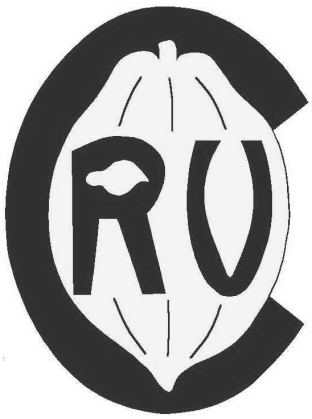
Annual Report 2004



Cocoa Research Unit
The University of the West Indies
St. Augustine, Trinidad and Tobago
2005

CONTENTS

Introduction

Research on cacao at the Cocoa Research Unit (CRU) continues to be centred on the valuable germplasm resources in the International Cocoa Genebank, Trinidad (ICG,T). As in recent years, our activities are summarised in the Overview (next section) and have been grouped under the headings of conservation, characterisation, evaluation and utilisation. However there is considerable overlap and interdependence among these categories so that, for example, characterisation and evaluation depend on conservation, and utilisation depends on effective evaluation. All the current activities in CRU have been mentioned in the Overview, but all our work is not reported in detail every year. Detailed reports are presented from areas where there have been significant findings or progress, so an individual activity may only be reported once every few years.

Details of the Cocoa Research Advisory Committee, staff, publications and visitors and a complete list of acronyms are given at the end of the report. In the text, acronyms will also be defined, normally only at their first mention.

CRU is a department in the Faculty of Science and Agriculture (FSA) of the University of the West Indies (UWI). Core activities in CRU are made possible by financial support from the Government of the Republic of Trinidad and Tobago (GORTT) and the Biscuit, Cake, Chocolate and Confectionery Association, UK (BCCCA). Sources of additional support for special projects and collaboration from other organisations are listed on the inside front cover of this report.

Projects

The final workshop for the project entitled *Cocoa germplasm conservation and utilisation: a global approach* took place in Reading, UK in March 2004.This project was supported by the United Nations Common Fund for Commodities (CFC), the Supervising Body was the International Cocoa Organisation (ICCO), and it was co-ordinated by the International Plant Genetic Resources Institute (IPGRI). It will be referred to in this report as the "CFC/ICCO/IPRGI Germplasm Utilisation Project". Since its initiation in 1998, satisfactory progress was made in all aspects of the project activities, and we extend our appreciation for the funding provided not only by the CFC, but from co-financiers (the BCCCA, Centre de coopération internationale en recherche agronomique pour le développement (CIRAD) and the World Cocoa Foundation (WCF) (previously the American Cocoa Research Institute (ACRI)). For the most of the activities in CRU, the majority of funding for this project came from counterpart contributions through support by the GORTT and the BCCCA.

Phase 2 of the project to *Evaluate cocoa germplasm for resistance to Witches' Broom disease* is continuing with support from the World Cocoa Foundation (WCF). During this phase, resistance to Witches' Broom disease (WB) is being confirmed and quantified for promising clones selected in Phase 1 (from 1998 to 2003). Cacao clones from a wide range of origins are being identified with WB resistance, which will contribute towards the achievement of durable resistance in countries affected by the disease.

CRU is participating in the project *To develop a DNA[1] fingerprinting database for all major cacao collections in the Americas* with the United States Department of Agriculture (USDA),

1 Deoxyribonucleic acid

through a tri-partite agreement between CRU, the BCCCA and USDA. The project is referred to as the USDA/BCCCA/CRU Fingerprinting Project in this report. Initial results indicate that there is relatively little duplication of germplasm in the collection of original trees in Marper Farm, demonstrating that this is a valuable reference for identification of cacao accessions.

A one-year extension of the project *To establish the physical, chemical and organoleptic parameters differentiating fine and bulk cocoa* was approved by the CFC for the period February 2004 to January 2005. The project began in 2001 and involves three other cocoa producing countries (Ecuador, Papua New Guinea and Venezuela). In addition to financial support from the CFC, co-financing is being provided for Trinidad and Tobago by the Ministry of Agriculture, Land and Marine Resources (MALMR), the Guittard Chocolate Company, USA, and Lindt and Sprüngli, Switzerland. The ICCO is the Supervising Body, and the Project Executing Agency is the Instituto Nacional Autonomo de Investigaciones Agropecurias, Ecuador (INIAP). The project will be referred to as the "CFC/ICCO/INIAP Flavour Project" in this report.

A new 5-year project entitled *Cocoa productivity and quality improvement, a participatory approach* began in June 2004. This project is supported by the CFC, the Supervising Body is the ICCO, and it is co-ordinated by IPGRI. It will be referred to in this report as the "CFC/ICCO/IPRGI Cocoa Productivity Project". The project involves partners in 12 cocoa producing countries and has two main components. The first is largely concerned with on-farm participatory research, and the second is largely concerned with building on progress achieved in the CFC/ICCO/IPGRI Germplasm Utilisation Project. A major new initiative for CRU in the new project is a germplasm enhancement programme for WB resistance.

Staff news

Neerupa Ramnath returned to CRU in January 2004 as a Technical Assistant to carry out fermentation and drying trials and other duties in the CFC/ICCO/INIAP Flavour Project.

Annelle Holder joined the staff in July 2004 as a Technical Assistant. Her time is divided between the WCF project on screening germplasm for resistance to WB and the germplasm enhancement for WB resistance in the CFC/ICCO/IPRGI Cocoa Productivity Project.

Naveta Persad (Technical Assistant) resigned in September 2004 to take up a teaching position. She had been working on morphological characterisation since she joined the staff in 2002.

Junior Bhola was appointed in November 2004 as a Laboratory Assistant to replace Naveta Persad in the morphological characterisation group.

Visitors for training

David Secco came to CRU in November 2003 to be trained in aspects of plant pathology for a 7-month period. He is a graduate student from the University of Tours, France and worked on an early screening test for WB with Jean-Marc Thévenin. During his time in CRU, he also undertook training and participated as a member of a sensory panel to assess cocoa liquor flavour.

Meetings and events

Three representatives from CRU (David Butler, David Iwaro and Jean-Marc Thévenin) participated in two workshops held in Reading, UK in March/April 2004. The first of these was

the final workshop of the CFC/ICCO/IPGRI Germplasm Utilisation Project, which was officially closed on 31st March 2004. The second was the initial workshop of the CFC/ICCO/IPGRI Cocoa Productivity Project. Working protocols were agreed for this project and a starting date was proposed. Initially funding from the CFC was agreed for 18 months from the starting date, and this was extended to five years later in 2004.

In June 2004, David Butler and Darin Sukha attended a workshop in Mérida, Venezuela, to assess progress in the CFC/ICCO/INIAP Flavour Project. The meeting achieved valuable interactions between participants from Ecuador, Trinidad and Tobago and Venezuela in comparing and contrasting results from these countries.

In July and August 2004, Darin Sukha spent two months at the University of Hamburg, Germany (UH) on study leave. During this period, he undertook chemical analysis of cocoa bean samples and made use of the library facilities at UH. While in Europe, he also visited various organisations for discussions on collaboration with CRU. These were; Chokladforum, Sweden, Lindt and Sprüngli, Switzerland, Valrhona, France and the Museum of Cocoa and Chocolate, Brussels. A direct outcome of his visit to UH is a Memorandum of Understanding between CRU and the Department of Useful Plants and Plant Ecology, Biocenter of Klein Flottbek.

One outcome from the workshop in Mérida was an agreement to hold an advanced training workshop on sensory assessment of cocoa liquors. This workshop was hosted by CRU with assistance from the Guittard Chocolate Company, USA, in September 2004. The week-long event provided an opportunity for the exchange of cocoa liquors from the participating countries, and for each panel member to gain an appreciation of marked differences in characteristic flavours from different fine or flavour producing countries.

In October 2004, David Butler attended the World Cocoa Foundation partnership meeting in Washington DC, USA. He was a panel member in the session on Cocoa Germplasm and he was presented with an award for Cocoa Research, in appreciation for his dedication to sustainable cocoa production.

In December 2004, David Butler participated as a member of the ICCO expert panel on fine or flavour cocoa at a meeting in London, UK. He was invited to make a presentation on "Characteristics of fine or flavour cocoa", drawing on results from the CFC/ICCO/INIAP Cocoa Flavour Project.

The Cocoa Research Unit – an overview

Cocoa, obtained from cacao (*Theobroma cacao* L.), makes a unique contribution to the flavour and textural properties of chocolate that holds an almost universal appeal to people of all ages. The international cocoa community generally classifies cocoa beans into two broad types. The first is Forastero cocoa, with highly pigmented beans, used in the manufacture of cocoa butter and high volume chocolate lines. These beans, referred to as bulk cocoa, make up over 95% of the world production. The second type is Criollo cocoa, mainly grown in Central and northern South America, whose white or pale violet beans are used to manufacture chocolate of the highest quality. Trinitario is a hybrid of the two types that originated in Trinidad but is now grown in many locations. It provides specific flavour distinctions in fine chocolate. Criollo and Trinitario beans are collectively known as 'fine or flavour' cocoa. There are however exceptions to this generalisation such as Nacional cocoa from Ecuador, which is believed to be a Forastero type classified as fine or flavour. Another group is Refractario, which comprises germplasm selected in Ecuador in the 1920s and 1930s. Selections were made of the few survivors among seedlings that had been infected by Witches' Broom disease.

Cacao was introduced into Trinidad around 1575 and ever since that time has been an integral part of the history of Trinidad and Tobago. Cocoa first became a staple product of Trinidad at the start of the 18th century and from the 1860s to the 1920s it played an essential role in the social and economic development of the society. In 1921 cocoa production in Trinidad and Tobago reached 34,000 metric tonnes per year, making the country amongst the world leaders in cocoa exports. Given the prominent position of Trinidad and Tobago in the international cocoa market at that time and the outbreak of Witches' Broom disease in 1928, a Cocoa Research Scheme was established in Trinidad to provide support for local and international cocoa production.

Cocoa research began in Trinidad at the Imperial College of Tropical Agriculture (now UWI) in 1930 and has continued uninterrupted since that time. CRU is responsible for maintenance of the ICG,T around which on-going research activities in the Unit are centred. Cacao germplasm has to be conserved as a living collection, since seeds do not remain viable if they are frozen and the costs of other methods of cryopreservation are prohibitive. The ICG,T is situated at the University Cocoa Research Station (UCRS), a 33 ha site, originally part of the La Reunion Estate at Centeno. Work to establish the ICG,T began in 1982 with support from the European Union (EU), by propagating trees using rooted cuttings from existing collections in Trinidad. These collections had been established at different locations on the island using selected varieties from Trinidad and Tobago, from other national collections and from numerous missions to collect primary germplasm. They include the Imperial College Selections (ICS) which resulted from an exhaustive survey of Trinidad and Tobago carried out by F.J. Pound between1930 and 1935. About 50,000 high-yielding trees were selected and those bearing small and thick-shelled pods were eliminated. The 100 most productive trees (ICS 1 to 100) were selected from the resulting 1,000 using exact criteria from detailed observations.

A main source of original material for the ICG,T was Marper Farm at Manzanilla, east Trinidad, established by F.J. Pound following his expeditions to the upper Amazon between 1937 and 1942. The trees at Marper are now old and have suffered periods of neglect, however they still serve as an important anchor in confirming the identity of clones in the ICG,T and in replacing material which has proved difficult to establish. In addition, germplasm was available from other expeditions such as the Anglo-Colombian expedition in 1952-53 and Chalmers'

expeditions between 1968 and 1972. By 1994 over 2,000 accessions had been planted in the ICG,T and additional clones are added as they become available. The genebank contains one of the most diverse collections of cacao germplasm in the world and has been designated a Universal Collection by IPGRI.

Since the ICG,T was established, research activities in CRU have been centred on the collection. The ICG,T is considered to be of major importance to the future of world cocoa production, but the potential of the collection cannot be fully exploited unless the accessions are characterised, evaluated, and made available to end users in cocoa-producing countries. Furthermore, information related to the germplasm must be well documented and made readily available in a user-friendly format.

CRU has an interest in all aspects of cacao cultivation, including quality. Our mission is to provide support for the provision of varieties suited to sustainable cocoa production, both locally and globally, by making planting material available with improved traits for high yield potential, disease resistance, high fat content and with good flavour characteristics.

Research efforts at CRU over the last 10 years have been directed towards the task of characterising and evaluating all the accessions in the ICG,T, selecting those with desirable traits and undertaking pre-breeding to produce genetically diverse populations with enhanced characters (such as disease resistance). Below is a summary of achievements and an outline of plans for future research in the medium-term time frame.

Conservation

Maintenance and propagation

If the ICG,T is not well maintained, research progress would become limited, so a balance is necessary between funds directed towards the genebank maintenance and research.
Apart from routine maintenance such as weed control, pruning, shade management, irrigation, security/firewatch, there is a continuous need for re-propagation of clones. When the ICG,T was established, 16 trees of each accession were planted in each plot, however, in the majority of cases, not all the trees grew and some accessions proved very difficult to establish as rooted cuttings. The situation now (11-19 years after establishing the plots) is that plots contain anything from 1 to 16 trees, and some accessions have no survivors. Plots with less than three living trees are considered at risk to genetic erosion. The urgent need to conserve these clones by grafting their budwood onto rootstocks is being addressed, and the grafted plants are being established in clonal gardens. In cases where there is no survivor in UCRS, but the original tree in Marper Farm or elsewhere is still alive, budwood from the original tree is being grafted onto rootstocks. Once established, cuttings can be taken from the grafted plants and rooted to fill gaps in the ICG,T with plants on their own roots. It is important to make a concerted effort to raise plants from rooted cuttings to avoid potential confusion in the future with chupons from rootstocks.

New introductions

The ICG,T is considered to be a dynamic germplasm collection. We are continuously adding accessions from collecting expeditions (when the opportunity arises) or from other national collections. The objective of these inputs is to increase the representation of genetic groups that are currently under-represented in the genebank, thereby creating a balanced collection with maximum genetic diversity. Towards this end, recent acquisitions (since 1990) are Trinitario

populations from other islands in the Caribbean and Central America, Lower Amazon material from French Guiana and Venezuela, wild Criollo material from Belize, and genetically diverse Upper Amazon clones from the John Allen collection, Ecuador. Until 2003, new material was introduced through the Barbados Cocoa Quarantine Station (BCQS) however this activity has been suspended due to financial constraints. Material is now being introduced to Trinidad through the intermediate quarantine station, Reading, UK.

Further acquisitions are proposed when funding permits, from Mexico (Criollo/Trinitario), Costa Rica (CATIE) (Criollo), Guyana (Lower Amazon), French Guiana (Lower Amazon), Columbia, Ecuador and Peru (Upper Amazon) and Brazil (Lower Amazon).

Documentation

New introductions, difficulties of establishment, and filling gaps in the ICG,T mean that field maps and databases need to be continuously updated. Each tree has been assigned a unique number to accurately record the source of samples for research and other purposes. This will avoid confounding issues if trees are identified as off-types subsequent to a research activity, since it will always be possible to return to the same tree within a plot. From 1998 to 2001, we completed the task of drawing up-to-date maps, and in numbering plots within fields and trees within plots. All this information has been organised in a database, to enable notes about individual trees to be included, and this information is being continuously updated.

Verification

The task of establishing the ICG,T from ageing trees by use of rooted cuttings was complex and there was ample opportunity for mislabelling to occur. Steps in which errors may have arisen include:

- Collection of budwood for cuttings during the clonal propagation of trees from Marper Farm prior to their planting in the ICG,T or on campus. The budded trees in Marper Farm were already old when the multiplication process started in the 1980s. Many of the trees had multiple trunks, which included rootstock as well as scion material. In addition, some trees have fallen and re-grown in new locations, so these are difficult to identify from the field maps. In other cases, seed may have germinated at the base of the original tree, in which case trunks of seedlings would be difficult to distinguish from the trunk of the original tree.
- Mislabelling of plants in the greenhouse after clonal propagation, e.g. when rooted cuttings were moved from the propagation bin to harden off, or from the hardening-off area to another part of the greenhouse or from the greenhouse to the genebank.

Some off-types have been recognised from the pod morphology, and these trees are being tagged to avoid their mistaken use in research. In recent years, further off-type trees have been identified using DNA sequencing methods and, it is now recognised that all trees being used for research or distribution should be verified by DNA fingerprinting to ensure their correct identity.

Initially, molecular verification was undertaken using random amplified polymorphic DNA (RAPD) analysis, this being the technique available in CRU when the work started in 1997. Results from the RAPD analysis showed that approximately 70% of the trees tested were true to type. However, more recently results from some RAPD analyses have been shown to be

inconsistent, so it is possible that the 30% off-types identified by this technique is an over-estimate. Since 2001, we have adopted microsatellite analysis (otherwise known as Simple Sequence Repeats, SSR) for the verification work. We use two techniques to visualise SSR results; either agarose gels with ethyl bromide staining or polyacrylamide gel electrophoresis (PAGE) with silver staining, which gives much better resolution of bands. SSR analysis for DNA fingerprinting is reported to be reliable, with consistent results between different laboratories.

The task of verifying every tree in the ICG,T (over 11,000 trees) is enormous, so it is necessary to set priorities to arrive at achievable targets in the short- and medium-term. Clones identified as having desirable traits (such as disease resistance, good yield potential, high butterfat content or beans of superior flavour) will be given a high priority for the verification of individual trees within plots.

Characterisation

Morphological characterisation

About half of the accessions in the ICG,T have yet to be fully described. To address this problem, a concerted effort is being made to systematically document each accession using morphological descriptors. Work started in 1990 using a complete list of 65 morphological descriptors developed by the International Board for Plant Genetic Resources (IBPGR, now IPGRI) in 1981, but initial progress was slow and this was superseded by a short list of 22 morphological descriptors developed at CRU. The list includes detailed descriptions of leaves, flowers and fruit for traits that aid identification and/or affect economic yield. It remains a large task even with the short list of descriptors, and the work was further streamlined in 2000 by reducing the sample size of pods from 20 to 10 and that of flowers from 15 to 10. Full descriptions of over 1,132 accessions have now been completed. As they are recorded, the descriptors are entered in a local database and are also sent to the International Cocoa Germplasm Database (ICGD), Reading, UK, for global distribution.

Having reached a point where large numbers of accessions in the ICG,T have been characterised, analyses are possible to examine phenotypic variation among various groups of cacao (such as Upper Amazon Forastero, Refractario, Lower Amazon Forastero, and Trinitario). Furthermore, this large volume of carefully catalogued data should form the basis of new avenues of work. Recently developed techniques allow the possibility of gene association between specific traits (recorded as morphological characters) and well-identified parts of the cacao genome. Such information could lead to rapid advances in selection for desirable traits in plant breeding programmes of the future.

Molecular characterisation

From 1994 to 2001, molecular characterisation was carried out using RAPD analysis, with the completion of over 600 accessions. This technique provided information used to assess the genetic diversity within the germplasm collection. Genetic diversity studies can be used to identify cacao types that are over- or under-represented in the ICG,T, to assess the degree of homogeneity within accession groups, and the genetic distances between them. For cacao, the term population normally used to refer to accessions sharing the same collection name, but here the term “accession group” will be used. The geographic origin within an accession group could vary from a small estate to a large region. This would naturally affect its genetic diversity.

This work took a new direction in 2001 when the USDA Fingerprinting Project was initiated. In this project we are generating a DNA fingerprint of each accession in the ICG,T (2,300 accessions), taking a sample from the most original tree of each clone. The analysis is using 15 SSR primers, selected to cover most of the cacao genome (9 of the 10 chromosomes) and to give good differentiation between clones. The results of these analyses are not only providing a means of positively identifying each clone, but also provide data for genetic diversity studies. DNA has been extracted in CRU from each accession, and the samples are being analysed in USDA, Beltsville with an automatic sequencer. In our previous work with RAPD, we analysed 600 accessions in 6 years, and now we expect to analyse 2,300 accessions in 3-4 years. This collaborative effort will therefore accelerate the rate of progress in genetic diversity studies by a factor of six.

Information on genetic diversity within and between populations will be vital to the selection of populations for inclusion in germplasm enhancement and breeding programmes of the future.

Evaluation

To assess the value of accessions in the ICG,T, traits that affect the economic yield need to be evaluated. Examples of these traits are disease resistance, bean size, pod index (the number of pods needed to produce 1 kg of dry beans), cocoa butterfat content and flavour potential.

Disease resistance

Two important diseases that affect cacao in Trinidad are Black Pod disease (BP), caused by *Phytophthora* spp., and Witches' Broom disease (WB), caused by *Crinipellis perniciosa* (Stahel) Singer.

Mass screening for resistance to BP was started in 1996 using a detached pod inoculation method, which distinguishes pre- and post-penetration types of resistance. Inoculations are carried out with *P. palmivora*, the more aggressive of two species of *Phytophthora* found in Trinidad (*P. palmivora* (Butler) Butler and *P. capsici* Leonian). So far, over 1,400 accessions have been screened at least once and the inoculation has been repeated on 967 accessions. Overall, about 13% of the clones tested are either resistant or moderately resistant to BP, although the proportion of resistant clones is greater in the Forastero group than in the Trinitario group.

In addition to screening by controlled inoculation, the incidence of BP in the field has been observed in the ICG,T. This combination of detached pod inoculations in controlled conditions with field observations over a number of years will provide sound evidence on host resistance to BP.

Mass screening for resistance to WB is being undertaken using a spray inoculation method. This work was started in 1998 using young grafted plants, replicated up to five times to allow inoculations of the same clone to be repeated. The inoculation method had to be adapted for use with grafted plants (as opposed to seedlings) and to the environmental conditions in Trinidad, so early progress in this project was slow. However, about 700 accessions were inoculated in the first phases of this project by July 2003. Results from this work identify clones that are susceptible to WB, but there is a need to verify true resistance to WB where few or no symptoms developed after inoculation. This is because escapes are common with the spray inoculation method.

Recently, an optimised agar-droplet method was developed that allows resistance to WB to be

quantified. We are therefore verifying the resistance of promising accessions identified by the spray method, using agar-droplet inoculations, using either seedlings or clones (grafted plants). These results will also be combined with field observations in the ICG,T over a number of years.

Quality traits

The percentage butterfat has been determined in over 400 clones from the ICG,T and further determinations are being made in selected clones.

Assessment of flavour is an aspect of evaluation of particular value to cocoa farmers in Trinidad and Tobago who produce 'fine or flavour' cocoa. Sensory assessments are carried out using trained panellists to investigate effects of various post-harvest processes on the flavour attributes of selected accessions. Recent work has demonstrated the consistency of trained panels to give quantitative sensory assessments, and flavour profiles are being documented for a range of accessions. We plan to extend this effort to determine flavour profiles of clones with other desirable traits such as good yield potential and/or disease resistance.

The heritability of flavour traits is a new area of investigation in CRU. Work is underway to explore the relative contributions of the growing environment, the environment during post-harvest processing and pollen to flavour.

Utilisation and application

Distribution

Selected cacao accessions from a diverse genetic background with desirable agronomic traits are being distributed to cocoa-producing countries via intermediate quarantine at the University of Reading, UK. After satisfying the required period in quarantine, these elite accessions will be distributed to a range of cocoa-producing countries, including participants in the CFC/ICCO/IPGRI Germplasm Utilisation Project. In the future, selected populations from germplasm enhancement programmes (below) will be distributed in a similar way.

Germplasm enhancement

From 1998 to 2002, over 90 accessions were used in a pre-breeding programme to accumulate genes for resistance to BP. Parents were selected by considering their genetic diversity, geographic origin and economically important traits, as well as disease resistance.

The progeny from crosses in the pre-breeding programme were evaluated for BP resistance with a leaf inoculation method. This permits early selection of seedlings and comparison of the disease resistance of the parents and progeny at an early stage. The most resistant individuals in the progeny were planted in field trials and are being evaluated for performance, not only in terms of BP resistance, but also for precocity, vigour, productivity and WB symptoms. Initial results from field observations and the detached pod inoculation method confirm substantially improved resistance in these selections compared to unselected populations. The main objective of the pre-breeding programme is to produce enhanced germplasm that will introduce resistance genes to conventional breeding programmes in various cocoa-producing countries throughout the world.

A similar pre-breeding programme was initiated this year for WB. Progeny from crosses between WB resistant clones will be screened with the agar-droplet inoculation method. Work is also in progress at CRU to develop alternative techniques for early screening of resistance to WB.

Marker assisted selection

Research at CRU in the CAOBISCO[1] project (1995-2000) identified quantitative trait loci (QTL) for resistance to BP based on results of the leaf inoculation method. Selected plants from the same progeny were planted in the field, and we plan to validate the leaf inoculation method with field observations and detached pod inoculations once the plants come into bearing. Confirmation of the QTL would open the possibility of marker assisted selection in future breeding programmes for BP resistance.

Other work (outside CRU) is underway to search for QTL for resistance to other diseases such as WB and Frosty Pod disease (FP, caused by *Moniliophthora roreri* (Ciferri & Parodi) Evans *et al*.). When this has been completed, it should be possible to use marker assisted selection for germplasm enhancement even for diseases not present in Trinidad (such as FP).

It is likely that other advanced molecular techniques such as ESTs[2] and microarray analysis will lead to other selection methods in the future. However, the application of such techniques is entirely dependent on reliable datasets for traits of interest. The painstaking ground work at CRU on morphological characterisation, disease resistance screening and evaluation for quality traits has the potential to form a rigorous basis for such future investigations.

Conclusion

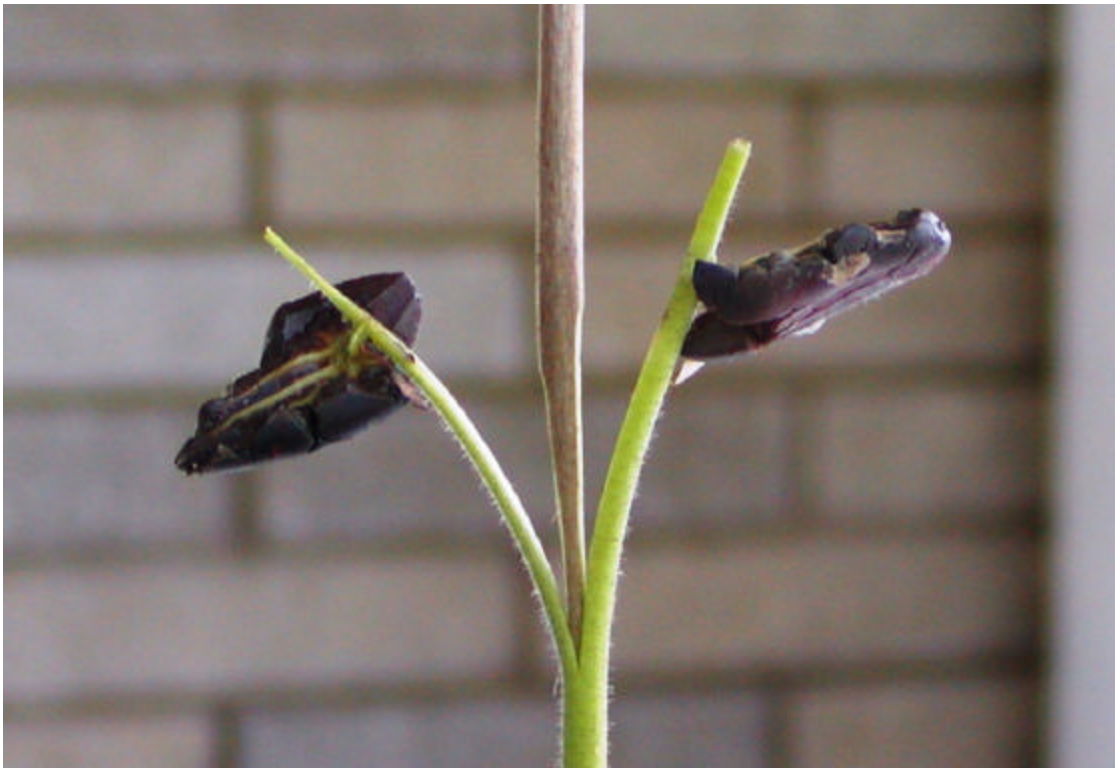
Since establishing the ICG,T, substantial progress has been made in research at CRU. A large body of information has been accumulated and documented, some of which has immediate applications, and some of which will form the basis for future investigations. For example, the list of 100 priority clones available in the ICG,T that are part of the "CFC/ICCO/IPGRI Project Collection" is being transferred to intermediate quarantine in Reading. This is the end-point of a large body of research in CRU, including morphological and molecular characterisation, evaluation for BP and WB (screening and field observations) and cocoa butterfat determinations. The selected clones will soon be available for further distribution to many cocoa-producing countries.

As the work of characterisation and evaluation continues, further selections of priority germplasm will be possible. In addition, practical results from the germplasm enhancement programme will soon be forthcoming after completing some basic field observations. Selections from BP resistant populations will then be sent to intermediate quarantine for further distribution.

The utilisation of the substantial body of information resulting from on-going activities in the development of novel selection methods provides the prospect of an exciting future for cocoa research. The possibility of molecular based selection techniques, together with well-documented information on genetic diversity, could lead to unprecedented progress in cocoa breeding in the foreseeable future.

1 Association des industries de la chocolaterie, biscuiterie et confiserie de l'UE
2 Expressed sequence tags

Conservation



New cacao introductions into the International Cocoa Genebank, Trinidad

D.R. Butler and J. Joseph

Cacao germplasm that was transferred to Trinidad from Barbados in 2003 (Butler and Sukha, 2004), is being multiplied in a greenhouse at UWI.

The clones from French Guiana listed in the Annual Report 2003 were wrongly named (Philippe Lachenaud, personal communication). The correct names are shown in Table 1.

Table 1. Correct names of clones recently introduced into Trinidad, originating from French Guiana.

Name in given in Annual Report 2003	Correct name	Name in given in Annual Report 2003	Correct name
ELP 1 (T3)	ELP 1/A	ELP 34 (T7)	ELP 34/S7
ELP 1 (T4)	ELP 1/S4	ELP 35 (T4)	ELP 35/S4
ELP 10	ELP 10/T[1]	ELP 35 (T8)	ELP 35/B
ELP 10 (T6)	ELP 10/S6	ELP 40 (T6)	ELP 40/B
ELP 11 (T1)	ELP 11/S1	ELP 40 (T9)	ELP 40/S9
ELP 11 (T3)	ELP 11/S3	ELP 41 (T5)	ELP 41/S5
ELP 16 (T3)	ELP 16/S3	ELP 7 (T2)	ELP 7/S2
ELP 16 (T7)	ELP 16/S7	ELP 7 (T7)	ELP 7/S7
ELP 18 (T9)	ELP 18/S12	ELP 8 (T3)	ELP 8/S3
ELP 2 (T4)	ELP 2/S4	ELP 8 (T7)	ELP 8/A
ELP 20 (T3)	ELP 20/S3	ELP 9 (T2)	ELP 9/S2
ELP 20 (T4)	ELP 20/A	ELP 9 (T4)	ELP 9/S4
ELP 22 (T10)	ELP 22/S10	GU 192	GU 192/A
ELP 22 (T6)	ELP 22/S6	GU 202	GU 202/A
ELP 28 (T4)	ELP 28/S4	GU 32	GF 32
ELP 28 (T6)	ELP 28/S6	YAL 1 (T2)	YAL 1/S2
ELP 30 (T5)	ELP 30/S5	YAL 5	YAL 5/T[1]
ELP 32 (T3)	ELP 32/S3	YAL 6 (T3)	YAL 6/S3
ELP 32 (T4)	ELP 32/S4	YAL 6 (T4)	YAL 6/S4
ELP 34 (T6)	ELP 34/S13		

[1]Clone for which the tree number in French Guiana was not recorded. The progeny name followed by "/T" is used to signify "Trinidad".

Trees are being planted in the field after establishing at least five plants of each accession in the greenhouse. During 2004, some of these new introductions were planted in Campus 8 at UWI and in Field 5A at UCRS (Table 2).

Table 2. Number of trees planted in fields at UWI (Campus 8) and at UCRS (Field 5A) in 2004.

Preferred Name	No. at UWI	No. at UCRS	Preferred name	No. at UWI	No. at UCRS	Preferred name	No. at UWI	No. at UCRS
RIM 23 [MEX]	2		ELP 7/S7	2	4	LCT EEN 37	2	
RIM 52 [MEX]	2		ELP 8/S3	2		LCT EEN 370	2	
RIM 9 [MEX]	2	3	ELP 8/A	2	6	LCT EEN 403	2	
AMELONADO 2/0-17 [MAY]	2	3	ELP 9/S2	2		LCT EEN 409	1	3
BE 2	2	5	ELP 9/S4	2		LCT EEN 411	2	8
BLZ 5 [BLZ]	2	3	EQX 69 [EQX]	2		LCT EEN 413	2	
C 15/61 [TRI]	2	4	GDL 2		3	LCT EEN 49	2	
CC 57	2		GDL 3	2		LCT EEN 57	2	
COCA 3348/52 [CHA]	2	4	GDL 7	2		LCT EEN 60	1	
CRIOLLO 54 [CRI]	2		GS 32	2		LCT EEN 63	2	3
ELP 1/A	2		GU 192/A	2		LCT EEN 81	2	
ELP 1/S4	1		GU 202/A	2		LCT EEN 91	2	
ELP 10/T	2		GF 32	2		MA 12 [BRA]	2	
ELP 10/S6	2		LCT EEN 107	2		P 19 [MEX]	2	3
ELP 11/S1	2	5	LCT EEN 122	2		PH 1-1	2	
ELP 11/S3	2		LCT EEN 133	2		PH 1-2	2	
ELP 16/S3	2		LCT EEN 188	2		PH 1-3	2	
ELP 16/S7	2		LCT EEN 189	2	8	PH 1-4	2	3
ELP 18/S12	2		LCT EEN 193	2		PH 2-1	2	4
ELP 2/S4	2		LCT EEN 195	2	3	PH 2-2	2	3
ELP 20/S3	2		LCT EEN 218	2		PH 2-3	2	
ELP 20/A	2	4	LCT EEN 220	1		PH 2-4	2	
ELP 22/S10	2		LCT EEN 221	2	6	PH 2-5	2	
ELP 28/S4	2		LCT EEN 227	1		PH 2-8	2	
ELP 28/S6	2		LCT EEN 249	2	4	RB 33/3 [BRA]	2	
ELP 30/S5	2	4	LCT EEN 255	2		RB 39 [BRA]	2	4
ELP 32/S3	2		LCT EEN 267	2		RB 46 [BRA]	2	5
ELP 32/S4	2		LCT EEN 278	2		SIAL 44	2	6
ELP 34/S13	2	3	LCT EEN 30	1		TAP 10 [CHA]	2	
ELP 34/S7	2		LCT EEN 312	2		TAP 7 [CHA]	2	3
ELP 35/S4	2		LCT EEN 32	1		UF 221	2	
ELP 35/B	2		LCT EEN 33	2		UF 676	2	
ELP 40/B	2		LCT EEN 333	2		YAL 1/S2	2	
ELP 40/S9	2		LCT EEN 36	2		YAL 5/T	2	
ELP 41/S5	1		LCT EEN 362	2	3	YAL 6/S4	2	9
ELP 7/S2	2		LCT EEN 368	2				

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Validation and optimisation of SSR-PCR and SSR detection in agarose gels

L.A. Motilal

Introduction

Microsatellite markers (SSR) are internationally utilised as molecular tools and are currently in use at CRU to detect off-types within the ICG,T. The existence of online SSR data in the ICGD (www.icgd.rdg.ac.uk) as well as the USDA/BCCCA/CRU Fingerprinting Project have allowed comparisons to be made between results obtained using agarose and sequencers. DNA fingerprinting is based on the comparison of DNA lengths (product size) as visualised after electrophoretic separation. Resolution of alleles would therefore be an issue between these two systems, with sequencer results being more precise. Two types of errors may be made when comparing two bands on agarose from different individuals – bands may be classified as similar when they are actually different (resolution efficiency) and bands may be thought to be different when they are in fact similar (artefact). Here this scenario is investigated and, in addition, ways in which the efficiency of the agarose system may be improved are examined.

Material and Methods

A selection of accessions from the ICGD and/or from the USDA/BCCCA/CRU Fingerprinting Project were assessed for microsatellites known to work well at CRU and which were common to these databases. Data from Johnson *et al*. (2004) were also examined for product sizes. Basepair (bp) differences of increasing sizes for a range of ICS accessions were chosen; SSR-PCRs[1] were run and products were resolved on agarose gels for pairwise comparisons.

The potential to maximise resource efficiency was examined by assessing: (a) the possibility of re-using buffers and microplates, (b) different DNA concentrations, (c) type and concentration of enzyme and (d) the potential for multiple loading of agarose gels.

Verification work included assessing reference ICS trees in the Cheesman Field at the San Juan Estate for those accessions which were represented by more than one tree across the five blocks.

Results

Resolution Comparison

The accessions JA 1/9 [POU], JA 1/17 [POU], JA 1/18 [POU] and LP 4/37 [POU] were shown in the USDA/CRU Fingerprinting Project, using 15 microsatellite primers, to be equivalent to one another. Similarly, CRU 104, CRU 111, ICS 8 and SCA 10 were found to be identical to each other. In the former case, twelve different SSR primers were used at CRU, and eleven were utilised for the latter group. Two primers (mTcCIR56 & 58) separated the former group into two different pairs: JA 1/9 [POU] was equivalent to JA 1/17 [POU] and JA 1/18 [POU] was equivalent to LP 4/37 [POU]. In the second group one primer (mTcCIR38) detected a difference,

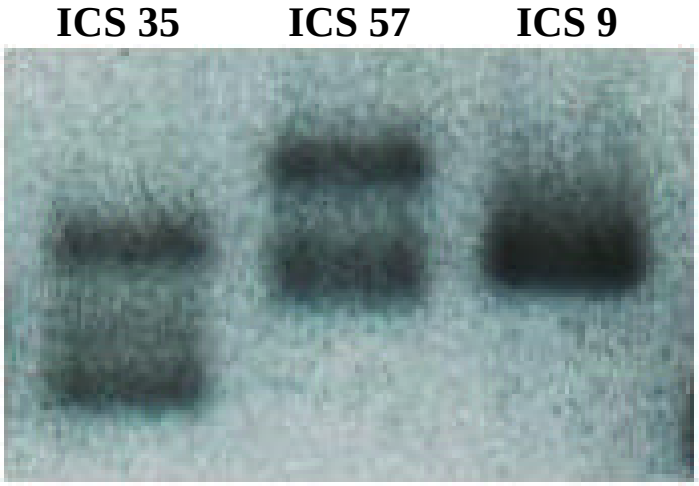
[1]PCR – polymerase chain reaction

separating CRU 104 and CRU 111 (one group) from ICS 8 and SCA 10.

The accessions EET 400 [ECU], ICS 1, ICS 95, SCA 6 were assessed with the primers mTcCIR1, 7, 12, 15, 37, 40 and 60. Accession profiles were repeatedly consistent. However, there were several inconsistencies found by the present study between product sizes on gels and those given in the ICGD from sequencer data. For instance, ICS 1 and EET 400 [ECU] for mTcCIR1 gave similar heterozygote patterns to match the product sizes of 131/143. SCA 6 and EET 400 [ECU] for mTcCIR37 are reportedly homozygous at 133 bp and 146 bp, respectively. However on agarose, the product of SCA 6 appeared lower than the 131 bp band of ICS 1 and EET 400 [ECU], and the 146 band of EET 400 [ECU] was at a lower position than the 143 band of the heterozygote. There is a good possibility that DNA from different trees could have been used in CRU as opposed to sequencer databases, since EET 400 [ECU] is reportedly homozygous at mTcCIR7 but a distinct heterozygous pattern was obtained in this study.

A combination of select reference ICS clones and SSR primers were utilised to generate PCR products of differing sizes to compare with sequencer data from Johnson *et al*. (2004). At least 47 anomalies were observed which could be grouped into four categories: (a) difference in genetic state, (b) width between heterozygous bands less than expected, (c) band position inconsistent with given sizes for different accessions with same or different SSR primers and (d) band position inconsistent with given sizes for a particular accession but with different primers. An example is provided in Figure 1.

Figure 1. Bands on agarose for three ICS clones compared to allele sizes (below) for the same primers reported in Johnson *et al*. (2004).



Primers:	mTcCIR6	mTcCIR9	mTcCIR9
Sizes (bp):	224/246	274/285	285/285

Of the 47 anomalies, two cases were obtained where a difference in genetic state was observed. ICS 9 for mTcCIR3 was reported as homozygous but a distinct heterozygote was found. ICS 57 for mTCCIR15 was reportedly heterozygotic but was found to be homozygous even though samples were taken from the same trees. Six instances were encountered where the width between heterozygous bands was less than expected. For example with ICS 1, an 11 bp

band width was reported for mTcCIR3 and an 18 bp width for mTcCIR15 (Johnson *et al.*, 2004). However, a wider separation was obtained with mTcCIR3 than with mTcCIR15 on agarose. Eight instances were encountered where the same accession analysed with different primers yielded inconsistent results. For example ICS 11 for mTcCIR6 reportedly has alleles at 228 and 246 bp whereas with mTcCIR15, alleles at 232 and 250 were expected. When run on agarose however, two distinct bands were observed for each accession but the upper band of mTcCIR15 was at a lower position than that of mTcCIR6. In the last category, (band position was inconsistent with given sizes for a particular accession but different primers), 31 instances were observed. A good example of this category was illustrated for mTcCIR9: ICS 46 and ICS 57 were reportedly heterozygous with alleles at 274 bp and 285 bp whereas ICS 9, 14, 63 and 80 were homozygous at 285 bp. Visualisation in agarose, however, revealed the lower band of ICS 46 and 57 at the same position as the 285 bp homozygotes.

Resource utilisation

It was found that x1Tris-borate-EDTA tank buffers could be re-used at least twice. There was an increase in the ratio of potential difference:current (p.d.:I ratio) with continual use, resulting in a longer runtime (2^{nd} use – 22 minutes more) but without adversely affecting the resolution of bands on agarose gels. New microplates from USA Scientific Inc. were found to withstand repeated sterilisation (ten times) there being no false positive products compared to new plates for ten SSR primers in the absence of template DNA. Subsequent PCR reproducibility for the accessions MXC 67 and EQX/JS [CHA] were assessed with seven SSR primers on new versus once-used plates sterilised either one or ten times. There were no differences among product profiles.

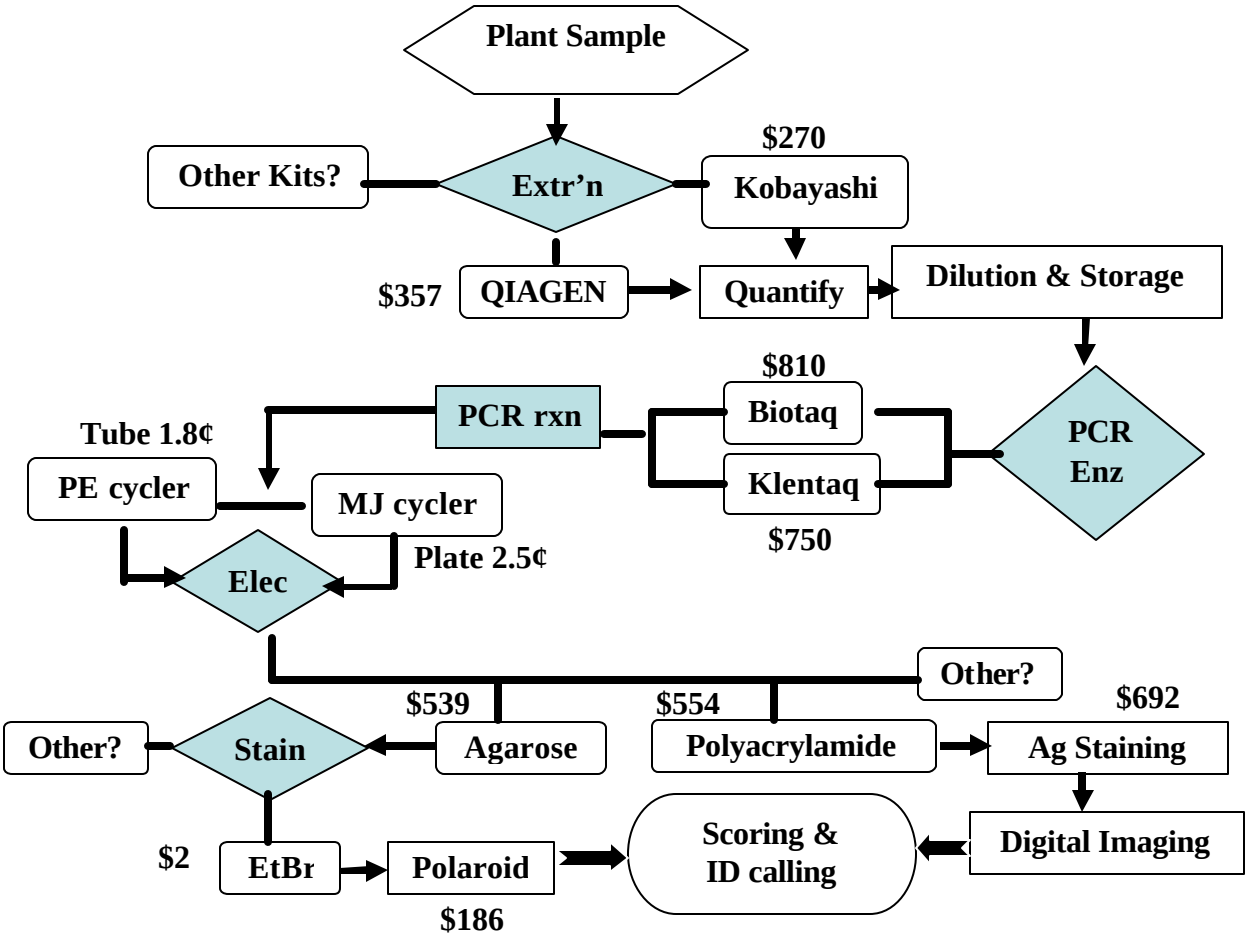
Repeatable and consistent amplification was found over a wide range of DNA concentrations from 0.25 – 500 ng in 25 µL of reaction mix for the accession AM 2/38 [POU] with the primer pair 43/44 at an annealing temperature of 51°C. There was an increase in staining intensity from 25 ng onwards with few multiple bands and without any artefact bands. Although there was an increased incidence of smearing, this did not affect band position or clarity. The accessions PA 165 [PER] and CRU 263 were consistently amplified for the SSR primer pairs mTcCIR3, 11 and 56 for concentrations of 5, 10, 20 and 45 ng of PA 165 [PER] DNA per reaction volume, and for 10, 50, 100, 250, 500 ng of CRU 263 DNA per reaction volume.

DNA polymerase enzyme from Biotaq and Klentaq were assessed over 0.5 - 6.5 Units, increasing in 0.5 steps for ICS 1 with the SSR primer pair 240/241. Both enzyme forms generated the same profiles, with an increased band thickness as enzyme concentration was increased. However, better background resolution was obtained at lower enzyme concentrations, with bands under Klentaq conditions tending to decrease in staining intensity when more than 3 Units were present. A concentration of 0.5 Units per 25 µL reaction was supported. Fine assessment of the effect of enzyme concentration was checked by evaluating 0, 0.025, 0.05, 0.1, 0.2, 0.4 and 0.5 Units for the accession AM 2/38 [POU] with mTcCIR12 as well as the accession ICS 65 with mTcCIR15. There was an increase in staining intensity with increasing enzyme concentration but this was not very different at values greater than 0.1 Units. Multiple or artefact bands and smearing were absent. To obtain a satisfactory result with efficient use of resources, it is recommended that an enzyme concentration of 0.1 Units/25 µL be utilised.

Mixed loadings of PCR products could be resolved on agarose gels but there were instances where confusion arose, especially when the products sizes were not known beforehand. Staggered loading yielded better results. A second loading was applied after approximately one hour had elapsed. This resulted in easily readable gels as the front positions did not overlap. More than two loadings may be possible but the time between loadings must be reduced to allow sufficient runtime.

A flowchart of control processes where costing may be critical is presented in Figure 2.

Figure 2. Flowchart for stages involved in verification work for cacao using SSR markers. Cost estimates (US$) for each stage are for the assessment of 100 plants with 15 SSRs without multiplexing or multiple loading.



Examination of Figure 2 reveals that the Kobayashi method is a cheaper alternative to that involving use of QIAGEN kits. However, cost savings must be weighed against the necessity of buffer preparation and the time required for dissolution of DNA (especially with crude DNA) in the former method as opposed to the resultant aqueous DNA solution as the end product from the QIAGEN kits. Substantial cost savings are possible if reduced enzyme concentration and/or if

Klentaq instead of Biotaq polymerase is utilised. Further savings can be achieved by using plates instead of tubes and by re-using these microplates. The cost of staining was much more for polyacrylamide gels than for agarose gels. Revelation of SSR profiles on polyacrylamide gels with silver nitrate staining should therefore be reconsidered since there is the tendency to have more artefact bands in polyacrylamide gels, the large polyacrylamide gels are unwieldy, the glass plates are difficult to clean, there is more waste for disposal by incineration and there is a higher degree of neurotoxicity with polyacrylamide gels.

Identification of mislabelled trees at the San Juan Estate

Reference ICS trees at the San Juan Estate were checked for off-types. Twenty-seven accessions, represented by 68 trees were evaluated with at least seven SSR primer pairs (Table 1). Morphological evaluation was approximately 63% coincident with molecular results, which gave an error rate of 33% by accessions and 13% by trees. These results indicate that three of the trees (ICS 10, 16 and 100) used for the field guide of Johnson *et al*. (2004) may have been off-types.

Table 1. Assessment of identities of select ICS accessions at the San Juan Estate.

		Morphological study		SSR study		
Accession	**Leaves from Blocks**	**Photos from Blocks**	**[1]Decision**	**# SSR loci evaluated**	**# SSR loci differing**	**Decision**
ICS 4	2 & 5	None	None	11	0	B2 = B5
ICS 10	3 & 5	3 & 5	B3 fits B5 differs	7	5 or 6	B3 ? B5
ICS 11	1 & 2	None	None	19	Probably 2	B1 = B2
ICS 14	1, 2 & 3	2	B2 fits	19	4 or 5	B1 ? B2 = B3
ICS 16	2, 4 & 5	2, 4 & 5	All OK	11	3	B2 ? B4 = B5
ICS 30	1, 2 & 5	1, 2 & 5	Only B1 & B5 fit	10	10	B1= B5 ? B2
ICS 36	4 & 5	None	None	11	0	B4 = B5
ICS 44	1 & 5	1 & 5	Both OK	20	0	B1 = B5
ICS 65	3 & 4	3 & 4	Both OK	17	Probably 2	B3 = B4
ICS 66	1 & 3	1 & 3	Both OK	11	0	B1 = B3
ICS 69	1, 2 & 4	1, 2 & 4	3 types?	20	0	B1 = B2 = B4
ICS 75	1, 2 & 4	1, 2 & 4	B4 differs?	20	0	B1 = B2 = B4
ICS 84	2, 3, 4 & 5	2, 3 & 4	4 types?	18	Probably 1	B2 = B3 = B4 = B5
ICS 86	2 & 4	2	Both OK	18	0	B2 = B4
ICS 87	1 & 2	None	None	11	0	B1 = B2
ICS 89	1 & 3	1 & 3	B1 ? B3	11	0	B1 = B3
ICS 92	1, 3 & 4	1, 3 & 4	Only B1 & B3 fits	7	3	B1 = B4 ? B3
ICS 93	3, 4 & 5	3, 4 & 5	All OK	22	Probably 3	B3 = B4 = B5
ICS 94	3 & 4	3 & 4	Both fit	22	Probably 1	B3 = B4
ICS 97	1, 2 & 3	2 & 3	B2 better; B3 differs	7	6	B1 = B2 ? B3
ICS 100	2 & 5	2 & 5	B2 fits B5 differs	7	5 or 6	B2 ? B5

Identification of mislabelled trees in the ICG,T

Twenty-five accessions represented by 114 trees were evaluated, and 13 accessions contained at least one off-type in the plots with a total of 40 off-type trees. Off-type trees detected are presented in Table 2. ICS 5 in Field 6B had four off-types which were similar to each other

Table 2. Off-type trees in the ICG,T as determined from SSR alleles resolved on agarose gels.

Accession	[1]No. of trees in ICG,T	No. SSRs used	SSRs detecting differences - mTcCIR	Off-types
AM 2/38 [POU]	3	24	None	None
DE 52/B [TTO]	4	4	None	None
EQX/JS [CHA]	1 (2 Trunks)	25	None	None
ICS 5	5	23	1, 11, 12, 15, 33, 40, 42, 49, 56, 57	Field 6B B124 T4, 8, 11, 12
ICS 16	3	10	None	None
ICS 28	4	3	None	None
ICS 30	3	10	1, 6, 7, 11, 12, 29, 32, 42, 56, 60	Field 6B E364 T13
ICS 40	8	5	1, 6, 12	Field 6B E287 T1, 4, 6, 9, 10, 11, 14
ICS 42	4	2	10	None
ICS 46	7	16	1, 6, 11, 12, 15, 29, 37, 42, 44, 49, 57, 58, 61	Field 6B E289 T1, 3, 7, 10, 11, 14, 15
ICS 73	3	13	6, 30, 44, 56, 57, 61	Field 4A C308 T1, 2, 3
ICS 82	3	17	None	None
ICS 97	1	10	1, 6, 11, 12, 15, 29, 30	Field 6B B110 T4
IMC 13	1	20	17?, 29?, 42?	None
IMC 47	12	26	1, 2, 3, 6, 8, 9, 11, 12, 15, 29, 37, 42, 44, 49, 55, 56, 57, 58, 60, 61	UWI Campus 11 x5y2; Field 6B F401 T1, 3, 4, 6, 9, 10, 11, 12, 13, 14
JA 5/41 [POU]	2	4	None	None
JA 5/47 [POU]	2	4	None	None
MXC 67	6	16	1, 6, 12, 15, 29, 37, 49, 56, 58, 61	UWI Campus 12 x4y5
NA 699	5	6	1, 3, 11, 58	Field 6B F448 T3
PA 120 [PER]	13	20	6, 10, 12, 15, 29, 37, 44, 61	UWI Campus 9A x15y9
PA 169 [PER]	12	10	None	None
PA 218 [PER]	6	14	None	None
SCA 11	1	5	12	UWI Campus 11 x4y19
SP 1 [VEN]	2	15	None	None
VEN B 47 [ICT]	2	17	2, 3, 6, 7, 11, 12, 15, 29, 40, 56, 58	Field 6B B90 T2, 7
? = 25	? = 114			? = 40

[1]Reference tree from Marper farm or San Juan Estate not included; ICG,T covers both UWI & UCRS fields

except perhaps T4 which appeared to be different with the primer pair mTcCIR56. ICS 30 of Field 6B had one off-type which was identical to the off-type ICS 30 of Block 2 in the San Juan Estate. All the ICS 40 off-types in Field 6B were identical to each other, as had previously been reported for different primers (Motilal, 2004). ICS 46 in Field 6B was heterogenous with three off-type groups: (a) T1, (b) T3 and (c) T7, T10, T11, T14 and T15. ICS 73 in Field 4A was also

heterogenous with T1 and T2 being similar to each other but different from T3. IMC 47 off-types were separated into four groups: (a) UWI C11 x5y2, Field 6B F401 T13, T14 and perhaps T6, (b) Field 6B F401 T1, T3, T4 and T11, (c) Field 6B F401 T9 and (d) Field 6B F401 T10. VEN B 47 [ICT] in Field 6B, is represented by two trees but these are not identical to each other.

Consideration of all SSR data to date revealed error rates in tree identities of 4.9% (8/164) at UWI, 27.8% (83/298) at UCRS, giving a value of 19.0% (91/462) in the ICG,T (UWI and UCRS fields together).

Discussion

The verification work at CRU has highlighted a number of areas which are critical for genebank management.

Firstly, reference trees must be carefully selected, since mistakes at this point affect all downstream processes. This is well illustrated by the presence of errors in the Cheesman Field at the San Juan Estate, where trees which belong to the same accession had been thought to be correct. This assumption was later shown to be wrong when error rates of 33% and 13% were found in accessions and trees. Caution is needed to avoid the use of misidentified material as standards for reference samples, by assessing both morphological characters and molecular profiles before selecting in confirming the identity of reference trees.

Secondly, the choice of primers appears to be critical especially when resolving on agarose gels. For instance, ICS 73 was reportedly a homogenous off-type plot at UCRS Field 4A (Motilal, 2004) but the use of the primers mTcCIR1, 6 and 56 separated this supposedly uniform group of trees. The accession VEN B 47 [ICT] is a known mixed plot as the two trees have different coloured pods; however earlier work with the primers mTcCIR29 and 45 did not easily resolve these, unlike the present study (Table 2) . The issue concerning the choice of primer pairs have been raised (Motilal and Boccara, 2004). The problem may not be restricted to the lower resolution agarose system since two reportedly homogenous groups from sequencer data could be further split by three primers not present in the sequencer data and which were resolvable on agarose gels. These primers were mTcCIR38, 56 and 58. Modification of the set of primers used internationally may be worthwhile as this may prevent the lumping of accessions which are not genetically equivalent. A usable set which may bring lower resolution data more aligned with sequencer data is provided in Motilal and Boccara (2004).

The third aspect is perhaps the most disturbing, in that there was an unexpectedly high level of mismatching between allele size calling by Johnson *et al*. (2004) and that observed on agarose gels. One would have expected that the agarose system would have been consistently underscored. However, the differences observed seemed to reflect inconsistencies between the results on gels and allele sizes reported from the use of a sequencer. This could be due to a large degree of variation between and within gels or to a mix-up of samples. The former explanation is probably unlikely since runs with the same sample/SSR primer pair combination at various times (different gels/buffer/p.d.:I ratio) have resulted in consistent scoring in this laboratory since the start of SSR work at CRU. Mixing up samples during PCR is a possible source of error, but experiments were repeated at least once and the same type of profile appeared repeatedly. It is still possible that problems may have originated at the level of the DNA inventory either at CRU or elsewhere and/or sample confusion occurred at some stage in the sequencer run.

Fourthly, it would appear that substantial cost savings can be achieved with the agarose system making it even more attractive to low-end users. It would appear that preliminary work on genebank material can be performed on agarose to detect easily discernible off-types at a genotype level. The remaining true-type trees can then be accepted as such until so established from sequencer data. This scenario would result in a relatively rapid generation of results and a reduced demand on sequencer resources.

Lastly, the error rate in tree identity within the ICG,T is currently estimated at 19%. The sample size may be biased and is indeed small (<5%) and so is unlikely to reflect the true error rate within the ICG,T. However, this estimate may be used as a working value until such time that a more reliable figure is obtained.

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Progress in resolving identity issues of the cacao resources held in Trinidad

M. Boccara

Introduction

Conservation of genetic resources has become a priority for the world cocoa community. However, the multiplicity of sources of material, of locations of collections and the wide dispersal of end users has resulted in misinterpretation of identities in many instances. Recent molecular techniques for identification of cocoa clones cannot be efficient if collaborative work between research centres is not implemented. The ICG,T is a living reference for verification of material now planted all over the world; nevertheless, to be useful, information collected and published must be precise and correct. Collaborative projects and common strategies could help the efficiency of the work being carried out.

An international collaborative project on DNA fingerprinting of cacao germplasm was started in 2001 with an aim to obtain a fingerprint of each living accession in the Americas. DNA extracted from the most original tree of each accession present in Trinidad has been shipped to the USDA Beltsville Laboratory for analysis by microsatellite markers. Duplicate trees of the UCRS fields have also been tested for mislabelling. Each branch from where sample leaves were taken, was clearly identified (tagged) with a specific blue label, and an FP# (fingerprinting number) was dedicated to each sample. Collections of extra leaf samples have been also undertaken for comparison to existing data.

Priority is given to the analysis of Upper Amazon material such as the "Parinaris" (PA) since these are of particular interest to the international cocoa community.

The PA accession group

It has been reported that the PA accession group originated from 20 pods collected by Pound in 1938 and that 277 PA accessions were planted in Marper Farm from 1939 to 1941 (Pound, 1943). Records available in CRU show that in 1943, 133 PA accessions were recorded in Block D and 11 in Block C, making a total of 144 established clones.

Currently, 92 trees labelled PA are still alive in Block D, including 2 adjacent PA 13 [PER] trees and 2 PA 187 [PER] clones in positions 737 and 482 respectively.

Nine accessions are still alive in Marper Block C and among them PA 293 [PER] which is also present in Marper Block D.

A total of 101 samples were collected including 98 different original trees and 3 replicated trees (PA 13 [PER], PA 187 [PER] and PA 293 [PER]).

Sixteen samples of trees now absent from Marper were collected from the UCRS field as well as 41 replicated samples to check their conformity.

Results

Original trees in Marper fields

The molecular profile from 15 microsatellite primers of the 2 adjacent trees PA 13 [PER] planted in Marper D158 and D159 are identical, showing that they are replicates. The same was found for both examples of PA 293 [PER], one planted in Marper C817 and the other in D762. However, analysis of the data concerning PA 187 [PER] (Marper D737, D482) shows that the trees do not share the same profile (13/15 SSR, D. Zhang, personal communication). Furthermore, leaf samples provided to USDA Miami (J.C. Motamayor, personal communication) shows that the tree in D737 is identical to the tree PA 189 [PER] in position D489. As an interesting footnote, a question mark had been inserted in the notes for this tree dating from 1943.

The DNA profile of PA 205 [PER]suggests that it belongs to the Trinitario group, and it is possible that the leaf sample was obtained from the surviving rootstock.

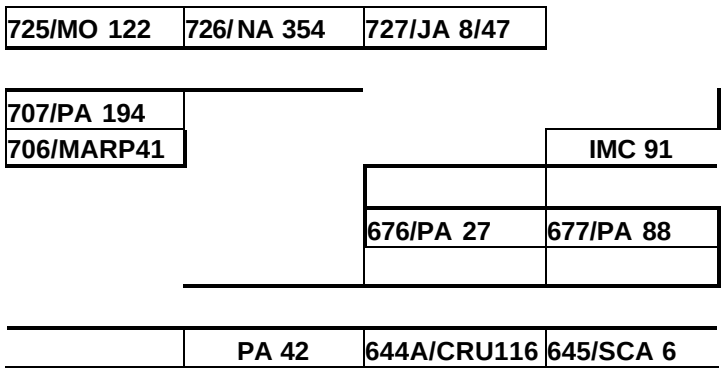
Though no other irregularity has been shown with microsatellite markers among the PA accession group planted in Marper Farm, controversial results could occur if DNA were to be extracted from samples collected at other times to the definitive reference samples sent to Beltsville.

Duplicated trees in UCRS fields

PA 27 [PER], PA 88 [PER] and PA 194[PER]

PA 27 [PER], PA 88 [PER] and PA 194 [PER] were planted contiguously in Marper Block D (Figure 1). PA 194 [PER] has since died. The results of DNA analysis have confirmed that confusion must have occurred during the propagation of these clones when the UCRS fields were established. PA 27 [PER] planted in 5B originates from PA 88 [PER] (Marper D677), while PA 194 [PER] planted in 5B was propagated from PA 27 [PER] (Marper D676). It is interesting to note that the profile of PA 88 [PER] in the ICGD database for RUQ 34 is different from all the above. This clone was transferred to Reading quarantine from the Royal Botanical Gardens, Kew.

Figure 1. Excerpt of the Block D field map in Marper Farm (trees are shown by position number/clone name).



Mislabelling of other accessions

Many different cases of mislabelling can occur, especially when records are not available, labels are misplaced and maps are wrongly interpreted.

Suspected mislabelling in Marper Farm

The preliminary results of the USDA/BCCCA/CRU Fingerprinting Project showing accessions with identical profiles suggested that mislabelling of some trees probably occurred at the time of planting in Marper Farm. That may be the case for a few neighbouring clones, however results have to be carefully interpreted as sampling leaves for DNA extraction could also be a reason for errors (Table 1).

Table 1. Neighbouring trees in Marper Farm sharing the same DNA fingerprinting profile. For each row of the table, accession I was found to be identical to accession II.

Accession I			Accession II		
Clone name	**Fingerprinting number**	**Marper location**	**Clone name**	**Fingerprinting number**	**Marper location**
AM 1/19 [POU]	FP#2145	C923	AM 2/92 [POU]	FP#1606	C924
AM 2/17 [POU]	FP#2022	C440	CLM 65	FP#2302	C438
AM 2/3 [POU]	FP#1439	C105	MOQ 6/46	FP#1296	C106
AM 2/68 [POU]	FP#1275	C258	CL 78/9	FP#2007	C297
AM 2/70 [POU]	FP#1338	C236	LX 20	FP#695	C192
B 8/8 [POU]	FP#1602	C1068	LX 41	FP#2089	C1088
LP 1/56 [POU]	FP#2156	C916	SJ 1/33 [POU]	FP#2582	C917

Suspected mislabelling in UCRS fields

As all the material planted in the UCRS fields is a multiple replication of an original tree, other mislabelling could have occurred during propagation and establishment of these clones.

The accession names used for some plots in the UCRS fields are shown to be invalid when the DNA fingerprinting profile of the tree (accession I) matches that of another accession (accession II), and the original mother tree of "accession I" had either never been recorded in Trinidad, or had died long before the establishment of the UCRS plots (Table 2). In these cases, the mistake must have been made in writing the wrong accession name.

Identity verification of individual trees within plots of UCRS is being confirmed by comparing the SSR marker profiles with the reference tree where possible, or by comparing individual trees within the same plot.

Table 2. Mislabelled plots in the University Cocoa Research Station, Centeno.

Accession I				Accession II			Original tree		
Clone name	Finger-printing number	Location in UCRS Field	Plot	Clone name	Finger-printing number	Position in Marper	Clone name	Position in Marper	Status
AM 1/29 [POU]	FP#1716	5B	I804	AM 1/10 [POU]	FP#1969	C360	AM 1/29 [POU]	C577	Dead
B 22/7 [POU]	FP#1257	5B	A32	B 22/17 [POU]	FP#52	D127	B 22/7 [POU]	D162	Dead
JA 3/39 [POU]	FP#1702	5B	F512	JA 3/37 [POU]	FP#2081	C1120	JA 3/39 [POU]	C1123	Dead
JA 5/19 [POU]	FP#1712	5B	F427	JA 5/18 [POU]	FP#2032	C420	JA 5/19 [POU]	C804	Dead
LP 4/45 [POU]	FP#1673	4A	E473	LP 4/41 [POU]	FP#2172	C965	LP 4/45 [POU]	C879	Dead
LX 1[a]	FP#1682	4A	D335	MOQ 6/5	FP#1294	C92	LX 1	C91	Dead
MOQ 2/17	FP#1570	5B	C197	MOQ 4/17	FP#1804	C984	MOQ 2/17	C865 and C866	Dead
AM 2/60 [POU]	FP#1559	5B	I760	AM 1/60 [POU]	FP#1313	C179	AM 2/60 [POU]	Was never in Marper	
B 5/5 [POU]	FP#1942	5A	B99	B 5/3 [POU]	FP#1599	C1023	B 5/5 [POU]	Was never in Marper	
B 2/34 [POU]	FP#1569	5B	A45	B 23/4 [POU]	FP#1489	D383	B 2/34 [POU]	Was never in Marper	
IMC 81	FP#1635	6B	F421	MO 81[b]	FP#1188	D192	IMC 81	Shouldn't exist	
NA 33[c]	FP#1669	4A	D371	NA 833	FP# 297	D640	NA 33	Was never in Marper	

[a]LX 1 scions were taken from the neighbouring tree in Marper C92.
[b]The accession MO 87 present in 4A, recently added to the CRU database, is also a replicate of MO 81.
[c]The accession NA 33 planted in field 4A, selected as a priority clone in the CFC/ICCO/IPGRI Germplasm Utilisation Project is identical to NA 833. However the SSR profile does not match the ICGD NA 33 (RUQ 822), provided by CIRAD. There is no record of NA 33 ever having been planted at Marper Farm.

Conclusion

Analysis of SSR profiles of the PA accession group showed that there are very few mistakes with the identity of original trees. More results regarding upper Amazon material will add to our knowledge of the genetic diversity of this group. International exchange of data has greatly improved the effectiveness of resolving identity ambiguities.

References

Pound, F.J. (1943) First Report on the selection of cacao trees for resistance to Witches' Broom disease. Unpublished report, Ministry of Agriculture, Trinidad.

Characterisation

Examining phenotypic relationships among Upper Amazon Forastero clones

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Introduction

The objective of this study was to examine the phenotypic relationships among several Upper Amazon Forastero (UAF) accession groups, *viz*., IMC, MO, NA, PA and SCA in relation to some of their known progenitors, POUND accessions (Pound, 1943a). This is in keeping with our mission to generate information on the germplasm conserved in the International Cocoa Genebank, Trinidad that will facilitate its efficient utilisation, management and improvement (Bekele, 1999; Bekele *et al*., 1994; Bekele and Bekele, 1996; Bekele *et al*., 2005; Iwaro *et al*., 2003a). The UAF clones were selected for this, the first of several such planned studies, because they account for close to 60% of cocoa cultivation globally and are widely used in breeding in Trinidad and elsewhere, e.g. IMC 67, SCA 6, POUND 7 [POU], as well as selections from the PA accession group (Eskes and Lanaud, 2001; Wood and Lass, 1985). These clones possess many desirable traits, which justify their continued prominence in germplasm enhancement programmes such as those described by Iwaro *et al*. (2003b).

The POUND clones were collected as budwood from Loreto, Peru, near Iquitos and Nanay in 1942 (Pound, 1943) by the late Dr. F.J. Pound of the Trinidad Department of Agriculture. Collecting from trees, with no obvious signs of Witches' Broom (WB) disease, was usually restricted to 2-3 hundred yards inland from the respective river (Table 1). Three or four plants were propagated at Iquitos, Peru and later shipped to the Barbados Quarantine Station. Originally, each collection was assigned an alpha-numeric code (*e.g.* POUND 1 [POU]), but suffixes were subsequently appended in Trinidad to produce designations such as POUND 1/B [POU] since variants among some of the replicated original trees were observed over time. The suffixes may refer to plants originating from budwood collected on adjacent trunks growing at the same site or to seedlings originating from the original tree.

Morphological data are available at CRU for the following POUND accessions: POUND 1/B [POU], 2/B, 4/A, 5/B, 5/C, 7/A, 7/B, 7/C, 8/C, 9/B, 10/B, 10/C, 12/A, 15/A, 16/B, 18, 18/A, 25/A, 26/C, 27/C, 31/A, 31/C, 32/A.

Table 1. Collecting sites of some of the POUND clones featured in this study.

POUND clone	Collection site
POUND 1 – 8, 15	Río Nanay, river bank
POUND 9 -14, 16, 17, 19, 20, 30	Río Nanay
POUND 18	Island south of Iquitos
POUND 21	Island river bank
POUND 31, 32	Río Ucayali, Contamana

The POUND mother trees of the other UAF groups under study were identified by Pound (1943a) as:

IMC – POUND 18 and 21;
NA – POUND 1[1], 2[1], 3[1], 4[1], 5, 6, 7, 8, 9, 10, 11, 12[2], 15, 19, 20, 30 and 32[1];
SCAVINA – POUND 31;
PA and MO – unspecified: ? 20 trees and 1 tree, respectively.

IMC clones

The IMC clones were collected directly opposite the town of Iquitos in Loreto, Peru, on a large island in front of Iquitos, 73.12W, 3.5S (Pound, 1938). Pods were collected from at least two groups of trees free of WB disease in 1937. They were probably progenies from POUND 18 and 21, collected and propagated as clones in 1942 (Pound, 1943a). One hundred and twelve IMC clones were planted at Marper Farm (Pound, 1943b). Three main pod morpho-types for IMC clones were recognised by Pound:

Pod type 1: Very large 'lagarta calabacillo', oval shape and smooth, normal blanco;
Pod type 2: Large, somewhat warty at stem end, but smooth and round at the apex with large, flat 'lozenge' shaped seeds;
Pod type 3: Small oval and warty. (The trees bearing these pods were flowering heavily, as is typical of self-incompatible trees).

MO clones

These clones were collected in Loreto, Peru, on the Río Morona, 77.17W, 4.12S (300 miles from Iquitos). The pods may have been collected from one tree free of WB from a group of 25 trees. Eighty MO clones were subsequently planted at Marper Farm Pound (1943b). Accessions with large numbers (>100) could be MOQ clones (Bartley, personal communication in 1993, cited in the ICGD by Wadsworth and Harwood, 2000), but this does not agree with records in CRU dating from 1943. The pods are typically short, oval, and warty. Some have a red tint.

NA clones

The collection site for the NA clones is given as Loreto, Peru, along the Río Nanay, about 15km from Iquitos, 73.17W, 3.38S. The pods were probably collected from 14 (Pound, 1938) or 17 trees (Pound, 1943a) free of WB and laden with fruit. Nine hundred and eight NA clones were originally established at Marper Farm between 1939 and 1941 (Pound, 1943b). The fruits are typically completely unpigmented, half blanco, long oval, and slightly warty with no conspicuous point or bottleneck. Some NA trees currently appear to have several different characteristics from their original descriptions. This is postulated to be a result of the generation of seedling progenies of the original collections (Bartley, personal communication in 1995, cited in the ICGD by Wadsworth and Harwood, 2000).

[1] Noted as being disease-free in 1937 (Pound, 1938)
[2] POUND 12 was collected on the Río Nanay not far from the area where the IMC trees were collected (Pound, 1943)

PA clones

Pound (1938) reported the collection site of the PA clones to be Loreto, Peru, lower Río Maranon, Parinari, 74.60W, 4.6S. Pods were collected from some 7-20 trees free of WB. Two hundred and seventy seven PA accessions were transferred to Marper Farm (Pound, 1943b). Pound described the pods as being long, generally warty, with a pronounced bottleneck and conspicuous point. Some pods were almost smooth with five, shallow furrows. The pods varied in appearance from blanco to pure green. Six morpho-types were distinguished by Pound (1938).

SCA clones

These clones were collected in Loreto, Peru, along the Río Ucayali, 75.00W, 7.21S. The pods were reputedly collected from one tree at Contamana, and 27 plants were generated. Twenty two clones were eventually planted at Marper Farm (Pound, 1943b). Pound (1938) described the pods as green or slightly blanco, with a central type that was mid-green (such as SCA 6), only moderately warty, with a definite but blunt point and lacking a bottleneck. Pound also recorded a lagarta type, which was mostly warty with a less pronounced point and generally no bottleneck constriction. Variants were also observed; more warty or of the smooth, five-furrowed type. The latter were the only ones slightly susceptible to WB.

Materials and Methods

Morphological or phenotypic data were collated over several years for the UAF clones included in this study. Details on the methodology are provided in previous Annual Reports of the Cocoa Research Unit, and by Bekele and Butler (2000); Bekele *et al*., (2005) and Iwaro *et al*. (2003a). Four datasets were analysed each for the 25 descriptors or variables employed from the International Board for Plant Genetic Resources (now IPGRI) descriptor list for cacao (Anon., 1981) (Table 2):

1. 74 accessions – 20[1] IMC, 20[1] NA, 11 SCA and 23 POUND;
2. 40 accessions – 17 MO and 23 POUND accessions;
3. 90 accessions – 67[1] PA and 23 POUND accessions;
4. 111 accessions - 20[1] IMC, 20[1] NA, 17 MO, 20[1] PA, 11 SCA and 23 POUND.

The purpose of analysing individual UAF groups with the available POUND accessions was to afford potential users of the germplasm the opportunity to closely examine phenotypic relationships among the accessions so that informed choices could be made during selection. The relationships within and between the aforementioned accessions groups were examined using the multivariate technique, Cluster Analysis (NTSYSpc Ver. 2.10b, NTSysPc, 2000). The data were first standardised to eliminate the effects of different scales of measurement. Similarity matrices were generated and clustering was performed. Dendrograms (trees) were used to depict, in two dimensions, the inter-relationships among the accessions studied, and were displayed using the

[1] Generated as random numbers from the available data

graphics facility of NTSYSpc.

The group average method (UPGMA-unweighted pair-group method using arithmetic means) was used to perform cluster analysis (CA) as recommended for this type of dataset (mixed continuous and categorical) (Mardia *et al*., 1979; Sneath and Sokal, 1973).

In order to test whether the clusters obtained in each analysis were valid, a cophenetic value matrix of the tree matrix, produced after clustering using the SAHN[1] procedure of NTSYSpc, was computed using COPH[2], and the MXCOMP[3] module was used to compare the cophenetic value matrix with the original matrix that was clustered. Principal components analysis (PCA) was also performed on the data obtained for the 111 accessions, representing all of the accession

Table 2. Descriptors used for morphological characterisation - their states and sample sizes (n).

Descriptor	State
Flower, anthocyanin intensity in column of pedicel	1=green, 2=reddish, 3=red [n=10].
Flower, sepal length (mm) [n=10]	
Flower, anthocyanin intensity on ligule	0=absent, 3=slight, 5=intermediate, 7=intense [n=10]
Flower, ligule width (mm) [n=10]	
Flower, anthocyanin intensity in filament	0=absent, 3=slight, 5=intermediate, 7=intense [n=10]
Flower, style length (mm) [n=10]	
Flower, ovule number [n=10]	
Fruit, shape	1= oblong, 2= elliptic, 3=obovate, 4= orbicular [n=10], 5= other.
Fruit, basal constriction	0=absent, 1=slight, 2=intermediate, 3=strong, 4=wide shoulder [n=10]
Fruit, apex form	1=attenuate, 2=acute, 3=obtuse, 4=rounded, 5=mammillate, 6=indented [n=10]
Fruit, surface texture (rugosity or degree of wartiness)	0=absent, 3=slight, 5=intermediate, 7=intense [n=10]
Fruit, anthocyanin intensity in mature ridges	0=absent, 3=slight, 5=intermediate, 7=intense [n=10]
Fruit, ridge disposition	1=equidistant, 2=paired [n=10]
Fruit, primary ridge separation	1=slight, 2=intermediate, 3=wide [n=10]
Fruit, pod wall hardness [n=10]	3= = 1.6 MPa, 5 = > 1.6 MPa = 2.0 MPa, 7= > 2.0 MPa
Fruit, length (cm) [n=10]	
Fruit, width (cm) [n=10]	
Seed, number [n=10]	
Seed, shape	1=oblong 2=elliptic 3=ovate
Seed, cotyledon colour	1=white, 2=grey, 3=light purple, 4=medium purple, 5=dark purple, 6=mottled [n=40]
Wet bean weight (total) (g) [n=10]	
Cotyledon length (cm) [n=20].	
Cotyledon width (cm) [n=20].	
Cotyledon weight (g) [n=20]	
Pod index (the number of pods required to produce 1 kg of dried cocoa) [n=10]	

[1] Sequential agglomerative hierarchical nested clustering
[2] Cophenetic module
[3] Matrix comparison module

groups studied. For further validation, it was then ascertained whether clusters in the dendrogram (Figure 1) coincided with groupings in the three-dimensional PCA plot (Figure 2).

Descriptive statistics for each descriptor studied were generated using MINITAB Release 12 (Minitab Inc., 1997). The significance of the between accession group variation in Pod Index value (PI) (Table 2) was tested using the one-way ANOVA[1] module of MINITAB.

Results

Considerable phenotypic diversity was observed among the UAF clones studied (Tables 3 and 4), and this coincided with observations on other samples of germplasm from the ICG,T (Bekele *et al*., 1994; Bekele and Bekele, 1996; Bekele *et al*., 2005; Iwaro *et al*., 2003a).

Table 3. Minimum and maximum values observed in this sample of germplasm (111 UAF clones) for the 25 descriptors used.

Descriptor	Minimum value	Maximum value
Ligule colour	0.00	7.00
Filament colour	0.00	7.00
Pedicel colour	1.00	3.00
Sepal length	5.29	9.12
Ligule width	1.83	3.83
Ovule number	33.0	62.0
Style length	1.33	3.56
Mature pod ridge colour	0.00	5.00
Pod shape	1.00	3.00
Pod basal constriction	0.00	3.00
Pod apex form	1.00	6.00
Pod surface texture	0.00	7.00
Pod ridge disposition	1.00	2.00
Pod ridge (pair) separation	1.00	3.00
Husk hardness	3.00	7.00
Bean colour	2.00	5.00
Bean shape	1.00	3.00
Pod length	11.1	20.0
Pod width	6.0	11.1
Wet bean weight (less pulp)	26.0	82.2
Bean number	26.6	59.3
Cotyledon weight	0.50	1.19
Cotyledon length	1.64	2.68
Cotyledon width	0.86	1.38
Pod index	17.0 (IMC 96)	65.4 (SCA 11)

The variation between accession groups was highly significant ($F = 6.01$, $P<0.0001$). IMC was the most promising accession group in terms of PI. A similar result was also found in a study by Bekele *et al.* (2005), which included Trinitario and Refractario germplasm.

[1] Analysis of variance

Table 4. Descriptive statistics of Pod Index values for the accession groups studied.

Accession group	Mean Pod Index[1]	Standard error	Coefficient of variation	Minimum value	Maximum value
IMC	23.8	0.8	15.2	17.0	29.5
NA	29.6	1.1	15.7	23.9	42.7
POUND [POU]	30.4	1.7	26.4	17.8	46.7
PA [PER]	31.3	1.4	20.5	19.7	48.0
MO	34.2	1.5	17.8	25.4	46.0
SCA	35.1	3.8	36.2	20.2	65.4

[1]The number of pods required to produce 1 kg of dried cocoa

Cluster analysis (CA) of all UAF accession groups

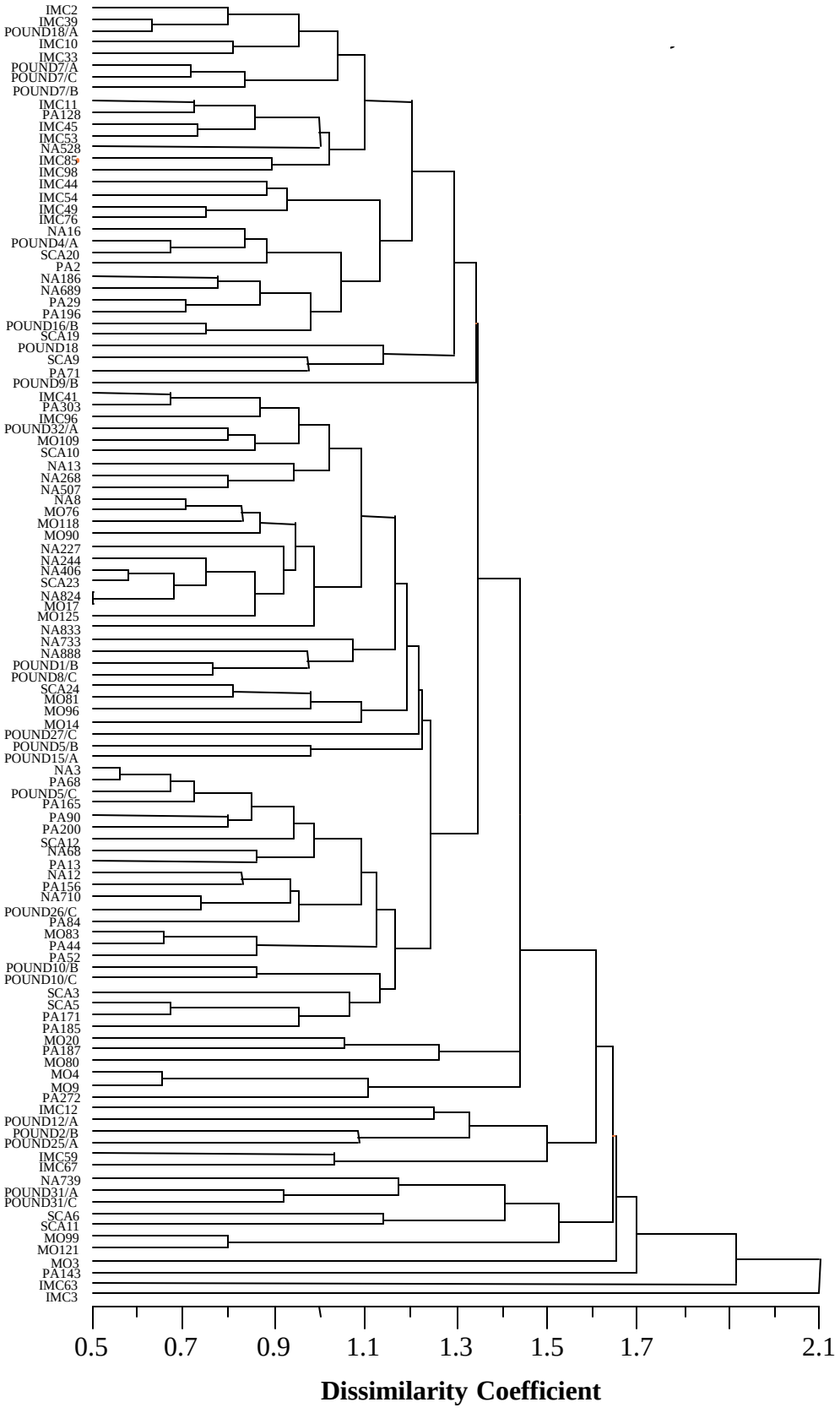
The phenotypic relationships among all of the UAF groups analysed using CA are depicted in Figure 1. The cophenetic correlation, calculated as a measure of goodness of fit for the cluster analysis, suggested a good fit (r = 0.81). Some of the main observations of the relationships depicted in Figure 1 are listed below:

IMC clones tended to clump together;
IMC 39 and POUND 18/A [POU] were very closely linked;
IMC 59 and 67 were fairly closely linked;
NA clones formed several small clusters together, interspersed with SCA, PA and MO clones;
NA 824 and MO 17 were phenotypically identical (Dissimilarity Coefficient = 0.5)[1];
Some PA clones are grouped in small homogenous clusters e.g. PA 165 [PER], 90 and 200;
NA 406 and SCA 23 were very closely related;
NA 3 and PA 68 [PER] were very closely related;
PA 44 [PER] and MO 83 are closely linked. The latter was distinct from other MO clones;
PA 303 [PER] and IMC 41, and PA 171 [PER] and SCA 5 were closely linked;
MO 4 and 9, and MO 99 and 121were closely linked;
SCA 6 and SCA 11were fairly closely linked, and were distinct from other SCA clones;
POUND 7/A [POU], 7/C and 7/B were closely linked;
POUND 10/B [POU] and 10/C were fairly closely linked;
POUND 31/A [POU] and 31/C were fairly closely linked;
POUND 18 [POU] was fairly closely linked to PA 71 [PER] and SCA 9;
POUND 4/A [POU] and SCA 20 were closely linked;
MO 3, PA 143 [PER] and IMC 63 and 3 were the most distinct clones; ungrouped until a Dissimilarity Coefficient > 1.9 was applied.

(All of the clones formed one cluster at Dissimilarity Coefficient = 2.1)

[1] Smaller values of the Euclidean Distance or Dissimilarity Coefficient incidate more similarity between clusters.

Figure 1. Dendrogram depicting inter-relationships among the 111 UAF clones studied.

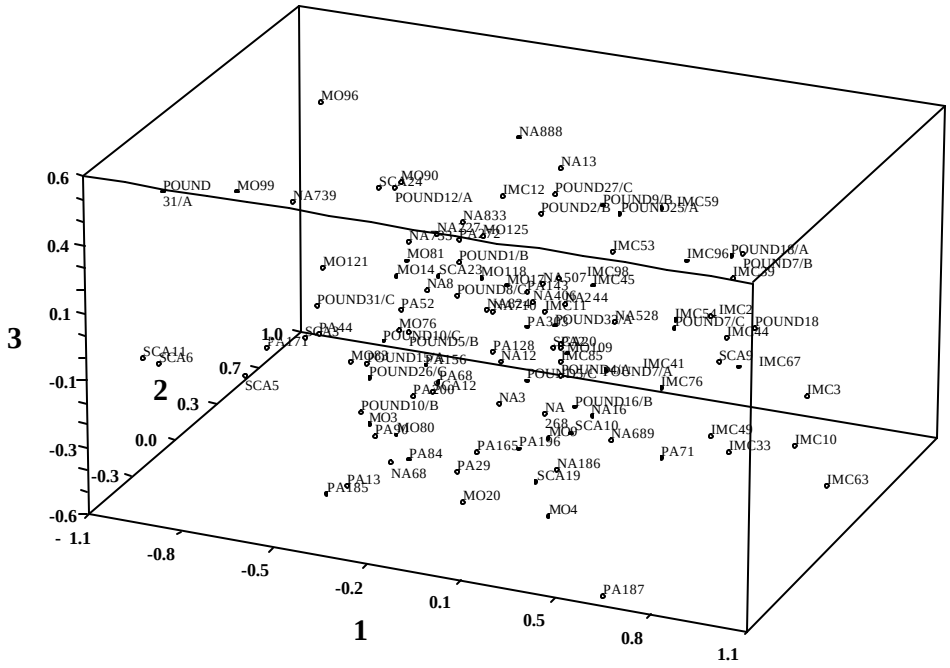


Comparison of the CA and PCA results for all the UAF clones studied

Congruent groupings for both analyses

POUND 7/A [POU], 7/B and 7/C were rather close in the PCA plot (Figure 2) as in the dendrogram (Figure 1) as were:
POUND 10/B [POU] and 10/C;
SCA 6 and SCA 11; and
POUND 4/A [POU] and SCA 20;
POUND 31/C [POU] and 31/A were in the same general area of Figure 2, and close in Figure 1;
POUND 31/C [POU] and 31/A were in the vicinity of SCA 6, 11, 3 and 5 (the other SCA clones were dispersed in Figure 2);
POUND 18 [POU], SCA 9 and PA 71 [PER] were fairly close in Figures 1 and 2;
NA clones were grouped closely in the PCA plot and were interspersed with SCA, PA and MO clones as in Figure 1;

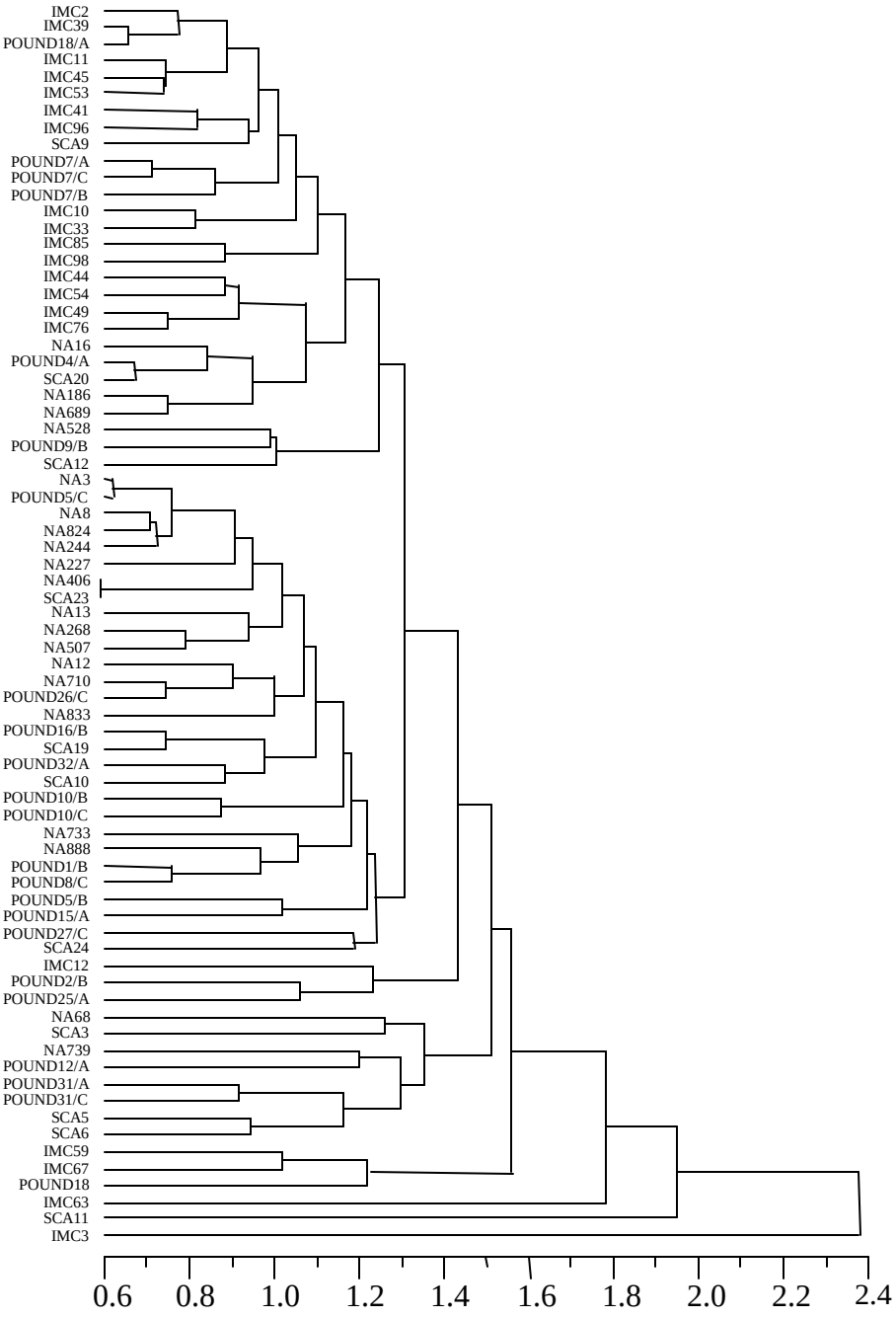
Figure 2. Principal component plot of the 111 UAF accessions studied.



IMC clones tended to group together (IMC 49, 33, 3, 10, 63; IMC 44, 67 and 54) in the PCA plot, and were discrete compared to the NA, PA, SCA and MO clones, as in Figure 1;
PA clones formed several tight groups in the PCA plot (Figure 2) as in Figure 1;
IMC 3 and 63 were among the most disparate clones in the PCA plot as they were in Figures 1 and 3.

In addition, IMC 67 and POUND 18 [POU] were grouped closely together in Figure 2, as they were in Figure 3.

Figure 3. Dendrogram depicting inter-relationships among the POUND, IMC, NA and SCA clones studied.



Some unique observations in the PCA plot of the 111 clones

MO 96, PA 187 [PER], SCA 6 and SCA 11, and IMC 10 were among the most disparate clones

in the PCA plot (IMC 3 and 63 were distinct in the PCA plot as well as in the dendrogram (Figure 1));
MO 90 and POUND 12/A [POU] grouped together closely as did:
POUND 9/B [POU] and 27/C;
NA 733 and MO 81;
SCA 23 and NA 8;
MO 3, 80 and POUND 10/B [POU];
MO 9 and SCA 10;
POUND 7/C [POU] and IMC 54;
POUND 16/B [POU] and NA 16;
POUND 4/A [POU] and 5/C;
IMC 41 and MO 109;
POUND 5/B [POU] and MO 76;
PA 84 [PER] and NA 68;
SCA 19 and NA 186;
POUND 18/A [POU] and 7/B;
POUND 7/A [POU] and 32/A;
MO 109 and IMC 85;
PA 68 [PER] and SCA 12;
POUND 31/A [POU] and MO 99;
MO 14, 81 and 121;
MO 118 and 17.
POUND 18 [POU] and 18/A were rather close unlike in the dendrograms (Figures 1, 3, 4 and 5).
POUND 5/B [POU] and 5/C were in the same general vicinity in Figure 2.

Phenotypic relationships observed in the dendrograms (Figures 1, 3, 4 and 5)

POUND 7/A [POU], 7/B and 7/C were closely clustered together in all of the dendrograms generated by CA. POUND 10/B [POU] and 10/C were closely linked in two of the dendrograms.

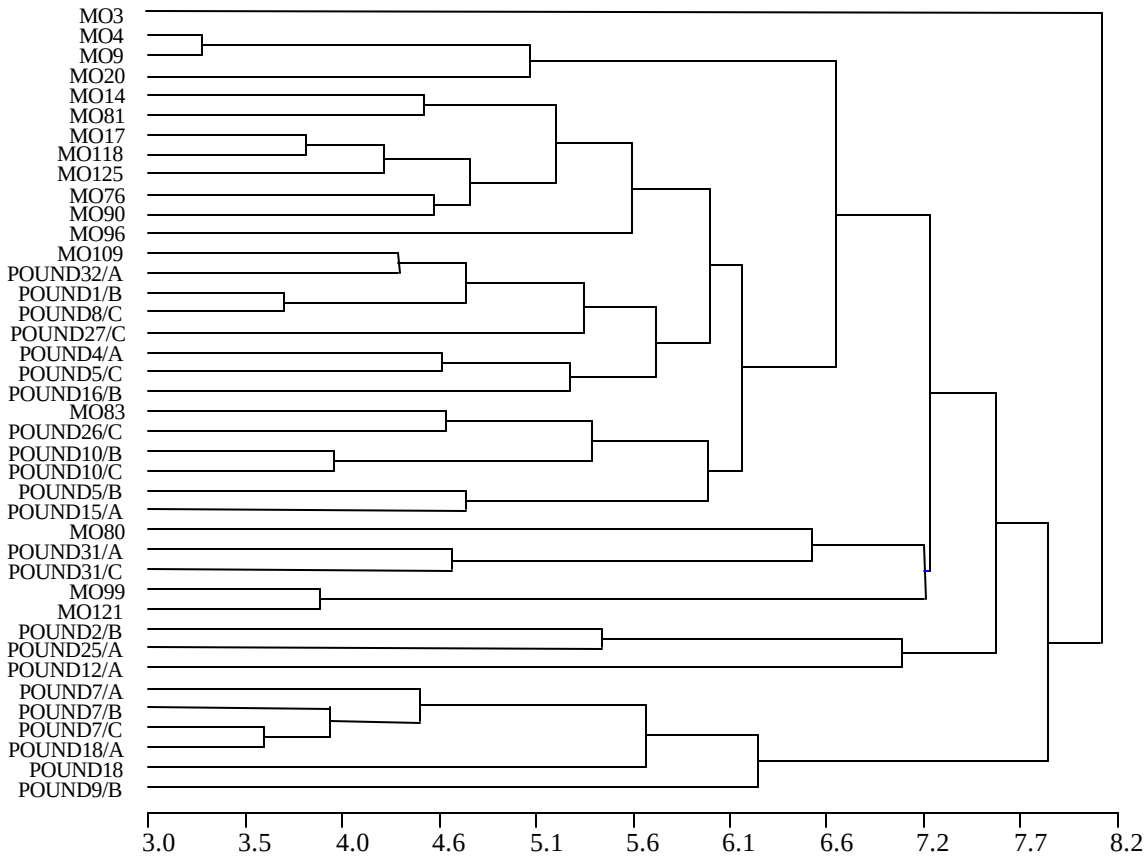
POUND 31/A [POU] and 31/C were closely linked in at least two of the dendrograms. POUND 18 [POU] and 18/A and POUND 5/B [POU] and 5/C were well separated in all the dendrograms.

IMC 3, 63, MO 3 and PA143 [PER] were found to be the most distinct clones overall in all of the dendrograms, and are thus considered prime candidates for conservation. IMC 3 and 63 were also found distinct with PCA. Sounigo *et al.* (2001a) also found MO 3 to be distinct from the MO clones studied with RAPD markers.

Mother trees versus 'Putative Progenies'

POUND 18 [POU] appears to be phenotypically related to its putative progenies, IMC 67 and 59 (Figures 2, 3). IMC 44, 2, 54, 67, 39, 96, 3, 76, 41, 49, 53, 45, 10, 33 and POUND 18 [POU] and 18/A were all in close association in the PCA plot (Figure 2). No data were available for POUND 21 [POU] and therefore no comparison could be made for this other putative mother

Figure 4. Dendrogram depicting inter-relationships among the POUND and MO clones studied.



tree of the IMC clones.

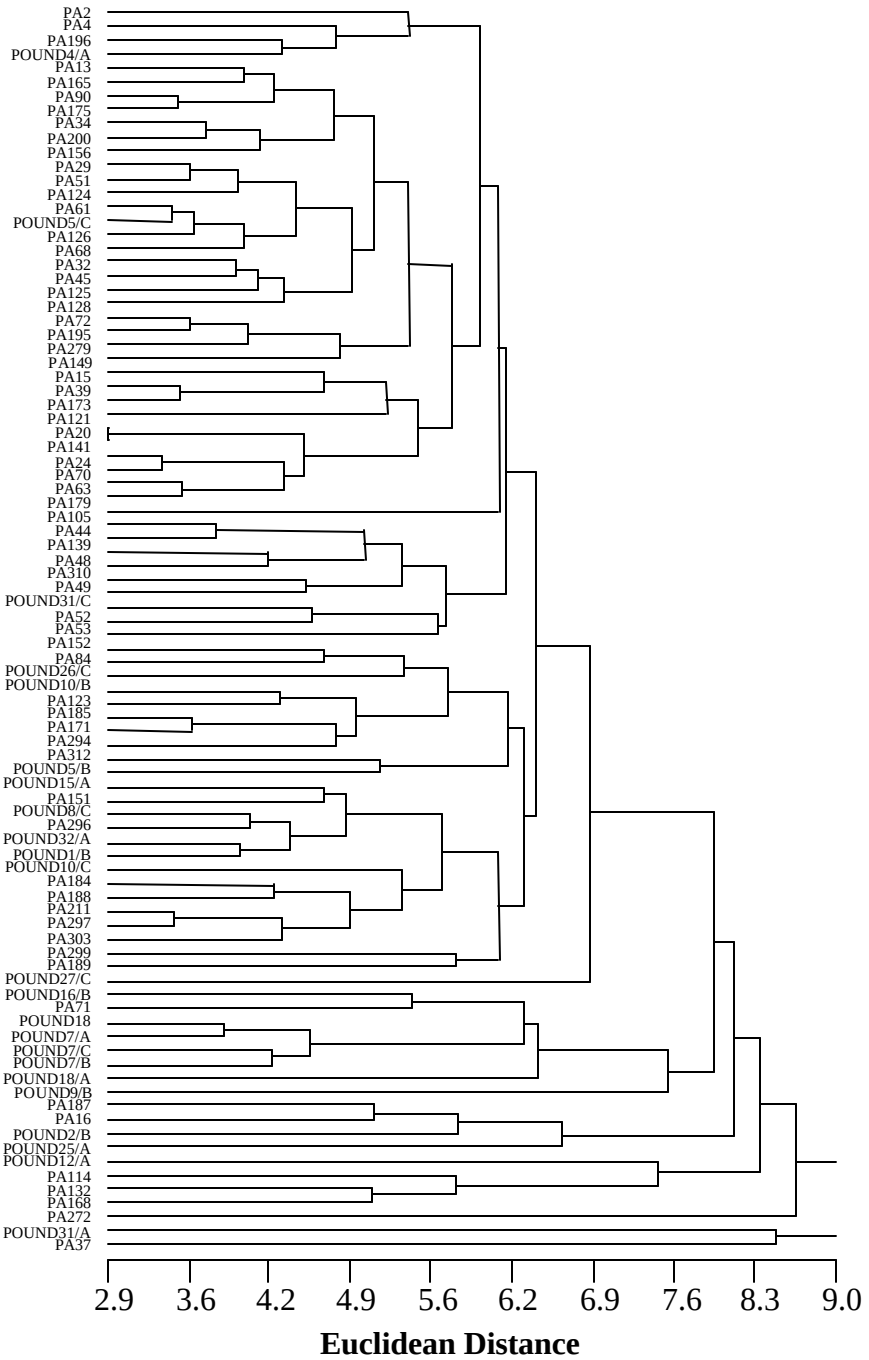
NA 888 and 733 were fairly closely related to POUND 1/B [POU] and 8/C, two putative NA mother trees, in Figure 1. NA 733, 227 and 833 were in close proximity to POUND 1/B [POU] in Figure 2. No other NA clones grouped closely with stated POUND mother trees (no data were available for POUND 19 [POU], 20, or 30).

Some SCA clones, such as SCA 6, 5, 3 and 11 grouped fairly closely with POUND 31/A [POU] and 31/C, the putative mother tree(s) of SCA clones (Figures 1, 2 and 3).

Summary of findings

POUND 7/A [POU], 7/B and 7/C were closely clustered together in all of the dendrograms and were in the same general vicinity in the PCA plot. Conversely, POUND 18 [POU] and 18/A were well separated in the dendrograms though they were much less so in the PCA plot. In the

Figure 5. Dendrogram depicting inter-relationships among the POUND and PA clones studied.



CA and PCA of the IMC, NA, SCA and POUND clones, the IMC clones tended to group together, and separately from the SCA and NA clones (Figures 1, 2 and 3). However, IMC 12, 59, 67, 63 and 3 were well separated from the other IMC clones in Figure 3. IMC 12 was also

more removed from other IMC clones in Figure 2. Clumping together of NA clones occurred frequently (Figure 1, 2 and 3). POUND 31/A [POU] and 31/C were closely linked (Figures 1, and 3), and SCA 6 (Figures 1 and 3) and SCA 5 (Figure 3) were closely associated with the latter two clones. SCA 6, 11, 3 and 5 were fairly close to POUND 31/C [POU] in the PCA plot (Figure 2), but further removed from POUND 31/A [POU]. Motomayor (personal communication) recently indicated that POUND 31/A [POU] and 31/C were not closely linked when analysed using SSR markers. This was somewhat corroborated in Figure 2.

There was also a fairly close association among IMC 59 and 67 and POUND 18 [POU] (Figure 3). A close relationship is evident between the latter two clones in Figure 2. POUND 10/B [POU] and 10/C were closely linked in Figures 3 and 4, and to a lesser extent in Figure 2. MO 3, IMC 3, 63, and PA 37 [PER] (Figures 1 and 5) and PA 143 [PER] were the most distinct clones in their respective accession groups (Figure 1). MO 96, PA 187 [PER], IMC 63, SCA 6 and SCA 11, IMC 3 and IMC 10 were the most disparate clones in the PCA plot (Figure 2).

According to Pound, the genotype collected on the Nanay River as POUND 12 [POU] grew not far from the area where the IMC trees were collected. Our results suggest that Pound 12/A [POU] is not phenotypically very closely related to the IMC clones although it was not far removed from IMC 12 in Figure 2.

Discussion and Conclusion

These results are interesting in the context of the origins of the clones, as described by Pound (1938; 1943a), Cheesman (1944) and others. The structuring of the phenotypic variation in this sample of germplasm appears to be influenced by geographic origin (the river basin where collection was made) as evident from the grouping of IMC, MO, NA, PA and SCA clones in various homogeneous clusters or groups (Figures 1, 2 and 3). The IMC clones formed 3 or 4 homogeneous clusters (Figure 1).

Most of the diversity was expressed in the NA, PA, MO and SCA groups; as evident from the many small homogeneous clusters, which were widely dispersed (Figures 1-5). It must be noted that recent diversity studies on Upper Amazon Forastero genotypes showed that the genetic distance between Nanay (NA) and IMC clones was relatively small (Sounigo *et al.*, 2001b). Sounigo *et al*., (2003) obtained diversity index values for several UAF accession groups based on RAPD analysis as shown in Table 5. The genetic diversity value for the NA group is not explained by the number of putative mother trees. This group has the most mother trees, but was found by Sounigo *et al.* (2003) to be least diverse genetically (Table 5). However he only sampled a relatively small number of clones in each accession group, which may not reflect the true diversity of each group. Nevertheless, another possible explanation of the anomaly in the genetic diversity observed by Sounigo *et al*. (2003) is that the widespread self-incompatibility in UAF clones and the possibility of mixed pollinations provided opportunities for a large number of pollen parents to contribute to the genepool and so create the possibility of large diversity among half-sib progenies, as was also observed by Lockwood and End (1993).

Table 5. Shannon Weaver diversity index values for UAF groups after Sounigo *et al.* (2003).

Accession group	Shannon Weaver diversity index[1]
IMC (5 clones)	0.36
MO (4 clones)	0.36
NA (8 clones)	0.31
PA (11 clones)	0.32
POUND (4 clones)	0.36
SCA (3 clones)	0.36

[1]Shannon and Weaver (1949)

In this study, the phenotypic diversity pattern observed is better explained by the number of mother trees identified by Pound (1943a) for NAs and PAs (more diverse), and IMCs (less diverse) compared to that of the genetic diversity (Table 5).

The phenotypic diversity pattern for the SCA and MO groups seems not to be explained by a single mother tree source. Sounigo *et al*. (2001a) drew a similar conclusion for MO and SCA in terms of genetic diversity; and Bartley (1968) and Mooleedhar (1986) for SCA. In the latter case, Mooleedhar identified eight compatibility alleles among the 11 SCA clones he studied, and proposed that at least four parents are involved.

Detailed consideration of the phenotypic relatedness among the clones studied along with information on their allelic richness or gene frequencies will be useful for selecting core collections, and when formulating strategies for future collections in the wild. In addition, the identification of potentially heterotic groups will be informative for further germplasm enhancement.

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Evaluation

Evaluation of cocoa germplasm for resistance to Witches' Broom disease

R. Umaharan, J-M Thévenin, A. Holder and J. Bhola

Introduction

The second phase of the WCF Witches' Broom disease screening project began in July 2003, and the project is being extended on an annual basis. In phase two of the project, revised targets were set in line with levels of financing and technical limitations encountered in phase one, (see Umaharan *et al*., 2004). The new targets are to complete mass screening of 60 new accessions and to confirm the resistance of 30 accessions per year. Emphasis is on the confirmation and quantification of the level of resistance of clones found to be putatively resistant during preliminary mass screening.

The most salient conclusions drawn from the first five years of the screening programme (phase one) were that considerable variation of resistance to WB disease exists within the ICG,T and therefore with careful screening for the disease it was possible to identify numerous clones showing some level of resistance. Also inferred from previous observations was the need to use additional criteria for assessing resistance. Hence in addition to percentage infection, symptom severity based on the broom diameter and incubation period were used to provide a quantitative measure of resistance. The results also suggested that a two-tiered screening system was needed. Therefore the protocol for WB screening now involves initial mass screening and selection of promising clones using a spray inoculation system, followed by the confirmation and quantification of resistance of the selected clones using the agar droplet technique (Surujdeo-Maharaj *et al*., 2003). This report gives a summary of the guidelines now adopted for evaluation of the results of WB resistance screening at CRU.

Material and Methods

Inoculation

Spray - mass screening

Inoculation of clones for mass screening is being carried out manually using a Preval Sprayer (Precision Valve Corp., NY, U.S.A), which delivers a fine spray. When spraying manually, inoculum is delivered in a downward direction from the top of the plants, in a regular and even movement.

Approximately 1 mL of inoculum at a concentration of 350,000 basidiospores/mL is applied to each plant. After inoculation, plants are left undisturbed for two and a half days in the dark at 25°C and high relative humidity to facilitate germination of basidiospores and host penetration by germ tubes. Relative humidity and temperature are monitored with a data logger throughout the incubation period.

Agar droplet - verification

Screening of clones for confirmation of resistance to WB is carried out using the agar droplet technique (Surujdeo-Maharaj *et al*., 2003), where a single 30? L drop of basidiospore suspension

in 0.3% agar is delivered to the growing point of shoots via a micropipette. The inoculum concentration is 350,000 basidiospores/mL and incubation conditions are the same as for spray inoculation.

Material to be inoculated for confirmation of resistance is either propagated clonally by micro-grafting or grown as seedlings from open pollinated pods. For clonal propagation, young, green budwood is collected and grafted onto six-week-old seedlings. Inoculations are carried out when the grafts are at least six months old. Fifteen replicates of each clone are grafted, out of which ten plants per clone are inoculated.

For seedlings, 30 plants per clone are pruned at 5 weeks old and inoculated at 8 weeks.

Analysis

Selection of promising clones is based on more than one measure of resistance, since percentage infection alone commonly shows inconsistencies. Complimentary studies (Surujdeo-Maharaj, *et al.*, 2003) have shown that variables such as incubation period and broom-base diameter were found to be highly correlated with resistance.

Symptoms are assessed in two ways:

1. By calculating the percentage of shoots showing symptoms out of the total number of shoots inoculated and
2. By quantitatively assessing two variables to indicate symptom severity; broom-base diameter and number of days from inoculation to the first appearance of symptom (incubation period).

<u>Selection of putatively resistant clones from mass screening</u>

Clones are identified as putatively resistant from mass screening based on the following criteria:

Percentage infection

1. Those with at least three replicates per accession inoculated with at least four shoots per replicate and
2. Those for which the total percentage of symptoms is less than 20% or,
3. Those showing absence of brooms after 16 weeks of observation in the greenhouse.

Further analysis of those clones which produced symptoms is undertaken and clones are selected according to the following criteria.

Broom development and broom-base diameter

1. At least three replicates per accession inoculated
2. At least four shoots per replicate plant inoculated
3. No brooms developed
4. Have a broom-base diameter less than the most resistant control or,
5. Have a broom-base diameter of less than 6mm (most brooms seem to show this size as the minimum base diameter)

Broom-base diameter is taken at the time of maximum broom development.

Results for symptom severity (incubation period and broom-base diameter) are evaluated by ANOVA using the general linear model and the Tukey-Kramer Multiple comparison test

(MINITAB Release 14 or NCSS 2001 software).

Criteria for selecting and ranking clones - confirmation screening

Clones are confirmed as resistant based on the following:
1. At least three replicates per accession inoculated
2. Having a broom-base diameter less than the most resistant control or,
3. Having a broom-base diameter of less than 6mm
4. No brooms
5. Delayed onset of the first symptom.

For the Tukey-Kramer Multiple comparison test:
- ? Significantly different from susceptible control and/or statistically similar to the resistant control for both incubation period and broom-base diameter (or no brooms).
- ? No brooms developing from shoots with swellings.
- ? Significantly different from susceptible control and/or statistically similar to the resistant control for incubation period alone.
- ? Significantly different from susceptible control and/or statistically similar to the resistant control for broom-base diameter alone.

Clones are then ranked according to the level of resistance, with those clones which show good performance for both incubation period and broom-base diameter/ no broom being designated as the most resistant.

Table 1. Accession groups which contain clones selected for further confirmation of WB resistance and clones which have been confirmed.

Accession group	No. of clones	No. confirmed	Accession group	No. of clones	No. confirmed
AM [POU]	4		LX	1	
AMAZ [CHA]	2	1	LZ	2	
B [POU]	11		MATINA	1	
C [TRI]	1		MO	1	
CL	4	1	MOQ	8	
CLM	1		NA	6	2
CRU	7	2	POUND [POU]	5	1
DOM	3		PA [PER]	12	5
E [ECU]	1		PLAYA ALTA [VEN]	1	
EET [ECU]	6	1	REDAMEL	1	
GS	2		SCA	2	1
GU	1		SJ [POU]	3	
ICS	16	2	SLA	2	
IMC	16	5	SLC	2	
JA [POU]	1		SP [VEN]	1	
LCT EEN	4		SPEC	1	
LP [POU]	8	2	UF	4	
LV [POU]	3				

Overall results

Having developed and fine-tuned the above criteria for screening over the last seven years it is now possible to routinely evaluate the results of WB screening with a good level of accuracy and consistency.

Since the commencement of WB screening in July, 1998, over 777 accessions have been spray-inoculated and screened for WB resistance in the greenhouse, and a total of 145 promising clones have been selected for further confirmation of resistance. These clones belong to thirty five (35) accession groups (Table 1) represented in the ICG, T so cover a good range of the genetic diversity available in the genebank,

Promising clones were selected by considering both percentage infection and the development of brooms (either no brooms or those with a small base diameter). Thirty eight (38) of these clones have been inoculated by the agar droplet method to confirm and quantify their resistance, with a total of 23 clones (Table 1) confirmed with resistance to WB, under the experimental conditions at CRU.

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Utilisation

Enhancing cacao germplasm for resistance to Black Pod disease

A.D. Iwaro and V. Singh

Background

A Germplasm Enhancement Programme (GEP) was initiated in 1998 as part of the CFC/ICCO/IPGRI Germplasm Utilisation Project. Between 1998 and 2001, 136 resistant/moderately resistant genotypes were selected and used in 96 bi-parental crosses (36 Forastero, 17 Refractario, 20 Trinitario and 23 mixed). Progenies (3,486 seedlings) were raised in the greenhouse and screened for resistance to *P. palmivora* using a leaf disc test (Nyassé *et al*., 1995). Between 2000 and 2003, 1,026 plants including resistant, moderately resistant and susceptible genotypes (control) were established under cacao trees in Field 14 at the La Reunion Estate, Centeno. In addition, 70 parental genotypes involved in the first two batches of crosses in 1998 and 1999 were planted in Field 14. A replicate of the population in Field 14 was planted in a newly established field (Field 7) at the University Cocoa Research Station (UCRS) to increase pod production for the evaluation of pod resistance and bean traits (bean number and bean weight). In 2004, genotypes bearing pods in Fields 14 and 7 were assessed for resistance to Black Pod disease (BP) under field conditions and in the laboratory using the detached pod test (Iwaro *et al*., 2003). In addition, all the genotypes established in the two fields were assessed for resistance to Witches' Broom disease (WB) under field conditions. The methods adopted and the results obtained are discussed below.

Methodology

Field observations for resistance to Black Pod and Witches' Broom diseases

From October to December 2004, the plants established in Fields 14 and 7 were assessed for the following characteristics:

- Number of healthy pods per tree/genotype
- Number of diseased pods due to *Phytophthora* infection per tree/genotype
- Number of trees/genotypes free from WB
- Number of trees/genotypes with WB
- Number of brooms per tree/genotype

The percentages of genotypes infected by BP and WB and the levels of infection per genotype were determined from the data collected.

Assessment for resistance to *P. palmivora* using the detached pod test

Fully grown unripe pods (2 - 4 pods per genotype) were harvested and evaluated for resistance to *P. palmivora* using the detached pod test described by Iwaro *et al.*, 2003.

Results and Discussion

Field observations for resistance to Black Pod and Witches Broom diseases

Among the 1,026 plants (progeny population) established in Field 14 between 2000 and 2003, 890 plants have survived to date (Table 1). In Field 7, 779 plants have survived from the 960 plants (progeny population) established between 2001 and 2002 (Table 2). The field survey showed a 13% loss of plants in Field 14 and 19% in Field 7 from the progeny population (Tables 1 and 2). Losses were mainly due to attacks by termites (*Neotermes* sp.) and cocoa beetles (*Steirastoma breve*).

Table 1. Field observations conducted in Field 14 (Centeno).

Batch	Date of establishment	No. of genotypes established	No. of genotypes alive	No. of genotypes flowering	No. of genotypes with pods	No. of genotypes with BP	No. of genotypes with WB
1	2000	316	261	185	148	3 (2%)	118 (45%)
2	2001	339	281	157	100	4 (4%)	94 (33%)
3	2003	182	170	2	0	0 (0%)	10 (6%)
4	2003	189	178	3	0	0 (0%)	6 (3%)
	Total:	1,026	890				

BP - Black Pod disease
WB - Witches' Broom disease

Table 2. Field observations conducted in Field 7 (UCRS).

Batch	Date of establishment	No. of genotypes established	No. of genotypes alive	No. of genotypes flowering	No. of genotypes with pods	No. of genotypes with BP	No. of genotypes with WB
1	2001	204	169	110	83	1 (1%)	1 (1%)
2	2002	171	123	45	18	0 (0%)	3 (2%)
3	2002	310	263	45	10	0 (0%)	0 (0%)
4	2002	275	224	52	23	0 (0%)	0 (0%)
	Total:	960	779				

BP - Black Pod disease
WB - Witches' Broom disease

Seven plants (3%) were observed with BP symptoms among the 248 genotypes bearing pods in Field 14 (Table 1). Levels of infection were generally low (0 - 4%) among the four batches of selected plants established in Field 14 from 2000 to 2003 (Table 1). In Field 7, BP symptoms were observed on one plant (1%) among the 134 plants bearing pods (Table 2). Levels of infection were extremely low (0 - 1%) among the four batches of selected plants established in Field 7 (Table 2). The low levels of infection in the two fields suggest that inherent resistance among the selected plants is independent of the differences in site conditions. Although Field 14, which contains old cocoa trees, has a higher level of inoculum than Field 7 (a newly established field), the levels of infection by BP were low at both the sites (3% : Field 14; 1% : Field 7).

In contrast, 228 plants (26%) had WB among the 890 genotypes in Field 14 (Table 1). The level of infection varied with the date of establishment of the four batches of selected plants. Higher levels of infection were observed in the first two batches of plants established in 2000 (45%) and 2001 (33%) than in the latter two batches established in 2003 (6%, 3%). The level of infection was lower (1%) in Field 7. Infection levels varied from 0 to 2% among the four batches of plants established between 2001 and 2002 in Field 7 (Table 2). The results show a marked difference in the levels of infection at the two sites, probably due to differences in inoculum pressure.

Assessment of resistance to *P. palmivora* using the detached pod test

Among the 90 genotypes (progeny population) evaluated for resistance to BP using the detached pod test, 26 (28%) were found to be resistant (disease rating 1 - 3) (Figure 1). No visible lesions (disease rating 1) were observed on four genotypes, while 10 had 1 - 5 localised lesions (disease rating 2). Six to fifteen localised lesions were observed on 12 genotypes (disease rating 3). Thirty-two genotypes (36%) were found to be moderately resistant to *P. palmivora* (disease rating 4 - 5). Twenty-three of the 32 genotypes had more than 15 localised lesions (disease rating 4), while 9 had 1 - 5 expanding lesions (disease rating 5). Thirty-two genotypes (32%) were classified as susceptible. In this category, 22 genotypes had 6 - 15 expanding lesions (disease rating 6), while 10 had more than 15 expanding lesions (disease rating 7).

Figure 1. Distribution of scores for resistance to *P. palmivora* among 90 genotypes (progeny population).

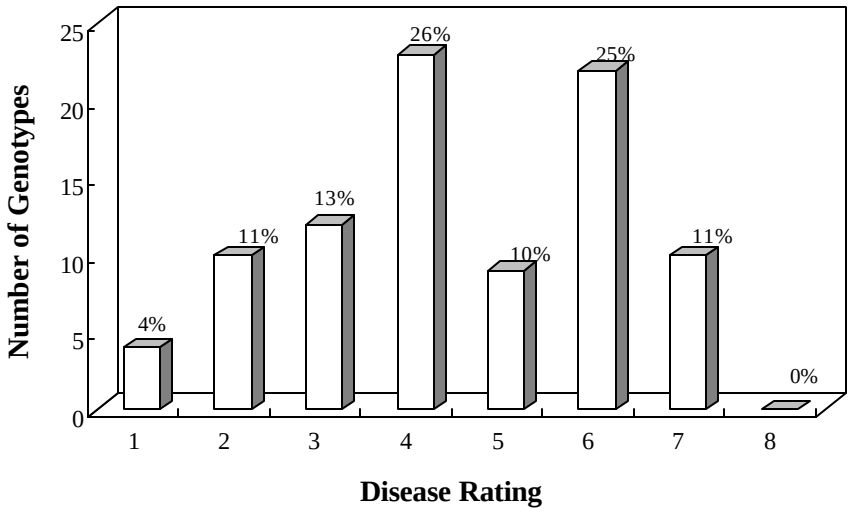
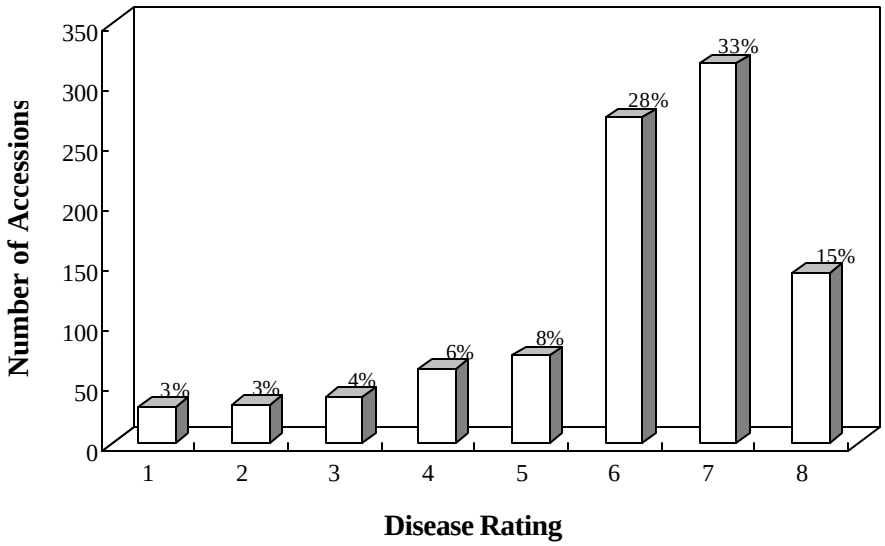


Figure 1 shows that the sub-set of the progeny population (90 plants) possessed 28% resistant (disease rating 1 - 3) and 36% moderately resistant genotypes (disease rating 4 - 5), compared to a subset of the ICG,T population (967 genotypes), which had 10% resistant and 14% moderately resistant genotypes (Figure 2). The subset of the progeny population (Figure 1) shows a considerably higher frequency of resistant genotypes and, consequently, a higher level of

resistance alleles than the base population (Figure 2). The sub-set of the progeny population (Figure 1) shows a significant reduction in the frequency of susceptible genotypes (36%) as compared to the sub-set from ICG,T (Figure 2), which had 76% susceptible genotypes. This shows a significant improvement in BP resistance in the composition of the new population. It further shows the effectiveness of the selection criteria imposed on the base population for the selection of the base parents by the detached pod test and further selection within the progeny population using the leaf disc test. When completed, further selection based on the results of the detached pod test and field observations, will facilitate the identification of resistant genotypes/populations to be used as base parents for the next cycle of the germplasm enhancement programme.

Figure 2. Distribution of scores for resistance to *P. palmivora* among 967 genotypes in ICG,T.



Although emphasis is placed on resistance to BP, information on field resistance to WB should also facilitate the identification of plants resistant to WB. Plants that are free from WB infection will be confirmed for their inherent resistance using the agar droplet method. Consequently, the strategy being adopted in the programme should facilitate the identification of genotypes with BP resistance and those that combine BP and WB resistance. This will allow end-users of the enhanced population to combine good yield potential with an acceptable level of resistance to both BP and WB diseases.

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Trends in flavour profiles and pyrazine compounds of cocoa liquor samples evaluated over three growing seasons for the CFC/ICCO/INIAP Flavour Project

D.A. Sukha, N. Ramnath and D.R. Butler

Introduction

The CFC/ICCO/INIAP Flavour Project "To establish physical, chemical and organoleptic parameters to differentiate between fine and bulk cocoa" is in its fourth and final year and the majority of results from the different analyses for each quality variable have been generated. We now have flavour profiles and trends in pyrazine compounds present after roasting from various accessions evaluated over three crop years (2002 – 2004). As a continuation of a report on preliminary results (Sukha *et al*., 2004), it is useful to examine a more complete dataset that is available from these two variables and revisit the original objectives of the CFC/ICCO/INIAP Flavour Project to examine overall trends of interest. Are we any closer towards finding clear classifications for "fine" and "bulk" cocoas? This report compares and contrasts some of the more important trends for flavour profiles and pyrazine compounds from local cacao accessions and commercial estates evaluated over three crop years in relation to the project objectives and expected outputs.

Project objectives and outputs

It is important to examine the results in the context of the central objective of the CFC/ICCO/INIAP Flavour Project which is to provide universally accepted criteria to differentiate between fine and bulk cocoas through a series of scientific evaluations of physical, chemical and organoleptic parameters. The project also aims to provide technologies that enable the evaluation of cocoa quality in relation to genotype and the environment, as well as, to provide and disseminate methodologies, standards and instruments to be used in the evaluation of cocoa quality.

Three main outputs expected from this project include: 1) reliable information on physical, chemical and organoleptic characteristics which differentiate fine from bulk cocoa; 2) methodologies to measure and compare the main variables that define quality of fine cocoas and 3) standards and instruments to evaluate the quality of fine cocoa thereby contributing towards improving the competitive position of fine cocoa as a distinctive product.

Materials and methods

Coding

The identities of the commercial Trinidad Selected Hybrids (TSH) used in this project have been coded with "CRU" accession codes in compliance with an agreement between MALMR and CRU governing the presentation of results for these accessions. In this report, these accessions will be referred to as local clones. Samples taken from commercial estates have also been alpha-numerically coded to protect the true identities of these estates.

Sample preparation

Detailed accounts of the protocol followed for sample preparation *viz.* fermentation and drying (primary processing) and roasting and milling (secondary processing) as well as the organoleptic assessment procedure can be found in the proceedings of the workshop to establish working procedures for the CFC/ICCO/INIAP Flavour Project (Sukha, 2001a, 2001b) and in previous reports by Sukha *et al*. (2003, 2004).

Pyrazine analysis

Pyrazines are a group of compounds containing rings composed of four carbon atoms and two nitrogen atoms. Pyrazines are thought to be the major flavour compounds eliciting typical roasted flavours in certain highly heated food systems. Recently, pyrazines have been used as the pertinent volatile compounds in peanuts to establish roasting models to determine how variable roasting factors affect roasted nut and peanut flavour (Baker *et al.*, 2002). In the context of the CFC/ICCO/INIAP Flavour Project, pyrazines are being assessed as a possible class of volatile compounds that can be used to distinguish "fine" from "bulk" cocoa. Solid phase micro extraction (SPME) was used together with gas chromatographic mass spectrometry (GCMS) to measure significant differences in pyrazine concentrations of a variety of cocoa bean samples.

SPME was developed in the early 1990's and uses a fused silica fibre that looks like a modified syringe coated with a thin film of several stationery phase polymers to act like a sponge to concentrate organic analytes on the surface of the fibre by adsorption and absorption. After a suitable extraction time, the fibre is withdrawn and injected directly into a gas chromatograph to elucidate and identify the organic analytes (Kataoka *et al*., 2000).

SPME-GCMS analysis was conducted at the Queensland Department of Primary Industries (QDPI), Australia on both unroasted and roasted (140°C for 30 minutes) cocoa bean samples that had been fermented for 7 days and sun-dried. The specific conditions used in this analysis were as follows: SPME fibre - Supelco StableFlex^{TM}, Divinylbenzene/Carboxen^{TM}/polydimethylsiloxane, using headspace sampling mode after 30 minutes at a temperature of 20°C, with a matching gas chromatography column. Compounds were identified by spectra searches matching to National Institute of Standards and Technology libraries and results for matching pyrazine compounds were then expressed in terms of peak area units (QDPI personal communication).

Data analysis

Pyrazine data analysis

Pooled data over three crop years as well as individual crop year data were examined by principal component analysis using GenStat 7.0 (VSN International) and graphical representation was done in Microsoft Excel. Each crop year represents a repetition with time.

Organoleptic data analysis

Data from the three crop years were pooled and ANOVA conducted using MINITAB Release 14 (Minitab Inc.). The significance of treatment effects and interactions as well as mean flavour profiles and the standard errors of the mean (SE) were calculated. Average flavour profiles are presented graphically as bar graphs with SE indicated by lines at the top of the graphs.

Results and discussion

Table 1 summarises ANOVA of flavour profiles for local clones for each individual crop year and for all three crop years combined and Figure 1 shows the average flavour profiles of local clones over three crop years (2002 – 2004) compared to the bulk reference from Ghana.

Table 1. ANOVA of flavour profiles for local clones for 2002, 2003 and 2004 crop years and all three crop years combined.

Flavour attribute	Significance			
	2002 crop year	2003 crop year	2004 crop year	2002 -2004 crop years combined
Cocoa	NS	***	***	NS
Acidity[1]	NS	***	***	***
Astringency	NS	NS	**	*
Bitterness	*	NS	NS	***
Fruity	NS	NS	NS	*
Floral	***	***	***	*
Nutty[2]	*	NS	**	**
Raw/beany/green	NS	NS	NS	*

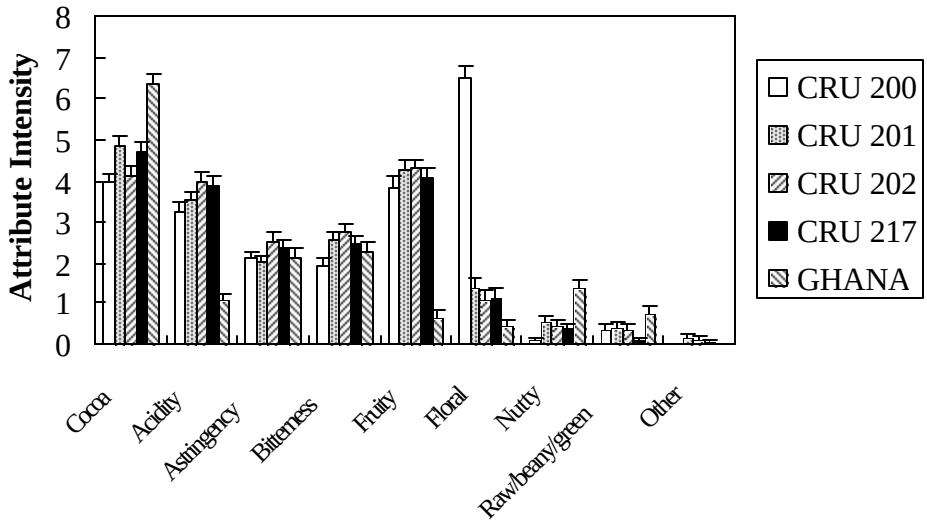
* *P*?0.05 ** *P*?0.01 *** *P*?0.001 Not Significant (NS) *P*? 0.05

[1]Varied significantly (*P*=0.001) within clones and between the three crop years

[2]Varied significantly (*P*=0.05) within clones and between the three crop years

The average flavour profile trends for the local clones over the three crop years showed that all flavour attributes with the exception of cocoa flavour varied significantly (*P*=0.05 to *P*= 0.001) over the three crop years combined (Table 1). The ANOVA presented in Table 1 revealed that with the exception of fruity and raw/beany/green, all flavour attributes varied significantly within any given crop year. Floral was the only flavour attribute that varied significantly (*P*=0.001) for each individual crop year investigated. Acid and nutty attributes exhibited a clone × crop year interaction where they varied significantly (*P*=0.001 and *P*=0.05, respectively) both within the local clones and between the three crop years. Figure 1 reveals that there were only slight variations (*P*=0.05) between the average scores for the individual local clones over three crop years. CRU 200 was the only exception to this since there was a very strong floral note that differed significantly (*P*=0.001) from the other three local clones. The data presented in Table 1 point towards a strong seasonal effect on flavour where in some crop years certain flavour attributes were expressed in different intensities than in other years. The only flavour attributes that did not show any significant differences within each crop year were fruity and raw/beany/green, but combined crop year analysis reveals that there was some variation (*P*=0.05) between crop years. The impact of seasonality on quality is not a new concept and is well established for wine and other crops but it is poorly understood in cocoa. These findings represent one of the first instances where a seasonal effect phenomenon has been demonstrated by scientific investigation on the flavour attributes of cocoa and is one of the many important findings to emerge from the CFC/ICCO/INIAP Flavour Project.

Figure 1. Flavour profiles for local clones averaged over 3 crop years (2002 – 2004) compared to the Ghana Reference.



In all instances, the flavour profile trends in Figure 1 show that the local clones had consistently higher fruity and acid scores than the Ghana reference sample which is characterised by the highest cocoa and nutty scores. These profile trends have been observed consistently (Sukha *et al*., 2004) and highlight strong organoleptic differences between what can be considered "bulk" and "fine" cocoa. The profiles also demonstrate that organoleptic analysis is as a reliable method to discriminate between fine and bulk cocoa in spite of possible seasonal effects on flavour. This also is another important finding to emerge from the CFC/ICCO/INIAP Flavour Project that satisfies the main project objective and expected project outputs.

The average flavour profiles for commercial estate samples compared to the Ghana reference sample for the three crop years combined and the average flavour profiles for all the commercial estate samples combined over each of the three crop years are presented in Figures 2 and 3, respectively. Cocoa, acid, fruity, floral, nutty and "other" flavours varied significantly (P=0.05 and P= 0.001) amongst the commercial estates (Figure 2) whilst all the flavour attributes except cocoa flavour differed significantly (P=0.05 to P= 0.001) between the three crop years (Figure 3). The trends in the average flavour profiles for the commercial estates showed a similar but slightly more variable general trend to the local clones in all flavour attributes except the intense floral attribute of CRU 200. All the commercial estates also differed significantly (P=0.001) from the Ghana reference sample especially in acidity and fruitiness. Cocoa flavour was the only flavour attribute that did not vary significantly between the different crop years for both the local clones and commercial estates. Cocoa flavour can be considered a "basal flavour" which is present in all well fermented and dried samples. However, it is easily masked by other more dominant flavour attributes but commonly remerges as a residual flavour at the end of the tasting experience during organoleptic evaluation.

Commercial estates E2 and E3 consistently produced the most fruity samples and E3 the most floral samples over the three crop years, whilst commercial estate E1 and E5 consistently had the

Figure 2. Flavour profiles for coded commercial estate samples averaged over 3 crop years (2002-2004) compared to the Ghana reference sample.

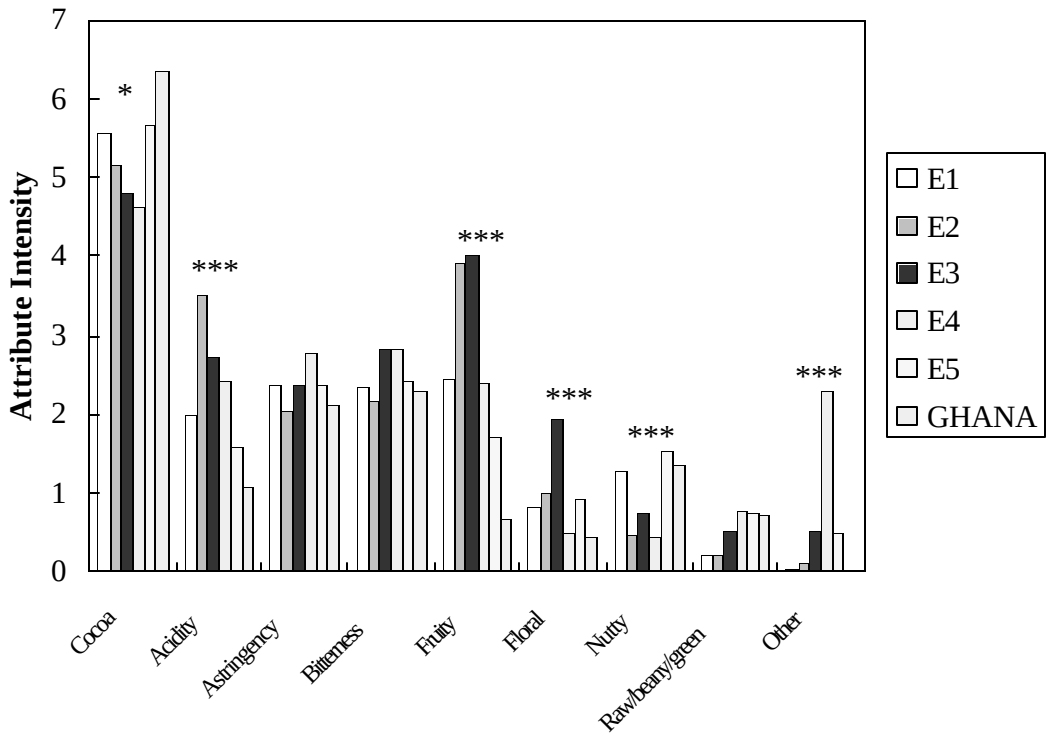
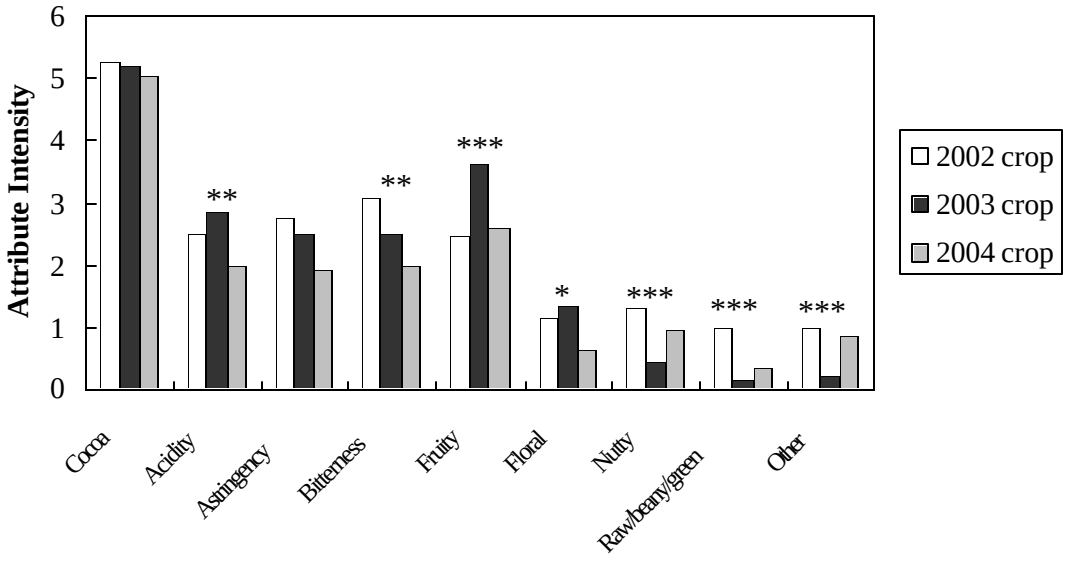


Figure 3. Average flavour profiles for all the commercial estate samples combined for each of three crop years.



* *P*?0.05 ** *P*?0.01 *** *P*?0.001 Not Significant (NS) *P*? 0.05

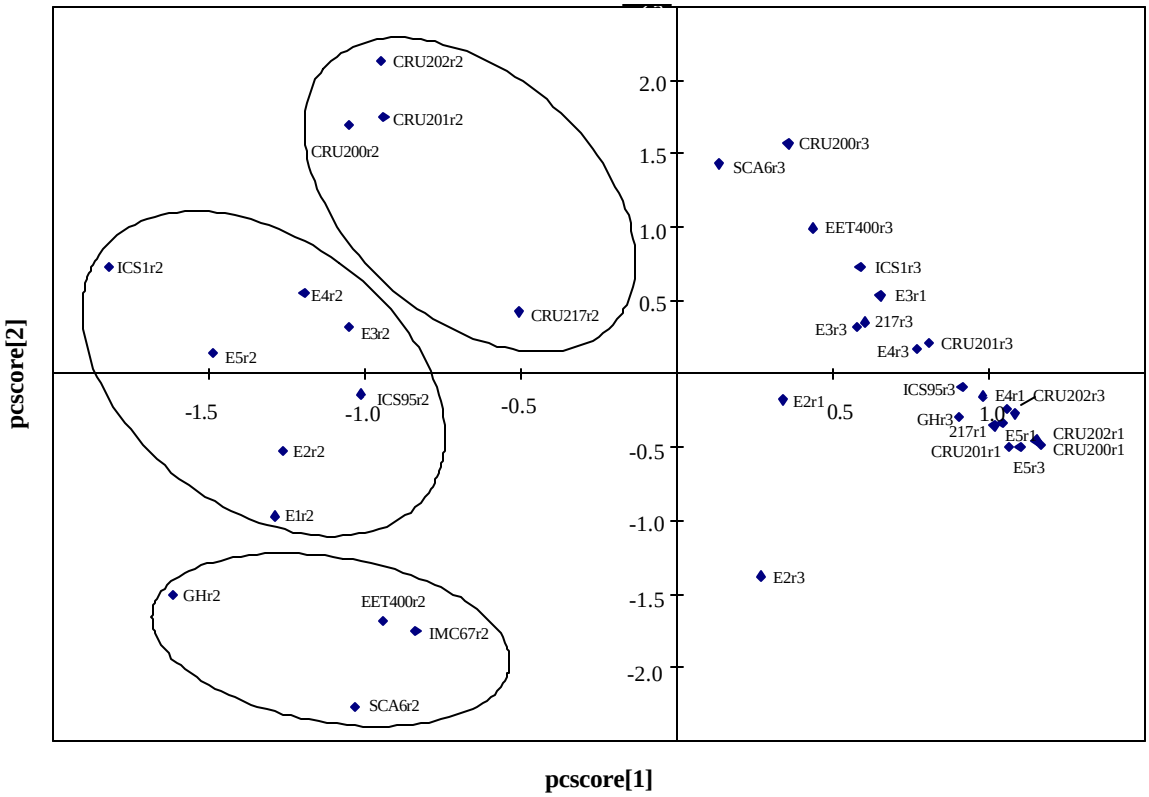
lowest acid scores and the most balanced flavour profile in terms of all the major flavour attributes. The commercial estate E4 had a smoky off-flavour note recorded under "other"

flavours, and the problem worsened in the 2004 crop year. Reasons for the trends in flavour profiles of the commercial estates were discussed in Sukha *et al*., (2004). There were significant (P=0.05 to P=0.01) estate × crop year interactions for cocoa, acid, fruity, floral, nutty and other flavour attributes (data not presented), and a strong seasonal effect was demonstrated between crop years for the combined commercial estate scores presented for each individual crop year (Figure 3).

All the flavour attributes with the exception of cocoa flavour and astringency varied significantly (P=0.05 to P=0.001) over the three crop years with the 2003 crop year being distinct from the other two crop years for a number of attributes. The results presented in Figures 1 – 3 clearly demonstrate a strong seasonal effect on the expression of flavour attributes in cocoa and the differences between fine and bulk cocoa.

The results from the pyrazine analyses over the three crop years combined are presented as a PCA plot (Figure 4). Successive crop years are distinguished by the suffixes r1 (2002), r2 (2003) and r3 (2004). The Ghana reference samples (GH) are presented with other common clones for the CFC/ICCO/INIAP Flavour Project (IMC 67, ICS 1, EET 400 [ECU] and SCA 6).

Figure 4. Principal component analysis plot pyrazines in CFC/ICCO/INIAP Cocoa Flavour Project samples from Trinidad over three crop years.



The trends in results from the PCA plot of pooled pyrazine data show that two principal components explained 88.1% of the variation. Principal component 1 represented 63.3% of the

Table 2. Flavour attributes associated with selected pyrazine compounds responsible for the variation observed in the PCA plot.

Pyrazine compound	Flavour attributes of pyrazine compound
2-Methylpyrazine	Chocolate, grass, green, nutty and roasted notes
2,5-dimethylpyrazine	Chocolate, roasted nuts and earthy flavours
2,3-Dimethylpyrazine	Caramel, nutty, green, sweet, malt and chocolate notes
2-Ethyl-6-methylpyrazine	Butter scotch, nutty, earthy, raw potato and roasted notes
2,3,5-Trimethylpyrazine	Baked potato, grass, musty, nutty and roasted peanuts
3-Ethyl-2,5-dimethylpyrazine	Roasted peanut flavours
2,3,5,6-Tetramethylpyrazine	Cocoa, chocolate, nutty and burnt almond notes
2,3,5-Trimethyl-6-ethylpyrazine	Roasted peanut and cocoa flavours

(Source: Leffingwell & Associates, 1999)

variation and principal component 2 represented 24.8% of the variation due to the eight pyrazine compounds presented in Table 2. Principal component 1 clearly separated the second (2003) crop year from the first and third crop years. Principal component 2 separated the local clones, commercial estates and the common clones in 2003. There is no clear overall separation between "fine" and "bulk" cocoa (especially in 2002 and 2004) from this PCA plot but rather a separation according to crop year, which can be interpreted as a seasonal effect similar to the earlier observations from the sensory data.

In the second crop year, the distinction between local clones, the commercial estates and the common clones may indicate a possible separation between "bulk" and "fine" types of cocoa. Both the pyrazine data and sensory results (Figure 4 and Table 1) showed that the second crop year stood out as being most different for the commercial estates over the three crop years. There were also significant clone × crop year interactions for acid and nutty flavour attributes in the local clones. The flavour attributes associated with the important pyrazine compounds responsible for the variation observed in the PCA are all associated with "nutty" and/or "cocoa" flavours (Table 2).

Pyrazine formation during roasting has been found to vary depending on the geographical origin of cocoa beans and substantially more pyrazines are formed in well fermented beans than in under-fermented beans (Reineccius *et al*., 1972). Additionally it has been observed that the same pyrazines can be present in different samples but their contents after roasting can vary considerably, even among different lots of the same type of bean. These differences may reflect the impact that subtle variations in the fermentation processes caused by seasonal effects can have on the production of reducing sugars and free amino acids which are the precursors of pyrazines (Rohan and Stewart, 1967a and b; Reineccius *et al*., 1972). Different genotypes of mixed or hybrid origin (such as the Trinitarios) may have subtle differences in their fermentation requirements and the effect of slight over- or under-fermentation can also affect the formation of pyrazine precursors. The combined influences of genotype, growing environment (climatic and edaphic) and processing (fermentation and drying) on the flavour and quality attributes of cocoa samples can therefore affect the pyrazine contents of the same types of cocoa and can be manifested as strong seasonal effects. This could account for the agreement between the results from the PCA plot and organoleptic observations regarding the second crop year results compared

to the other two crop years. It could also explain why there is a possible confounding effect on the separation between "fine" and "bulk" cocoa in all years from this pooled PCA plot.

Of particular interest is the fact that 2,3,5,6-tetramethylpyrazine was the only pyrazine compound consistently present in the unroasted bean samples (data not presented). It has been shown (Reineccius *et al*., 1972) that tetramethylpyrazines in fermented but unroasted beans might arise during fermentation through thermally initiated biochemical reactions or by microbial synthesis. The temperature of the sweat box during fermentation in Trinidad typically crosses 50°C (Sukha, 1997) and therefore generation of tetramethylpyrazines through heating during fermentation may be possible. It is also likely that 2,3,5,6-tetramethylpyrazine forms during fermentation through microbial action, as demonstrated by Kosuge and Kamiya (1962) and Hashim *et al*. (1997) where tetramethylpyrazines were generated as a metabolic product of *Bacillus subtilis* grown on certain types of media. Several species of *Bacillus* have been identified in the fermenting mass of Trinidad cocoa beans (Ostovar, 1971, Ostovar and Keeney, 1973 and Zak *et al*., 1972).

These organoleptic and pyrazine results clearly demonstrate a strong seasonal effect on the expression of flavour attributes in cocoa. The flavour profiles also demonstrate that organoleptic analysis is a reliable technique to discriminate between fine and bulk cocoa over and above these seasonal effects. The pyrazine results however seem to be somewhat less reliable in discriminating between fine and bulk cocoa every year. It appears that subtle variations in the fermentation processes, conditions during fermentation and the fermentation requirements of different genotypes may affect the production pyrazine precursors. However, these variations raise a number of questions concerning the relative importance of genotype, growing environment (climatic and edaphic) and processing (fermentation and drying) on the flavour and quality attributes of cocoa.

The results also highlight close links between sensory attributes and chemical compounds in different cocoa samples. Such links between chemical, organoleptic and even physical attributes investigated in this project provide a holistic approach for future research towards isolating and investigating each major factor affecting flavour and quality in cocoa.

Are we any closer towards finding clear classifications for "fine" and "bulk" cocoas? From the subset of results presented here, the answer to this question is a positive one. As more complete results from the CFC/ICCO/INIAP Flavour Project become available we hope to add further clarity to these complex issues, and we will almost certainly raise some intriguing questions. It appears that progress is being made in resolving this long standing issue, which will contribute towards an improved competitive position of fine cocoa as a distinctive product.

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Papers presented

Butler, D.R. 2004. Characteristics of fine or flavour cocoa. Presented at the ICCO ad hoc panel on fine or flavour cocoa, 9 December 2004, London, UK.

Iwaro, A.D., Bharath, S.M., Bekele, F.L , Butler D.R. and Eskes, A.B. Germplasm enhancement for resistance to black pod disease: strategy and prospects for the new project 'Cocoa productivity and quality improvement: a participatory approach'. Presented at the closing workshop of the CFC/ICCO/IPGRI Project on "Cocoa germplasm utilisation and conservation: a global approach", 28-31 March 2004, the University of Reading, U.K.

Iwaro, A.D. and Thévenin, J-M. Validation studies on detached pod test and leaf disc inoculation. Presented at the closing workshop of the CFC/ICCO/IPGRI Project on "Cocoa germplasm utilisation and conservation: a global approach", 28-31 March 2004, the University of Reading, U.K.

Iwaro, A.D., Thévenin, J-M. and Umaharan, R. Problems and solutions adopted for clone multiplication. Presented at the closing workshop of the CFC/ICCO/IPGRI Project on "Cocoa germplasm utilisation and conservation: a global approach", 28-31 March 2004, the University of Reading, U.K.

Iwaro, A.D. Areas for technological research to increase productivity in the cocoa industry in Trinidad and Tobago. Presented at a seminar organised by the Tobago House of Assembly on the improvement of cocoa production in Tobago. May 28, 2004.

Sounigo, O. and Butler, D.R. CFC/ICCO/IPGRI Project Collection. Presented at the closing workshop of the CFC/ICCO/IPGRI Project on "Cocoa germplasm utilisation and conservation: a global approach", 28-31 March 2004, the University of Reading, U.K.

Sukha, D.A. A brief overview of cocoa in Trinidad and Tobago and activities of the Cocoa Research Unit. Presented at: Cocoa Group Colloquium ,7 July 2004, Institute of Applied Botany, Biocenter Klein Flottbek and Botanical Garden, University of Hamburg, Germany.

Sukha, D.A. The influence of processing location, growing environment and the effect of pollen donor on the flavour attributes of cocoa (*Theobroma cacao* L.). Presented at: Post graduate research seminars, 27 April 2004, Faculty of Engineering, the University of the West Indies, St. Augustine.

Sukha, D.A., Bharath, S.M., Straker, S.S. and Ramnath N. Cocoa liquor preparation and organoleptic quality assessments – activities and results. Presented at: Review of CFC/ICCO/INIAP Flavour Project "To establish the physical, chemical and organoleptic parameters to establish the difference between fine and bulk cocoa", 3-5 June 2004, Mérida, Venezuela.

Sukha, D.A., Bharath, S.M., Straker, S.S. and Ramnath, N. Chemical quality assessments – activities and results. Presented at: Review of CFC/ICCO/INIAP Flavour Project "To establish the physical, chemical and organoleptic parameters to establish the difference between fine and bulk cocoa", 3-5 June 2004, Mérida, Venezuela.

Sukha, D.A., Bharath, S.M., Straker, S.S. and Ramnath, N. Fermentation and drying trials – activities and results. Presented at: Review of CFC/ICCO/INIAP Flavour Project "To establish the physical, chemical and organoleptic parameters to establish the difference between fine and bulk cocoa", 3-5 June 2004, Mérida, Venezuela.

Thévenin, J-M. and Umaharan, R. Evaluation of cacao disease resistance under natural conditions of infection. Presented at the closing workshop of the CFC/ICCO/IPGRI Project on "Cocoa germplasm utilisation and conservation: a global approach", 28-31 March 2004, the University of Reading, U.K.

Umaharan, R and Thévenin, J-M. Evaluation of cacao resistance to Witches' Broom disease: screening under controlled conditions. Presented at the closing workshop of the CFC/ICCO/IPGRI Project on "Cocoa germplasm utilisation and conservation: a global approach", 28-31 March 2004, the University of Reading, U.K.

Thévenin, J-M. and Umaharan, R. Witches' Broom resistance screening methods: Which, how and why? Presented at the opening workshop of the CFC/ICCO/IPGRI Project on "Cocoa productivity and quality improvement, a participatory approach", 1-3 April 2004, the University of Reading, Reading, U.K.

Posters presented

Zhang, D., Leamy, E., Mischke, S., Boccara, M. and Butler, D.R. Verification of genetic identity in *Theobroma cacao* germplasm using microsatellite markers. Presented at the Mid-Atlantic Molecular Biology Symposium, Maryland, USA. 19-20 August 2004.

Zhang D., Boccara, M. Mischke, S., and Butler, D.R. Identification of mislabelling in T*heobroma cacao* germplasm using microsatellite markers. Presented at the World Cocoa Foundation meeting, Washington DC, USA. 14-15 October 2004.

Visitors to CRU in 2004

Ia Orre Montan	Elle à la Carte, Stockholm, Sweden
Niclas Ohlsson	V&S Domaine Rabiega, France
Duane Dove	Restaurang Sjögräs, Stockholm, Sweden
Kampta Persaud	San Juan Estate, Gran Couva
Pierre Costet	Valrhona 26602 Tain l'Hermitage, France
Tony Lass	BCCCA, UK
Philippe Petithuguenin	CIRAD-CP Montpellier, France
Edward S. Seguine	Guittard Chocolate Co., California
Dapeng Zhang	USDA-ARS Beltsville, MD 20705, USA
Mott Green	The Grenada Chocolate Company Ltd
Juan Carlos Motomayor	USDA Masterfoods, USA
Sarah Gingold	USDA Beltsville, MD, USA
Juan Carlos Jiménez	INIAP, Ecuador
Alejandra Saltos Icaza	INIAP, Ecuador
Freddy Amores Puyutaxi	INIAP, Ecuador
Alexis Zambrano Garda	INIA, Venezuela
Alvaro Gomez Morales	INIA, Venezuela
Gladys Ramos Carranza	INIA, Venezuela
W.D. Chalmers	21 Maple Drive, Taverham, Norwich NR8 6TF, UK
Sharon Jones	Green Ventures, Kiligwyn Estate, Canaan, Tobago
José Rodrigo Salgado	Solis 2032, Buenos Aires, Argentina
Francois N. Mouttet	Lot 9A, Diamond Vale Ind. Estate, Diego Martin
Beryl Hunter	22 Low Croft, High Bentham LA2 7FD, England

Acronyms and abbreviations

ACRI	American Cocoa Research Institute, USA
ANOVA	Analysis of variance
BCCCA	Biscuit, Cake, Chocolate and Confectionery Association, London, UK
BCQS	Barbados Cocoa Quarantine Station
bp	base pair
BP	Black Pod disease
CA	Cluster analysis
CAOBISCO	Association des industries de la chocolaterie, biscuiterie et confiserie de l'UE
CATIE	Centro Agronómico Tropical de Investigatión y Enseñanza, Costa Rica
CFC	United Nations Common Fund for Commodities
CIRAD	Centre de Coopération Internationale en Recherche Agronomique pour le Développement, France
CIRAD-CP	Centre de Coopération Internationale en Recherche Agronomique pour le Développement -Culture Pérennes, France
COPH	Cophenetic module
CRU	Cocoa Research Unit, Trinidad and Tobago
DNA	Deoxyribonucleic acid
ESTs	Expressed sequence tags
EU	European Union
FP	Frosty pod disease
FP#	Fingerprinting number
FSA	Faculty of Science Agriculture, UWI, St. Augustine, Trinidad and Tobago
GCMS	Gas chromatographic Mass Spectrometry
GEP	Germplasm enhancement programme
GORTT	Government of the Republic of Trinidad and Tobago
IBPGR	International Board for Plant Genetic Resources, Rome, Italy
ICCO	International Cocoa Organisation, London, UK
ICGD	International Cocoa Genebank Database
ICG,T	International Cocoa Genebank, Trinidad
INIA	Instituto Nacional de Investigaciones Agricolas, Venezuela
INIAP	Instituto Nacional Autonomo de Investigaciones Agropecurias, Ecuador
INGENIC	International Group for Genetic Improvement of Cocoa
IPGRI	International Plant Genetic Resources Institute, Rome, Italy
MALMR	Ministry of Agriculture, Land and Marine Resources, Trinidad and Tobago
MXCOMP	Matrix comparison module
P	Probability
PAGE	Polyacrylamide gel electrophoresis
PCA	Principle component analysis
PCR	Polymerase chain reaction
p.d.:I	Potential difference:current ratio
PI	Pod Index
QDPI	Queensland Department of Primary Industries, Australia
QTL	Quantitative trait loci
r	Correlation coefficient
RAPD	Random amplified polymorphic DNA
SAHN	Sequential agglomerative hierarchical nested clustering
SE	Standard error
SPME	Solid phase micro extraction
SSR	Simple sequence repeats
TSH	Trinidad Selected Hybrid
UAF	Upper Amazon Forastero

UCRS	University Cocoa Research Station
UE	Union Européenne
UH	University of Hamburg, Germany
UPGMA	Unweighted pair-group method using arithmetic means
USDA	United States Department of Agriculture
UWI	The University of the West Indies
WB	Witches' Broom disease
WCF	World Cocoa Foundation, USA