heterozygosities per locus per population were also estimated. In Table 10, the average gene diversity within the populations ranged from 0.643 to 0.703. The minimum gene diversity value of 0.643 was recorded for the hybrid population, whilst the highest (0.703) value was recorded for the 'T' clone population. The overall average genetic diversity estimated for the farmer's selection was 0.648, which is less than that for the breeder's collections of 0.667.

Table 9: Measures of genetic diversity within and among cocoa Genetic parameters for SSRs markers.

Locus	H_O	H_E	H_T	D_{ST}	G_{ST}	F_{IS}	N_m	C
mTcCIR 1	0.330	0.522	0.548	0.026	0.047	0.369	5.069	0.460
mTcCIR 3	0.749	0.822	0.845	0.023	0.028	0.088	8.678	0.838
mTcCIR 6	0.537	0.616	0.631	0.014	0.023	0.129	10.619	0.771
mTcCIR 9	0.495	0.530	0.539	0.009	0.016	0.067	15.375	0.874
mTcCIR 12	0.825	0.849	0.865	0.016	0.019	0.028	12.907	0.945
mTcCIR 15	0.757	0.752	0.756	0.004	0.005	-0.006	49.750	1.012
mTcCIR 17	0.350	0.408	0.433	0.025	0.058	0.142	4.060	0.751
mTcCIR 18	0.735	0.799	0.815	0.015	0.019	0.080	12.907	0.851
mTcCIR 19	0.744	0.678	0.686	0.008	0.012	-0.097	20.583	1.214
mTcCIR 21	0.728	0.703	0.713	0.010	0.014	-0.035	17.607	1.072
mTcCIR 24	0.301	0.286	0.290	0.004	0.012	-0.052	20.583	1.109
mTcCIR 25	0.762	0.818	0.821	0.002	0.003	0.069	83.083	0.870
mTcCIR 26	0.812	0.745	0.751	0.006	0.008	-0.090	31.000	1.197
Overall	**0.625**	**0.656**	**0.669**	**0.013**	**0.019**	**0.048**	**22.478**	**0.920**

Ho = observed heterozygosity, H_E = expected heterozygosity, H_T = total parimitic heterozygosity, D_{ST} = interpopulation gene diversity, G_{ST} = coefficient of genetic differentiation, F_{IS} = heterozygote deficit, Nm = gene flow, C = frequency of cross pollination

Table 10: Gene diversity values within each of the five cocoa population at the 13 SSR loci.

Primer	pop1	pop2	pop3	pop4	pop5
mTcCIR 1	0.402	0.595	0.631	0.469	0.516
mTcCIR 3	0.840	0.798	0.817	0.817	0.843
mTcCIR 6	0.541	0.496	0.667	0.723	0.660
mTcCIR 9	0.582	0.394	0.549	0.504	0.625
mTcCIR 12	0.878	0.845	0.828	0.837	0.857
mTcCIR 15	0.804	0.675	0.802	0.716	0.765
mTcCIR 17	0.212	0.447	0.501	0.233	0.651
mTcCIR 18	0.826	0.718	0.818	0.821	0.817
mTcCIR 19	0.604	0.671	0.686	0.700	0.726
mTcCIR 21	0.789	0.723	0.677	0.601	0.727
mTcCIR 24	0.328	0.230	0.267	0.262	0.346
mTcCIR 25	0.796	0.809	0.814	0.804	0.871
mTcCIR 26	0.764	0.765	0.717	0.737	0.741
Mean	**0.643**	**0.628**	**0.674**	**0.632**	**0.703**

Pop 1 = Controls, Pop 2 = Series II hybrids, Pop 3 = Amazonia

Pop 4 = Clones, Pop 5 = Progenies.

Table 11: Matrix of Nei's genetic distances for 5 cocoa populations.

Population	1	2	3	4	5
1	*****				
2	0.0475	*****			
3	0.0731	0.0414	*****		
4	0.0562	0.0486	0.0227	*****	
5	0.0806	0.0556	0.0134	0.0146	*****

1 = Controls, 2 = Series II hybrids, 3 = Amazonia

4 = Clones, 5 = Progenies.

Genetic association among the cocoa populations

Genetic differentiation

There was a low level divergence among the 5 populations as indicated by the coefficient of genetic differentiation (G_{ST} = 0.019). Wright's Fixation Indices (F_{IS}), was 0.048 showing heterozygote deficit in the accessions. The value of the interpopulation gene diversity D_{ST} was 0.013, whereas gene flow (N_m) estimate was 22.5.

Genetic distance and cluster analysis

The genetic distance among populations analysed using UPGMA cluster analysis based on Nei's (1978) unbiased minimum distance identified two main clusters at the 0.0895 dissimilarity coefficient (Fig. 2). The first

group consisted of two populations (Series II hybrids and the control/susceptible). Group 2 consisted of the Upper Amazon accessions; 'T' clones, progenies and farmers selections. Thus the farmers' selections clustered with breeders collections from Bechem. At 0.072, the Series II hybrids separated from the controls. Further down at a dissimilarity of 0.0446, the 'T' clones separated from their progenies.

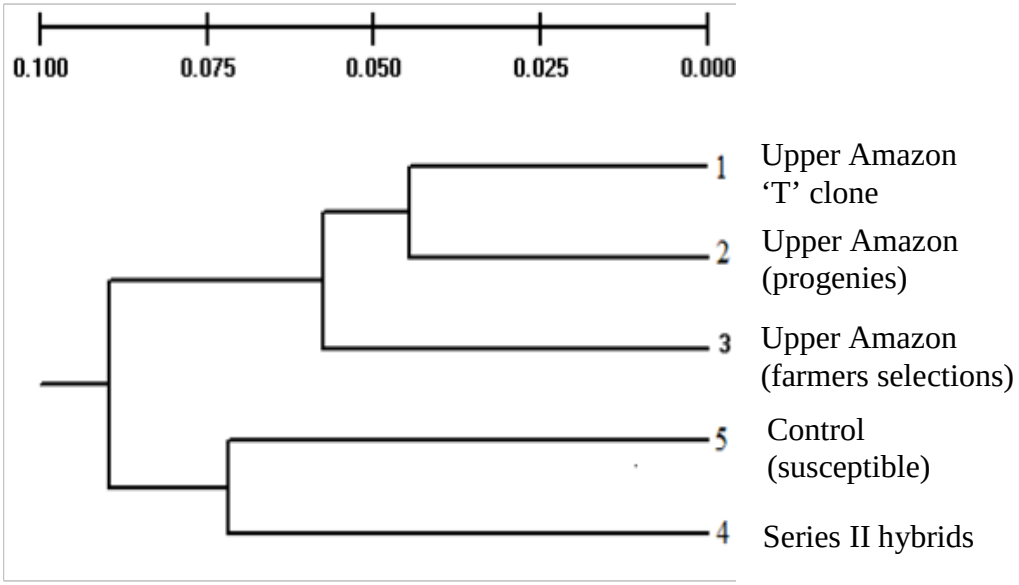

Fig. 2: Dendrogram of dissimilarity coefficient among the five cocoa populations.

The genetic distance among individuals of the population's analysed using UPGMA (Power Marker V3.25) cluster analysis based on Nei's and Takezaki (1983) unbiased minimum distance identified also identified two main clusters (Fig. 3). The Upper Amazon materials clustered into a group (group 2) while the Hybrid and the susceptible controls also form a group (group 1). There was however some individuals scattered within the groups (Fig 3.)

Fig. 3: Dendrogram (tree) of dissimilarity coefficient among the 204 cocoa accessions.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

Discussion

Results from the analyses of resistance and microsatellite data are discussed in this chapter. Conclusions are also drawn and recommendations for further research are suggested.

Analysis of resistance values

Leaf disc scores showed significant ($p<0.05$) differences in susceptibility levels among the five populations. The 'T' clones were the most resistant among the populations with a mean score of 1.24, which is within the resistance range of 0 to 2.50 (Opoku *et al.*, 2004). The current findings confirm earlier reports by Adu-Ampomah *et al*. (2002) that the 'T' clones, which are Upper Amazons in origin, show high levels of resistance to cocoa diseases such as CSSV and black pod. The higher mean score value of 1.51 recorded for the Inter-Upper Amazon hybrids, which are progenies of the 'T' clones indicates that not all resistant traits/genes were passed on from the parents to their offsprings.

Population 3 had the third highest mean score value of 1.68, indicating an overall resistance. However, 4% of the population were susceptible. This could be due to some misidentification or wrong labelling by farmers during selection. This value compared to that of the population 5 (breeder's progenies) shows a mean score difference of 0.17. Farmers' interview for the study indicated that planting materials were collected from among themselves instead of from seed gardens. This might have accounted for the difference observed.

Population 2 (Series II hybrids) had a mean score value of 1.97, which was higher than the mean for the population 3 (1.68). This is because 21.3% of the individuals of the population were scored as susceptible with resistant values in the score range 2.5 to 3.5. The susceptible of one of the parent to black pod disease (Thresh *et al.*, 1988) could explain the higher mean score value for the Series II hybrids. Population 1(susceptible controls) recorded the highest mean score of 3.33, indicating high susceptibility. Sixty five percent of its individuals scored as susceptible and 35 % as highly susceptible. This validated the fact that one of the parents is susceptible and also confirms farmers' observations.

Microsatellite analysis

Twenty –three unique alleles were associated with the best 20 black pod-resistant trees (Table 9), which were not found the trees susceptible to black pod disease. A preliminary work on genetic fingerprinting of cocoa done

at CRIG showed that some cocoa trees contained alleles that conferred resistance to black pod (unpublished). This suggests that the unique alleles revealed in the current study might have conferred the resistance to black pod disease in these trees. However, this requires further research to confirm.

Genetic variation among cocoa populations

The 13 microsatellite markers used in this study were highly polymorphic among the five populations. All 13 microsatellite markers gave 100% polymorphic loci. The genetic distance (Nei, 1978) between the populations ranged from 4.4% to 8.9%. This shows that there is a low genetic base in the cocoa accessions used in the study. However, Nei's observed heterozygosity (0.625) was smaller than the expected (0.656) indicating a heterozygote deficit of 3.1% in the accessions. This was confirmed by the F_{IS} (heterozygote deficiency) value of 0.048 obtained. Positive F_{IS} values suggest heterozygote deficiency as a result of non-random mating (Gehring and Lindhart, 1992).

The probability of deviation from Hardy-Weinberg proportions (Haldane, 1954) value of 0.189, also confirmed the presence of non-random mating and that transfer of genes from one generation to another was not under the influence of evolutionary forces. This is because most of the populations are Upper Amazon in origin and possess self-incompatible alleles. Manual pollinations are used for crosses between specific clones at the seed garden to produce seedlings for planting. The progenies obtained as a result of the crosses are cross-compatible. The value recorded for frequency of cross-

pollination (0.92) (Wrights, 1952) confirmed the self-crossing nature of the populations.

Work done by Engels (1986), Laurent *et al*. (1993a, b), Laurent *et al*. (1994) and N'Goran *et al*. (1994) on the Upper Amazon basin materials showed that there is considerable variation in morphologically and physiologically. This agrees with the results obtained in the current study, where majority of the genetic variability observed was contributed by the Upper Amazon materials.

The Series II hybrid population had the least gene diversity and heterozygosity of 62.8% and 62.1%, respectively. Parents of this population include Amelonado cocoa, which have a narrow genetic base due to their self-compatible nature. This might have accounted for the lower gene diversity observed in the hybrids.

Comparison of the overall mean heterozygosity, as well as gene diversity of breeders collections, with that of the three farmers populations also showed a significant ($p<0.03$) heterozygote deficit in the farmers collections. Failure of farmers to collect planting materials from seed gardens could account for this observation. The effective number of alleles, among the 124 alleles recorded for all the 13 SSR loci, was 61% indicating that majority of the alleles revealed in the analysis contributed to the heterozygosity and genetic distances existing in the cocoa collections.

Genetic diversity estimated according to Nei's (1978) method showed high genetic variability in the accessions. The average gene diversity values ranged from 62.8% to 70.3%. Moreover, Nei's coefficient of genetic

differentiation (Gst), was 0.019. Gst values approaching 1 indicate a significant variation between populations with values approaching zero indicating significant variation within populations (Nei, 1978). Thus an overall 1.9% genetic differentiation was observed among the cocoa accessions while the greater percentage of total genetic diversity of the accessions was observed within accessions. In the current study, the percentage gene diversity within each population was 62.8%, whereas the gene diversity between the populations was 7.5% indicating further that there was much variation within populations than between populations.

Also, the mean heterozygosity of individual populations was 64.5%, whereas the range of heterozygosity between the populations recorded 6.17%. Thus, there was a low genetic divergence among the populations. Nei's (1978) unbiased genetic distances between the populations which ranged from 1.34% to 8.06% also showed that there was a narrow genetic base for the cocoa populations. This suggests that the populations could come from common parents, which agrees with the findings.

Adomako and Adu-Ampomah (2000) have reported that Posnette's introduction of the Upper Amazon cocoa from Trinidad was meant to broaden the genetic base of cocoa germplasm used in breeding programmes in Ghana. A survey conducted by Opoku *et al*, (2007) in all the cocoa growing regions of Ghana, including the Central Region, where farmers materials used in this work were collected, indicated that 69% of materials in farmers fields were made up of Inter-Upper Amazon hybrids. This indicates that the majority of the genetic variability observed in this work could come from the Upper

Amazon materials.

Genetic association among the cocoa populations

The low G_{ST} value of 0.019 over all the five populations indicated a low genetic divergence among the populations. This supports the low genetic divergence observed among the various populations (6.17%) as against high genetic divergence within the populations. This confirms that most of the populations come from a common source, such as seed gardens.

The cluster analysis also showed separation of the farmers collection (Inter-Upper Amazon) from that of the breeder's materials at a dissimilarity coefficient of 0.06. This indicates that majority of planting material in the selected area of the Central Region were similar to the breeder's materials. The breeder's collections (Upper Amazon and Inter-Upper Amazon) also clustered together at a much lower value of dissimilarity. This was expected since crosses between the Upper Amazon clones gave rise to the Inter-Upper Amazon progenies.

However, at a dissimilarity coefficient of 0.071 the farmer's collections (Series II hybrid and the susceptible accessions or controls) clustered together but separated from the Upper Amazon collection. Apart from the fact that the Upper Amazon materials are disease resistant, the Upper Amazon collections in this work showed considerable resistance to the black pod disease as compared to the hybrids and the controls from the result of the leaf disc test. This could explain why the Upper Amazon collections clustered together and separated from the hybrids and controls, which showed less resistance to black pod. The high susceptibility of one of the parents of the

hybrid and some of the controls also explains why they were more susceptible to black pod disease and, subsequently, clustered together. A factorial analysis of correspondence plot of the 5 populations grouped into one major cluster with few outliers, confirming the similarity of the materials and low genetic distance among the populations.

Conclusions

The findings of this work revealed a considerable resistance level to black pod disease among the cocoa accessions from breeders and farmers fields. These could serve as potential source of trees resistant to *P. megakarya.* The breeder's material showed the highest resistance, while some farmer's materials also recorded comparable resistance to that of the breeder's material; an indication in favour of evaluating selections made by farmers for low black pod disease incidence. This also indicates that farmers' knowledge is a useful input in cocoa breeding programmes. The Upper Amazon materials showed a higher resistance than Series II hybrids to the incidence of the pod rot disease. The susceptibility of one of the parents of the Series II hybrids to black pod disease could partly account for the level of susceptibility observed.

This study has revealed the existence of a genetic diversity based on resistance of cocoa accessions. The values of genetic parameters obtained in this study, mainly the values of heterozygosity indicate that the genetic base of cocoa accessions used in this study was narrow. This indicates the need to further broaden the genetic base of the cocoa germplasm collections to ensure effective breeding and development of improved varieties. The 23 unique

alleles revealed in this work could account for the high resistance recorded for some of the selected trees.

Breeder's collections recorded a significantly ($P<0.03$) higher diversity than collections from farmers fields. Thus, there was a deficit of heterozygotes in farmer's field collections due to the collection of planting materials from unapproved sources such as their own fields instead of from seed gardens.

The Upper Amazon introductions from Trinidad known as 'T' clones, remain the most reliable source of resistant planting material, which were being used in new breeding programmes at Cocoa Research Institute of Ghana.

Recommendations

This study should be extended to cover all the cocoa growing areas in Ghana in other to access the resistance base of farmer's selections which had promising revelation in this work.

Quantitative trait loci analysis also should be carried out on the best trees to identify the genetic markers associated with resistance in the identified trees. This could lead to isolation of these unique genes and their subsequent introduction into the marker-assisted breeding programmes at the Cocoa Research Institute of Ghana.

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APPENDICES

Appendix A

COCOA MOLECULAR MARKERS FARM SURVEY QUESTIONNAIRE (REVISED)

A. Demographics

1.Name: _____

2.Owner or Caretaker_____

3.Town/Village:_____

B. Characteristics Of Cocoa Plantings

Area planted with cocoa (ha)1: _____

2. How much do you harvest from the entire plantation: Not known

Known per year (indicate total amount):2005/06

_____2006/07_____2007/08_____ (farm passbook) (Estimate)

C. Problems Encountered In the Cocoa Plantation

1. Diseases present: (a) Black pod (b) CSSV (c) Other: _____

2. Extent of losses due to disease: mild medium severe

3. Insect pests present: Mirids (*Akate*) Bathycoelia (*Atee*) Stem borer (*Akokono*) other: _____

4. Extent of losses due to insect pest: mild medium severe

E. Planting Material Present on the Farm

Name of varieties grown: not know, known (types): _____

2**. Origin of planting material:** (a) not known (b) from own farm (c) from extension service/government (d) from private nursery (e) From other farmer (f) other: _____

3**. Level of satisfaction with planting materials:** High medium low

4**. Do you have any outstanding trees in your farm?** Yes /No

If Yes, notably for*:* high yield (indicate number of outstanding trees):_____

not affected by disease (specify which disease and number of trees):

not affected by pests (specify which pest and number of trees):

5**. Do you agree to share some budwood or seeds from outstanding trees in your farm? Yes /No**

F. Farmers' Interest In New Varieties

1**. Are you interested in other new varieties than indicated above?**

Yes, specify which variety and why: _____

No , specify why: _____

2**. What features are most important in new varieties**?

a. Early yielding b. High yielding c. Big pods d. Big beans

e. Smaller trees f. Bigger trees

g. Less susceptible to disease (specify to which disease): _____

h. Less susceptible to pests (specify to which pests): _____

i. Good quality (specify for which type of quality):_____

j. Other (specify reason): _____

[1] NB: 1 ha. = 2.5 acre

Appendix B

One-way ANOVA: ‘T’ Clones, progenies, Amazonia, Series II hybrid, Control

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Analysis of Variance

Source	DF	SS	MS	F	P
Factor	4	67.098	16.775	117.01	0.000
Error	200	28.673	0.143		
Total	204	95.771			

Level	N	Mean	StDev	Individual 95% CIs For Mean Based on Pooled StDev
‘T’ Clones	48	1.2408	0.1708	(-*)
Progenies	40	1.5105	0.3736	(-*)
Amazonia	50	1.6878	0.3941	(*-)
Series II hybrid	47	1.9706	0.5302	(*-)
controls	20	3.3310	0.2635	(--*-)

Pooled StDev = 0.3786

----+---------+---------+---------+-

1.40 2.10 2.80 3.50
