Newsletter

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IN THIS ISSUE

From the Editor's Desk:

The past year was eventful for INGENIC, climaxing in October 2003 with the 4th INGENIC Workshop on ***Cocoa Breeding for Improved Production Systems*** in Accra, Ghana. The detailed *Synthesis and Conclusions* of this two-day Workshop, included in this issue, are testimony to the usefulness of the deliberations. Not only was the forum productive in matters pertinent to INGENIC's mission, but I'm sure all will agree that the camaraderie was delightful, and delegates enjoyed the hospitality of the hosts and the beauty of Ghana. The INGENIC Committee wishes to extend its gratitude to the local organising Committee of CRIG, and for the tremendous support received from the Cocobod, besides that of the other sponsors of this meeting (BCCCA, CTA, German Association of Cocoa and Chocolate Confectionary Industry, USDA, WCF and Masterfoods).

The Minutes of the General Assembly Meeting of INGENIC, held on Monday 20 October 2003 in Accra, on page 56 of this issue, highlight some of the other recent activities of our Group.

Prior to the Workshop, on Sunday 19th, there was a meeting of the informal Cocoa Genomics Group, which was chaired by Ray Schnell of USDA/ARS. This was the culmination of months of dialogue on activities for this Group. We are pleased to announce the formalisation of the *"INGENIC Study Group in the Molecular Biology of Cocoa* (INGENIC-*Mol-Biol*)*"*, which is also described herein, and to welcome its elected Chairman, Mark Guiltinan of Penn State University, as a member of the INGENIC Committee.

As a result of the session on the conclusions of the Workshop, an *Ad-Hoc* meeting was convened to discuss proposals for enhanced regional collaboration for the improvement of cocoa. The Minutes of this Meeting are also enclosed in this issue, as well as the minutes of the follow-up meeting held in Reading, UK, on April 3 2004.

The quality, quantity, diversity and pertinence of the contributions to 9th issue of the INGENIC newsletter enable us to feel certain that INGENIC has indeed "come of age". Our mission to promote the exchange of information and ideas as well as collaborative efforts in cocoa breeding and genetics is being realised because of the involvement, input and support of all our members. As we approach the 10th anniversary of this publication, we express our gratitude to the more than 260 INGENIC correspondents scattered over the globe, and urge you to continue working together with the INGENIC Board for the good of all partners in the international cocoa industry.

Please submit succinct contributions for the next issue of the NL to me at fbekele@fans.uwi.tt; louisebekele@yahoo.co.uk or louisebekele@hotmail.com on or before March 1st 2005.

Readers are invited to visit the temporary INGENIC website at *http://guiltinan.cas.psu.edu/publications.htm* to learn more about recent INGENIC activities including those of INGENIC-*Mol-Biol*.

We look forward to another year of useful discussions on further partnerships and collaborative research activities among our readership.

With best wishes from the editor of your INGENIC Newsletter and the other members of the INGENIC Committee!

Frances Bekele

Synthesis and Conclusions of the INGENIC Workshop on "Cocoa Breeding for Improved Production Systems" held October 19-21, 2003, at the Miklin Hotel, Accra, Ghana

Edited by Bertus Eskes

Topic 1. General Cocoa Breeding Aspects

Cocoa Production Systems

- Cocoa production systems vary greatly within and between countries. Most cocoa production is still based on low-input management systems. There are, however, tendencies to increase inputs (fertiliser, labour) to reduce overhead shade, to adopt pruning to limit tree height and canopy density, and to associate cocoa with other profitable trees (fruit trees, timber).
- Cocoa planting materials are still largely made up of unselected seedling populations. Selected hybrid varieties are cultivated on less than 30% of the global area under cocoa. Often farmers rely on their own sources of planting material. The use of cocoa clones is increasing in Asia and America.
- Strong competition between cocoa trees is often observed in older cocoa plantations, which may be a factor in yield decline. Common cocoa varieties may be too vigorous, especially so under favourable growing conditions and at commonly used planting densities.
- Little information is available on the possible interaction between cocoa genotypes and production systems (shaded versus unshaded, high input versus low input cultivation, pruned versus unpruned, etc.).
- There is a tendency to involve farmers directly in the identification of promising genotypes and to carry out validation of new varieties in farmers' fields (*e.g.* as demonstrated in the presentation of Uilson Lopes, CEPLAC, Brazil). Such would help to ensure that new varieties are adapted to the farmers' growing conditions.

Cocoa Breeding Programmes

- Objectives of major cocoa breeding programmes include: yield potential, resistance to major diseases and pests, production uniformity and stability, easier and less costly management and improvement of quality characteristics.
- To be successful, a breeding programme should be comprehensive, balanced, and with sufficient magnitude of scale. Continuity and sustainability are of utmost importance. These conditions are only rarely being fulfilled in any of the cocoa producing countries.
- There is an increased tendency to select clonal cocoa varieties rather than hybrid varieties. Clonal varieties allow for more rapid genetic progress that can be fixed in asexually propagated varieties. Selection of clones is to be considered even in Africa, where traditionally only seed varieties are grown.
- Growing of clonal cocoa varieties requires important changes in cocoa cultivation practices. Although adoption of clonal cocoa may be difficult under some conditions, availability of outstanding clonal cocoa varieties may be a stimulus for the farmers to improve production and management practices.
- Clonal cocoa varieties are mostly multiplied through budding or grafting. This poses the question of the possible effects of the rootstocks on the scion, which is still poorly understood in cocoa (see Topic 5).

Recommendations

- *Similarities between cocoa producing countries in the same region (America, Africa and Asia) justifies regional approaches to cocoa breeding.*
- *Well-supported regional centres or regional programmes would ensure that the critical mass of scientists and research activities required for success in cocoa breeding is obtained.*
- *A long-term commitment from donor agencies, the chocolate industry and producing countries is required to sustain cocoa breeding programmes.*
- *The workshop participants recommended that INGENIC set up a committee to propose regional breeding efforts and study the feasibility of establishing regional centres, regional programmes and/or regional projects.*

Topic 2: How to select superior mother trees in heterogeneous progenies for more productive clone or hybrid varieties

Causes and consequences of interplant variation in cocoa

- Highly correlated variation is observed in yield and vigour between trees in traditional cocoa hybrid trials. For example, in Ghana 17 to 27% of the trees were found to produce no useable pods, and the coefficient of variation (C.V.) between trees for pod yield varied from 30 to 76%.
- In Papua New Guinea (PNG), high between-tree variation was observed for the number of flowers produced, for pollination efficiency and for the percentage of cherelle wilt. High yielding trees appeared to be able to bring a higher proportion of cherelles to maturity.
- In Côte d'Ivoire, variation for early yield between trees can be as high within clones as in hybrid progenies. This would suggest that the environment is a major factor inducing large tree-to-tree variations in yield. However, it was noted that in other datasets the tree-to-tree variation varied according to the genetic uniformity of the population (Lockwood, pers. comm.).
- Individual tree selection for yield in experimental plots, aiming at clone selection or at selection of mother trees for creating new seedling progenies, has so far been shown to be relatively inefficient.
- Visual observations in intensively managed clonal cocoa plantations would suggest that between-tree variation in

yield could be reduced if uniform tree management is applied, including canopy pruning. However, more data analysis on this aspect would be required to draw firm conclusions.

Statistical approaches for improving individual tree selection

- Statistical methods that are helpful for increasing the genetic value of the observations in heterogeneous stands are: "combined individual-family selection", "smoothing methods" derived from spatial statistics and "longitudinal data analyses".
- New experimental designs based on the use of more than one individual per progeny plant will facilitate further increase in the genetic component of the variation observed. Such designs can be based on splitting the cocoa seeds into two parts ("true twins"), or on rapid vegetative multiplication of seedlings in the nursery as rooted cuttings, buddings or graftings ("accelerated hybrid clone selection").

Correlations between yield of individual trees in progeny trials and clones grown from them

- Correlations between early yield of individual trees and clonal progenies grown from them appeared to be low and non-significant in trials conducted in Côte d'Ivoire and in Malaysia.
- This is seemingly in contradiction to the relatively high heritabilities that have been reported for yield in some progeny trials.
- Other factors may therefore interfere when comparing yield performance of seedling trees with that of plagiotropic budded clones. It is important that these factors be better understood.

Correlations between mean yield of hybrid progenies and clones selected from them

- In Malaysia, ortet selection for yield efficiency was less effective than had been hoped, but nonetheless helpful. Family level selection for yield efficiency was more effective.
- In PNG, significant correlations were found between the family means and the yield of clones selected from these crosses in two sets of data, but not in another set of data.
- In Côte d'Ivoire, the average yield of mature hybrid progenies tended to be significantly correlated with that of the clones grown from individual trees from the same progenies, suggesting that family selection can be successful.

Correlations between yield of clones observed in collections or in clone trials, and general combining ability for yield of the same clones used as parents in hybrid progeny trials

- The best set of data presented come from Malaysia (BAL Plantations), where significant correlations were observed between yield of clones tested in trials and general combining ability (gca) for the same trait.
- In PNG, a good correlation was also found between the yield of ten Trinitario parental clones and the average yield of the clones derived from progenies resulting from crosses between these ten clones.
- Less conclusive results were presented from Côte d'Ivoire and Brazil, both for yield and yield efficiency. However, in Brazil positive correlations were observed for yield efficiency when comparing genetically different sets of clones in the CEPLAC collection, whereas genetically uniform clone sets produced non-significant correlations for the same trait.

Recommendations

- *More studies are needed to obtain a better understanding of the causes of tree-to-tree variation, comparing variation in seedling families with that in clones.*
- *Good experimental designs, sound agronomic practices and efficient data handling in breeding trials are fundamental to securing selection progress for yield.*
- *Ortet selection, based on family means, can be recommended to find better clones for yield and for yield efficiency. However, the correlation between family means and clones grown from them is not always significant.*
- *Ortet selection, based on individual tree yield alone, has generally not been reliable for predicting the yield of clones grown from them.*
- *However, individual tree values may be useful to select for other more heritable traits such as disease resistance (as shown for Black Pod resistance in Côte d'Ivoire and for Witches' Broom resistance in Brazil) and quality.*
- *The use of rapid vegetative multiplication of seeds or seedlings ("true twin" and "accelerated hybrid clone selection") is recommended to speed up, and possibly increase, the efficiency of ortet selection in new segregating populations.*
- *Individual clone values for yield, as obtained in clone trials, can be good predictors for yield of the hybrid progenies grown from these clones, as well as for the average value of the clones derived from these hybrids, provided that planting densities are near the optimum for the different clones. In other words, good clones are expected to produce good hybrids and good clones can be selected from those hybrids.*
- *Use of statistical methods may provide additional information on the genetic value of individual trees for quantitative traits by introducing the family value and spatial variation parameters to correct individual tree values.*
- *However, more studies are required for a better understanding of the relationship between yield of seedling trees (or families) and that of plagiotropic clones grown from these trees (or families). These studies could involve determination of the possible influence of plant growth habit (erect versus more horizontal plagiotropic branches, erect being favourable for clones whereas the growth habit seems less important for seedling trees) and of inter-plant competition (planting density, pruning intensity).*

Topic 3. Factors affecting yield x vigour relationships in cocoa

Scope and definition of yield efficiency (YE)

- Yield is considered to be the product of assimilate production and partitioning to the harvestable part of the trees.
- Efficiently producing trees are characterised by a favourable distribution of assimilates to the harvestable crop in relation to total dry matter of the plant.
- Increases in production efficiency have facilitated dramatic increases in yield per area associated with higher planting densities in a large number of crops.
- High variability in the partitioning of biomass in cocoa creates an opportunity for the breeder, while taking into consideration the need to optimise planting density.
- In addition, higher production efficiency would reduce the need for pruning to keep the trees small (which is needed in cocoa to maintain profitable yields and to manage the crop more easily).
- Total dry weight production of a tree is correlated with the trunk cross-sectional area (cm^2).
- Terms used to express biomass partitioning are "harvest efficiency", "yield efficiency", "cropping efficiency" or "harvest index".
- Hereafter, yield efficiency (YE) is defined for cocoa as dry bean weight divided by the increment (in cm^2) of the trunk cross-sectional area measured at a uniform height above the soil over a certain period of time.
- Calculations of YE may either be based on dry bean yield and the increment in stem girth over a specific number of harvests, or on total yield and the final stem girth attained in the experiment.

Correction for vigour in cocoa breeding trials

- Large variation in tree size and vigour exists in cocoa genotypes (size of the adult tree canopy may vary 4-6 fold).
- Relative differences in vigour between small and large clones increase with time (PNG).
- Selection of clones based on yield alone will tend to favour the more vigorous clones, which may, however, not be adapted to the planting density in which they were selected.
- In breeding trials, yield values should therefore be corrected by the plant vigour in order to evaluate the YE of the genotypes, and to include smaller genotypes in the selection process.
- Depending on the genotype, optimal planting densities for clones may vary seven-fold (700 to 5000 trees per ha), as has been demonstrated at BAL Plantations in Malaysia. Optimal densities depend also on general growing conditions, such as soil fertility and management (*e.g.* in PNG).
- In selection or validation trials, cocoa varieties with known differences in vigour should be planted at different planting densities. Such is done routinely in clone trials in PNG.

Genetic and environmental factors affecting YE

- Large additive components of genetic variance have been observed for YE as well as for yield and vegetative vigour (Malaysia).
- Although yield and YE (as defined above) are generally quite closely correlated, the best progenitors for yield are not necessarily the best progenitors for YE, and the best yielding clones may not have the highest YE.
- In PNG, YE is more responsive to increases in planting density than is yield.
- Furthermore, YE decreased after three cropping years in PNG for "small" as well as for "big" clones. This is because stem girth increased with time, but yield reached a peak after three cropping seasons. This behaviour appears to be quite common in fruit crops.
- In PNG, smaller-sized clones at higher densities appear to have, on average, better yield and YE than larger clones planted at lower densities.
- However, it is not well known whether it is a general trend that YE decreases with increase in tree vigour. In fact, there is evidence that genotypes with large statures can also be rather efficient (*e.g.* BR 25 clone).
- Little is known so far about other environmental and agronomic factors (pruning) that may affect YE.

Physiological traits related to efficiency of cocoa trees

- Total dry matter production is the product of photosynthetic capacity and the quantity of solar irradiation intercepted by the canopy, which are affected by the leaf area index and the canopy architecture.
- Higher photosynthetic capacity may not necessarily lead to higher productivity, as other factors may interfere, such as partitioning of assimilates between vegetative and generative parts.
- More open canopies would favour light distribution in the canopy. Such canopies have lower light extinction coefficients.
- Exploitation of variation for canopy architecture and tree vigour in breeding would allow for selection of genotypes adapted to high-density planting that may be highly productive.
- Genotypes may be found with a relatively high photosynthetic rate under low light intensity that could be better adapted to yield well under high shade conditions.

Effects of inter-tree competition

- A general competition effect between neighbouring trees is observed in cocoa stands planted at normal densities. This effect has been observed quite early (*e.g.* 18 months at a density of 1667 trees per ha), and may then not be due to vegetative vigour, but to other causes such as attractiveness to insect pests.
- Inter-plant competition, generally attributed to an imbalance between vigour and planting density, is increasing with time and may be partly responsible for "yield decline", as observed in aging plantations in many countries.

- Inter-plant competition may disappear with a drastic reduction in the planting density (thinning). Thinning of 50% of the trees may drastically increase individual tree yield to the extent of maintaining or even increasing the same yield per ha as before thinning (*e.g.* in Côte d'Ivoire, French Guiana).
- Under the conditions of the French Guiana trial reported, cocoa families could be classified as "aggressive", "stimulating", or "passive" in relation to their neighbours.
- Interplant competition effects of families are best explained by variations in the trunk cross-sectional area, and not by the yield or by the yield components. Hence, it should be possible to select non-aggressive, high yielding families.

Recommendations

- *Aiming at obtaining more efficient cocoa trees, YE is a trait that should be taken into account in any breeding programme.*
- *It is therefore recommended that stem girth be routinely measured in all breeding trials and even in collections, in order to be able to calculate YE over certain periods of time.*
- *It is recommended that studies be conducted to obtain measurements of YE that are less correlated with yield itself. Such would be useful to obtain a variable that carries more weight in selecting for highly efficient cocoa trees.*
- *Official recommendations for commercial cocoa varieties should be done for groups of hybrid progenies or of clones exhibiting similar vigour level and growth habit, and for which the optimum planting density has been identified.*
- *Genetic and plant husbandry (pruning, planting density) pathways need to be explored further to decrease competition effects between neighbouring trees and optimise YE.*

Topic 4. Possibilities to use rootstocks for developing more efficient and compact cocoa trees

General

- The development of more compact cocoa trees is desirable to increase yield and YE as well as to facilitate tree management (pruning, harvesting, spraying).
- Compact cocoa trees may be developed through the selection of less vegetatively vigorous trees and possibly also through the use of "dwarfing rootstocks".

Lessons learnt with temperate fruit trees

- The development of dwarfing rootstocks in temperate fruit tree cultivation is the single most important factor increasing YE by reducing the size of scion varieties, and allowing for high density planting.
- Dry matter partitioned to fruit in dwarfing *Malus* (apple) rootstock can be 70%, compared to 40-50% in more "vigourating" rootstock varieties.
- Positive effects of selected rootstocks also include increased precocity, fruit size and quality, tolerance to climatic stress and pest and disease resistance (including resistance to *Phytophthora* sp.).
- The rootstock-scion dwarfing effect on growth is independent of cropping; it is not a simple change in yield efficiency. Most dwarfing rootstock x scion combinations have smaller amounts of roots than less dwarfing combinations.
- The vigour of the rootstock when grown on its own is not always related to the degree of dwarfing induced by the rootstock. Some "dwarfing rootstocks" can be quite vigourous when grown on their own roots, and not all low-vigour genotypes have dwarfing effects when used as rootstocks.
- Grafting higher up the rootstock stem can enhance dwarfing effects of low-vigour rootstocks.
- Dwarfing effects can also be obtained by using inter-stem grafting.
- Mechanisms of dwarfing rootstocks are not yet fully understood. However, hormonal factors, variation in nutrient and water movement, and partial obstruction at the graft union have all been suggested to be involved.
- Dwarfing rootstocks in apple generally appear to have a high phloem-xylem ratio. This trait may possibly be of value as a selection criterion to find new dwarfing rootstocks in other fruit crops also.
- Alternative approaches to induce dwarfing effects include the use of growth regulators, restriction of root growth and of irrigation at critical times.

Rootstock experiences in cocoa

- Rootstocks are used in a wide range of crops to compensate for deficiencies in otherwise excellent scions, such as difficulty in rooting and long juvenile periods. Very often these scions have qualities that are highly valued in the market. These considerations do not apply to bulk cocoa.
- However, there may be interest to try to increase yield efficiency in cocoa, allowing for high-density plantings, and to obtain resistance to root disease by selecting suitable rootstock varieties.
- Rootstocks for commercial cocoa clones are generally seedlings obtained by open-pollination. In some American countries, rootstock varieties with resistance to *Ceratocystis fimbriata* have been selected and used.
- Most relevant yield data using seedling rootstock varieties of cocoa come from three trials carried out in Malaysia. In two trials, there was no main effect of hand-pollinated or open-pollinated rootstock varieties. In another larger trial, there was a significant variation in early yield and vigour but not in YE among twelve hand-pollinated rootstock varieties, but interaction with three scion varieties was generally absent. The SCA 6 x SCA 12 rootstock produced the largest scions and also the highest yield, but below average YE.
- Only one experiment has been reported with clonal rootstock varieties (Murray and Cope, 1959, Report on Cacao Research, Imp. Coll. Trop. Agr. Trinidad, pp.29-

35). The authors of this paper concluded that there was no better prospect of producing higher yields with particular combinations than with single clone plants, that it is possible to increase the yield of a weak clone like ICS 45 by growing it on a vigorous stock, and that yields on a "mutant" dwarf were extremely poor.

- However, as pointed out during the workshop by some participants, the results presented in the above paper might be interpreted differently when analysed from the perspective of yield efficiency. The rootstocks had significant effects on YE (with ICS 1 having 50% lower YE than ICS 60), and the highest yield and YE were obtained when grafting the best scion (ICS 1) onto the best rootstock (ICS 60). The least vigorous rootstock clone (ICS 45) produced the lowest yields but not the lowest YE. This may suggest that choosing appropriate densities for rootstock/scion combinations giving high YE can further enhance yield.

Prospects of using mutant seed varieties as rootstock in cocoa

- In PNG, a mutant genotype, MJ12-226, was found among progenies of the SCA 12 x NA 149 cross. It has small and narrow leaves, a smaller root system and strong branching habit, suggesting weak apical dominance. The mutation appears dominant with possible interactions with the cytoplasm.
- Mutant seedlings, when used as rootstocks, produced a dwarfing effect on the scions in the nursery. This effect persists at least until one year after field planting. No data are yet available for older grafted plants.
- The MJ 12-226 mutant is also of potential value as a scion or seedling, interesting for its multi-stem, orthotropic growth habit.
- In Ghana, the potential of the crinkle leaf mutant (CLM) as a possible source for dwarfing rootstock is being investigated. CLM is characterised by small crinkled leaves, short internodes, a smaller root system and smaller pods and beans. The CLM condition appears to be inherited as a dominant trait, with a 2:1 ratio being observed in hand pollinations when using the CLM as female parent.
- Initial field studies (not yet conclusive) show that CLM has potential for reducing the shoot growth of the scions.

Recommendations

- *In the search for compact cocoa varieties, there are favourable prospects to select for more efficient and compact scion varieties. In the discussions, the urgent need for developing such better scion varieties was generally stressed.*
- *Opinions varied on the prospects and need for developing "dwarfing" rootstocks for cocoa. Some participants considered that this is not a priority because the advantages expected from dwarfing rootstocks can possibly be developed also in good scions, irrespective of the rootstock.*
- *Other participants considered, however, that developing good rootstock varieties (dwarfing and with other important traits) is also important, because of increasing interest in the commercial use of clonal varieties that are generally multiplied by grafting or budding. The high vigour of otherwise good commercial clones (e.g. PBC 123 and CCN 51) might be corrected by adequate rootstocks.*
- *Rootstocks with uniform positive effects on the scions would be more interesting than rootstocks that cause important interactions in different combinations.*
- *Rootstocks have still been poorly studied for cocoa; more understanding is required on how "dwarfing rootstocks" can be developed in cocoa.*
- *Research experience related to rootstocks in apple may possibly help to accelerate the development of good rootstock varieties in cocoa. For example, studies on phloem/xylem ratio and on grafting at different heights of low-vigour rootstock seedlings could be tried.*
- *Care must be taken with grafting of vigorous scions onto low-vigour rootstock. If budding or grafting is done too low (e.g. hypocotyls-type of budding or grafting), the scion may develop its own roots and the rootstock effect may be lost (experience in French Guiana).*
- *Crosses with related species in the same genus deserve retrying using a wide range of combinations for introgression of important traits, including rootstock effects.*

Topic 5. Analysis of genotype by environment (GxE) interactions in cocoa

Stability of cocoa varieties over sites

- Information presented on other occasions suggested that there are relative, small interactions between hybrid cocoa varieties and sites between cocoa growing regions in the same country (*e.g.* Brazil, Côte d'Ivoire and Ghana).
- Multilocational trials at two sites in PNG failed to show significant interactions between clones and sites.
- However, strong interactions between clones and sites were observed in a recent trial in Indonesia. These interactions may, however, be partially explained by different plant husbandry standards applied at the sites, with high experimental errors observed at one of the two sites. For some clones, the interaction with sites appeared to be related to differential susceptibility to VSD, *Helopeltis* and *Phytophthora*. Four clones showed stable and good yields over both sites.
- Early results on recent multilocational clone trials in Brazil suggest larger GxE interactions for yield than for Witches' Broom resistance. The use of 10 trees per clone and per site results in similar selection progress as for 20 trees per clone for both traits.
- The influence of soil factors on GxE interactions for early yield appeared to be more important than that of geographic distance and climatic factors.
- Ongoing research (International Clone Trial in the CFC/ICCO/IPGRI project) within the next five years is expected to elucidate the stability of traits of cocoa clones over ten different countries.

- Little is known yet about the magnitude of interactions of cocoa genotypes with production systems (low against high input, shaded versus unshaded) and pruning regimes.

GxE interactions for planting density

- As indicated above, earlier results have shown a wide variation in the optimal planting density for cocoa clones.
- Such has been clearly demonstrated in Trinidad, in the early 1990's, for three TSH clones planted at three densities (748, 1495 and 2990 trees per ha). One clone was specifically well adapted to the high planting densities (TSH 919).
- In Ecuador, the EET 19 clone also had the best adaptation to a higher planting density (1000 compared to 666 and 500 trees per ha).
- Recent results from Indonesia and PNG showed little interaction between clones and planting density. This could possibly be ascribed to the relatively low variation in planting densities used in these trials (625 and 1000 trees per ha in PNG and 800 to 1333 trees per ha in Indonesia).

High density commercial planting

- Good yields (1.5 to 2 tonnes per ha) can be sustained over many years by high density planting of TSH clones or of TSH seedling progenies in Trinidad. The high inputs required are considered as economically advantageous in relation to "improved low density planting".
- Adoption rates of high density planting by smallholders in Trinidad have, however, been low.

Recommendations

- *Further studies into the interaction between genotypes and environment are required to match planting materials with local growing conditions and plant husbandry regimes (e.g. planting density, pruning).*
- *Promising new clones need to be tested over a wide range of environments before general recommendations can be made on the range of adaptation of individual clones.*
- *Clones can be found with good adaptation to many sites, however, the possibility of selecting clones adapted to specific environments should not be excluded.*
- *High density planting can be economically advantageous in cocoa, provided that adequate plant husbandry practices are applied, including intensive pruning.*

Topic 6. Other perspectives related to development and multiplication of new planting materials

Farmer-researcher participatory on-farm selection of new varieties

- In Brazil, integration of the knowledge of farmers and breeders in selection of new varieties has been shown to be successful in dealing with an emergency situation that necessitated finding good Witches' Broom resistant varieties.
- Results of an on-farm survey on planting materials in Nigeria have implications for participatory breeding and for adoption of research results by farmers. Farmers tend to give priority to trees that yield well throughout the entire year and are relatively less vigorous. The ideal tree was considered to possess a mixture of traits found in Amelonado and Upper Amazon cocoa types.
- Nigerian farmers were further able to identify trees with a reduced incidence of Black Pod.

Prospects for rapid multiplication of scion and clonal rootstock varieties for cocoa

- Results in Trinidad and Malaysia have shown similar yield potentials for clones that are grafted onto seedling rootstock using either plagiotropic or orthotropic budwood.
- Use of plagiotropic grafted clones appeared superior to that of plagiotropic rooted cuttings for adult tree yield in one trial in Ecuador. Further studies on this subject would be useful.
- Commercial production of orthotropic plantlets directly through somatic embryogenesis is viewed as very expensive. This must be combined with downstream nursery multiplication to improve its feasibility when utilised for germplasm transfer or other purposes.
- Bending of orthotropic shoots in the greenhouse or in a budwood garden has been used with success on a large scale, be it experimentally, for rapid multiplication of orthotropic budwood or grafts. Simple heading pruning of low-forming jorquettes can be used to avoid canopies forming at different, and too low, heights.

Recommendations

- *Use of farmers' knowledge of planting materials and direct involvement of farmers in the selection process are important new approaches in the development of better yielding and more resistant cocoa varieties. Such approaches are expected to result in higher adoption rates of selected varieties by farmers.*
- *Comparison between orthotropic versus plagiotropic rooted cuttings should be considered if clonal dwarfing rootstocks are to be developed.*
- *Rapid multiplication of orthotropic cuttings, budwood or graftwood is probably best obtained by bending orthotropic shoots in budwood gardens.*

Tackling Mislabelling in Cocoa Germplasm Collections

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Introduction

Cocoa is unusual amongst crop species in that its germplasm has to be maintained as live collections. This is because its recalcitrant seeds are ill suited for long-term storage. Incorrectly labelled accessions have always been a problem in these collections, and until recently, the tools have not been available to clearly identify mislabelled clones. However, the emergence of reliable genetic fingerprinting techniques has enabled great strides to be made in the molecular characterisation of cocoa germplasm held in *ex situ* collections. Incorrectly labelled accessions can be broadly divided into the following three categories: a) synonymous designation, where the same genotype carries different names; b) homonymous designation, where different genotypes carry the same name; and c) non-designation, where accessions lack any name. Cocoa is not unique in having a significant mislabelling problem in *ex situ* collections. Indeed, mislabelled clones have been identified in collections of a variety of tree species including Eucalyptus (Keil and Griffin, 1994), Sitka spruce (Wilhelmina *et al.*, 1995) and oil palm (Purba *et al.*, 2000), and even in crop genebanks such as potato (Huamán *et al.*, 2000) and enset (Negash *et al.*, 2002).

Preliminary work performed on material held in the International Cocoa Genebank collection in Trinidad (ICG,T) using RAPD analysis suggested that up to 30% of trees might be mislabelled (Christopher *et al.*, 1999). However, more recent work by Motilal (2004) revises this estimate downwards to around 12% on the basis of the more reliable SSR technique. There is no intrinsic reason to suggest that there are fewer mislabelling events elsewhere, particularly since ICG,T has historically acted as a major donor of cocoa material for other collections. Motilal and Butler (2003) provided circumstantial evidence in support for this view when they estimated the global figure of putative off-types to stand at about 30% on the basis of a survey of the International Cocoa Germplasm Database (ICGD). DNA fingerprinting, together with an increasing body of morphological descriptors for cocoa now make it possible for curators to identify mislabelled clones within their collections with some ease. Once a tree within a collection has been identified as being mislabelled, a simple mechanism is needed for re-naming this individual in a clear and unambiguous way. Here, we propose a system for renaming mislabelled clones, intended as a guide for curators of all cocoa germplasm collections. If the same system is implemented widely, it is hoped this will minimise the potential confusion that could arise from unstructured approaches to the renaming of clones around the world.

How can mislabelling occur?

There are a number of ways in which mislabelling can occur:

- Plants may lose their labels or the labels may become illegible;
- Plants may be moved before being properly labelled;
- Labels may get mixed up during vegetative propagation;
- Detached labels on the ground may be re-attached to the wrong plant;
- Dieback may result in labels being lost when a dead branch breaks;
- Chupons may grow from the rootstock and be confused with the scion;
- Established seedlings may be confused with the original tree;
- Some plants may be mislabelled in the greenhouse (human error);
- Introduction of synonymous germplasm (with different names) from abroad;
- Simple transcription errors can occur during plant propagation or label replacement.

Genotypes are difficult to distinguish on the basis of appearance during greenhouse propagation and when initially planted in the field since there are usually no flowers or pods. Consequently, identification at this stage relies heavily on the plant labels and field maps. However, maps become outdated when trees die or are replaced, or even when an old tree falls and a new "main trunk" becomes established in a new location. Furthermore, hand annotation of maps is prone to misinterpretation and the problem can be confounded by high planting densities and irregular-shaped field plots with unclear boundaries; this can lead to ambiguities if individual trees are not labelled.

What is being done in Trinidad (ICG,T)

Trees in original plantings:

Many original trees, which are located at Marper Farm, Manzanilla, east Trinidad, act as vital reference genotypes for many international clones that have been distributed around the world. A number of steps have been taken to tackle the mislabelling problem in this collection:

- Trees found growing in Marper Farm with no labels were given new labels and assigned new names (CRU 1 to CRU 155).
- While revising maps in 2001-2002, more trees were found with uncertain identities and assigned new names (MARPER 1 and MARPER 55).
- More recently, it has been possible to re-assign original clone names to some "CRU" and "MARPER" accessions using historical hand-written records.
- Even where the original identities of accessions have been lost, we still know something about the germplasm; it is either Pound's Refractario collection from Ecuador (1937) or his Upper Amazon collection from Peru (1938).
- Furthermore, it may soon be possible to learn more of the genetic origin of these clones by their classification/grouping based on DNA fingerprints (Sounigo *et al.*, 1996; J.-C. Motamayor, pers. comm.).
- Using 116 PA accessions maintained at Marper farm and 57 ICS accessions maintained at the Cheesman field as two test cases, we have assessed the extent of mislabelling in the reference genotypes. The result of SSR fingerprinting showed that only 2-3% of the PA and ICS trees were synonymously mislabelled (the same genotype given different names). Moreover, for all the mislabelled trees, comparing the SSR profile of the mislabelled ones with its neighbours allowed us to make corrections. This result shows that there is a trustworthy reference that can serve as a baseline for individual identification.

Trees in replicate plantings:

Many clones have been replanted in replicated blocks at ICG,T. Here too, a number of steps have been taken to tackle mislabelling, which can occur amongst replicates in individual clonal plots:

- It is usually possible to check the identity of trees replicated in a new location by comparison with the original tree:
  - Initially fruit and flower morphology are compared;
  - In cases of doubt, DNA fingerprints can be compared.
- Where such comparisons are feasible, it is possible to determine which trees are true to type and which are off-types with confidence.
- It is often difficult to confirm the identity of a replicate where the "original" tree no longer exists. Passport data can help enormously, though this has also been lost in many cases.
- However, general characteristics of accession groups can be used to confirm that a clone fits into an appropriate group (such as NA, PA and IMC) in the absence of a reference tree.

How should we proceed?

In order to fully identify mislabelling within cocoa collections, morphological and molecular characteristics of clones for all accessions in the international collections need documenting:

- CRU has been recording morphological characteristics of accessions in the ICG,T for the last 12 years;
- DNA has been extracted from all the accessions in the CATIE collection (about 800 clones) and the ICG,T (about 2,300 clones); in all cases sampling the most original tree. These two collections have been designated as Universal collections by IPGRI. These DNA samples are being used by USDA Beltsville to generate genetic fingerprints that will make it possible to compare replicate trees in the two collections. To date, about 700 CATIE accessions and 900 ICG,T accessions have been genotyped.
- The entire collection at the University of Reading intermediate quarantine facility has been fingerprinted.

Similar procedures should be followed for all cocoa collections worldwide, to obtain reference DNA fingerprints from as many original trees as possible. This is an urgent matter, since many collections are aging.

Most of the laboratories involved in genetic fingerprinting are already using the same CERVUS software package (Marshall *et al.*, 1998; Slate *et al.*, 2000) and other statistical tools to compare profiles that have been generated by a set of standard markers provided by CIRAD, France (Lanaud *et al.*, 1999; Risterucci *et al.*, 2000). The community has agreed to use a set of standard alleles provided by Nick Cryer, as controls for calling allele size, for the international set of markers. It has also been agreed that when using other microsatellites, standard genotypes should be used to compare output from different laboratories.

The results we obtained so far confirmed that the set of 15 pairs of SSR primers could give sufficient statistical power for cocoa cultivar identification.

Using microsatellite data for the confirmation of clone identity is dependent on the existence of a known example of that genotype. We must define one clone for each accession to be the true-type specimen (the original tree if available or the source of distributed material).

What should happen to mislabelled plants?

There are three possible ways of dealing with a mislabelled plant once it has been identified:

- If positively matched to a known clone that is already growing in the collection, the plant could be removed;
- If positively matched to a known clone that is not growing in the collection, the plant could be relabelled with its proper clone name;
- If the plant does not match a known clone, it should be given a new name.

Although the curator of the germplasm collection will make the final decision, off-types that do not match a known clone should not automatically be removed since they may have valuable agronomic traits. Several of the "CRU" accessions have been shown to have useful traits subsequent to their establishment in the ICG,T. Similarly, resistance to *Moniliophthora* has been found in two accessions in CATIE that are now known to be off-types.

Renaming mislabelled clones

There is a need for a coordinated policy on the renaming of mislabelled accessions that can be followed by all curators of cocoa germplasm collections. Newly assigned names should be unique to the clone, they should have some meaning and should assist in documenting the origin of an off-type.

We propose the following procedure to avoid future confusion and provide mislabelling information for other users of the clone:

- The new name should begin with a unique identifier used within the collection. This would typically be the collection accession number, for example CRU 263;
- In addition, the new clone name would include further information in brackets to indicate that the clone was originally misidentified:
  - The identifier MIS denotes that the clone has been mislabelled;
  - This is followed by a 7 letter code identifying the country and collection in which the mislabelled clone was found, *e.g.* ICG,T in Trinidad is TTOICGT (already used by ICGD and CocoaGenDB);
  - The full name on the original label is retained at the end of the new clone name, for example POUND 12/B [POU]. This could be 'UNKNOWN' or left blank if, for example, the plant had lost its label;
- These parts would be separated by underscores (not previously used in clone names) for clear identification of the parts, so the full clone name might be:
  CRU 263 (MIS_TTOICGT_ POUND 12/B [POU])

Although this new name may seem very long, for discussions and correspondence the short first part of the name (CRU 263) can be used since it is unique. The full name need only be used in written records (publications, labels, *etc.*) in order to highlight the mislabelling event. The shorter version could be used in publications, particularly in tables and graphs where space is limited, if the full name is referred to earlier in the article.

It is also possible that some off-types may only be renamed temporarily, since a positive identification might be possible once a comprehensive database of DNA fingerprints is available.

The role of the International Cocoa Germplasm Database (ICGD)

The ICGD will include detailed notes providing information on any mislabelling, including the technical data that provided evidence for the mislabelling and, where possible, the most likely identity or similarity based on genetic fingerprinting information.

If the mislabelled clone (*e.g.* POUND 12/B [POU]) is found to be identical to another (e.g. CLONE A/1), then this is the name that should be used (*i.e.* CLONE A/1). Notes would be included in ICGD to say that this clone was mislabelled at the particular station (*e.g.* CLONE A/1 mislabelled as POUND 12/B [POU] at TTOICGT) together with a reference and relevant dates.

If there were a period of time between identifying a mislabelled clone and finding its true identity, then the name given to the mislabelled accession would become a synonym of the true clone name. For instance, in the example given above CRU 263 (MIS_TTOICGT_ POUND 12/B [POU]) would still be maintained in the database, but as a synonym of CLONE A/1.

The entire collection of the University of Reading Intermediate Cocoa Quarantine Facility has been fingerprinted using the international set of 15 microsatellite markers and the data has been included in the ICGD. These profiles are also being submitted to CocoaGenDB (http://cocoagendb.cirad.fr), a new cocoa genomics database being developed through a collaborative project involving ICGD, CIRAD (Centre de Coopération Internationale en Recherche

Agronomique pour le Développement, France) and USDA (United States Department of Agriculture, USA). It is hoped that the large number of profiles being generated in other laboratories using SSR markers will soon be submitted to CocoaGenDB (e-mail: tropgenedb@cirad.fr) and that this will continue as new fingerprinting projects develop. ICGD Online (http://www.icgd.rdg.ac.uk) will continue to include microsatellite profiles generated from the standard 15 markers to complement morphological data used for clone identification.

Summary

The level of mislabelling of cocoa clones in germplasm collections is fairly high. Although not a new problem, the availability of genetic fingerprinting technologies has allowed projects to be developed that use microsatellite markers to compare clones. However, it is important that when clones with the same name are found to have different profiles, one is determined to be the true type (with reference to the most original material). Off-types should be kept for their potential agronomic value, but with a unique new name.

We have proposed a format for renaming that highlights the mislabelling event and which includes references to the source germplasm collection and the name originally given to the clone.

Acknowledgements

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Red Pods in Progenies from the Euleupousing River in French Guiana

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It is generally accepted that the pods of Amazonian Forastero cocoa trees are completely yellow when ripe, without any red pigmentation (Urquhart, 1955; Braudeau, 1969; Mossu, 1990). However, some authors are not so categorical, though they remain quite vague (Thirion, 1950; Enríquez, 1985; Lanaud *et al.*, 1999), whilst others explicitly mention exceptions to that rule. Of the latter, the forerunner and most well-known was F.J. Pound, a great collector, who reported on the existence of cocoa trees that he enigmatically called “Criollos de la montagne” (in French in the text) encountered on the banks of the Napo and Ortegaza rivers, whose pods were “blanco, warty lagarta, with splashes of red pigmentation on the ridges” (Pound, 1938). He also said “there seems to be no doubt that this criollo de la montagne type represents the wild cocoa population of the whole region from the Rio Napo to the foothills of the Cordilleras to the North”. Lastly, speaking of the entire Amazon valley, he adds, “Throughout the whole valley the pods were either green or blanco and only when approaching Colombia was the presence of pigment on the pods encountered.”

Since Pound, authors have generally considered the existence of traces of red pigment in wild Forastero trees as an undeniable reality, albeit not very common. According to Cheesman (1944): “The only fruit characters that all the members of this group seem to have in common are absence of red pigment from the pod wall, and flattish beans with dark purple cotyledons. Even these may not be entirely constant characters. Absence of red pigmentation from the pod wall is not absolute because slight traces of pigmentation on the ridges of the pod may sometimes be detected in trees that certainly belong to the Amazonian Forastero group. Nevertheless, for practical purposes, the absence of red pigmentation in the pod can be taken as at least a very common characteristic of Amazonian Forastero populations.” Baker *et al.* (1954), then Soria (1970), accepted and shared those views, though somewhat placing in context the importance of this "criollo de la montagne", which they more realistically called “criollo del monte” (i.e “forest cocoa”), and whose wild nature they were not so sure about. Toxopeus (1985) also resorted to Pound’s descriptions.

Other collectors, such as Barriga *et al.* (1986); Allen (1988); and de Almeida *et al.* (1996) also mentioned the existence of reddish pigment on the cuticle of pods in some populations. Both Brazilian authors pointed out that anthocyanic pigments were often found at some sites in the States of Acre and Rondônia, particularly on fruits exposed to sunlight.

However, in all the previously mentioned cases, it was a matter of reddish or pinkish (Enríquez, 1985) patches at the most, or “tanning” of the parts of fruits exposed to sunlight. Nevertheless, the existence of totally red fruits is mentioned, albeit more rarely, and scarcely documented; for instance, R. de Lemos Fróes is said to have collected a specimen near the river Jandiatuba (Amazonas state), though apparently without leaving any published trace (Bartley, pers. comm.). Moreover, one variety is known for its totally red pods, the "Red Amelonado" (Red, or Red Amel) received in Trinidad from Belém and of Amazonian origin, but without any further information (Bartley, pers. comm.; Lockwood and Gyamfi, 1979). In Belém, the Museu Goeldi apparently possesses “a tree with red fruits in its collection from the Amazon” (Bartley, pers. comm.).

As far as the wild cocoa trees of French Guiana are concerned, long years of observing hundreds of individuals derived from open-pollinated progenies of mother trees from the basins of the Camopi and Tanpok rivers have convinced us that their pods are solely yellow when ripe, without any pink, red or purple “tanning”. The same applies for the “Borne 7” and “Ker” populations from the Upper-Oyapok (Lachenaud and Sallée, 1993).

However, during the 1995 expedition on the banks of the Euleupousing River, pods with a pinkish pigmentation of the cuticle were collected, and some mother trees also displayed pale beans (Lachenaud *et al.*, 1997). Seedlings from the harvested pods were planted in a plot at Paracou-Combi (French Guiana) in 1996 and, as soon as the first pods appeared, it could be seen that some had a very marked red pigmentation, even at the cherelle stage, in (at least) the two progenies Elp 28 and Elp 32 (Elp 28 came from a mother tree with pale beans) (Figures 3a & b). Since 2001, a tree with entirely red pods has even been found in progeny Elp 28 (Tree Elp 28-A, Figure 3 b).

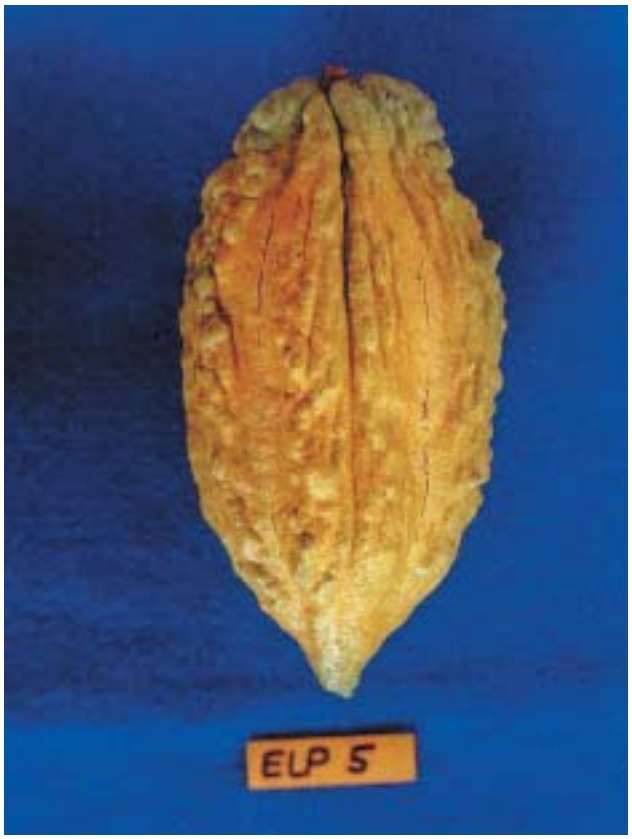

Figure 1: ELP 5 pod

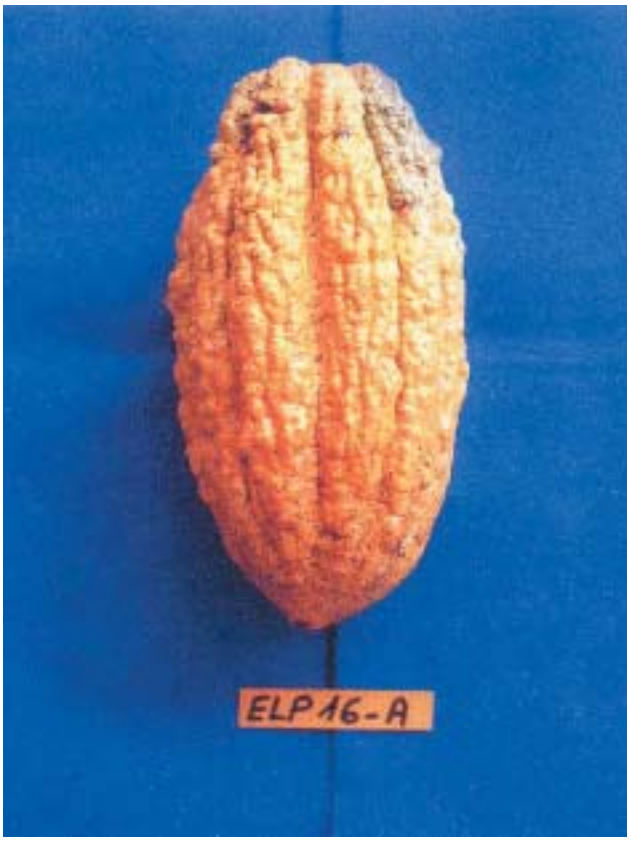

Figure 2 (b): Pod of ELP 16-A

Figure 2 (a): ELP 16 pod

Figure 3 (a): Unripe pod of ELP 28-4

Figure 3 (b): ELP 28-A pod

The Euleupousing River is located in a very remote region, in a protected Indian zone with regulated access, hundreds of kilometres from the coast and from old plantations abandoned for decades. The Amerindians also shun it, making it a virtually virgin territory and a natural sanctuary (Lachenaud *et al.*, 1997). For all these reasons, pollution by "Trinitario" material is highly unlikely. To be completely sure, leaf samples were taken to compare trees of 12 Elp progenies (including the one with entirely red pods) with 34 controls of the Trinitario and "Criollo" types. The following analyses were carried out in the USDA Miami laboratories where thirteen markers were used to fingerprint 1,098 accessions belonging to 75 diverse cocoa populations from South and Central America. To determine the genetic origin of tree Elp 28-A, an *assignment test* was performed as described in Paetkau *et al.* (1995). Two Trinitario clones, ICS 1 and ICS 95, with red pods were used as controls. The assignment test involved calculating the expected frequency of each of the three individuals' genotypes in the 75 populations, including the Elp (Figures 1 & 2) and Trinitario populations, and subsequent assignment of each individual to the population where its expected genotype frequency was highest. Absences of alleles specific to a given population and the subsequent 0 frequency were treated using the zero avoidance device described in Titterington *et. al.* (1981). Twelve individuals from the Elp population and 34 individuals from the Trinitario population were fingerprinted to calculate genotype frequencies in these two populations. The product of the expected genotype frequency for the 13 microsatellites was calculated to determine the expected frequency of each genotype in the Elp and Trinitario populations.

As a result, individual Elp 28-A was assigned to the Elp population. In other words, its expected genotype frequency calculated from the use of 13 microsatellites was higher in the Elp population than in any other of the 75 populations fingerprinted in the USDA including the Trinitario population. The controls, ICS 1 and ICS 95, were both assigned to the Trinitario type using the assignment test. Table 1 indicates the genotype frequency of individuals Elp 28-A, ICS 95 and ICS 1 in the Elp and Trinitario populations. The ratio of the frequency of these genotypes in the Trinitario population compared to the Elp population is also presented.

Table 1 clearly shows that individual Elp 28-A belongs to the Elp population when compared with individuals ICS 1 and ICS 95, which clearly belong to the Trinitario group.

These results underline the fact that traits, thought to be specific to the Criollo group and subsequently to the Trinitario group (because of its hybrid character), can also be found in other populations, as is already known for the white bean colour. This work highlights the importance of molecular markers in discriminating between cocoa genetic groups.

Table 1: Expected genotype frequencies of individuals Elp 28-A, ICS 1 and ICS 95 within Elp and Trinitario populations

	Elp	**Trinitario**	**Elp/Trinitario**
Elp 28-A	5.44×10^{-7}	1.13×10^{-36}	2.07×10^{-30}
ICS 1	1.47×10^{-37}	5.44×10^{-10}	5.44×10^{27}
ICS 95	2.11×10^{-45}	2.91×10^{-7}	1.38×10^{38}

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West African Cocoa: A Pilot Study on DNA Fingerprinting of the Germplasm from the Cross River State of Nigeria

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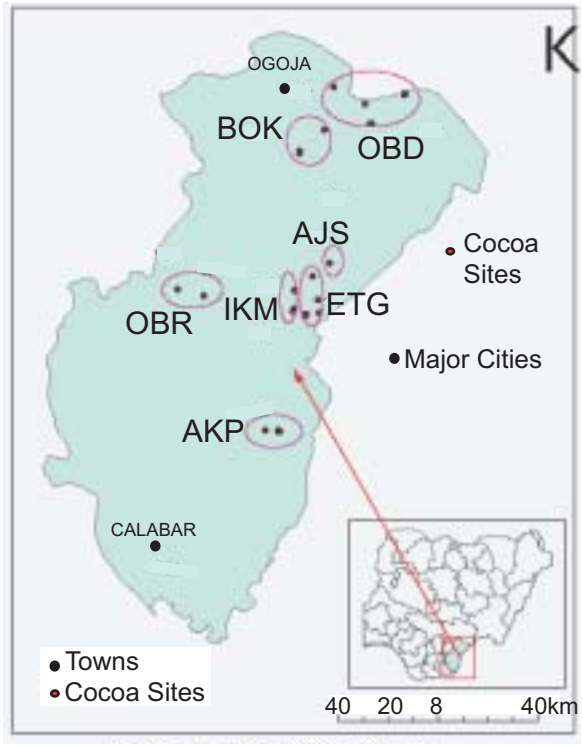
Introduction

Currently, the major area in the world under cocoa cultivation is West Africa, which contributes more than 70% of the world's cocoa production. Therefore, the chocolate industry depends on West African cocoa production. However, cocoa cultivation in West Africa is under serious threat from two major diseases, Black Pod and Swollen Shoot, along with deforestation and natural calamities. Genetic information on local germplasm will provide information needed to select the best parents for long-term cocoa improvement breeding programmes. Furthermore, there is a problem of duplication and/or misidentification of cocoa germplasm in West Africa due to the fact that several introductions were made in this region, and it is impossible to identify duplicates or mislabelling without genetic fingerprinting.

Therefore, an initiative has been launched by USDA/USAID-Masterfoods in collaboration with the International Institute of Tropical Agriculture (IITA) and national cocoa research institutes in the above-mentioned countries to assess the range of genetic diversity, and also to identify duplicate/mislabelled accessions. This report is based on a pilot study, undertaken by the first three authors, on DNA fingerprinting of approximately 350 accessions from Cross River State of Nigeria using 11 microsatellite markers (SSRs) (Table 2). Cross-River State borders with Cameroon, where a different variety of cocoa is grown. There is a possibility, therefore, that exchange of germplasm has been taking place among cocoa farmers across the border, resulting in a higher genetic diversity in this region than in the rest of the cocoa growing regions of Nigeria. Generally, Nigerian cocoa is Upper Amazon Forastero while Cameroon cocoa is Trinitario. The results from this study will subsequently be compared with those from the entire Nigerian cocoa germplasm collection.



Map of Cross River State of Nigeria showing the Cocoa study sites

Figure 1: Map of cross River state of Nigeria showing the locations of farmers' fields from where cocoa accessions were collected

Materials and Methods

Plant Material and DNA Extraction

Three hundred and forty-six accessions of field grown cocoa trees were sampled as leaf tissue from the Cross River region (Figure 1) and the field genebank of the Cocoa Research Institute of Nigeria (CRIN). The list of cocoa accessions collected is presented in Table 1. Accessions were classified as:

Parental clones (six populations of parental clones consisting of 25 accessions collected from the field gene bank of CRIN),

Breeder's collection (five populations of breeders' materials from CRIN, comprising 87 accessions that are mainly F1's and F2's, according to the generation of originally introduced Amazon material), and

Farmer's collection (234 accessions representing eight populations collected from farmers' fields covering all cocoa growing regions of Cross River state (Figure 1).

The selection of accessions from the farmers' fields was based on the *Farmer's Participatory Approach* wherein trees were selected based on the individual farmer's perception of 'best performing' and 'worst performing' trees as well as that of morphological variability.

Leaf tissue was collected from the selected trees and preserved in Eppendorf tubes containing NaCl-CTAB-Azide solution (Rogstad, 1992). DNA was extracted from leaf samples following a modified extraction procedure described by Bhattacharjee *et al.* (2004).

Table 1: Cocoa accessions collected from Cross River state and the field gene bank of CRIN

Farmers' Colection (accession names in codes	Collection sites – (Local govt' names converted to accession codes)	Hnb (over all loci)	Ho (over all loci)	$P_{0.95}$	A	B
AJS (10)	Ajassor	0.5183 (0.085)	0.4542 (0.014)	0.72	3.1	35
BOK (30)	Boki	0.3699 (0.111)	0.2536 (0.022)	1.00	4.5	21
ETG (58)	Etung	0.6033 (0.130)	0.4365 (0.040)	1.00	5.2	31
OBD (24)	Obudu	0.4229 (0.099)	0.3139 (0.019)	1.00	4.4	26
OBR (48)	Obubra	0.5560 (0.113)	0.4763 (0.030)	1.00	6.0	34
AKP (11)	Akamkpa	0.5325 (0.073)	0.4393 (0.101)	1.00	3.9	34
IKM (49)	Ikom	0.4574 (0.151)	0.3968 (0.048)	1.00	5.1	31
ETM (4)	Etomi	0.4481 (0.055)	0.4545 (0.042)	0.54	1.5	17
Breeder's Collection	**CRIN field bank**	**Mean 0.4885 (0.110)**	**0.4031 (0.077)**	**0.91**	**4.2**	**2.0**
CESA (38)	CRIN Elite Selected Accessions	0.5513 (0.169)	0.5176 (0.045)	1.00	6.2	36
CSA (15)	CRIN Selected Accessions	0.6254 (0.083)	0.5999 (0.112)	1.00	4.5	38
AMAZ10 (1)	Amazon 10 (Upper Amazon)	0.7272 (0.022)	0.7272 (0.089)	0.89	1.9	17
CTIS (23)	CRIN Trinidad Introduction and selections	0.6209 (0.123)	0.5022 (0.045)	1.00	5.0	41
CS2H (10)	CRIN Series 2 Hybrids	0.5799 (0.045)	0.5714 (0.056)	1.00	3.7	34
Parental Clones	**CRIN Field Bank**	**Mean 0.6209 (0.118)**	**0.5837 (0.099)**	**0.98**	**4.3**	**1.41**
COT (11)	C-Clones (Upper Amazon and Trinitario)	0.5912 (0.067	0.5567 (0.063)	1.00	4.5	42
CFS (2)	CF-Clones (Trinitario)	0.8030 (.036)	0.8182 (0.051)	1.00	3.0	33
PAS (2)	Parinari-Clones (Upper Amazon)	0.5606 (0.071)	0.5909 (0.049)	0.73	2.0	22
SCA (1)	Scavina 6	0.3636 (0.004)	0.3636 (0.014)	0.40	1.4	14
TOS (5)	T-Clones (Upper Amazon)	0.3915 (0.049)	0.3970 (0.027)	0.70	2.2	22
AMEL (4)	Amelonado	0.5413 (0.039)	0.4261 (0.082)	0.91	2.7	30
		Mean 0.5419 (0.137)	**0.5314 (0.121)**	**0.79**	**2.6**	**1.43**
Overall Mean ± SD		**0.5504± 0.378**	**0.5061± 0.0512**	**0.89± 4.45**	**3.73± 1.1**	

Note: The values in parentheses indicate the number of accessions collected in each population. Hnb = unbiased gene diversity (Nei, 1978); Ho = observed heterozygosity; $P_{0.95}$ = proportion of polymorphic loci when the most frequent allele does not exceed 95%; A = mean number of alleles per locus; B = effective number of alleles per locus. Standard deviations are indicated in parentheses.

PCR Optimisation and SSR Marker Amplification

A set of 48 accessions, selected from the region, was first screened with 11 pairs of SSR primers (Table 2), developed at CIRAD, and later identified for fingerprinting purposes at USDA-ARS (Miami, USA) (Schnell, pers. comm.) in order to optimise PCR components. Optimisation was done following the strategy of Cobb and Clarkson (1994), which is based on the Taguchi method (Taguchi, 1986). The optimised PCR reaction was then used to screen all the 346 accessions with the 11 pairs of SSR primers. All PCRs were preformed in either 0.2ml strip tubes or 96-well plates with a total volume of 10μL in a gradient cycler PTC 200 (MJ Research, USA) using the following cycling parameters: 94 °C for 4 min. (initial denaturation) followed by 35 cycles of melting at 94 °C for 30 s, 51°C/ 46°C annealing for 1 min. (depending on the primer) and elongation at 72°C for 1 min. This was followed by further incubation at 72°C for 7 min. The PCR products were then stored at 4°C. They were separated on an ABI 3100 automated genetic analyser through capillary electrophoresis. The forward primer for each of the chosen markers was labelled at the 5'end of the oligonucleotide with one of the following fluorescent dyes; (HEX) 4,7,2',4',5',7'-hexachloro-6-carboxyfluorescein, (6-FAM) 6-carboxyfluorescein and (NED) 7',8'-benzo 5'-fluoro-2',4,7 trichloro-3-carboxyflouresein. Easily interpretable pherograms with stutter peaks were produced, and primers generated either one or two alleles in each accession.

Electrophoresis and Data Collection

Amplification products were separated by capillary electrophoresis in a 36 cm capillary array containing POP 4 (Performance Optimised Polymer) matrix with urea. A modified run module, which consisted of a 60°C run temperature, 10 sec injection time, 15kV run voltage and 20 min run time, was used for all samples. Each PCR sample was separated with 0.1μL of an internal size standard (X-Rhodamine Mapmaker, Bio Ventures Inc.). The data were automatically collected by 'Data Collection' software v1.01 and analysed using 'GeneScan' software v3.7 (Applied Biosystems). Fragment sizes were automatically calculated to two decimal places using the "local Southern" algorithm with reference to the internal size standard ranging from 35-500 bp (base-pairs). All 114 SSR allele sizes were then estimated by using 'Genotyper' software v3.7 (Applied Biosystems) after defining the marker category. This produced a single or pair of peak (s), as expected for co-dominant markers such as microsatellites. For co-dominant markers, any individual genotype will have two peaks recorded if it is heterozygous for that marker, or a single peak if it is homozygous. In some genotypes, more than two peaks were also recorded, and in such cases only the two highest peaks were scored. The dataset of 114 alleles from 11 SSRs, scored as base pairs (bp) for individual accessions, was then used for subsequent biometrical analyses. All polymorphic and monomorphic fragments were included in data analysis.

Data Analysis

The genetic diversity among accessions was estimated with the following statistics: mean number of alleles per polymorphic locus, effective number of alleles per locus, average observed and expected heterozygosity (H_o and H_e), percentage of polymorphic loci and average gene diversity (Nei, 1978). To refer to the informativeness of microsatellites, PIC (polymorphism information content) values were calculated using the formula: $PIC_i = 1 - \sum p^2_{ij}$ where p_{ij} is the frequency of the *j* th microsatellite allele for accession i. This value is referred to as heterozygosity and gene diversity (Weir, 1990; Anderson *et al.*, 1993). The *genetic similarity* (gs) between individual accessions was calculated based on Nei's (1978) unbiased genetic distance. Cluster Analysis was performed considering all 346 accessions individually, and a dendrogram was generated using the neighbour-joining (NJ) (Saitou and Nei, 1987; Studier and Keppler, 1988) method of clustering. The genetic similarity and distance matrix, as well as dendrograms were obtained using Genetix 4.04, TreeMaker 1.0.1 and TFPGA 1.3 software. Genetic differentiation was quantified by the F-statistics estimator (Fst) (Wright, 1951), as described by Weir and Cockerham (1984), using Genetix 4.04. F_{st} values were estimated per allele, per locus and overall. Genetix performs bootstrapping (Efron, 1982) over loci and provides rigorous testing of hypotheses of genetic differentiation. The SSR data were subjected to Principal Component Analysis (PCA) using SAS version 8.0.

Table 2: Microsatellite sequences with repeat motifs, number of alleles and fragment size range with observed and expected heterozygosity, polymorphic information content and F-statistics detected in 346 cocoa accessions

Primer	Sequence (5' – 3')	Repeat Motif	No. of alleles	Allele size range (bp)	H (O)[1]	PIC[2] Values	H (E)[3]	Fst[4]
SSR943-NED mTcCIR40	**F**:AATCCGACAGTCTTTAA **R**:CTAGGCCAGAGAATTGA	$(AC)_{15}$ 284	7 (0.023)	260-284	0.460 (0.023)	0.460	0.505 (0.010)	0.037
SSR982-HEX mTcCIR8	**F**:CTAGTTTCCCATTTACCA **R**:TCCTCAGCATTTTCTTTC	(tc) $(tt)_5(tc)_{17}$ (tt) (ct)4	9	287-305	0.250 (0.030)	0.373	0.406 (0.017)	0.032
SSR984-NED mTcCIR10	**F**:ACAGATGGCCTACACACT **R**:CAAGCAAGCCTCATACTC	$(TG)_{13}$	7	201-212	0.688 (0.106)	0.693	0.724 (0.097)	0.033
SSR985 FAM mTcCIR11	**F**:TTTGGTGATTATTAGCAG **R**:GATTCGATTTGATGTGAG	$(TG)_{13}$	10	287-314	0.546 (0.034)	0.658	0.697 (0.021)	0.033
SSR991-FAM mTcCIR18	**F**:GATAGCTAAGGGGATTGAGGA **R**: GGTAATTCAATCATTTGAGGATA	$(GA)_{12}$	13	331-354	0.574 (0.022)	0.594	0.621 (0.136)	0.032
SSR996-HEX mTcCIR24	F:**TTTGGGGTGATT TCTTCTGA** **R**:TCTGTCTCGTCTTTTGGTGA	$(AG)_{13}$	9	184-210	0.122	0.132	0.135 (0.009)	0.033
SSR990-HEX mTcCIR17	F:**AAGGATGAAGGATGTAAGAGAG** **R**: CCCATACGAGCTGTGAGT	$(GT)_7N4$ $(GA)_{12}$	7	271-289	0.244 (0.113)	0.296	0.317 (0.048)	0.034
SSR998-NED mTcCIR26	F:**GCATTCATCAATACATTC** **R**:GCACTCAAAGTTCATACTAC	$(TC)_9C(CT)4$ $TT(CT)_{11}$	13	284-308	0.435 (0.070)	0.663	0.701 (0.011)	0.034
SSR997-NED mTcCIR25	F:**CTTCGTAGTGAATGTAGGAG** **R**:TTAGGTAGGTAGGGTTATCT	$(CT)_{21}$	21	118-164	0.650 (0.028)	0.687	0.716 (0.168)	0.031
SSR883-HEX mTcCIR1	**F**:GCAGGGCAGGCTCAGTGAAGCA R:**TGGGCAACCAGAAAAAGCT**	$(CT)_{14}$	6	126-139	0.432 (0.032)	0.350	0.426 (0.051)	0.032
SSR980-FAM mTcCIR6	F:**TTCCCTCTAAACTACCCTAAAT** **R**:TAAAGCAAAGCAATCTAACATA	$(TG)_7(GA)_{13}$	12	224-250	0.558 (0.013)	0.550	0.622 (0.019)	0.031
Mean± SD			**10.4± 0.167**		**0.446 (0.026)**	**0.496**	**0.534 (0.108)**	**0.033**

1. H (O): Observed Heterozygosity, 2. PIC: Polymorphic Information Content, 3. Expected Heterozygosity, 4. F-Statistics

Standard deviations are indicated in the parentheses

Results and Discussion

Genetic variation at 11 SSR loci was assessed in the 346 cocoa accessions under study, which were grouped into the three "genetic" categories, defined above. In the entire population, a total of 114 alleles were recorded for all the SSRs used. The number of alleles observed at each locus ranged widely from 6 (mTcCIR1) to 21 (mTcCIR25) (Table 2).

The genetic diversity parameters for each population are presented in Table 1. The unbiased mean genetic diversity (Nei, 1978) among all the three genetic groups was recorded as 0.5504 ± 0.0378, and the highest was recorded for the accessions from the breeders' collection (0.6209) that consisted of F1 and F2 hybrids. Though the mean gene diversity for accessions from the farmers' collection was recorded as 0.4885, the mean observed heterozygosity (Ho), in comparison to accessions from the breeders' collection and parental clones, was low, representing a deficit of heterozygotes in the farmers' collection. Similarly, the average number of alleles was highest for accessions from the breeders' collection (4.3), which also had the highest percentage of polymorphic loci and observed heterozygosity (Table 1). The effective mean number of alleles was also determined by considering those alleles whose frequencies were equal to or higher than 0.05. It was found that the mean effective numbers of alleles for the farmers' collection, breeders' collection and parental clones were very low; 2.0, 1.41 and 1.43, respectively. However, C-clones (Table 1) had the maximum number of effective alleles (42 alleles out of 114 alleles), followed by accessions belonging to the CTIS hybrids (41 alleles).

The results from this study displayed a pattern demonstrating that the accessions from the breeders' collection, mostly derived from parental clones (except AMAZ), represent relatively high genetic diversity with

high heterozygosity, which is expected in hybrids, while the accessions from farmers' selections were comparatively less diverse. The latter might be related to the origin of farmers' selections and to the farmers' preference to continually plant seeds from their selected/ preferred trees or from trees within neighbouring farms.

The mean number of alleles per locus over all the populations was low in comparison to results from other studies on cocoa from Central and South America (Motamayor *et al.*, 2002), and averaged 3.73 ± 1.11. This reflects the difference in allelic richness for the accessions from West Africa in comparison to that of those from the centre of diversity. However, the results obtained in the present study are comparable to those from other West African studies (N'Goran *et al.*, 2000) where the effective mean number of alleles was 2.7.

Interestingly, in our study, the accessions from the so-called "Amelonado" population had a high percentage of polymorphic loci (91%) with high gene diversity (0.5413). However, studies of other researchers have shown that lower Amazon Forastero or Amelonado accessions are mostly homozygous with low genetic diversity (N'Goran *et al.*, 1994; Motamayor and Lanaud, 2002; Motamayor *et al.*, 2003). The high heterozygosity we observed may be due to the fact that the Amelonado accessions used in the present study were collected from a so-called 'Amelonado' plot, but are in fact off-types or mixtures of germplasm or the result of mislabelling/misidentification. Therefore, a better picture might be obtained when the entire germplasm collection from Nigeria is analysed.

Figure 2: Scatter plot of SSR marker diversity in cocoa accessions derived by plotting the first two principal components (Dimension 1 and 2)
(The arrows indicate the direction of different principal coordinates)

The scatter plot of the first two principal components obtained after subjecting the data to PCA is depicted in Figure 2. Principal components 1 and 2 accounted for 32% and 35% of the total variance, respectively. PCA clustered all the accessions into one major group with a few accessions from the breeders' collection (CS2H) and farmers' collection (ETG) diverging. The genetic association among populations was further analysed using Neighbour-Joining Cluster Analysis (Figure 3), and two major clusters were formed, one grouping the parental clones with the farmers' collection and the other the breeders' collection with a few parental clones.

Table 2 represents the SSR sequences with their repeat motifs, number of alleles detected per locus along with their fragment size range, observed and expected heterozygosity with polymorphic information content and F-statistic values. The mean number of alleles across all loci was 10.4 ± 0.167 with the highest number of alleles detected for locus mTcCIR25 (21 alleles) and the least for locus mTcCIR1 (6 alleles). Most loci showed a significant deviation from the Hardy-Weinberg equilibrium ($p < 0.05$), and had a deficit in heterozygotes ($H_E > H_O$) except for locus mTcCIR1. The Fst estimators of genetic differentiation, averaged over all loci, were 0.033 as estimated through jackknifing, with a confidence interval of 99% (Table 2). The results revealed a low level of differentiation among genotypes, and Fst values were almost equal to zero indicating that the populations are mostly identical in their allele frequencies.

The results from this pilot study clearly show that there is a very low allelic richness in the accessions under study, which is evident from the effective number of alleles recorded in each population. In general, it was apparent from the results and also from the collection survey that farmers are not extensively using improved material from the national research institutes, and they tend to use materials with a narrow genetic base. Results from this study confirm the findings of Aikpokpodion *et al.* (2003) that farmers generally use seeds from selected 'mother trees' for successive generations while establishing new farms or plantings, rather than using germplasm from research stations. These 'mother trees' were often derived from improved materials collected from research stations or the Government's Tree Crops Unit, which is responsible for distribution of improved seedlings to farmers. This may explain the clustering of a few parental clones with the farmers' collection, as shown in Figure 3.

The genetic characterisation of accessions from other eco-regions of Nigeria is currently in progress, and will be compared with the results of this study to estimate the overall genetic diversity present in the cocoa germplasm of Nigeria. Once the entire Nigerian cocoa collection is analysed, a more complete appreciation of the extent of genetic diversity will be obtained, and selection of potential parents and populations for use in future breeding programmes will be facilitated.

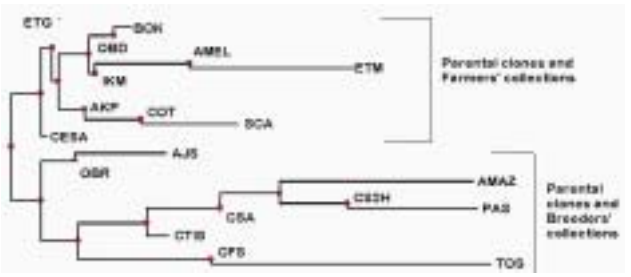


Figure 3: Neighbour-Joining (NJ) tree of 19 cocoa populations based on Nei (1978) genetic distances

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Screening and Evaluation of SSR Primers in Gel Systems for the Detection of Off-types in Cocoa Field Genebanks

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Abstract

The set of SSRs recommended by J. Saunders, formerly of USDA, for verification purposes, *i.e.* unique identification of accessions in field genebanks, is discussed. We suggest that twelve primer pairs be a minimum for verification studies using gel systems as done at CRU. The selection of SSRs to be used is suggested as an issue requiring further discussion. Based on our studies, the mTcCIR primers 1, 6, 8, 11, 12, 15, 29, 42, 58, and 61 are proposed as ideal candidates for use in such verification studies.

Introduction

Currently, SSRs (microsatellites or simple sequence repeats) are used widely among researchers in genomics for gene tagging, mapping, diversity studies and fingerprinting. SSRs are codominant markers obeying Mendelian inheritance, but are non-functional and not subjected to strong selection. These microsatellites are tandem repeats of bases flanked by conserved DNA sequences and occur frequently and randomly in all eukaryotic nuclear DNAs. SSR polymorphism is due to changes in the lengths of the repeat sequences (see Gupta and Varshney, 2000 and references therein). There are a number of different strategies (hybridisation or PCR based) to which SSRs lend themselves, and these are well reviewed by Gupta and Varshney (2000).

At CRU and presumably in other cocoa research laboratories, microsatellite-primed PCR (MP-PCR) is utilised. In this technique, two inversely oriented microsatellites (provided as synthetic oligonucleotides) are present within an amplifiable distance from each other, and upon provision of proper reactants, the inter-repeat sequence is amplified (Gupta *et al.*, 1994). SSRs will theoretically generate zero (null allele), one (homozygous) or two (heterozygous) bands for a given genotype. In the latter case, both loci generate products of distinct lengths. SSR alleles in cocoa reportedly differ in size by 2-10 bases (Wilkinson, 2001).

The detection of mislabelled plants (different genotype(s) within an accession plot) at CRU is currently achieved through the use of SSRs that unambiguously distinguish different trees. Reliance on morphological characteristics alone is at best tentative due to environmental variation and the unknown range of expression for any character for a particular accession. The unique nomenclature needed for misidentified germplasm should not rely solely on morphological characteristics since phenotypically similar material may be genetically different.

The use of SSRs as a tool for the identification of mislabelled cocoa accessions in field genebanks has been advocated by Wilkinson (2001) and Risterucci *et al.* (2001), among others. From a study of 20 genotypes assessed with 19 SSRs, Risterucci *et al.* (2001) suggested that 15 loci are sufficient for verification of identities. Both J. Saunders (formerly of USDA-ARS, Beltsville) and N. Cryer (University of Reading) (pers. comm.) have used 15 SSRs with the latter indicating that additional SSRs are utilised as required. However, Swanson *et al.* (2003) suggested that 11 SSRs may be sufficient for fingerprinting cocoa accessions.

The ongoing verification programme at CRU has highlighted the need to determine how many, and particularly, which SSRs are required to uniquely identify an accession.

How many SSRs are required to uniquely identify an accession?

Lelly and Stachel (1998) were able to distinguish bread wheat varieties using 1 SSR per chromosome arm (total 42). Senior *et al.* (1998) from a study with 70 SSRs were able to uniquely identify 94 elite maize inbred lines with as few as 5 SSRs. North American soybean cultivars reportedly have a limited genetic base due to the contribution of not more than 20 introductions and repeated use of related parents in breeding (Gizlice *et al.*, 1994). Nevertheless, thirty-five soybean genotypes accounting for 95% of alleles in North American soybean were distinguished by 20 SSRs (Diwan and Cregan, 1997). In another study, 11 closely related, visually indistinguishable barley cultivars were distinguished by 5 SSRs (William *et al.*, 1997). In cocoa, Charters and Willkinson (2000) used six SSRs and were able to distinguish all but three pairs of 62 accessions.

Sackville Hamilton *et al.* (2002) proposed that instead of asking if the accessions are identical, the question for genebank managers should rather be how similar should the accessions be before being considered identical? The critical question may be recast as: "What level of similarity should be utilised as a cut-off point to demarcate distinct accessions? –

Should we utilise a 95%, 99%, 99.9%, 99.99% or 99.999% level? The FBI laboratory routinely types 13 short tandem repeat loci (STR), which are estimated to generate a random match probability for unrelated persons of less than 1 in a trillion (Budowle *et al.*, 2000). Balding (1999) recently suggested that 11 human STR loci would be sufficient to assert uniqueness at a 99.9% confidence level. D. Zhang (USDA) has indicated that the use of 15 SSRs will achieve a probability of identification of 1 in 10,000 (pers. comm.), *i.e.* a confidence level of 99.99%. These values were based on data obtained on a sequencer for cocoa samples representing various genetic backgrounds, assuming full-siblings as the closest relationship. Fifteen SSRs are therefore considered quite sufficient for the determination of off-types in cocoa.

Which SSRs do we choose?

Information from N. Cryer (e-mail of October 11th 2002) indicated that both USDA-ARS and the University of Reading cocoa research laboratories are using the same set of primers. The properties of these SSR primers are provided in Table 1. At CRU, Motilal (2003) has demonstrated that several of these primers, when assayed in gel systems, either have no amplification (mTcCIR 26), weak amplification (mTcCIR 7, 18 & 24) or have several multiple bands (mTcCIR 1, 7, 15, 22, 24 & 33). Swanson *et al.* (2003) provided results where more than two alleles were scored for the same genotype for particular primers on a sequencer system. In addition, these authors reported poor amplification for four primers, one of which was mTcCIR33. The other three are usable at CRU. Multiple bands may be attributed in part to primer binding at DNA regions other than the cloned site from which the SSR was developed. The existence of multiple bands (excluding stutter bands) for several SSR primers may therefore not be restricted to a gel-based system. There are instances, however, when extraneous bands are produced which are consistently reproducible. These may be used as additional bands for scoring. William *et al.* (1997) have utilised an extraneous SSR band to differentiate two closely related barley cultivars.

Given a particular primer pair and the same DNA, we would expect that, irrespective of the detection system used, the same information profile would be obtained provided that the amount and size of products were sufficiently resolvable. Grivet and Noyer (2003) state that the length of the cocoa microsatellite primers (20 to 25 nucleotides) dictates an infinitely low probability of randomly amplifying another sequence in the genome. If so, then differences among laboratories should not be attributed solely to the detection system. The reaction conditions must also be critically examined. The concentration of reactants in the amplification mix (especially template DNA, Mg^{2+} and SSR primers) may prove different among laboratories. Work at CRU has also demonstrated that increasing the annealing temperature to 51°C for several primers, which were supposed to be amplified at 46°C, reduced the existence of multiple bands, as did the use of a touchdown amplification program.

We are concerned that coverage of chromosome 5 is completely lacking with the suggested set of 15 SSRs (Table 1). This may be inconsistent with the desired end-result of detecting off-types. In a study of 105 bread wheat varieties, closely related varieties could be discriminated using selected microsatellites located on different chromosomes (Manifesto *et al.*, 2001). Similarly, 42 SSRs, one for each of the 42 chromosome arms of the wheat genome were found to be effective for distinguishing varieties (Lelley and Stachel, 1998 in Gupta and Varshney, 2000). Risterucci *et al.* (2001) also indicated that for verification of identities in cocoa germplasm there should be a choice of SSR loci on different chromosomes. There is then supporting evidence in the literature for the use of one SSR primer pair per chromosome. Each of the ten chromosomes of cocoa may therefore be represented by at least one SSR for identification purposes. Saturation of a chromosome by SSRs in order to detect crossover events may, however, be more suitable in cases where trees are more homozygous or are suspected of being closely related. Hence, a given set of markers for verification purposes should not be restricted to 10 SSRs.

Forty-six additional primers were shown to be reproducible when used in gel-based systems at CRU (Motilal, 2003). Several accession plots within the ICG,T were shown to have off-types in the verification programme. Trees from these plots were compared with a maximum of 35 primers. Several SSRs, not included in Saunders' list, proved quite valuable in detecting off-types (Table 2). Details of these primers are provided in Table 3. The data in Table 2 indicate that not all SSRs will detect differences amongst the same set of accessions. Interestingly, the percentage success of the additional primers as a set was similar to that of Saunders' set, except in the case of ICS 30 (Table 4). It seems that an off-type may be widely separated by utilising particular SSRs, which would vary from accession to accession. However, these SSRs would not be known before the analysis. This difficulty may be overcome by selecting SSRs most likely to detect off-types. This can only be achieved using information provided by a larger data set. However, the use of the set of 12 primers recommended in this study resulted in comparable or better detection of off-types relative to the set circulated by J. Saunders.

Manifesto *et al.* (2001) selected SSR primers for identification purposes in bread wheat cultivars based

on (i) high Polymorphism Index Content (PIC), (ii) repeatability and clarity of the banding pattern, (iii) absence of close linkage to any other locus and (iv) ability to separate cultivars not differentiated by other SSR primers. Applying these principles to the detection of off-types on a given electrophoretic system for material from a field genebank, we would obviously have to utilise the primers which are scorable for that system. Furthermore, it is rational to utilise primers which are more likely to detect differences. Since we are dealing with a field genebank, there is no guarantee that we know the type of identification errors associated with the establishment of any particular plot. Thus, the primers utilised should be as versatile as possible in order to detect as much variation as possible. Currently, we cannot determine the absolute minimum number of primers that should be used. However, a minimum set of primers that can generate scientifically acceptable results is a reasonable starting point. Based on information in the literature, it would seem that at least ten primers, one per chromosome, are required. The primers mTcCIR 1, 6, 8, 11, 12, 15, 29, 42, 58, and 61 appear promising in this regard. These SSR loci would cover all linkage groups except 3 and 7 in the cocoa genome. Two additional primers (e.g. mTcCIR 49 and 56) may be selected to comprise a final set of 12, which covers all linkage groups and which would be more versatile in detecting off-types, in addition to providing an expectedly higher level of confidence.

Additional primers may be utilised if other observations *e.g.* of morphological traits or disease resistance suggest that variation is present in material providing similar SSR profiles. The protocol to be used would be dependent on the number of trees involved. If there are only two trees to be differentiated, then gross differences in morphology may be used to supplement the molecular data. When two or more trees exhibit similar phenotypes and are genotypically equivalent based on available data, the use of additional primers is recommended. It may be that, like Manifesto *et al.* (2001), SSR primers with a low PIC but with the ability to differentiate cultivars need to be included alongside those with high PIC but with inability to differentiate the same cultivars.

Conclusion

The cocoa genome is relatively small (approximately twice that of the *Arabiodopsis* genome according to Crouzillat *et al.*, 1996), and reportedly has little repetitive DNA (Wilde *et al.,* 1992), with many of the cocoa groups having a narrow genetic base. Lanaud *et al.* (1999) stated that SSRs were screened on several cocoa genotypes but the actual numbers or diversity of these plants were not indicated. This may account for the low number of alleles detected with some SSRs. Nevertheless, useful polymorphisms could still be detected. Furthermore, the existence of repeatable extraneous bands can increase the discriminating power from SSR-PCR. However, these should be used cautiously and only upon rigorous confirmation that they are usable products.

We recommend that SSRs that span the ***entire*** genome be utilised, but that the primers mTcCIR 22, 24 and 26 be discarded and replaced by others taken from Table 3 for use in gel-based detection systems. A confidence level of at least 99.99% is also suggested as the target in verification work. The number and types of SSRs used will determine the number of detectable variants. The choice of primers is therefore a critical issue. A set of 12 may be sufficient to detect differences for verification purposes. Statements concerning the number of SSRs used should probably be qualified thus: "n individuals were found to be equivalent from the profiles of x SSRs" where n and x are the numbers involved. The identity of the SSRs should also be given. We suggest to the cocoa community that the primers mTcCIR 1, 6, 8, 11, 12, 15, 29, 42, 58, and 61, in addition to two more primers (mTcCIR 49 and 56) covering linkage groups 3 and 7, be utilised for the detection of off-types in gel-based systems.

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Table 1: Properties of cocoa SSR primers recommended by J. Saunders (formerly USDA-ARS)

SSR primers			Chrom. no.	Ann. Temp. (°C)	Motif	Allele size (bp) range & (average size)
AGTC	mTcCIR	EMBL				
7/8	1	Y16883	8	52	$(CT)_{14}$	133-149 (143)
27/28	6	Y16980	6	47	$(TG)_7(GA)_{13}$	226-253 (231)
33/34	7	Y16981	7	50	$(GA)_{11}$	153-167 (160)
35/36	8	Y16982	9	47	$(TC)_5TT(TC)_{17}TTT(CT)_4$	274-309 (301)
43/44	11	Y16985	2	46	$(TC)_{13}$	293-321 (298)
45/46	12	Y16986	4	46	$(CATA)_4N_{18}(TG)_6$	168-258 (188)
53/54B	15	Y16988	1	49	(TC)19	219-265 (254)
64/65	18	Y16991	4	52	$(GC)_{12}$	335-359 (345)
82/83	22	Y16995	1	49	$(TC)_{12}N_{146}(CT)_{10}$	278-296 (289)
86/87	24	Y16996	9	50	$(AG)_{13}$	190-208 (198)
92/93	26	Y16998	8	46	$(TC)_9C(CT)_4TT(CT)_{11}$	280-303 (298)
118/119	33	AJ271826	4	51	$(TG)_{11}$	268-349 (285)
132/133	37	AJ271942	10	46	$(GT)_{15}$	139-190 (150)
148/149	40	AJ271943	3	51	$(AC)_{15}$	247-287 (286)
250/251	60	AJ271958	2	51	$(CT)_7(CA)_{20}$	188-214 (207)

Table 2: SSR primers and their ability to detect off-types in selected accessions

SSR	EQX 3161	ICS 5	ICS 30	ICS 40	ICS 73	ICS 95	PA 120	PA 150	SCA 11	ELP 22 vs. ELP 35
7/8	**Y**	**–**	**Y**	**Y**	**N**	**N**	**N**	**Y**	**Y**	**N**
13/14	–	–	N	–	–	–	N	Y	–	N
21/22	N	–	N	–	–	–	–	–	–	Y
27/28	**–**	**N**	**Y**	**Y**	**Y**	**N**	**Y**	**Y**	**N**	**Y**
33/34	**Y**	**N**	**Y**	**N**	**N**	**N**	**–**	**–**	**–**	**–**
35/36	**Y**	**N**	**N**	**N**	**Y**	**Y**	**N**	**Y**	**–**	**Y**
39/40	N	N	N	–	N	N	Y	N	–	N
43/44	**–**	**Y**	**Y**	**–**	**Y**	**N**	**N**	**Y**	**–**	**Y**
45/46	**–**	**Y**	**Y**	**–**	**Y**	**Y**	**Y**	**–**	**Y**	**N**
53/54B	**–**	**N**	**N**	**Y**	**Y**	**N**	**Y**	**Y**	**Y**	**Y**
62/63	–	–	–	–	–	–	–	–	–	Y
64/65	**–**	**N**	**–**	**–**	**N**	**Y**	**–**	**–**	**–**	**–**
104/105	–	Y	Y	–	Y	Y	Y	Y	–	Y
112/113	Y	N	Y	–	Y	Y	N	–	–	Y
114/115	–	–	N	–	–	–	–	N	–	–
116/117	–	–	Y	–	–	–	–	–	–	–
118/119	**Y**	**–**	**Y**	**–**	**N**	**–**	**–**	**Y**	**–**	**–**
122/123	Y	–	–	–	–	–	–	Y	–	–
132/133	**–**	**N**	**–**	**–**	**?**	**Y**	**Y**	**–**	**–**	**Y**
136/137	–	–	Y	–	–	–	–	Y	–	–
148/149	**–**	**Y**	**–**	**–**	**N**	**N**	**–**	**Y**	**–**	**–**
150/151	–	–	Y	–	–	–	–	Y	–	–
164/165	Y	Y	Y	Y	N	N	Y	Y	–	Y
168/169	Y	N	Y	–	–	–	–	Y	–	–
176/177	–	–	Y	–	N	–	Y	Y	–	N
180/181	–	N	–	–	–	–	–	–	–	Y
192/193	–	Y	Y	Y	–	–	N	–	–	N
202/203	–	–	Y	–	–	–	–	Y	–	–
214/215	–	–	N	–	–	–	–	–	–	–
224/225	–	N	Y	Y	–	–	–	–	–	Y
226/227	–	N	Y	N	Y	–	Y	–	–	N
238/239	–	Y	N	N	Y	N	N	Y	–	N
240/241	N	N	N	Y	Y	Y	Y	Y	Y	Y
250/251	**–**	**–**	**Y**	**–**	**N**	**–**	**N**	**–**	**–**	**N**
262/263	–	N	Y	Y	Y	–	Y	Y	–	Y

Y - Difference(s) detected; N - no difference detected; ? - questionable profile; dash indicates that PCR was not performed. Bold entries are selected from SSR primers recommended by J. Saunders, and which work well at CRU.

Table 3: SSR primers, other than those suggested in Table 1, which detect off-types

SSR primers			Chrom. no.	Ann. Temp. (°C)	Motif	Average size of product (bp)
AGTC	**mTcCIR**	**EMBL**				
13/14	2	Y16978	5	51	$(GA)_3N_5(AG)_2CG(AG)_4$	(254)
21/22	3	Y16977	2	46	$(CT)_{20}(TA)_{21}$	(249)
39/40	10	Y16984	5	46	$(TG)_{13}$	(208)
62/63	17	Y16990	4	51	$(GT)_7N_4(GA)_{12}$	(271)
104/105	29	AJ271822	1	46	$(CA)_{10}$	(172)
112/113	30	AJ271823	9	46	$(CA)_{11}$	(182)
116/117	32	AJ271825	4	46	$(CA)_{10}$	(198)
122/123	34		10	46	$(GT)_{16}$	(256)
136/137	38		10	51	$(GT)_{20}$	(186)
150/151	41		10	46	$(GT)_{18}$	(138)
164/165	42	AJ271944	5	46	$(CA)_{21}$	(232)
168/169	43	AJ271945	4	46	$(TG)_5(TA)(GA)_{15}$	(206)
176/177	44	AJ271946	2	51	$(GT)_{10}$	(178)
180/181	45	AJ271947	8	51	$(GT)_9$	(284)
192/193	49	AJ271951	3	46	$(TG)_8$	(197)
202/203	51		5	46	$(GT)_{30}$	(216)
224/225	55	AJ271954	7	46	$(CA)_6(GCACAC)_5$	(234)
226/227	56	AJ271955	7	46	$(TC)_{14}N(TG)_{15}$	(354)
238/239	57	AJ271956	4	46	$(AC)_{13}$	(253)
240/241	58	AJ271957	9	51	$(GT)_{40}$	(266)
262/263	61	AJ271959	10	46	$(CA)_{18}$	(199)

Table 4: Comparison of recommended and additional SSRs for successful detection of off-types

Accession	Saunders' Set			Additional SSR primers			Recommended Set*		
	# Used	# Detected	%	# Used	# Detected	%	# Used	# Detected	%
ICS 5	9	3	33	12	4	33	11	4	36
ICS 30	9	7	78	21	12	57	12	9	75
ICS 40	5	3	60	7	5	71	9	7	78
ICS 73	12	6	50	9	6	66	11	9	82
ICS 95	9	4	44	6	3	50	9	4	44
PA 120	8	4	50	11	7	63	12	8	67
PA 150	7	7	100	14	12	86	9	9	100
ELP 22 vs. ELP 35	6	5	62	15	9	60	12	8	67

*Recommended set of 12 SSR primers: mTcCIR 1, 6, 8, 11, 12, 15, 29, 42, 56, 58 and 61.

Understanding the *Theobroma cacao-Crinipellis perniciosa* Interaction using ESTs and Proteomic Analyses

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Introduction

Crinipellis perniciosa (Stahel) Singer is a basidiomycete that causes Witches' Broom disease in members of the families Sterculiaceae, Solanaceae, and Bixaceae (Andebrhan *et al.*, 1999). The economic impact of the pathogen is mainly on *Theobroma cacao* L. Basidiospores infect meristematic tissues (shoots, flower cushions, single flowers, and developing fruits), inducing a range of symptoms depending on the organ infected and stage of development (Purdy and Smith, 1996). Hypertrophic growth of infected buds ('brooms') is the most dramatic symptom. Flower cushion infection usually leads to the production of vegetative shoots and abnormal flower development. Pod infections can directly result in seed losses approaching 90% of the potential annual production in some areas and seasons (Andebrhan, 1985). Pod set is further reduced indirectly by the infection of flower cushions and the general debilitation of the tree. The life cycle of the pathogen is completed by the production of basidiocarps on necrotic brooms and dried pods, which release basidiospores, the unique source of inoculum (Purdy and Smith, 1996).

Since 1989, Witches' Broom disease (WBD) has spread throughout Brazil, destroying cocoa plantations, and leading to important economical and social changes in affected areas such as the State of Bahia (Pereira *et al.*, 1989; Rocha *et al.*, 1993; Dos Santos Filho *et al.*, 1998). In order to safeguard cocoa plantations, numerous efforts have been made such as the development of new cocoa varieties, use of resistant clonal planting material or biological control of the disease (Rudgard *et al.*, 1993). Although these technical procedures have been efficient, they do not represent an adequate method of control for WBD. The high variability in the fungus, associated with several possible genetic recombinations in a short period of time, could break the resistance of cocoa (Wheeler *et al.*, 1988; Anderbrhan and Furtek, 1994) as observed for some hybrids derived from the resistant parent, SCA 6 (Rios-Ruiz *et al.*, 2001). For many pathogenic systems in plants, the study of gene interaction is a suitable approach to discover plant defense mechanisms (Bent,1996; Fluhr, 2001; Hammond-Kosack and Jones, 1996). The identification of differentially expressed genes between cocoa trees susceptible and resistant to Witches' Broom disease is essential to understand biological events of the cocoa x *Crinipellis* interaction.

The aim of the research developed in this laboratory is to acquire sound knowledge of the determinism of the interaction between the cocoa tree and the pathogen, C. *perniciosa*, based on functional genomic studies. One of the challenges associated with conducting this research is the isolation of intact nucleic acids, especially RNA. This is particularly difficult due to the high content of polyphenols and polysaccharides in the different tissues, which co-precipitate with nucleic acids upon isolation (Whistley *et al.*, 1956; Blakemore *et al.*, 1966; Figueira, 1994; Redgwell and Hansen, 2000). Moreover, the quantity of polysaccharides is higher in some organs such as meristems or fruits — which are unfortunately the organs infected by *Crinipellis* — than in leaves or seeds.

The quality of the isolated RNA we obtained was consistently high, and was used for subsequent applications such as cDNA library construction and functional genes studies. In this article, we report the first results concerning the sequencing of ESTs from the resistant interaction between *Theobroma cacao* and *C. perniciosa*. We also present an overview of the approaches developed in the Genetic and Bioinformatic Laboratories of the UESC to increase our understanding of the molecular basis of the cocoa-*Crinipellis* interaction.

Material and methods

Plant and Fungus material

Cocoa trees susceptible (Catongo) and resistant (TSH 1188) to WBD were cultivated in the greenhouse of the Cocoa Research Centre (CEPEC/CEPLAC) (Figure 1A), and spray inoculated (Figure 1B) by a pooled sample of *Crinipellis* spores (to represent the fungus variability). The meristems of non-inoculated control plants and inoculated plants were harvested from time zero up to 70 days after inoculation (necrosis symptoms/ presence of a dry broom). The fungal mycelia were grown in Petri dishes containing culture medium. Plugs of the material were harvested over a period of time, the RNA extracted, pooled and used for the construction of a cDNA library.

Figure 1: Cultivation and inoculation of plants at CEPEC. **A:** *Theobroma cacao* resistant (TSH1188) and susceptible (Catongo) to Witches' Broom disease were cultivated in the greenhouse of the CEPEC. **B:** Spray inoculation of the plants. **C + D:** *Crinipellis perniciosa* growing in artificial systems simulating the life cycle

RNA extraction from cocoa tissues with a new protocol using tert-Butanol

Several protocols exist for isolating RNA from recalcitrant plants (Schneiderbauer *et al.*, 1991; Schultz *et al.*, 1994; Burgos *et al.*, 1995; Chi-Manzanero *et al.*, 2000; Suzuki *et al.*, 2001) including those optimised for woody plants (Wang *et al.*, 2000), and only few recent studies concern the isolation of cocoa RNA (Kochhar *et al.*, 2001; Jones *et al.*, 2002; Laloi *et al.*, 2002). However, these methods use either a CsCl gradient and ultracentrifugation or result in a low RNA yield. The isolation of RNA from cocoa was first attempted in our laboratory using classical methods of extraction and several commercially available kits, without success. Then we developed a successful and reliable procedure for the isolation of RNA from cocoa organs infected and uninfected by C. *perniciosa* such as leaves, meristems and fruits (Gesteira *et al.*, 2003). This new protocol uses tert-Butanol and directly overcomes the problems associated with polyphenol and polysaccharide contaminations (Figure 2). Moreover, it uses a non-expensive method without ultracentrifugation, which could be easily applied in any laboratory. The quality of the isolated RNA thus obtained was consistently high, as judged by spectrophotometric analysis and by its separation on an agarose gel, and could be used for subsequent applications such as RT-PCR and cDNA library construction.

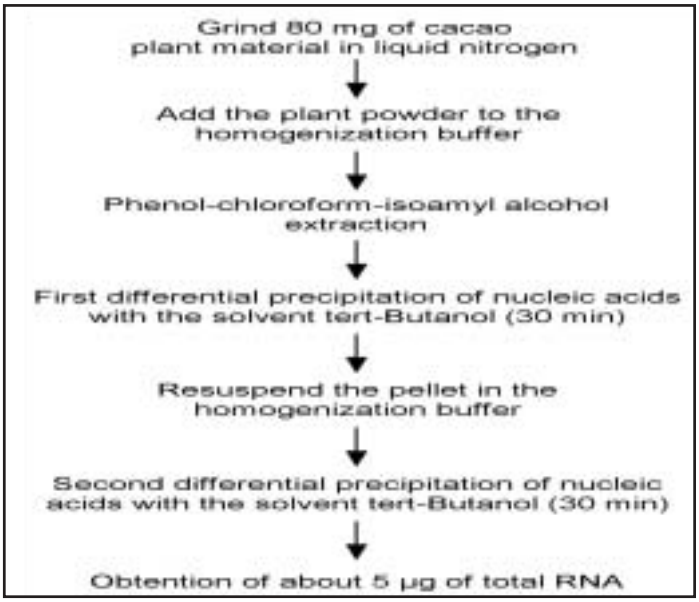

Figure 2: Purification steps for the isolation of total RNA from cocoa tissues (from Gesteira *et al.*, 2003)

cDNA library construction, EST sequencing and bioinformatic analysis of the sequences

Three cDNA libraries corresponding to resistant and susceptible interactions between *Theobroma cacao* and *C. perniciosa*, and the fungal life cycle were constructed from total RNA using the DB SMART Creator cDNA library kit as described by the manufacturer (Clontech). The cDNAs were cloned in the pDNR-LIB plasmid and the EST sequencing was performed on a Megabace apparatus in the Genetic

laboratory of the UESC. The resulting ESTs have been analysed in the Bioinformatic Laboratory of the UESC using the following methods: determination of the GC percentage of each sequence, codon usage, automatic blast against GenBank and *C. perniciosa* genome databases (Genome Project, http://bioinfo01.ibi.unicamp.br/vassoura/). These methods allowed us to distinguish cocoa sequences from those of *Crinipellis*.

Extraction of RNA from cocoa tissues

RNA extraction was successfully carried out using the new tert-Butanol method as described by Gesteira *et al.* (2003). Non-degraded total RNA was obtained using the newly developed method. Intact bands of 28S, 18S and 5S rRNAs were detected on DEPC-treated 1% agarose gel in all RNA fractions from the different organs of cocoa infected by *Crinipellis*, such as meristem and fruit, or uninfected, such as leaves and fruit. Unlike seeds, which are usually used in molecular biology studies of cocoa (Kochhar *et al.*, 2001; Jones *et al.*, 2002; Laloi *et al.*, 2002), these organs – in particular the meristem and fruit – contain high amounts of polysaccharides that could not be removed by conventional extraction procedures. The tert-Butanol method also enabled RNA extraction from almost dry brooms, which contained only a few intact cells (Gesteira *et al.*, 2003).

The quality of the RNA obtained from cocoa tissues was good as assayed by the A260/A280 ratio, which is approximately 1.85. Further assessment of RNA quality was obtained by visualising the major ribosomal RNAs following their separation on DEPC-treated 1% agarose gel. No degradation of the RNA was observed on these gels. Total RNA is obtained with a very good yield; about 5 μg for 80 mg of ground plant material used initially, i.e. about 60 ng of total RNA per mg of cocoa tissue (Figure 3). This protocol could be adapted for RNA extraction using greater amounts of cocoa plant material, and requires small quantities of reagents, uses less expensive consumables and could be easily applied in any laboratory. Furthermore, studies of the kinetics of the interaction of cocoa x *Crinipellis* (up to 70 days to obtain dry brooms) show that a fast isolation procedure is required that can be carried out in microcentrifuge tubes to prepare and analyse RNA from many samples simultaneously.

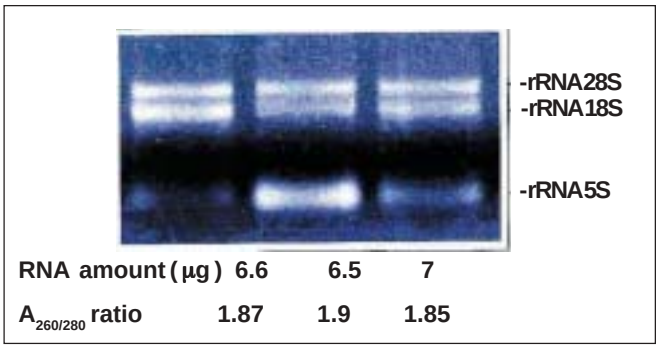

Figure 3: Total RNA was isolated from 80 mg of cocoa leaves using the tert-Butanol method. The quantity and quality of RNA were assayed by A_{260} and $A_{260/280}$ readings, respectively

cDNA library construction and EST sequencing

We generated two cDNA libraries corresponding to resistant (TSH 1188) and susceptible (Catongo) cocoa trees inoculated by a pooled sample of C. *perniciosa* spores. The sequencing of 5,000 ESTs from each cDNA library is in progress in our laboratory. The first ESTs sequenced have been compared to the public gene databases (GenBank), to *Crinipellis* genome sequences and to cocoa ESTs already published (Jones *et al.*, 2002). The preliminary results of the sequencing of the TSH 1188 ESTs are shown in Figure 4.

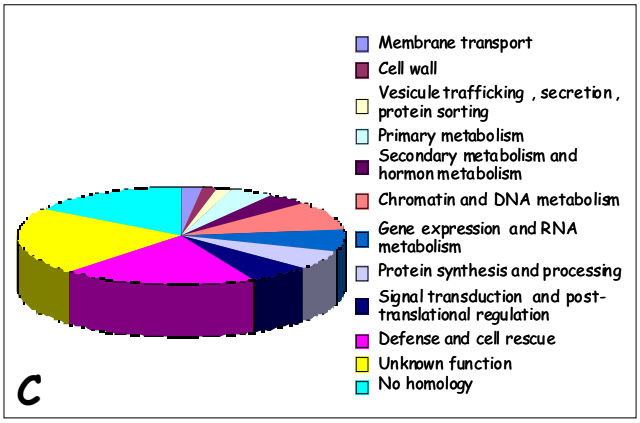

Figure 4: Preliminary results of the sequencing of the EST corresponding to the resistant (TSH 1188) cDNA library. Each gene category is indicated as a percentage of the total number of sequences

Fungal and cocoa sequences could also be discriminated according to their respective GC base percentage using a new informatics tool developed in the LABBI. This algorithm will be powerful enough to distinguish cocoa genes from those of *Crinipellis* before the end of the complete *Crinipellis* genome sequencing project (Genome Project, http:// bioinfo01.ibi.unicamp.br/vassoura/).

Proteomics

We are using proteomics to analyse secreted proteins from both the fungus and plant. This involves extracting apoplastic fluid proteins from infected and control plants, and also from culture medium where we grow C. *perniciosa* with different inductions (plant extracts, hormones, different media) (Figure 5). The antibodies raised against these proteins are currently being used to screen the cDNA libraries, and the differentially expressed ones will be identified by MALDI-TOF analyses at the UNICAMP Proteomics Laboratory (Campinas, Brazil). We expect to find genes involved either with resistance in *T. cacao* or with the virulence in *C. perniciosa*. In parallel, we are analysing the *C. perniciosa* sequences deposited at the genome databank in order to identify genes encoding for secreting proteins. We believe that the combined use of the genomics and proteomics approaches will accelerate elucidation of the molecular and biochemical bases of the *T. cacao-C. perniciosa* interactions. This work is part of two PhD programmes developed at UESC and UNICAMP.

Conclusions

Development of a new protocol of RNA extraction in cocoa

Using the tert-Butanol RNA extraction protocol, we succeeded, for the first time, in isolating RNA from cocoa organs infected by *C. perniciosa*. These results have led to new perspectives regarding the understanding, through genomic studies, of the cellular mechanisms of the infection of the cocoa tree by destructive pathogens such as *Crinipellis* or *Phytophthora* spp.

cDNA libraries of the cocoa-Crinipellis interaction and CP Life cycle

The collection of ESTs we obtained will provide new data about the physiology of the interaction between *Theobroma cacao* and *C. perniciosa*. In the short term, we plan to do a functional analysis of some candidate genes, which can also be used for mapping, to facilitate introgression strategies and to create new varieties resistant to *C. perniciosa*. These data may also be related to research on gene expression of cocoa challenged with other pathogens, such as *Phytophthora* spp.

All the approaches developed in the Genomic and Bioinformatic Laboratories could be summarised as described in Figure 6, and lead to new perspectives in understanding the *Theobroma cacao-C. perniciosa* interaction.

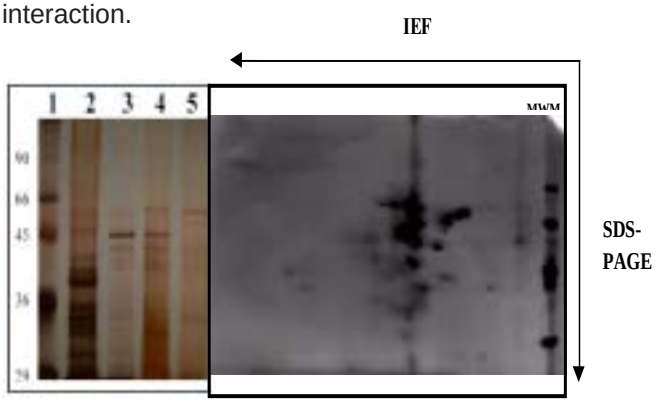

Figure 5: Secreted proteins of apoplastic fluid (AF): MWM (kKDa) (lane 1), total leaf protein (lane 2), healthy (AF) secreted proteins (lane 3) and infected (AF) secreted proteins (lanes 4, 5). There is a clear induction of different proteins in the infected tissues as shown in lanes 4 and 5, compared with lane 3. **Right**. Two-dimensional (pH interval 10–3) profile of *Crinipellis perniciosa* secreted proteins showing the diversity of proteins that were secreted into the medium

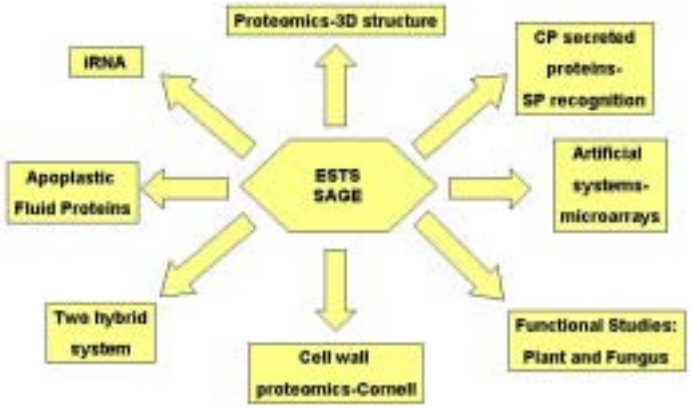

Figure 6: Proposed pipeline of the interaction in the genomic programme at UESC

Acknowledgements

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Imports and Availability of Accessions from the University of Reading Intermediate Cocoa Quarantine Facility

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Introduction

The University of Reading Intermediate Cocoa Quarantine Facility is the main resource for the international movement of cocoa germplasm. Material entering the facility undergoes a two-year quarantine period in accordance with international guidelines, during which it is virus indexed and inspected for fungal pathogens. The facility currently holds 450 accessions of which 350 have completed quarantine and are held in the collection for budwood export. The current focus for bringing in new accessions, and accessions that have been released over the past 12 months are described here.

Focus for imports of accessions into the facility

The primary focus for accessions entering the facility has been a priority list of clones selected during the CFC/ICCO/IPRGI project on "Cocoa Germplasm Utilisation and Conservation". To date the facility has 66 of these accessions, of which 30 are available for budwood export (Table 1). The quarantine and distribution of accessions on this list is a stated activity in the new CFC/ICCO/IPGRI project on "Cocoa Productivity and Quality Improvement: a Participatory Approach" and consequently we are working closely with CRU, Trinidad and other germplasm collections to import the remainder of these.

The second focus for germplasm imports is material with reported disease-resistant characteristics. Examples of recently imported accessions include two clones with resistance to *Moniliophthora* received from CATIE, Costa Rica (off-types mislabelled as "ICS 43" and "ICS 95"). We have also recently received clones from the Ucayali collection from *Universidad Nacional Agraria de la Selva*, Peru that have reported resistance to Witches' Broom disease.

Release of the material from quarantine

During the past twelve months, 19 clones have been released from quarantine and are now available for budwood export (Table 2). The full list of available accessions can be found at the following website: http://www.icgd.rdg.ac.uk/quarantine.htm.

Procedure for receipt of budwood

In order for institutions to receive budwood from the facility, we require a valid import permit at least two weeks before the date of export. This should be sent to the following address: School of Plant Sciences, The University of Reading, Whiteknights, Reading, RG6 6AS, UK or faxed to 00 44 118 9318160 or 00 44 118 988 7468. We are always interested in receiving feedback from germplasm recipients on the grafting success rate of material received from Reading.

Table 1: Accessions from the CFC/ICCO/IPRGI Collection held at Reading (where an accession is not currently available, the year of release from quarantine is given)

Available Accessions		**Accessions passing through quarantine**		
Accession	**Donor Genebank**	**Accession**	**Donor Genebank**	**Year Available**
AMAZ 15	ICG, T	NA 916	ICG, T	2005
AMAZ 5/2	ICG, T	SCA 10	ICG, T	2005
B 5/7	ICG, T	SPEC 41/6-18	ICG, T	2005
CL 19/10	ICG, T	AM 1/57	ICG, T	2006
COCA 3370/5	ICG, T	B 13/5	ICG, T	2006
EET 399	ICG, T	CC 71	ICG, T	2006
EET 59	ICG, T	CCN 51	INIAP	2006
GU 133/C	CIRAD	CRU 111	ICG, T	2006
GU 175/P	ICG, T	CRU 80	ICG, T	2006
IMC 20	ICG, T	CRU 89	ICG, T	2006
IMC 47	CIRAD	SILECIA 5	INIAP	2006
LCT EEN 37/1	Miami	FSC 13	ICG, T	2006
LCT EEN 162/S1010	ICG, T	GU 241/P	ICG, T	2006
LCT EEN 241	Miami	GU 261/P	ICG, T	2006
LCT EEN 302	ICG, T	ICS 35	ICG, T	2006
LCT EEN 46	Kew	ICS 55	ICG, T	2006
LCT EEN 62/S4	ICG, T	ICS 83	ICG, T	2006
LCT EEN 163/A	Miami	ICS 95	ICG, T	2006
MA 12	CATIE	IMC 31	ICG, T	2006
MAN 15/2	ICG, T	IMC 94	ICG, T	2006
MO 20	ICG, T	LCT EEN 212/S4	ICG, T	2006
NA 33	CIRAD	LCT EEN 261/S4	ICG, T	2006
PA 120	CIRAD	LP 3/4	ICG, T	2006
PA 169	ICG, T	LP 3/15	ICG, T	2006
PA 70	Kew	LX 43	ICG, T	2006
POUND 7/B	Kew	MATINA 1/7	ICG, T	2006
SC 1	Kew	MO 9	ICG, T	2006
SCA 24	ICG, T	MOQ 6/95	ICG, T	2006
SCA 9	ICG, T	NA 807	ICG, T	2006
PA 279	ICG, T	PA 121	ICG, T	2006
		PA 303	ICG, T	2006
		SJ 1/40	ICG, T	2006
		TRD 109	ICG, T	2006
		TRD 45	ICG, T	2006
		TRD 85	ICG, T	2006
		UF 613	ICG, T	2006

Table 2: Accessions released from quarantine during the past 12 months that are now available for export

Accession	Reading Code	Donor Genebank
B 7/14	RUQ 1082	ICG, T
F303	RUQ 1028	ICA, Palmira
GU 269/V	RUQ 1072	CIRAD
ICS 39	RUQ 1089	ICG, T
ICS 95	RUQ 1144	CIRAD
IMC 57	RUQ 1055	ICG, T
IMC 67	RUQ 1056	ICG, T
JA 5/24	RUQ 1124	ICG, T
NA 387	RUQ 1057	ICG, T
NA 804	RUQ 1116	ICG, T
PA 136	RUQ 1131	ICG, T
PA 137	RUQ 1081	ICG, T
PA 169	RUQ 1117	ICG, T
PA 279	RUQ 1119	ICG, T
PA 291	RUQ 1132	ICG, T
POR 3	RUQ 1062	ICG, T
SCA 9	RUQ 1064	ICG, T
SLA 16	RUQ 1092	ICG, T
VB 547	RUQ 1035	CEPLAC

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Frequency of Off-type Progeny among the Original ICS 1 x SCA 6 Reciprocal Families and Parental Clones used for Disease Resistance Selection in Trinidad

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2 CABI/CATIE/USDA/-ARS, based at the Cocoa Research Unit, The University of the West Indies, St. Augustine, Trinidad and Tobago, at the time of this study.
3 Masterfoods, c/o USDA, ARS, SHRS, 13601 Old Cutler Rd., Miami, FL 33158.

Abstract

The genetic integrity of a reciprocal full-sib family of *Theobroma cacao* L. was investigated using microsatellite markers. Of the 186 putative full-sibs analysed, 27.9 % of the trees were off-types based on 33 microsatellite loci. Significant differences were observed between the frequencies of off-types in the reciprocal families. In the SCA 6 x ICS 1 family, off-types accounted for 14.0% of the seedlings. Among these, two were maternal half-sibs (MHS) that most likely arose from pollen contamination during pollination, while 13 individuals were completely unrelated. The ICS 1 x SCA 6 family was more problematic with 46.8% of the seedlings classified as off-types. Of these, six were selfs of ICS 1, 13 were MHS, and 18 were unrelated. The six selfs and 13 MHS most likely arose from pollen contamination. The differences in frequency of off-type seedlings (maternal half-sibs and selfs), between the two families is striking, with only 2 of 94 (2.1%) for SCA 6 x ICS 1 vs. 19 of 61 (31.1%) for ICS 1 x SCA 6. Fifteen clones, known to have been used in breeding during the same time period, were evaluated to determine whether they were inadvertently used as parents and if a mistake in labelling occurred during pollination. It was not possible to identify any additional parents with certainty from the analysis. The results highlight the importance of strict control of pollinations and validating the identity of parental trees before performing genetic studies in cocoa.

Introduction

Witches' Broom disease, caused by *Crinipellis perniciosa* (Stahel), in cocoa has been the impetus for

much of the research on this crop in the New World. Discovered in Suriname in 1895, Witches' Broom was first reported in the Manzanilla district on the eastern coast of Trinidad in 1928. The Trinidad Department of Agriculture (currently the Ministry of Agriculture, Land & Marine Resources) funded expeditions to find Witches' Broom resistant genotypes after all methods of control failed. The first was by Mr. F. Stell in 1933 to Ecuador, followed by Dr. F. J. Pound, which culminated in Pound's expeditions to the head waters of the Amazon in 1937-38 (Pound, 1943). Seeds collected during these expeditions were quarantined in Barbados. Budwood of each seedling was sent, when it became available, to the St. Augustine Nurseries in Trinidad for grafting onto rootstock. Thus the original seedling in Barbados became a clone in Trinidad and was planted at the Marper Farm in Manzanilla for observations on Witches' Broom disease reaction (Pound, 1943). Approximately 2,500 clones were established at the Marper Farm during 1939 and 1940. By 1957, only six accessions, all derived from a single Scavina parent, were characterised as resistant (Montserin and de Verteuil, 1957). Two of these clones, SCA 6 and SCA 12, were high-yielding and were distributed to cocoa farmers in Trinidad.

Prior to his expeditions to Ecuador and the Amazon Valley, F. J. Pound conducted a statistical survey of the cocoa population in Trinidad to lay the foundations for selection of desirable types to be clonally propagated. One hundred trees, labelled ICS 1 to ICS 100, were planted in replicated field trials at River Estate in Diego Martin and the San Juan Estate in Gran Couva, Trinidad. The estates were chosen as representative of the soil types of Trinidad and Tobago. From these trials, ICS 1, 6, 60 and 95 showed the greatest promise.

In 1949, hybridisation of selected Trinitario and Amazonian genotypes was initiated to produce clones with a combination of a high degree of resistance to Witches' Broom disease, high yield, and good quality (Montserin *et al.*, 1957). In 1950, the first crosses between ICS 1, chosen because of its high yielding potential, and SCA 6, showing high resistance to Witches' Broom disease, were planted at the Marper Farm. A total of 460 progeny were planted under old trees with heavy Witches' Broom infection to subject the seedlings to high inoculum levels from the onset. Twenty-one trees showed no signs of Witches' Broom disease from 1950 to 1955 (Montserin and de Verteuil, 1957). A survey of disease incidence (scored as the number of brooms per tree), conducted in 2001, revealed trees with no sign of Witches' Broom infection. Thus, the surviving 195 trees were considered to be an excellent candidate population for mapping Witches' Broom resistance derived from SCA 6.

Recently, two Quantitative Trait Loci (QTL) associated with Witches' Broom resistance were identified in an F2 population that resulted from the self-pollination of Trinidad Select Hybrid (TSH) 516 (Brown *et al.*, 2003). TSH 516 is a hybrid from the ICS 1 x SCA 6 cross, and was selected in Trinidad. Unfortunately, the tree has died and is not among the 195 trees still in existence. Mapping of these QTLs in the original F1 population would complement the F2 mapping study, as segregating families useful for genetic studies with adequate phenotypic data are very rare. Our original objective was to produce a linkage map from this F1 family and to map QTLs for Witches' Broom resistance. It quickly became apparent that many of the individuals in these families contained non-parental alleles. We present here results obtained while ascertaining the source of the contamination, and investigating the hypothesis that different parental plants with the same or similar names could account for the non-parental alleles, or if there had been pollen contamination during the controlled crosses.

Materials and Methods

The sources of the trees used in the study are listed in Table I. Reciprocal F1 families, produced from controlled pollinations, were planted at Marper Farm in the 1950s. The SCA 6 x ICS 1 family originally contained 326 trees, of which 107 of the 110 remaining trees were sampled, and the reciprocal cross ICS 1 x SCA 6 originally contained 190 trees of which 79 of the 85 remaining trees were sampled. In addition, trees of the parental clones SCA 6 and ICS 1 were sampled from the St. Augustine Campus of the University of the West Indies (formerly the Imperial College of Tropical Agriculture), St. Augustine. Of the 36 trees labelled ICS 1, we sampled 13, and of the 30 trees labelled SCA 6, we sampled 17 (Table 1). Other cultivars known to have been used previously as parents in breeding were also sampled: Pound 18, IMC 67, ICS 6, ICS 10, ICS 60, "D 647" and SCA 12 (Table 1). Samples of SCA 6 and ICS 1 were obtained from the Cocoa Quarantine Unit at Reading, U.K. (Reading accessions RUQ234 and RUQ847, respectively) and from CEPLAC, Brazil (grandparents of the F2 population).

Leaf samples obtained from the clones listed in Table 1 were shipped to the USDA station in Miami, Florida and stored at 4^0C. DNA was extracted from 200 mg leaf tissue using the FastPrep 120 cell disrupter and the Fast DNA Kit (both from Bio101, Inc., Carlsbad, CA), following the manufacturer's directions. DNA was quantified from a subset of samples and 1:40 dilutions (of approximately 2.5 ng/μL) were prepared.

The PCR protocol described by Lanaud *et al.* (1999) was followed, using either a T_A of 46^0C or 51^0C. The reaction mix was optimised for use with samples from Trinidad by decreasing the amount of template to

approximately 2.5 ng DNA, and adding either betaine (Sigma, St. Louis, MO) or bovine serum albumin (BSA) as an enhancer. A 10 μL reaction consisted of 1 μL 10X buffer, 1 μL of either 5M betaine or BSA [10 mg/ml], 0.1 μL AmpliTaq (Applied Biosystems, Foster City, CA), 0.02 μL 100 mM dNTPs, 6.48 μL water and 1 μL template.

Post PCR multiplexing was accomplished by combining 1 μL PCR product from each of three samples (differing by MW and dye color) with 12 μL HiDi Formamide and 0.2 μL ROX500 (Applied Biosystems) molecular weight standard. Electrophoresis was carried out on an ABI3100 genetic analyser (Applied Biosystems) using the standard module for fragment analysis.

A set of 33 SSR markers was used to screen the two families. Twenty-eight microsatellite markers were developed by the Centre de Coopération Internationale en Recherches Agronomiques pour le Développement (CIRAD) and five at the Sub-Tropical Horticultural Research Station (SHRS). A complete list of the microsatellites used is presented in Table 2.

Parentage analysis was performed using the program CERVUS (Marshall *et al.*, 1998; Slate *et al.*, 2000). This software uses a simulation program to generate log-likelihood scores, producing a confidence statistic for assigning paternity. Log-likelihood values are expressed as LOD scores, which are the natural logarithm of the product of the likelihood ratios at each locus. A LOD score is calculated for each candidate parent, based on the genotypes of the candidate parents and putative offspring. A positive LOD score indicates that the candidate parent is more likely to be the true parent than a randomly chosen genotype, and a negative LOD score indicates the opposite. Since neither parent of the seedlings was available, the analysis was conducted to identify the most-likely parent. The most-likely parents identified in the first run are used as known parents in the second run. All seedlings and potential parents listed in Table 1 were used in the analysis.

Results and Discussion

SCA 6 and ICS 1 from Trinidad and Brazil had identical genotypes and were considered the correct standard for designating trees as SCA 6 or ICS 1. This result was further confirmed through population assignment studies performed in our laboratory (results not shown). Many different genotypes have been mislabelled as SCA 6 and ICS 1, and the molecular fingerprint and phenotypic data must be confirmed to ensure correct identification. Using all 33 markers, seventeen trees labelled SCA 6 at CRU were genotyped and found to be identical to SCA 6 samples from Reading and Brazil. In addition, CRU has confirmed that all 30 trees labelled SCA 6 on the St. Augustine Campus are the correct genotype. Five of the 13 trees labelled ICS 1 matched the fingerprint of the Reading and Brazil ICS 1. Of the eight trees remaining, two were identical but different from ICS 1 and six were each unique. Eight unique genotypes were therefore identified in the group labelled as ICS 1, with 38% (5 of 13) being the correct genotype. The off-types were labelled R177, R178, R179, R182, R183, R184, and R186 (Table 1). Further analysis by CRU has determined that 22 of the 36 trees (61%) labelled ICS 1 on Campus are the correct genotype. Three trees are still to be checked by CRU.

Of the 186 trees sampled in the SCA 6 x ICS 1 cross and its reciprocal, 134 were true hybrids (72.0%) based on the parentage analysis. ICS 1 and SCA 6 were assigned as the two most likely parents for 134 of the 186 individuals with 80% confidence. A partial output for the two-stage analysis of the ICS 1 x SCA 6 cross for the true progeny is presented in Table 3. In most cases, no parent-offspring mismatches occurred. A few progeny had non-parental alleles at a single locus, probably resulting from a mistaken call of the allele. This result may also indicate parentage with a Trinitario very similar to ICS 1. Trinitario genotypes are closely related and it often takes a high number of microsatellites to discriminate individuals (Motamayor *et al.*, 2003). In cases where ICS 1 or SCA 6 was not identified as the most likely parent, from 3 to 17 mismatching loci occurred (data not shown). The remaining 52 off-type trees could be categorised as selfs of ICS 1 (6 plants), ICS 1 x unknown (13 plants), SCA 6 x unknown (2 plants) and 31 plants that had neither ICS 1 nor SCA 6 as a parent (Table 4).

Making controlled crosses for genetic studies is essential for cocoa improvement, and this study demonstrates the need to confirm the integrity of cross progeny, using molecular markers, until the crossing technique used by any given research group is routinely accurate. Obviously, strict attention must be paid to avoid pollen contamination, as a high number of off-type plants renders these populations unsuitable for scientific studies, and can hinder breeding progress when an incorrect parent is assigned to a productive seedling. In the family studied here, we conclude that pollen contamination and mislabelling of trees were both responsible for the off-type trees.

Correctly identifying full-sib families is an important step in the estimation of heritabilities, predictions of genetic gain, and identification of superior parents. As demonstrated in this study, pollen contamination and mislabelling of plant families have created a serious problem in cocoa improvement programmes and in germplasm management. Other microsatellite analyses on segregating populations carried out in our laboratory

have shown an even higher proportion of off-type progeny (results not shown). Although laborious and relatively expensive, microsatellite analyses are continuously being used in the USDA cocoa breeding programme to check pollinations before establishing family or QTL trials.

Table 1: Sources of trees analysed

Name	Plot Location	Location of tree
SCA 6 & x ICS 1	Plot 1 in Field 10	Marper, Manzanilla
ICS 1 & x SCA 6	Plot 4B or 8B in Plot 10	Marper, Manzanilla
SCA 6[1]	X3Y15 in Campus B	Campus, UWI, St. Augustine
SCA 6A*	D644 in Field D	Marper, Manzanilla
SCA 12	D205 in Block D	Marper, Manzanilla
ICS 1	X6Y13 in Campus 1A	Campus, UWI, St. Augustine
R 177	X3Y8 in Campus IB	Campus, UWI, St. Augustine
R 178	X4Y8 in Campus IB	Campus, UWI, St. Augustine
R 179	X5Y8 in Campus IB	Campus, UWI, St. Augustine
R 182	X3Y10 in Campus IB	Campus, UWI, St. Augustine
R 183	X5Y10 in Campus IB	Campus, UWI, St. Augustine
R 184	X2Y12 in Campus IB	Campus, UWI, St. Augustine
R 186	X4Y12 in Campus IB	Campus, UWI, St. Augustine
ICS 6		San Juan Estate, Gran Couva
ICS 10	Block 5	San Juan Estate
ICS 60	Block 3	San Juan Estate
IMC 67	X6Y14 in Campus IB	Campus, UWI, St. Augustine
"D 647"	D647 in Field D	Marper, Manzanilla
POUND 18	Field B6	ICG, T (Centeno)

[1] One of 17 clonal SCA 6 trees on Campus, all with identical genotype
* SCA 6A was an off-type at Marper that may have been used in crosses as SCA 6.

Table 2: Microsatellite loci used in this investigation

CIRAD microsatellites[1]		SHRS microsatellites[2]
mTcCIR1	mTcCIR22	tcSHRS4
mTcCIR2	mTcCIR24	tcSHRS7
mTcCIR3	mTcCIR26	tcSHRS11
mTcCIR4	mTcCIR29	tcSHRS12
mTcCIR6	mTcCIR30	tcSHRS16
mTcCIR8	mTcCIR32	
mTcCIR9	mTcCIR35	
mTcCIR10	mTcCIR37	
mTcCIR12	mTcCIR40	
mTcCIR15	mTcCIR43	
mTcCIR17	mTcCIR44	
mTcCIR18	mTcCIR49	
mTcCIR19	mTcCIR53	
mTcCIR21	mTcCIR56	
	mTcCIR61	

[1]Pugh *et al.* (2004)
[2]Brown *et al.* (2003)

Explanation of acronyms used in this issue

ACRI	American Cocoa Research Institute
ARS	Agricultural Research Service
BCCCA	Biscuit, Cake, Chocolate and Confectionery Association (United Kingdom)
CATIE	Centro Agronómico Tropical de Investigación y Enseñanza, Costa Rica
CEPLAC	Commissao Executiva do Plana da Lavoura Cacaueira (Brazil)
CFC	Common Fund for Commodities
CIRAD	Centre de Coopération Internationale en Recherche Agronomique pour le Développement
CNRA	Centre National de Recherche Agronomique
CPA	Cocoa Producers Alliance
CRIG	Cocoa Research Institute, Ghana
CRU	Cocoa Research Unit
ICCO	International Cocoa Organization
ICGD	International Cocoa Germplasm Database
ICGT	International Genebank, Trinidad
IITA	International Institute of Tropical Agriculture
IPGRI	International Plant Genetic Resources Institute
IRAD	Institut de Recherche Agricole pour le Développement
MCB	Malaysian Cocoa Board
SCPS	Sustainable Crop Production Systems
STCP	Sustainable Tree Crops Program
UESC	Universidade Estadual de Santa Cruz
UNAS	Universidad Nacional Agraria de la Selva
USDA	United States Department of Agriculture
WCF	World Cocoa Foundation

Table 3: Two-stage parentage analysis of the ICS 1 x SCA 6 family for individuals identified as true hybrids based on 33 microsatellite loci

Offspring	1st candidate parent ID	Number of offspring 1st parent loci mismatching	2nd candidate parent ID	Number of offspring 2nd parent loci mismatching	LOD[a]	Delta[b]	Confidence
T1000	SCA 6	1	ICS 1	2	1.04E+01	3.11E+00	*
T1001	SCA 6	0	ICS 1	0	1.16E+01	8.55E+00	*
T1002	SCA 6	0	ICS 1	1	9.17E+00	5.51E+00	*
T1003	SCA 6	0	ICS 1	1	1.01E+01	5.40E+00	*
T1005	SCA 6	0	ICS 1	1	9.46E+00	3.34E+00	*
T618	SCA 6	0	ICS 1	0	1.09E+00	1.09E+00	*
T623	SCA 6	0	ICS 1	0	1.13E+01	6.81E+00	*
T628	SCA 6	0	ICS 1	0	1.43E+01	7.96E+00	*
T629	SCA 6	0	ICS 1	0	1.33E+01	1.63E+00	*
T630	SCA 6	0	ICS 1	0	1.15E+01	1.13E+01	*
T636	SCA 6	0	ICS 1	0	1.32E+01	4.84E+00	*
T640	SCA 6	0	ICS 1	0	1.28E+01	4.63E+00	*
T641	SCA 6	0	ICS 1	0	1.31E+01	9.11E+00	*
T649	SCA 6	0	ICS 1	0	1.14E+01	1.02E+01	*
T653	SCA 6	0	ICS 1	0	1.08E+01	9.47E+00	*
T660	SCA 6	0	ICS 1	0	1.15E+01	1.70E+00	*
T666	SCA 6	0	ICS 1	0	1.27E+01	6.07E+00	*
T680	SCA 6	0	ICS 1	0	1.28E+01	3.20E+00	*
T684	SCA 6	0	ICS 1	0	1.08E+01	6.23E+00	*
T708	SCA 6	0	ICS 1	0	1.27E+01	6.89E+00	*
T719	SCA 6	0	ICS 1	0	1.14E+01	1.14E+01	*
T720	SCA 6	0	ICS 1	0	1.25E+01	6.56E+00	*
T952	SCA 6	0	ICS 1	0	1.46E+01	1.14E+01	*
T955	SCA 6	0	ICS 1	0	1.20E+01	5.80E+01	*
T956	SCA 6	0	ICS 1	0	1.62E+01	8.44E+01	*
T957	SCA 6	0	ICS 1	0	1.40E+01	1.17E+01	*
T960	SCA 6	0	ICS 1	0	1.18E+01	6.32E+00	*
T961	SCA 6	0	ICS 1	0	1.30E+01	1.19E+01	*
T968	SCA 6	0	ICS 1	0	1.31E+01	8.42E+00	*
T970	SCA 6	0	ICS 1	0	1.21E+01	3.42E+00	*
T971	SCA 6	0	ICS 1	0	1.14E+01	8.45E+00	*
T972	SCA 6	0	ICS 1	0	1.33E+01	9.25E+00	*
T973	SCA 6	0	ICS 1	0	1.33E+01	7.04E+00	*
T977	SCA 6	0	ICS 1	0	1.31E+01	1.22E+01	*
T978	SCA 6	0	ICS 1	0	1.40E+01	4.71E+00	*
T979	SCA 6	0	ICS 1	0	1.25E+01	5.96E+00	*
T980	SCA 6	0	ICS 1	0	1.24E+01	1.13E+01	*
T981	SCA 6	0	ICS 1	0	1.27E+01	3.02E+00	*
T982	SCA 6	0	ICS 1	0	1.39E+01	6.37E+00	*
T984	SCA 6	0	ICS 1	0	1.27E+01	8.47E+00	*
T990	SCA 6	0	ICS 1	0	1.39E+01	6.36E+00	*
T991	SCA 6	0	ICS 1	0	1.17E+01	5.62E+00	*

a Sum of the log-likelihood ratios at each locus for the second candidate parent
b * = 80%

Table 4: Number of full-sibs, half-sibs, selfs, and misidentified seedlings for the reciprocal cross

Types	Family		Total
	SCA 6 x ICS 1	ICS 1 x SCA 6	
Full-sibs	92	42	134
Maternal half-sibs	2	13	15
Selfs	0	6	6
Unrelated seedlings	13	18	31
Total off-types	15	37	52
Total	107	79	186

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Genetic Mapping and Identification of Genomic Regions Associated with Witches' Broom Resistance Derived from Alternative Sources from the Brazilian Amazon (CAB genotypes)

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Introduction

This note describes the ongoing efforts to identify genomic regions associated with Witches' Broom resistance, caused by *Crinipellis perniciosa* (Stahel) Singer, derived from two cocoa genoytpes originally collected in the Brazilian Amazon. Genetic maps are being developed based on microsatellite markers (SSRs) for two populations involving two resistant genotypes (CAB 214 and CAB 208) and a susceptible parent (ICS 39).

The germplasm collection

The germplasm collection of CEPLAC established at the "Estação de Recursos Genéticos do Cacau José Haroldo" (ERJOH), located in Marituba, Pará state, results from an immense effort of the Brazilian government to systematically collect, as budwood or seeds, the genetic diversity of wild and semi-wild cocoa from the whole Brazilian Amazon region (Almeida *et al.*, 1987; 1995). The objectives of this initiative were to preserve and characterise the diversity of the cocoa in germplasm collections in the region. The search for Witches' Broom resistance was considered a top priority (Almeida *et al.*, 1995). The germplasm collection at ERJOH contains more than 1,800 accessions (denoted CAB - Cacao Amazon Brazil), of which 940 were

derived from clonal propagation, while 877 were derived from open-pollinated seedlings, representing 36 of the 186 Brazilian Amazon basins (Almeida *et al.*, 1995).

Genotype selection for Witches' Broom resistance

A systematic selection of Witches' Broom resistant materials was initiated in the 1990s, and the programme was conducted in two steps. Firstly, mature (12-15 year-old) field-grown trees were evaluated for the level of resistance under natural Witches' Broom infection. For four years (1990-1994), approximately 900 CAB accessions were kept under high inoculum pressure of *Crinipellis perniciosa* in Marituba, and the level of resistance was evaluated based on the number of infection sites (flower cushion and brooms) in the canopy (Table 1). During this initial screening, 300 clones presented promising results, with reduced levels of infection during the period of evaluation. Some of the results of this field screening were published by Fonseca and Albuquerque (2000).

The evaluation of resistance progressed to the next step, consisting of artificial inoculations under nursery conditions of seedlings derived from open-pollinated pods from the previously selected 300 accessions. Some of the resistance evaluations were conducted at CEPEC, Ilhéus, Bahia. Forty-day old seedlings were artificially inoculated by loading 40 µl of 10^5 basidiospores/ml on flushing apical meristems, followed by incubation for 24 h at high humidity (Albuquerque *et al.*, 1999). Each family was represented by 100 seedlings, and the disease reaction was compared to that of inoculated seedlings derived from open-pollinated pods of "Catongo" as a susceptible control for every experiment. Resistance was estimated for each family based on a series of symptoms in comparison with those of the "Catongo" controls. The level of resistance was summarised by the total number of seedlings exhibiting one or more of the evaluated symptoms, which included presence of terminal and/or axillary brooms; and/or seedlings exhibiting swollen stem and/or pulvinus (Table 2). The ten families with the lowest index of infection were selected for further experiments using controlled pollination (Table 2).

Families were obtained from controlled crosses involving the 10 most resistant accessions previously identified. For each controlled cross, 100 seedlings were evaluated for resistance using artificial inoculations under nursery conditions, in comparison to susceptible controls in various experiments for each experiment. These controlled crosses were performed mainly between the identified resistant genotypes, and limited segregation was observed since most of the families had very low levels of infection in comparison to the susceptible control (Table 3, first 4 rows). Depending on pod availability, other susceptible genotypes were also sometimes included, such as PA 195 or ICS 39. The lowest levels of Witches' Broom infection was observed for crosses derived from CAB 214 and CAB 208, and this resistance was stable (Table 3). These genotypes were originally collected along the Purus River, between Sena Madureira and Boca do Pauini, state of Amazonas in 1981 (Almeida *et al.*, 1987; 1995). Other interesting genotypes identified were CAB 270 (Tables 2 and 3), originally collected along the Solimões River, near Tefé in 1982, and RB 36, collected on Rio Branco, state of Acre (Soria, 1970). These genotypes with low levels of infection appeared very distinct from one other based on RAPD and microsatellite analyses (Silva *et al.*, 1998; Sereno *et al.*, 2001; Mota, 2003). The levels of resistance of these genotypes have also been confirmed using artificially inoculated clonal budded plants.

Contrasting population development and evaluation of resistance

This part of the project started in January 2002 with the development of specific mapping populations. The genotypes CAB 208, CAB 214 and CAB 270 were used as pollinators for the susceptible genotype ICS 39. At least 170 individuals were obtained for each family. The seedlings of each family were artificially inoculated as described above, and resistance was evaluated 40 days later (Table 3 shows partial data). For all the inoculations, susceptible controls ("Catongo") were included to ensure the efficiency of the method.

For the ICS 39 x CAB 208 population, approximately 38% of the seedlings presented some kind of symptom, while the rest were apparently resistant. For the ICS 39 x CAB 214 family, only 14% of the seedlings presented some Witches' Broom symptoms, while the susceptible seedlings represented 83% ("Catongo") or 77% (PA 195 x ICS 39) of infected seedlings (Table 3). The ICS 39 x CAB 270 cross resulted in 57% infected seedlings.

After resistance evaluation, the seedlings remained for at least 90 days in the nursery before being transferred to the field. In some cases, the seedlings had to be kept for up to 6 months for a suitable season to be transplanted to the field. The families have been established under field conditions from November 2002 through March 2004.

Genotyping

Two of the contrasting populations along with the parents have been genotyped using microsatellite markers developed by CIRAD. To date, 80 loci have been evaluated for the ICS 39 X CAB 208 population, and 40 loci for that of ICS 39 X CAB 214.

The microsatellite loci are being analysed either using denaturing polyacrylamide gels stained with

silver nitrate using a low-cost optimised protocol (Creste *et al.*, 2001), or fluorescent primers in an automatic DNA analyser (ABI 310). To reduce the cost of primer labelling and to give more flexibility for analysis, a system based on *fluorescent primer-tail* has been adapted. Primers with tails can be either used in conventional systems using silver nitrate staining or radioactive labelling followed by autoradiography, or for fluorescence detection in a DNA sequencer. In this system, one of the primers contains an extra 20 base-tail with an identical sequence with another fluorescent primer added to the PCR reaction, as suggested by Oetting *et al.* (1995). However, instead of using only a M13 sequence tail, three human sequences were adapted allowing the joint analyses of three loci with distinct fluorescence (Dario Grattapaglia, pers. comm.). This system allows multiplex analyses combining amplification products with three fluorescent dyes.

After the development of the genetic map for both populations, Quantitative Trait Loci associated with resistance at the seedling stage will be identified. A major objective is to compare the chromosomal location of the major resistance factors to be identified with the main one described for Scavina 6. According to recent unpublished work by Faleiro *et al.* (2004), the major QTL for Witches' Broom resistance described by Queiroz *et al.* (2003) was confirmed, and additionally, three microsatellite loci (*mTcCIR35, mTcCIR30* and *mTcCIR24*) were mapped to the same region. These microsatellites are located on chromosome 9 of the consensus map (Pugh *et al.*, 2004). Evaluation of resistance will also be conducted under field conditions for both populations for a number of years, and will include clonal replication of the individuals.

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Table 1: Number of infection sites in a sample of 10 clonal genotypes under field conditions after four years of high inoculum pressure of *Crinipellis perniciosa* illustrating the range of resistance found among CAB genotypes

Clones	Site of infection*	
	Shoots	Flower Cushion
CAB 16	103	30
CAB 20	68	30
CAB 21	292	10
CAB 23	166	40
CAB 27	167	15
CAB 92	185	79
CAB 207	25	214
CAB 208	5	8
CAB 214	1	4
CAB 270	6	10

*Average infection of 10 plants.

Table 2: Total number of inoculated seedlings; percent of infected seedlings; number of seedlings exhibiting some infection symptoms (terminal broom; axillary broom; shoot or pulvinus swelling) for various families derived from open-pollination, artificially inoculated with Witches' Broom

Parental genotype	No. of inoculated seedlings	% Infected plants Plants	No. of seedlings with any sympton	No. of seedlings with symptoms			
				Terminal Broom	Axillary Broom	Shoot swelling	Pulvinus swelling
Trial I							
Catongo	100	67	67	64	38	67	35
CAB 0268	100	95	95	92	31	95	47
CAB 0277	100	94	94	94	60	92	54
CAB 0283	100	82	82	80	31	82	41
CAB 0284	100	93	93	91	54	93	54
CAB 0316	100	88	88	83	50	88	62
CAB 0322	100	99	99	98	58	99	57
Trial II							
Catongo	120	42	50	41	50	21	50
CAB 0208	120	7	8	6	9	3	8
CAB 0214	120	10	12	8	12	5	12
CAB 0270	120	8	10	8	10	2	10

Table 3: Total number of inoculated seedlings; percent of infected seedlings; number of seedlings exhibiting some infection symptoms (terminal broom; axillary broom; shoot or pulvinus swelling) for various families derived from controlled cross pollination, artificially inoculated with Witches' Broom

Population derived from controlled pollination	No. of trials	No. of inoculated seedlings	% Infected plants Plants	No. of seedlings with any sympton	No. of seedlings with symptoms			
					Terminal Broom	Axillary Broom	Shoot swelling	Pulvinus swelling
Catongo	10	905	83	755	704	197	770	341
PA 195 x ICS 39	3	218	77	168	163	83	167	89
CAB 208 x CAB 214	3	140	9	12	7	0	11	07
CAB 270 x CAB 214	5	139	16	22	19	2	21	04
ICS 39 x CAB 208	7	293	38	112	76	16	93	18
ICS 39 x CAB 214	3	133	14	18	6	0	18	1
ICS 39 x CAB 270	9	525	57	298	230	41	285	79

A Rootstock-Scion Experiment with Cocoa Re-analysed for Yield Efficiency

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Scope

In cocoa selection and cultivation, emphasis is increasingly being placed on clonal cocoa varieties. Commercial multiplication of cultivated clones is mainly achieved through budding or grafting techniques. The expression of yield and yield efficiency of cocoa scions may be affected by the type of rootstocks used, as is the case with "dwarfing" apple rootstocks, which allow for high density planting of clonal rootstock x scion combinations that result in high yield efficiency in this crop (Wertheim, 1998). However, little information is available, so far, on the possibility of finding such "dwarfing" rootstocks in cocoa.

The most significant results can be expected from trials that use uniform rootstock varieties such as clones or homozygous seedling rootstocks rather than segregating families, for which results are more difficult to interpret. Therefore, we wish to re-analyse hereunder the following interesting article, which refers to one of the few finalised experiments with clonal rootstock-scion combinations in cocoa:

D.B. Murray and F.W. Cope (1959). A Rootstock-Scion Experiment with cocoa III", Report on Cocoa Research, 1957-1958. Imperial College of Tropical Agriculture, pp. 29-35.

The objective is to see if the results may have some bearing on prospects of finding good rootstocks for clonal cocoa varieties so that yield efficiency (YE) of otherwise good cocoa scions may be increased, allowing for higher density planting and easier management of cocoa trees.

Background information on the trial

The trial was planted in 1951 at a density of 1.83 x 3.66 m, using an incomplete block design with 3 replications of 4 blocks of 4 plots of six trees each, totalling 288 trees. Guard rows were planted only around the entire experiment, not between adjacent plots or blocks (Cope and Murray, 1953). Four clonal varieties (ICS 1, ICS 45, ICS 60 and ICS 95) were used in all possible scion x rootstock combinations (see Table 1). Rooted cuttings of each of the clones were used to bud the scion varieties (Cope and Murray, 1953). Results on girth of scions and rootstock measured seven years after planting, and on dry cocoa yield of the first five production years (1955-1958) were presented by Murray and Cope (1959) in the article cited above.

Results

Yield and vigour of the scion

Table 1 shows the results on yield and vigour of the 16 rootstock-scion combinations as presented in the paper by the aforementioned authors. Yields varied greatly between scion varieties, with the best yields being obtained with ICS 1 and ICS 60. ICS 45 is a very poor yielder as scion, and ICS 95 is intermediate. The best stock variety appears to be ICS 60, with ICS 45 also being the poorest yielding stock. Interaction between stocks and scions for yield was not statistically significant.

Stem girth above the graft union varied according to the scion variety (highest for ICS 60) and to the rootstock variety (lowest for ICS 45 and highest for ICS 1). Again, there appeared to be no statistically significant interaction effect between scions and stocks for girth.

The authors concluded that there was "no prospect of producing higher yields with particular combinations than with single clone plants" but that "it would be possible, however, to increase the yield of a weak clone (*e.g.* ICS 45) by growing it on a more vigorous stock". Furthermore, "the stock with the greatest dwarfing effect, ICS 45, gives poor yields even with a potentially high yielding scion". The authors further mention that the yields on a "mutant" dwarf ("crinkled dwarf", Cope and Murray 1953), not included in this experiment, were also extremely poor.

The above conclusions of the authors seem quite pessimistic with regard to the potential of developing clonal rootstocks with cocoa. However, we have re-analysed the data presented by the authors, by calculating the yield efficiency, and this seems to justify a revision of the conclusions of this article.

Table 1: Yield (dry bean weight) and girth (in cm, above the graft union) of four ICS clones grafted in all possible combinations (Murray and Cope, 1959)

Scion / Stock	ICS 1	ICS 45	ICS 60	ICS 95	Mean
ICS 1	1005	81	1000	647	**683**
	43	43	53	43	**45**
ICS 45	891	86	720	423	**530**
	33	29	39	30	**32**
ICS 60	1313	431	1197	778	**930**
	35	44	41	38	**40**
ICS 95	1080	212	1132	726	**787**
	38	32	43	34	**37**
Mean	**1072**	**202**	**1012**	**644**	732
	37	**37**	**44**	**36**	38.5

Analysis of Yield Efficiency (YE)

Yield efficiency (not analysed by Murray and Cope) has been calculated by us as the dry bean yield divided by the scion trunk cross-sectional area. YE is considered as an interesting trait selected for in tree crops (such as in apple), in order to obtain trees that are managed more easily and may be planted at a higher planting density. Using the data provided in the article of Murray and Cope, we have calculated the YE for the 16 combinations, as presented in Table 2.

YE varied five-fold for the scions, with ICS 1 being the best, ICS 45 the worst and ICS 60 and ICS 95 intermediate. YE varied about two-fold for the stocks, with ICS 60 being the best and ICS 1 the worst stock variety. The best YE (13.5) was obtained when the best scion (ICS 1) was grafted onto the best stock (ICS 60). For YE, this combination is about 100% better than when the best scion for yield is grafted onto its own roots (ICS 1), and approximately 50% better than when the second best scion for yield is grafted onto its own roots (ICS 60). The rootstock-scion effects seem to be mainly additive for YE, as was the case for yield and vigour (Murray and Cope, 1959).

We conclude from these calculations that, when aiming at maximum YE, the best scion clones for yield may not be necessarily the best for YE when a scion is grown on its own roots. Increments of 50 to 100% above that obtained when scions are grown on their own roots may be possible when the best scion-rootstock combination is selected. Secondly, the lowest-vigour rootstock (ICS 45), which was the worst stock for yield, produced a YE almost 50% superior to that of the most vigorous rootstock (ICS 1).

Table 2: Yield efficiency (dry bean weight divided by scion trunk cross-sectional area) of four ICS clones grafted onto their own stock (based on data from Murray and Cope, 1959)

Scion / Stock	ICS 1	ICS 45	ICS 60	ICS 95	Mean
ICS 1	6.83	0.55	4.47	4.39	**4.06**
ICS 45	10.28	1.39	5.95	5.90	**5.88**
ICS 60	13.46	2.80	8.94	6.59	**7.95**
ICS 95	9.39	2.60	7.69	7.89	**6.89**
Mean	**9.99**	**1.84**	**6.76**	**6.19**	6.12

Discussion

The analysis for yield efficiency of this interesting scion-rootstock experiment seems to draw attention to the potential for selecting better rootstocks for otherwise good scion varieties in cocoa, when the objective is to maximise YE. Furthermore, the best scion-stock combinations for YE would need to be further evaluated, at different planting densities, to determine the total gain possible for productivity on a kg/ha basis.

It is interesting to note that the best stock variety (ICS 60) for YE is the second most vigourous stock variety (after ICS 1). Therefore, the best stock varieties may not necessarily be the lowest vigour genotypes. However, the second best score for YE (10.3) is obtained when using the best scion (ICS 1) on the lowest vigour rootstock variety (ICS 45). Therefore, low-vigour stocks may still have potential as dwarfing stocks when aiming for more significant increases in "dwarfing effects" on the growth of the scion.

The good performance of ICS 60 as stock might be related to some special quality of the roots of this clone. Murray and Cope (1955) mention the relative tolerance of this clone to heavy soil in comparison to the ICS 1 clone. However, the experiments by Murray & Cope (1959) were conducted at River Estate in Diego Martin where the soils (loam) are among the best cocoa soils in Trinidad with "reasonably good physical characteristics and medium fertility" (Cope, 1953). Therefore, the good performance of the ICS 60 rootstock for obtaining high YE of scions may be a trait unrelated to its tolerance to heavy clay soils.

Finally, generalisation of the results must be reserved since only four clones of the same genetic group (Trinitario) were involved. Results with stocks from other genetic origins may provide different results.

Applications in cocoa

The use of varieties with high YE in cocoa seems a desirable characteristic in many situations. Such varieties may allow for higher density planting and for more easily managed canopies (e.g. with a lower requirement for pruning). Smaller trees would also facilitate easier control of diseases and pests, and may face reduced interplant competition, a possible cause of yield decline in adult plantations with overlapping canopies. This would further justify selection for high YE in cocoa, with or without the use of rootstocks.

Clonal trials, conducted earlier in Trinidad, indicated superiority for yield of good scions when grown as rooted cuttings, as compared to when budded onto seedling rootstock (Dodds and Cope, 1951). For ICS 1 and ICS 60, the budded material yielded 20 and 24% less than rooted cuttings of the same clones, respectively. This result further suggests that good rootstocks need to be found to fully exploit the yield potential of good clones, if these are to be multiplied by budding or grafting (which is currently the most commonly used practice for multiplication of commercial cocoa clones).

So far, very few promising rootstocks have been identified in cocoa. In further studies, it would be of interest to compare the yield and YE of commercial clones such as PBC 123 and CCN 51, grafted onto ICS 60 rootstock (plagiotropic and orthotropic) versus seedling rootstocks. It would also be of interest to compare the ICS 60 clonal rootstock with other promising rootstock varieties, *e.g.* the one under study in Papua New Guinea (Efron *et al.*, 2003). Such trials would need to include different planting densities so that the density at which the effect of the rootstock on the yield per ha is greatest may be identified.

Once good rootstocks are found for commercial clones, such may also prove to be good for other newly selected clones, if the above observed additivity between the effects of rootstock and scion is found to be a more general phenomenon.

When developing new clonal varieties, breeders would do best to select scions that intrinsically have adequately high YE, irrespective of the rootstock used. It may not be impossible to select scions, for which YE cannot be further improved, by using selected (dwarfing) rootstocks. The authors would, however, not exclude the possibility that, for optimisation of yield and YE, potentially vigorous and productive scions may be required, which through manipulation of their vigour (e.g. by using dwarfing rootstocks), would be able to transfer to pod production an even larger component of the energy that is normally allocated to vegetative growth. This hypothesis could be verified by carrying out rootstock trials using scions of similar yield potential, but with differing levels of vigour, under variable planting densities.

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Field Tests for Antixenosis and Tolerance of Cocoa towards Mirids

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Abstract

Field tests for antixenosis (feeding non-preference) and tolerance of cocoa trees to mirid damage were conducted as part of the CFC/ICCO/IPGRI Project, in the budwood garden of IRAD Nkolbisson Station.

Antixenosis was assessed by counting the number of mirid bites on the flush after 24 hours, while assessment of tolerance was based on regular observations of the evolution of mirid damage and of the ability of twigs to recover from it. An analysis of covariance, with the surface area of the flush leaves as a covariable, revealed a highly significant effect of the clone on the number of mirid bites, and SIC 5 and CATONGO showed a stronger effect of antixenosis than the other clones. The results obtained by observing the evolution of mirid damage and the sprouting of twigs revealed that the ability of clones to contain the extent of damage, and their ability to recover from it must be considered as two distinct components of genetic resistance to mirid attack. The analysis of covariance also revealed a highly significant effect of clones for these two components. UF 676 and IMC 60 showed the best ability to contain the degree of damage, while PLAYA ALTA 2, ICS 1 and UF 676 were the best clones in terms of ability to recover from mirid damage.

Introduction

Cocoa is one of the main sources of income for the rural population in the Cameroon's forest zone, where it is cultivated on about 200,000 cocoa farms. However, cocoa production is strongly reduced by damage caused by pests and diseases. Cocoa mirids, *Sahlbergella singularis* Hagl., and *Distantiella theobroma* Dist. (*Heteroptera: Miridae*) are the major pests of the cocoa tree in Africa, with 30 to 40 % losses in production in West-Africa. Their bite results in the drying up of flushes and young branches. In addition, the cankers that appear after a few days foster the development of parasitic cryptogamic fungi in the tree. Mirids and cryptogams cause damage resulting in premature aging of cocoa trees and important losses in production. In order to control these serious pests, IPM activities are under development by the Laboratory of Entomology of IRAD Nkolbisson station where one of the research priorities is to gain a better understanding of the mechanisms of genetic resistance to mirids, and to identify cocoa varieties resistant and/or tolerant to these pests. The CFC/ICCO/IPGRI Project has facilitated screening activities on local and international genotypes, based on the study of various components of the resistance. The goal of this paper is to present the method and the first results of field tests for antixenosis (feeding non-preference) and tolerance of the cocoa tree towards mirids.

Material and methods

Genotypes

Ten genotypes from Nkolbisson IRAD station budwood garden were tested in July, August and September 2003:

Two Upper Amazon Forasteros: T 79/501 and IMC 60,
Four Lower Amazon Forasteros: SIC 5, BE 10, CATONGO and IFC 5,
Three Trinitarios: PLAYA ALTA 2, ICS 1 and UF 676,
One clone from French Guiyana: GU 255/P.

Replications

The budwood garden is composed of four plots in which each clone is represented by one tree. The density is 4500 trees ha^{-1}, and there are no shade trees. Eight to 14 observations per genotype were carried out, distributed over the four trees of each genotype when they were available.

Choice of flushes

Approximately thirty terminal buds per genotype were marked with wool strands and their growth was watched regularly, until complete development of the last leaf. The age of the analysed flushes was calculated in this way. The width and length of the flush leaves were also measured in order to estimate their surface area. Fifty percent of the marked flushes were used as control.

Method of infestation with mirids

The tests were performed with fifth instar nymphs of *S. singularis*, the most common species in the study area, obtained from laboratory rearing. The larvae were kept in captivity without food for one night. The next morning, a mirid nymph was transferred and confined to a flush, in a nylon mesh sleeve cage, and allowed to feed on the plant for 24 hours, before being removed.

Measure of antixenosis

Antixenosis was assessed by counting the number of mirid bites on the flushes after 24 hours.

Assessment of tolerance

Evolution of mirid damage on the twigs

After removal of mirids, the physiological reaction of the flushes/twigs was examined twice weekly for 22 days, and assessed using a notation scale, ranging from 0 (healthy twigs) to 4 (dead flushes). For each twig, the mean value was calculated for all the scores given at the different times of the experiment. This *mean index of twig* therefore takes into account the speed as well as the final degree of degradation.

Ability to recover from damage

One month after infestation, and at the end of the dry season, i.e. 8 to 9 months after infestation, twig ability to recover from damage was estimated by observing sprouting. Each twig was assigned to one of the following classes typified by the symptoms:

1) The branch was sprouting normally,
2) The flush had dried but the flush was sprouting at its base, and
3) The twig dried up completely (die-back).

Control treatment

Half of the marked twigs were slightly punctured with a needle, and were not submitted to mirid feeding.

Statistical analysis

Correlation coefficients (Pearson), variances and covariances were estimated using SAS software (V8). Least Square Means were calculated and all pairwise comparisons were performed using General Linear Models (GLM).

Results and discussion

Antixenosis

The number of bites inflicted by the mirids bore no correlation to the age of the flushes (r = 0.03, non-significant).

There was a weak correlation between the number of bites and the estimated surface area of the flush leaves (r = 0.20, significant). This low correlation value indicates that the differences observed between clones in terms of the number of bites inflicted do not only result from differences in their flush surface area, but really reflects levels of antixenosis as well.

In order to measure antixenosis exclusively, the surface area of the flush leaves was used as a covariable for the analysis of the number of bites. The analysis of covariance revealed a highly significant effect of the clone (F = 2.94) and the ranking of the values, after correction by the use of the covariable, showed significantly different groups of clones (Table 1). Indeed, SIC 5 and CATONGO showed a significantly lower number of bites than BE 10, PLAYA ALTA 2, IFC 5 and GU 255/P, and consequently showed a stronger effect of antixenosis.

Tolerance

Evolution of mirid damage:

For each clone, the mean index of degradation of the twigs goaded with a needle (control) was consistently very low (approximately 1) and always differed very strongly from those of twigs damaged by mirids. This indicates that this assessment specifically measures the reaction of cocoa to mirid attacks and not to general stress.

The mean degradation index failed to show any correlation with the age of the flush (r = 0.17, non-significant), or with the estimated flush-leaf surface area (r = -0.03). On the other hand, a significant but rather low (r = 0.48) correlation was shown between the mean index of degradation of the twigs and the number of bites. This rather low correlation value indicates that the differences observed between clones for the evolution of damage do not simply result from the different numbers of bites they received. It shows that the ability to contain the extent of the damage must be considered as a component of the genetic resistance to mirids, independently from the antixenosis. As an illustration, the scatter plot (Figure 1) shows a higher mean degradation index for SIC 5 and CATONGO than for IMC 60 and UF 676, despite a higher mean number of bites for the last two clones. In addition, T 79/501 showed a slightly lower mean index of degradation than SIC 5 and CATONGO but a higher mean number of bites.

When the number of bites was used as a covariable, the analysis revealed a highly significant "clone" effect (F = 3.57 and 2.72 with and without the use of the covariable, respectively) for the mean index of degradation. The use of the covariable did not change the ranking for the three clones with the lowest mean degradation index: UF 676, IMC 60 and T 79/501. In both cases, UF 676 and IMC 60 showed a significantly lower value than most of the other tested clones. These two clones were also among the ones with the lowest numbers of bites. In both analyses, the four clones with the highest mean degradation index were the same: IFC 5, BE 10, ICS 1 and PLAYA ALTA 2. The use of the covariable resulted in ICS 1 showing the highest value instead of IFC 5 because ICS 1 had a rather high average mean number of bites.

Ability to recover from damages:

The correlation between the mean degradation index and the percentage of dried branches without sprouting was not significant (r = 0.11); showing that tolerance of the clones does not result exclusively from the ability to contain the damage.

As could be expected, a strong, significant negative correlation was found between the percentage of normally sprouting twigs per clone after one month and the degradation mean index (r = - 0.81, significant). Conversely, there was no significant correlation between the percentage of basal sprouting after one month and the degradation mean index (r = 0.45).

Figure 2 is a scatter plot showing the percentages of dried branches without sprouting after one month in relation to the mean degradation indexes. Some clones, such as PLAYA ALTA 2, ICS 1 and IFC 5, had a low level of dried branches without sprouting despite a high mean degradation index. These clones had a high percentage of sprouting at the base of the dried flushes, as shown in Figure 3. On the other hand, IMC 60 had a relatively high percentage of dried branches without sprouting, despite a low mean degradation index. Figure 3 shows a low percentage of sprouting at the base of the dried flushes for this clone. A similar relationship is observed between mean degradation index and percentage of dried branches without sprouting after the dry season, which was expected because of the strong positive and significant correlation (r = 0.8) between the percentages of dried branches without sprouting after one month and after the dry season. However, both Figures 3 and 4 show differences between this variable when measured at the two different periods for some of the clones. This is the case for T 79/501 and CATONGO, which showed a much higher value after the dry season than after one month. Two different situations are observed for these two clones. In the case of CATONGO, the increase in dried branches without sprouting results from the drying of the branches which showed a normal sprouting after one month, while, in the case of T 79/501, this increase results from the drying of new flushes issued from the base of the dried flushes. Other clones, such as ICS 1, UF 676, and IFC 5, did not show any increase in the % of dried branches without sprouting. Here again, a difference appears between ICS 1 and IFC 5 on one hand, and UF 676 on the other. Indeed, the same percentages of the three classes of branches are observed after the two periods in the case of ICS 1 and IFC 5, while the percentage of normal sprouting has decreased and the percentage of sprouting at the base of the dried flush has increased in the case of UF 676. The lowest level of tolerance is observed for BE 10, which had a low level of normal sprouting, agreeing with its high mean degradation index, combined with a low percentage of sprouting at the base of the dried flushes.

Conclusion

In this paper, we have described a relatively user-friendly method to assess the resistance of cocoa clones to mirids. Indeed, this method requires a limited number of mirids, since one single nymph can be used for several repetitions. In addition, this method can be applied under field conditions, the observations are not time-consuming, are easily repeatable, and do not require much handling that could affect the nymphs of mirids.

This assessment method also allowed us to confirm the existence of three distinct components of the reaction of cocoa to mirids: antixenosis, ability to contain the damages and ability to recover from damages.

Genetic differences were observed for these different components of the resistance to mirid attack despite the rather low number of cocoa clones assessed. These differences allowed us to identify some promising clones for the different components of resistance to mirids: CATONGO and SIC 5 for antixenosis, IMC 60 and UF 676 for ability to contain the evolution of damage, and PLAYA ALTA 2, ICS 1 and UF 676 for ability to recover from the damage.

Unfortunately, we failed to identify a single clone accumulating favourable alleles for the three components of resistance, but this might be possible by screening a larger number of them. In the absence of such clones, breeding programmes will need to consider making crosses between promising clones for the three resistance components in order to create progenies and progenitors accumulating favourable alleles for all components.

Table 1: The number of bites corrected by the use of the estimated flush-leaf surface area as a covariable and ranked according to pairwise Student T tests at a 5% significance level

Clone	Mean number of bites	
IFC 5	14.48	a
GU 255/P	14.09	ab
Be10	12.92	abc
PLAYA ALTA 2	12.23	abc
T 79/501	11.77	abcd
ICS 1	11.77	bcd
IMC 60	10.63	cd
UF 676	9.93	cd
CATONGO	9.04	d
SIC 5	9.01	d

Table 2: Values and ranking of the tested clones for the mean degradation index

Clone	Mean degradation index		Clone	Mean degradation index (with the number of bites as a covariate)	
IFC 5	3.11	a	ICS 1	2.97	a
BE 10	3.03	ab	BE 10	2.96	ab
ICS 1	2.98	ab	IFC 5	2.90	ab
PLAYA ALTA 2	2.87	ab	PLAYA ALTA 2	2.80	ab
GU 255/P	2.82	ab	SIC 5	2.78	ab
SIC 5	2.61	abc	CATONGO	2.71	ab
CATONGO	2.54	bc	GU 255/P	2.60	abc
T 79/501	2.53	bc	T 79/501	2.51	bc
UF 676	2.18	c	UF 676	2.28	c
IMC 60	2.14	c	IMC 60	2.21	c

Figure 1: Scatter plot of the degradation mean index in relation to the mean number of bites

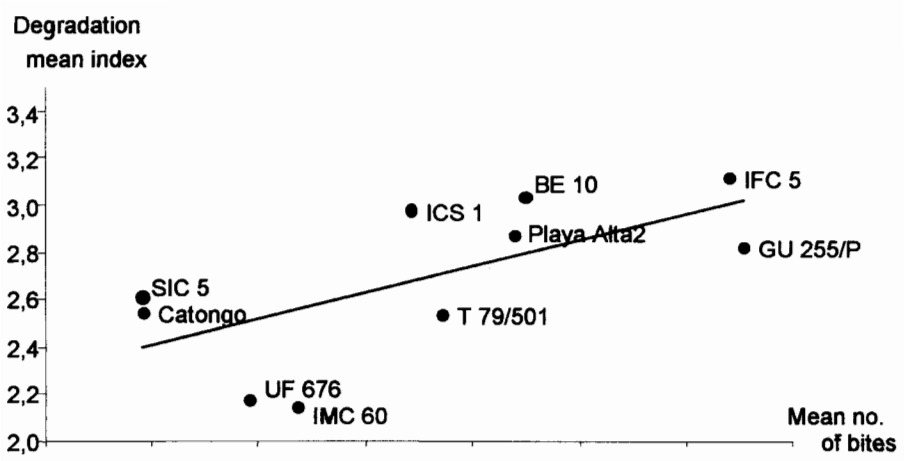


Figure 2: Scatter plot showing the % of dried branches without sprouting after one month in relation to the mean degradation index

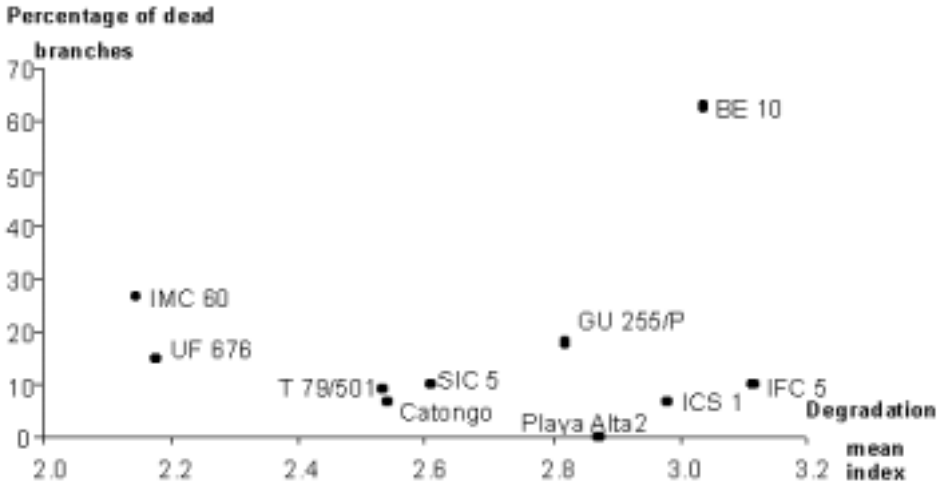


Figure 3: Pie Chart showing the percentages of the three categories of sprouting observed on each clone (black = normal terminal sprouting, grey = sprouting at the base only, and white = dried flushes without sprouting)

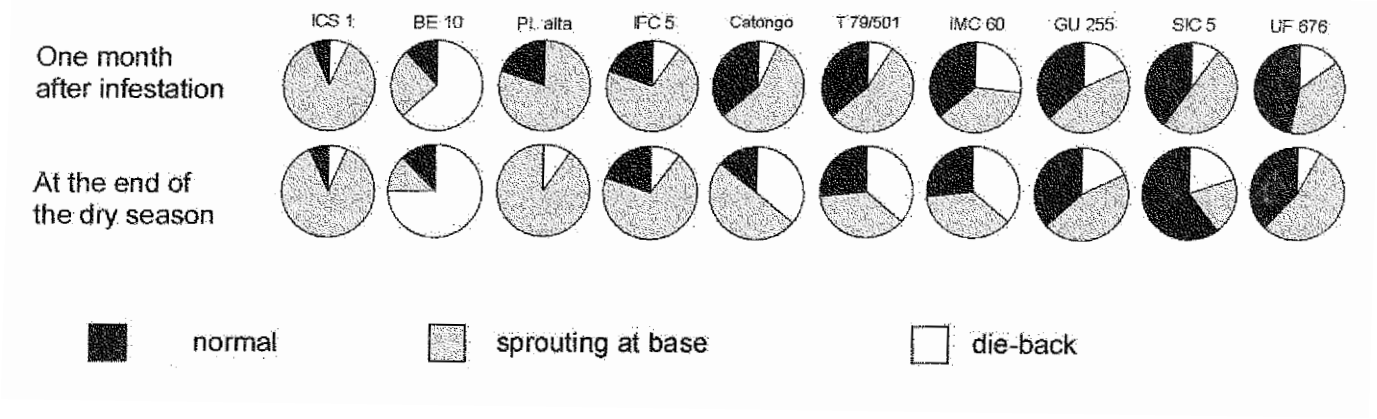
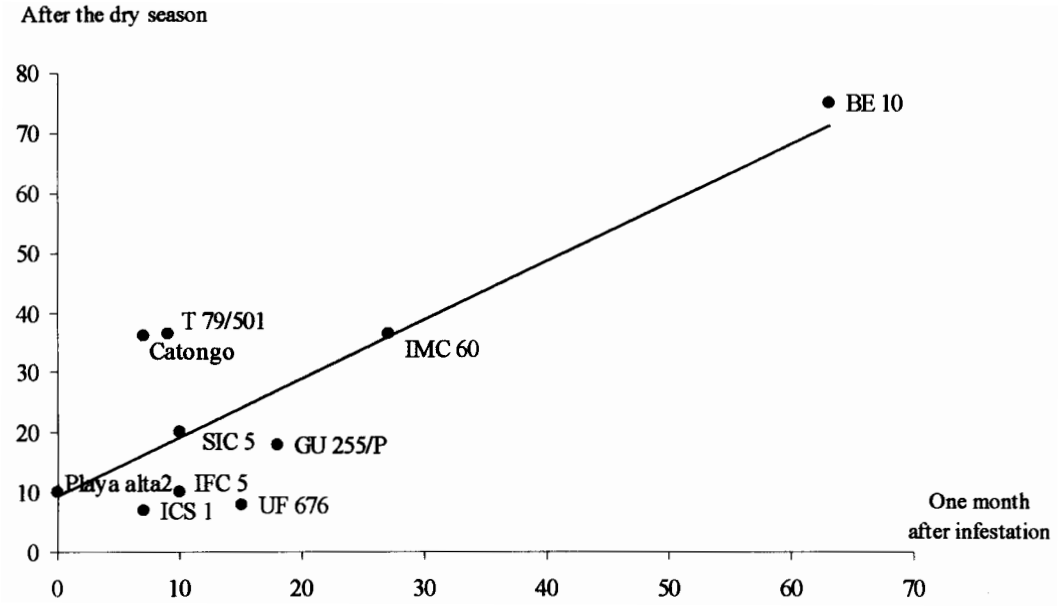


Figure 4: Scatter plot of the percentage of dried branches after the dry season in relation to the percentage of dried branches one month after infestation



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Selection in Terms of Pod Index and Disease Resistance of Promising Cocoa Trees in Peru

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3 Plant pathologist, UNAS, Tingo María, Perú

Introduction

In the Upper Amazon region of South America, the diversity of cocoa is large in wild as well as in cultivated populations. This valuable genepool is threatened by continuous genetic erosion, which will have unpredictable consequences.

In the 1990's, Peruvian cocoa production increased slightly, but productivity per ha remained stationary and with a tendency to decrease, with mean yields of about 375 kg dry cocoa ha^{-1} (García, 2000). This is related to the non-availability of improved varieties with high yield, resistance to the main diseases, better bean quality and wide adaptability.

Several researchers have reported on the narrow genetic base of clones and currently used cocoa hybrids (Cubillos, 1990; Lanaud *et al.*, 2001). Most of the cocoa cultivars have been derived from collections made by Pound in the Upper Amazon, and from Trinitarios selected in Central and South America (Cubillos, 1990).

UNAS maintains a collection of wild as well as cultivated cocoa genotypes (Ucayali and Huallaga accessions, along with introductions from other countries). Recent hybridisation and selection in segregating populations carried out at UNAS have made it possible to identify promising cocoa trees with low pod index and resistance to Witches' Broom (*Crinipellis perniciosa*) and possibly "Monilia" (*Moniliophthora roreri*).

Some results of the ongoing work at UNAS are presented hereafter. Results on dry seed weight suggest that transgressive segregation for this trait has occurred in some of the crosses.

Materials and Methods

Seedling progenies were planted in the Tulumayo's experimental station of UNAS in 1997 and observed until 2002. This site has a very humid-tropical forest climate, according to Holdridge's classification.

The genetic materials were "conventional" hybrids (crosses between introduced accessions), "regional" hybrids (Huallaga x Ucayali ; Huallaga x Huallaga and Ucayali x Ucayali crosses) and "miscellaneous" hybrids (Introduced x Ucayali and Introduced x Huallaga crosses). The parents were selected for their productivity features (PI = pod index) and disease tolerance. The inter-clonal crosses were made at the Cocoa Genebank of UNAS. The observation plots were comprised of 20 trees, spaced 3 x 3 m apart with intercropping bananas (*Musa* sp.) as temporary shade. One year later, we set up permanent shade with guava plants (*Inga edulis* L).

The assessment of yield potential of promising trees was made in the fourth and fifth year after planting using the pod index (PI = 1000 / SENU x SEWE), according to the Wood and Lass (1985) formula. Values of PI that were less than 15 were considered as very low, 15 to 22 as low, 22 to 33 as intermediate, 33 to 44 as high and above 44 as very high. The dry seed weight (with seed coat) of 30 seeds taken from 10 pods was obtained after oven drying of the wet seeds at 90 ^{o}C for 8 hours.

Preliminary assessment of Witches' Broom (WB) and Monilia (MO) attack was carried out until the fifth year. The presence or absence of WB on shoots, cushions and pods was scored. For Monilia, the percentages of infected fruits were recorded (number of fruits attacked/ total number of fruits). The assessment scale used was as follows:

1	resistant (R)	: 1 - 20%
2	moderately resistant (MR)	: 21 - 40%
3	intermediate (I)	: 41 - 60%
4	susceptible (S)	: 61 - 80%
5	highly susceptible (HS)	: 81 - 100%

Results and Discussion

In Table 1, the traits of seven promising cocoa trees are presented that highlight their high potential for having low pod index (PI). In particular, the selections SUP-1 (H-12 x ICS 6) and SUP-2 (IMC 67 x U 68) had very low PI values (15.5 and 15.8, respectively). The other selected trees had PI values ranging from low to intermediate, according to the proposed scale. The low values of PI are explained mainly by the contribution of very high dry seed weights.

Very low PI values have also been reported in Ecuador (Lizano, 1992) and in Costa Rica (Morera and Mora, 1992) in outcrosses with Upper Amazon clones (Silecia 1, SCA 6 and SCA 12) and (POUND 7 and PA 169), respectively.

Table 1: Pod index and reaction to Monilia (MO) and Witches' Broom (WB) diseases in seven promising trees

Selected trees	Cross progeny	SENU	SEWE	PI	Reaction to MO	Incidence of WB		
						S	C	P
SUP-1	H 12 x ICS 6	38	1.7	15.5	MR	A	A	A
SUP-2	IMC 67 x U 68	35	1.8	15.8	MR	A	F	A
SUP-3	U 12 x U 68	36	1.7	16.3	MR	A	A	A
SUP-4	U 68 x ICS 95	42	1.4	17.0	MR	F	F	A
SUP-5	U 43 x ICS 95	35	1.5	19.0	MR	F	A	A
SUP-6	IAC 1 x U 26	33	1.5	20.2	MR	F	A	A
SUP-7	ICS 1 x U 45	22	2.1	21.6	MR	F	F	A

SUP = Selection UNAS-Perú
SENU = seed number
MR = moderately resistant
P = pod
H = Huallaga
SEWE = dry seed weight
S = shoots
A = absent
U = Ucayali
PI = pod index
C = cushion
F = few (low)

The promising trees, SUP-2, SUP-3 and SUP-7, which had high dry seed weights (1.8, 1.7 and 2.1 g, respectively) could be transgressive segregants (Figure 1) because their parents had much lower dry seed weights (IMC 67 = 1.1g; U 68 = 0.7g; U 12 = 1.0g; ICS 1 = 1.3g and U 45 = 1.0g). This is surprising as the probability of finding such transgressive segregants for polygenic traits should be very low in small populations. This anomaly may be explained by the combining of relatively few favourable genes for seed weight.

Conversely, the low incidence of WB symptoms in these trees, both on vegetative and reproductive organs, was expected because of the parents used. U 43, U 68, U 45, U 26 and U 12 were collected for their moderate resistance or tolerance to this pathogen. Moreover, this response was corroborated later by observations in the UNAS collection by Arévalo *et al.* (1999). In addition, the IAC 1 clone has been reported to be resistant to WB in Brazil (Pires *et al.*, 1999) and the H 12 clone as tolerant (Coral, 1988).

With respect to MO incidence, this was low in all selected trees. Such behaviour could be explained by the presence of resistance genes and/or by relatively unfavourable conditions for disease development. However, there were similar observations for the parental clones U43, U68, U26, U45 and U12 in the Cocoa Genebank of Tingo María, where there were low percentages of diseased pods.

Likewise, the ICS 95 clone, which was used as parent in some crosses, has been reported as moderately resistant to WB (Phillips-Mora, 1999) and as resistant to MO (Brenes, 1983, cited by Enríquez and Soria, 1999), respectively. We think that these resistant cultivars might have contributed resistance genes to their progenies. Such findings make us optimistic about the chances of finding effective genetic control for these two diseases.

However, the concentration and spread of natural inoculum for WB and MO has not been high enough in the field plots for us to be fully certain of the disease reaction. It will be necessary to ensure conditions of higher inoculum pressure or to carry out inoculation tests to be able to confirm the observed resistance to these diseases. Such work will be carried out in the near future.

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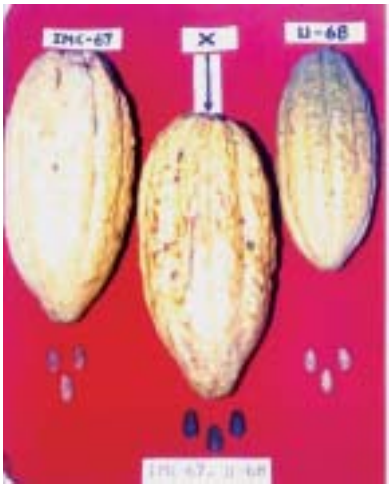
Figure 1: Transgressive segregant tree (M-18,18) derived from the IMC 67 x U 68 cross in Tulumayo's Station, Peru



INGENIC Study Group in the Molecular Biology of Cocoa

Mark Guiltinan

Department of Horticulture, Penn State University

Recent advances in molecular biology and the use of molecular markers in plant breeding have opened a new era for plant science research, the genomics era. In several laboratories throughout the world, efforts are being made to translate these advances to the field of cocoa research. The current progress in cocoa genomics was recently reviewed (Bennett, 2003). Together with other fields of cocoa research including breeding, biocontrol, integrated pest management, a complex international network of collaborating scientists and institutions has emerged, applying cutting edge science and technology in an integrated effort to improve the productivity of cocoa farming.

In order to support, promote and coordinate collaborative research in molecular biology of cocoa, a new INGENIC study group has been formed. This group was officially accepted at the last INGENIC workshop in Accra, Ghana in October 2003 by a vote of present INGENIC members. A steering committee and chairman of the committee were also elected. As part of INGENIC, the working group will benefit from enhanced linkage of molecular studies with plant breeding objectives. Some of the current collaborative projects involving members of the study group include:

1. ***Large scale sequencing of full length cDNAs from different tissues and genotypes of cocoa.*** The objectives are to sequence a large collection of cocoa full-length cDNAs. Project leader, Claire Lanaud, CIRAD, France; Collaborators: The Pennsylvania State University, USDA Beltsville and Miami; CEPLAC and DCB/UESC Brazil.

2. ***Use of molecular marker assisted selection for breeding of new cocoa cultivars.*** Development of molecular markers for various breeding projects. Project leader, Dr. Raymond Schnell, USDA, Miami, Collaborators: USDA-ARS, USA; Masterfoods Int.; CEPLAC, Brazil; CATIE, Costa Rica; EET, Ecuador; IITA, West Africa; CRU, Trinidad; CCRI, Papua New Guinea; GRIG, Ghana; The Pennsylvania State University.

3. ***Cocoa germplasm fingerprinting project: Standardisation of micro-satellite allele names and methods***. As part of the project, a ring test experiment is being conducted to unify and verify the reproducibility of results obtained by different labs using fingerprinting

techniques. Project leader, Dapeng Zhang, USDA-ARS, Beltsville, Collaborators: USDA-ARS, Miami, USA; Cocoa Research Unit, University of the West Indies, Trinidad; School of Plant Sciences, the University of Reading, UK; CIRAD, France and The Pennsylvania State University.

4. ***Creation and use of the INGENIC Cocoa Microarray.*** A cocoa microarray comprising of 3347 elements is being developed. This will allow the measurement of gene expression levels of many interesting cocoa genes simultaneously. Masterfoods Inc. supported oligonucleotide synthesis costs. Project leader: Mark Guiltinan, The Pennsylvania State University, Collaborators: USDA Beltsville and Miami; CIRAD, France; Masterfoods, UK.

5. ***Genomics of Cocoa-Crinipellis interactions***. This programme is sponsored by FAPESB, BNB and CNPq in Brazil. Genes expressed during the interaction of cocoa with Witches' Broom are being studied. Project Leader, Julio Cascardo, Collaborators: UESC, CIRAD, UNICAMP CEPLAC and BIOFABRICA.

How to become involved: We invite all interested scientists to participate in this working group. To become a group participant, simply sign up for the email discussion group and you will be included in all communications within this group.

Instructions to sign up for INGENIC MOL BIOL

INGENIC-Mol-Biol is the working group on cocoa molecular biology, a subgroup of the International Group for the Genetic Improvement of Cocoa (INGENIC). For more information about this list, contact Mark Guiltinan (mjg9@psu.edu) or Siela Maximova (snm104@psu.edu), Department of Horticulture at Penn State. An archive of messages is available at http://lists.cas.psu.edu/read?forum=ingenic-mol-biol

To subscribe to the INGENIC-Mol-Biol list, send a blank email to:

join-ingenic-mol-biol@lists.cas.psu.edu.

To unsubscribe from the INGENIC-Mol-Biol list, send a blank email to:

leave-ingenic-mol-biol@lists.cas.psu.edu.

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AGORA Update- May 2004

Rapid Adoption of "Access to Global Online Research in Agriculture"

Submitted by Mark Guiltinan

AGORA is the new Internet gateway that provides eligible institutions in 69 low-income countries with free access to over 500 international journals in agriculture, both in the biological and social sciences. Launched just six months ago, AGORA is a collaborative initiative led by FAO, in partnership with the world's leading academic publishers, Cornell University and the World Health Organization (WHO). Since AGORA went live last October, over 260 national agricultural research organisations (NARs) and universities in 50 countries have registered. (See list of eligible countries below). For the first time in history, students, faculty and scientists at these institutions have access to the latest scholarship in the field of agriculture, equivalent to what is available to their peers in developed countries. There are now 20 publishers contributing content, including a growing number of non-commercial publishers like the AAAS, National Academy of Sciences, and American Society of Plant Biologists. Each institution is assigned a unique username and password, which can then be shared with all affiliated students and staff. All of the NARs in qualifying African countries are now registered. Kenya, Moldova, Nigeria and Viet Nam are among the countries with the most number of institutions registered.

Mann Library at Cornell University is working together with FAO in AGORA training and outreach. The purpose of this update is to encourage all faculty at U.S. universities and partners overseas in agricultural development to promote greater awareness of AGORA. We urge you to browse the AGORA website at www.aginternetwork.org and contact us if you have any questions. A number of Land Grant universities have helped partner institutions overseas acquire the CD-ROM journal collection TEEAL (The Essential Electronic Agricultural Library), which is produced by Mann Library. Where bandwidth limits AGORA's use, which is the case at many African NARS and universities, TEEAL remains the most reliable and comprehensive source of journal literature. For more information about either program, please email Mary Ochs, TEEAL/AGORA Project Manager, Mann Library at mao4@cornell.edu. We would welcome your comments, feedback, questions.

AGORA-Eligible Countries (annual per capita Gross National Income of $1000 or less)

Africa

Angola, Benin, Burkina Faso, Burundi, Cameroon, Central African Republic, Chad, Comoros, Congo, Côte d'Ivoire, Dem. Rep. of Congo, Djibouti, Eritrea, Ethiopia, Gambia, Ghana, Guinea, Guinea-Bissau, Kenya, Lesotho, Liberia, Madagascar, Malawi, Mali, Mauritania, Mozambique, Niger,

Nigeria, Rwanda, Sao Tome & Principe, Senegal, Sierra Leone, Somalia, Sudan, Tanzania, Togo, Uganda, Zambia, Zimbabwe

Asia-Pacific

Afghanistan, Bangladesh, Bhutan, Cambodia, Dem. Rep. of Timor Leste, Kiribati, Lao People's Dem. Rep, Mongolia, Myanmar, Nepal, Papua New Guinea, Solomon Islands, Tokelu, Tuvalu, Viet Nam

Central and Eastern Europe & the Caucasus

Albania, Armenia, Azerbaijan, Georgia, Kyrgyzstan, Rep. of Moldova, Tajikistan, Turkmenistan, Ukraine, Uzbekistan

Latin America & the Caribbean

Guyana, Honduras, Haiti, Nicaragua

West Asia

Yemen

Book Release

Author: Dias L.A.S. (Ed.).
Title: Genetic Improvement of Cacao

Source: Editora Folha de Viçosa Ltda. xii + 578 pp. Translation by Cornelia Elisabeth Abreu-Reichart, Viçosa, M.G., Brazil; aided by the Editor and Peter Griffee of FAO and supported by FAO.

Abstract

In world literature to date there has been no work which deals exclusively with cacao genetic improvement. Until now, all initiatives in this respect have not gone beyond chapters inserted in books on the crop's agronomy or on genetic improvement of the species in general. This work, on the contrary, offers to be the first book which treats the subject exclusively and in depth; unique in the world. The original is in Portuguese, and the contributions are from mainly Brazilian scientists. Much of it is universally applicable to tree species, but it is focused on questions and solutions in Brazilian cacao cultivation. The objectives which drove the initiative were to: i) make available the accumulated knowledge to all the scientific community; ii) fuel the debate on the subject for all interested sectors (scientists, extension workers, students and producers, and iii) give more visibility to the scientific data on the subject, which otherwise would be restricted to a group of national and international scientists, who work in a few cacao research institutions around the world. This translation will greatly amplify accessibility globally.

The collaborators are internationally renowned international scientists in their specialities; all belonging to research institutions of national or international prestige. They were encouraged to give light to innovations on the state of the art of cacao improvement. Conceived to be encompassing and, at the same time, as profound as possible, the work is highlighted by its logical sequence and clarity of the themes developed in its 13 chapters. In Ch. 1, the principal aspects of cultivation and the strategies of environmental improvement are presented. In order to overcome the crisis which assails the cocoa economy, it deals with the socio-economic panorama that predicts changes of attitude of producers, researchers and institutions. Chs. 2, 3, 4 and 5 basically cover the collection, conservation and rational use of genetic resources of *Theobroma*, the genus to which cacao (*Theobroma cacao* L.) belongs. The diversity in *Theobroma* is focused on in Ch. 2 with a view to improvement by incorporation of genes from wild species into the genetic make up of the cultivated one. Ch. 3 presents a new scenario for the origin and distribution of cacao, with important reflections on the collection and conservation of germplasm. The ecology of natural populations in its most diverse aspects is dealt with in Ch. 4. How to collect, conserve, evaluate, characterise and use germplasm saved are topics developed in Ch. 5.

From Ch. 6 onwards, the book focuses on the actual genetic improvement. For the first time, the methodology of mixed mathematical models is introduced to cacao breeding, with a view to making it more precise and efficient. Chs. 7 and 8 cover the state of the art of resistance to diseases, in particular Witches' Broom, emphasising the heredity mechanism and the biochemical and physiological bases of this resistance. Asexual breeding is highlighted and covered in Ch. 9. The introduction of molecular markers in breeding and the possibilities open for these new tools are reported in Ch. 10. Another grey area, never really covered in cacao breeding, (Ch. 11) is research. In Ch. 12, breeding success is illustrated by the comparative results of improved cultivars against traditional ones. Finally, Ch. 13 capitalises on all improvement aspects, harmoniously integrating sexual and asexual improvement and biotechnology to project the future of breeding programmes.

Abstracts of some recent publications

Presented by Antonio Figueira

Mating System in a Natural Population of Theobroma grandiflorum (Willd. ex Spreng.) Schum. by microsatellite markers

Rafael. M. Alves; Angela S. Artero; Alexandre M. Sebbenn and Antonio Figueira. Genetics and Molecular Biology 26 (3) : 373-379. 2003. Brazilian Society of Genetics. http://www.scielo.br/pdf/gmb/v26n3/a25v26n3.pdf

The aim of this research was to study the mating system of a natural population of *Theobroma grandiflorum* (cupuassu)

from Nova Ipixuna, Pará state, using microsatellite markers. Eight polymorphic microsatellite loci were analysed in eight families, each represented by 10 six-month old seedlings derived from open-pollinated pods. The estimation for the multilocus outcrossing rate ($\hat{t}_m$=1.0) and individual outcrossing rate ($\hat{t}$=1.0) for this population suggests that *T. grandiflorum* may be a perfect outbreeding (allogamous) species. Likewise, for the studied population the estimate for single locus outcrossing rate ($\hat{t}_s$) was elevated (0.946), but lower than $\hat{t}_m$, confirming the likely outcrossing character of the species and suggesting the occurrence of a 5.4% biparental inbreeding rate ($\hat{t}_m$ - $\hat{t}_s$). The estimation of genetic divergence ($\hat{F}_{ST}$) between allelic frequencies in ovules and pollen revealed a deviation from random mating in 75% of the evaluated loci. Likewise, the estimate of correlation of paternity ($\hat{r}_p$= 0.930) and the mean coefficient of co-ancestrality within families ($\hat{\theta}_{xy}$ = 0.501) indicated that the outcrossings were predominantly correlated, and the offspring were full-sibs. These results suggested that for this particular population of *T. grandiflorum*, the sampling strategy for genetic conservation and breeding should adopt specific models for families derived from correlated outcrossing (full-sibs) and not the ones usually adopted in classic breeding programmes of outcrossing species (half-sibs).

Selection of morpho-agronomic descriptors for Theobroma grandiflorum germplasm characterisation

Rafael Moysés Alves; Antonio Augusto Franco Garcia; Eniel David Cruz and Antonio Figueira. Pesq. Agrop. Bras., Brasilia, Vol. 38, n7, p 807-818. July 2003. http://www.scielo.br/pdf/pab/v38n7/18202.pdf

The objective of this work was to select morphological and agronomic quantitative descriptors to characterise accessions of *Theobroma grandiflorum* (Willd. ex Spreng.) Schum. Fifty-three descriptors were evaluated, including 14 from leaves; 18 from flowers; 16 from fruits; and 5 agronomic traits. To discard redundant or non-discriminating descriptors, a two-step multivariate analysis of principal components was applied. The first phase included the selection of descriptors within each group of characteristics individually (leaf; flower; fruit; agronomic). Based on the descriptors selected in this first phase, a joint analysis of principal components was performed for the final selection. Three criteria for variable exclusion were adopted. Thirty-four descriptors were excluded, representing a reduction of 64%. A minimal list of descriptors for cupuassu was proposed, including leaf length; leaf thickness; leaf apex width; leaf base vein angle; flower bud length; flower peduncle length; flower peduncle diameter; ovary girth; number of ovules; flower petal lamina length; staminode length; seed width; flat seeds; acidity; brix ; pH; number of fallen flowers buds; number of abscised immature pods; and number of Witches' Brooms. Based on the selected 19 descriptors, it was possible to estimate the variability of accessions of the Cupuassu Germplasm Collection, Belém, using the average Euclidean distances and clustering by UPGMA and Tocher.

INGENIC GENERAL ASSEMBLY

Minutes of the Meeting held October 20th, 2003 at the Miklin Hotel in Accra, Ghana

Chairman: Bertus Eskes, Secretary: Frances Bekele

1. Minutes of the previous INGENIC General Assembly Meeting (GA), held on 16th October 2000 in Kota Kinabalu, Malaysia, were read by INGENIC's Vice-Chairman, Dario Ahnert, and were adopted.

2. The Editor presented her report on the status of the Newsletter. She reported that INGENIC had released the eighth issue of its Newsletter, in July 2003. It was noted that the newsletter had proven to be effective as a vehicle of communication, and in fostering links among cocoa breeders, geneticists and institutes. Thus far, roughly 78 articles covering a broad range of topics including breeding strategies, genetics and breeding studies, germplasm evaluation and diversity studies, and collaborative research had been featured, representing research or activities in 15 countries. The Editor expressed the appreciation of the INGENIC Committee for this vital input, and for the encouragement of the readership.

The Editor also highlighted the following:

- A suggestion was made last year that articles could be peer reviewed so that the newsletter could attain the status of a journal. The Editor's response to this was that the informal nature of the newsletter encouraged researchers to report their findings in a timely manner. These authors had the option to later refine their contributions into more detailed scientific reports suited for inclusion in journals.

- The production of the newsletter is a consultative process involving several members of the INGENIC Committee, and not just the Editor. By consultation, the Committee decides not to include any article considered at odds with INGENIC's mandate to promote useful discussion in cocoa genetics/breeding in an atmosphere of mutual respect and support. This does not preclude healthy debate. The Committee is grateful for the numerous constructive commentaries and responses to earlier articles received. E-mail addresses of authors are included in the newsletter to foster direct dialogue among interested parties.

- It has become necessary to limit the length of contributions in order to retain the nature of a newsletter, and to curtail the cost of production. Brief articles and short reports are invited, along with a feature article(s) for each issue, which may be peer reviewed.

More cocoa scientists were encouraged to avail themselves of this opportunity to share highlights of their research findings, and in so doing benefit from the experience of

others, and prevent duplication of effort. A special invitation was extended for news from supporting institutions, on collaborative research, and of upcoming events. The Editor underscored the point that the newsletter can only be dynamic (catering to our growing needs) with the input of all of the stakeholders. Those present were encouraged to contribute towards the future development of the newsletter, and to making it serve them better. The financial and/ or logistical support of the BCCCA, CIRAD, CRIG, CRU, MCB, UESC and WCF were recognised.

There was a suggestion (Raul Valle) that INGENIC publish its newsletter on a website or circulate it through a Yahoo newsgroup. The Chairman confirmed that this had already been discussed by the INGENIC Committee, but that a website host would have to be identified. Mark Guiltinan of Penn State University, U.S.A. volunteered to host the newsletter on a website managed by his University. The Committee agreed to study this kind offer.

The issue of the type of medium to be used for disseminating the Newsletter and the Proceedings of the Workshop was discussed. The consensus of opinion was that hard copies should be retained to facilitate those with limited access to computer facilities. The INGENIC Workshop Proceedings would also be released on CD-ROM, and the newsletters would be available on the Penn State website as pdf files. It was decided that the option to produce fewer hard copies for those requesting them would only be pursued if this were cost-effective.

The INGENIC Committee was complimented on its success in producing the newsletter (Rob Lockwood), but was urged to communicate with the publishers and Editor of the *Cocoa Growers' Bulletin* to notify them of the widespread interest among cocoa researchers to have that medium of communication maintained. It was emphasised that while the newsletter was an informal vehicle for disseminating news on cocoa breeding and genetics, the Cocoa Growers' Bulletin served as a cocoa journal. It was argued that if the Bulletin were discontinued, then the onus would be on the INGENIC Newsletter to fill the void. It was agreed that contacts would be made with the editor of the Bulletin, Mr. Tony Lass, to exchange proposals that could be of mutual benefit for both publications.

It was agreed that the newsletter would adopt a structure involving defined sections featuring items from various organisations within the cocoa breeding and genetics community such as the Reading Quarantine Station, The International Cocoa Germplasm Database, the Cocoa Research Unit and the International Cocoa Genebank, Trinidad, and the newly formed Study Group in the Molecular Biology of Cocoa.

The Chairman commented that only 50% of those on the 2002 INGENIC Mailing list had responded to his request to indicate their interest in having their names retained on the Mailing List. The assembly recommended that the non-responding members be contacted again, and that their names should be removed from the list if there were no response.

3. The Chairman delivered the Treasurer's report in the absence of Dr. Michelle End, and presented a summary of the INGENIC Accounts for 2000-2003, and the estimated Accounts for 2003-2006. It was stated that INGENIC had sufficient funds to cover its current activities including the 4th Workshop and the publication of the 9th issue of the Newsletter, but that more funds would likely be required to continue activities beyond 2004.

In keeping with its objective to encourage long-term cocoa breeding research, INGENIC secured funding for six young scientists to attend the 4th INGENIC Workshop. Five such researchers had been sponsored to attend the 3rd INGENIC Workshop.

INGENIC had not been able to pursue the offer of ACRI (now the World Cocoa Foundation - WCF) made in 2000 to audit the INGENIC accounts due to a change of the Director of Scientific and Regulatory Affairs of WCF. However, the Chairman reported that the current Director of the WCF, Dr. Bill Guyton, has reaffirmed the offer, and INGENIC will pursue this.

4. INGENIC's involvement in new collaborative initiatives:

4.1 The Chairman described the interaction of INGENIC with the *Global Programme for Sustainable Cocoa Production*. He indicated that even though INGENIC is not an institute or formal group, it had been invited to submit a list of priorities for the Global Cocoa Programme. INGENIC had formulated three proposals for consideration:

- *Cocoa Germplasm Conservation, Evaluation and Exchange;*
- *Development of More Efficient (Compact) Cacao Trees; and*
- *Regionally Co-ordinated Cocoa Breeding Programmes, including Participatory Selection of New Varieties.*

The research presented at the INGENIC Workshop and evident collaboration among participants were commended by a representative of chocolate manufacturers (Roger Dehnel). It was recommended that the needs of the end-users be well incorporated into cocoa research projects.

4.2. Dr. Mark Guiltinan of Penn State University, the newly elected Chairman of the INGENIC Study Group in the Molecular Biology of Cocoa (INGENIC-Mol-Biol), reported on the conclusions of the Meeting of the Group held on the previous day. He stated that the Group's goal is to promote collaboration among researchers in Molecular Biology. Three collaborative projects already in progress were outlined. They involve creation of EST libraries, micro-array studies, and large-scale cDNA sequencing, respectively. A Strategic Committee for the Study Group was formed with representatives from Penn State University, Masterfoods, CEPLAC, UESC and CNRA, Côte d'Ivoire, and was due to meet shortly under the auspices of USDA.

It was proposed that INGENIC-Mol-Biol become a sub-group of INGENIC, and that Dr. Guiltinan become a member of the INGENIC Committee to act as a liaison between the umbrella group, INGENIC, and the sub-group. This proposal was accepted by the Assembly.

5. Other new collaborative activities:

5.1. The Chairman announced that he had just been informed that the new Project Proposal on *Cocoa Productivity and Quality Improvement: A Participatory Approach*, which was submitted for approval to the Common Fund for Commodities, had been approved, and financing had been allocated for the first 1.5 years of the Project. This news was greeted with enthusiastic applause.

5.2. Dr. Maria Kolenosikova-Allen of IITA, Nigeria informed the Assembly of a new initiative in West Africa, involving collaboration among IITA, USDA in Miami, and cocoa research institutes in Côte d'Ivoire, Cameroun and Nigeria. The objective is to acquire knowledge of the existing diversity of cacao germplasm in this region. The emphasis is on molecular characterisation of breeders' selections and selections from farmers' fields. Morphological and quality characterisation data are also being collated. All of the information acquired will be made available to cocoa breeders worldwide.

6. The composition of the INGENIC Committee was brought up for debate. The Chairman informed the gathering that all of the Committee positions were up for election. Nominations for replacements for the existing Committee were invited, but none was forthcoming. It was accepted that the current members would retain their seats till the next GA, and that Dr. Guiltinan would join the Committee to represent INGENIC-Mol-Biol.

7. It was agreed that the INGENIC Committee would retain its informal structure.

8. Forthcoming events:

The Theme for the 5th INGENIC Workshop was discussed. Dr. Yaw Adu-Ampomah, Vice-Chairman, presented the theme formulated by the Committee prior to the General Assembly (GA), which is: *Cocoa Breeding to Meet Farmers' Needs*. This theme would embrace an assessment of the impact of breeding programmes at the farmers' level, participatory breeding efforts, and methods used in practical breeding programmes. The Chairman commented that the new CFC/ICCO/IPGRI Project might generate much data on this theme.

It was recommended that consideration be given to the production systems employed by farmers (Raul Valle). In addition, since the 15th Conference might be held in a consuming country, one participant (David Allaway) recommended that *Breeding for Consumer Needs* could be a relevant topic. Another proposal was *Sustainability of Cocoa Production* – a consideration of agro-forestry systems-since this would ensure a stable income for farmers (Howard Shapiro). The need to focus on the farmers' needs was reiterated by Dario Ahnert.

Dr. Adu-Ampomah advised that discussion on the theme for the next Workshop should continue before a final decision is taken.

9. Other business:-

9.1. Mislabelling of cacao germplasm.

The Chairman invited opinions on whether INGENIC should intercede in this problem. The Head of CRU, Dr. David Butler, informed the gathering that he was preparing a document on the status of the problem together with Prof. Paul Hadley of Reading University. It was decided that the INGENIC Newsletter could be used to disseminate the findings of this research.

9.2. Fingerprinting of Pound's collections

Juan-Carlos Motomayor informed the Assembly that a meeting would be convened in November to assemble fingerprinting data on Pound's collections, and to decide on the necessary action to follow.

9.3. It was recommended (Jeanne N'Goran) that future INGENIC Workshops should include more time for discussion since there were time constraints for this during the 4th Workshop. The Chairman agreed that this was always the intention of the organisers, but explained that there were many papers to be presented in a relatively short period of time.

9.4. The INGENIC Committee was acknowledged for its work, (Yoel Efron), and the Chairman expressed appreciation for this on behalf of the Committee, and pledged that it would continue to work towards fulfilling its mandate.

The Meeting culminated with the announcement from a representative from the chocolate manufacturing industry (Martin Gilmour) that the World Cocoa Foundation had publicly recognised the contribution of the Chairman of INGENIC, Dr. Albertus B. Eskes, towards ensuring the sustainability of cocoa production through his co-ordination of collaborative cocoa breeding initiatives. Dr. Gilmour then presented the Chairman with a plaque on behalf of the WCF.

The Meeting was concluded at 19.00 hours.

Minutes of the Meeting of the *Ad Hoc* INGENIC Working Group on Enhanced Regional Collaboration for the Improvement of Cacao held at La-Palm Hotel, Accra, Ghana on October 21st, 2003

Prepared by David Iwaro

In Attendance

Bertus Eskes	-	CIRAD, France (Chairman of the meeting)
Yoel Efron	-	CCRI, PNG
Badaru Kolawole	-	CRIN, Nigeria
Wilbert Phillips	-	CATIE, Costa Rica
Dario Ahnert	-	UESC, Brazil
Uilson Lopes	-	CEPLAC, Brazil
Juan C. Motamayor	-	USDA/Masterfoods, USA
David Iwaro	-	CRU, Trinidad and Tobago (Secretary of the meeting)

Background

Based on suggestions put forward during the INGENIC workshop, held on 20-21 October 2003 at the Miklin Hotel, Accra, Ghana, an *ad hoc* Working Group was formed to start discussions on enhanced regional collaboration for the improvement of cacao. The group met over dinner at La-Palm Hotel on 21st October, 2003 and deliberated for about 3-4 hours. Topics discussed were the benefits, needs and feasibility of setting up regional collaborative cacao improvement programmes, networks or projects addressing regionally important problems. The group also discussed briefly the possible mechanism for coordinating regional breeding activities.

Benefits of enhanced regional collaboration

The Working Group noted that each cacao producing region (America, Africa and Asia) has important production constraints in common that can be dealt with more efficiently by regionally coordinated research and development efforts than by efforts carried out by individual research institutes, that lack resources to do so. Such collaboration would foster good working relationships among regional cacao research institutions and thereby create opportunities to address common problems.

Specific benefits identified were:

- Facilitating the planning and implementation of long-term regional cacao breeding efforts (*e.g.* selection of high yielding clones with good resistance to regionally important diseases and pests, population improvement schemes, etc.);
- Facilitating the pooling of the available resources, thus enabling more cost-efficient use;
- Reducing duplication of research efforts within each region;
- Enforcing and promoting continuity of breeding programmes, which is the first requirement for obtaining significant and durable results;
- Allowing for sharing of the end products of breeding programmes within each region.

Requirements for defining regional programmes, projects or networking mechanisms

The Working Group agreed that enhanced regional cacao breeding efforts should be focused on providing better planting material for farmers. Most of the participants agreed that creation of mechanisms that build on the existing institutions and ongoing initiatives would be preferred over the creation of new structures (such as "regional cacao breeding centres").

The following requirements for the development of more concrete proposals were agreed:

- Need for identification of priorities in the area of cacao breeding to be addressed at the regional level, involving all interested stakeholders in the region. A better understanding of the individual government policies on cocoa improvement and on regional collaborative activities may be required;
- Identification of ongoing research activities and existing resources within each region;
- Identification of the need for complementation of existing resources and efforts to be able to achieve common goals;
- Formulation of regional programmes, projects and/or networks with clear objectives;
- Identification of the roles of the national, regional and international research and development institutions in the establishment and implementation of enhanced regional collaboration in cacao breeding;
- Development of working strategies and concrete plans which allow for the sharing of activities among the participating institutions based on comparative advantages and availability of resources;
- Identification of tentative budget requirements and possibly interested donors to complete regionally available resources;
- Identification of mechanisms for regional coordination. One suggestion put forward was to create coordination units that could use the facilities of one of the existing institutions as a basis for operation in each region;
- Identification of a mechanism for coordination, at the international level, between regional programmes, projects or networks.

Next steps

It was agreed that initial proposals would be elaborated within the next five months for each of the regions: America, Africa and Asia. These proposals would try to address the above-mentioned requirements at least in a general manner. The

group requested the following persons to coordinate the further development of such regional plans:

Region	*Coordinator*	*Institute*
Asia & Pacific	Yoel Efron	CRI, PNG
Africa	Yaw Adu-Ampomah	CRIG, Ghana
America	Juan Carlos. Motamayor	USDA/Masterfoods, USA

The Working Group requested that the above identified persons should consult with all cocoa research and development institutions, or other organisations, that have activities in the region to identify priorities for regional programmes and/or projects to present a possibly agreed mechanism or alternative mechanisms for carrying out these activities and also to present a preliminary idea of financial requirements.

The Working Group further requested Bertus Eskes, as chairman of INGENIC, to act as general coordinator for this initiative, with the task of stimulating the development of these plans and exchanging information between the regions and with other interested parties.

It was agreed that the regional proposals would be presented in a meeting to be organised on the occasion of the next CFC/ICCO/IPGRI project workshop (28 March - 3 April 2004 in Reading, UK). For this meeting other interested parties should be invited.

At the end of the meeting, the Working Group agreed that the minutes of the meeting be forwarded to Bertus Eskes, who would contact the "regional coordinators" for further action on the matters discussed at the meeting and also inform a wider INGENIC public of the outcome of the meeting.

Regional Collaboration in Cocoa Breeding

A note on the Meeting held April 3rd 2004 at The University of Reading, UK

Chairman: Roger Dehnel, Secretary: Michelle End

This meeting was organised on the occasion of the CFC/ICCO/IPGRI project workshop (28 March - 3 April 2004 in Reading, UK) following the suggestion made at the *Ad Hoc* INGENIC working group on *Enhanced Regional Collaboration for the Improvement of Cacao* meeting described above. The meeting was well attended with representatives from the institutes participating in the CFC/ICCO/IPGRI project, industry and donor organisations. The chairman gave an introduction covering the background to the meeting and an outline of some of the potential benefits that a regional approach could bring to cocoa breeding (as above). There was general agreement on these, particularly to the opportunities to share research and expertise, and for the creation of synergies through a multidisciplinary approach. The discussion moved on to design aspects and the need for resources and funding. Participants highlighted possibilities for linking regional programmes to existing collaborative efforts such as the CFC/ICCO/IPGRI regional variety trials, the STCP and SCPS initiatives in West Africa and to research centres where multi-national activities were already taking place. Some participants favoured a virtual base for a regional programme, at least initially, which might facilitate a rapid start to activities without heavy administrative and infrastructure costs. However, it was acknowledged that funds would be needed to maintain trials in the longer term in order to exploit their full potential.

The Chairman invited the three coordinators, nominated at the previous meeting, to give presentations on the possibilities for collaborative efforts in their regions.

Latin America

Dr. Juan Carlos Motamayor outlined how he had contacted cocoa breeders in the Americas and found much support for the creation of an initiative to continue efforts to coordinate long-term trials and consolidate international and regional efforts. Needs identified included; sharing of information, germplasm exchange, support for genetic analysis to exploit trial data, and preventative breeding against the risk of the spread of disease from area to area. The need for funding to support long-term and advanced trials was highlighted. The group had proposed that a coordinator and scientific committee be established, which might include a full-time breeder. The breeding objective would be to produce improved materials with characteristics which might include high yield, resistance to Monilia, Witches' Broom, homogeneity, fine flavour and suitability for high density planting. Specific objectives included improved information availability and management through genetic fingerprinting and web-based databases, genetic resource management and screening for useful characteristics and collaboration in research to establish and use early screening tests for disease reaction and incompatibility status. The aim would be to enhance existing breeding efforts and coordinate plans for mid and long-term efforts. The scientific committee would have a role in analysis of national plans and resource allocation to support advanced trials.

Africa

Dr. Yaw Adu-Ampomah gave a presentation on the possibilities for regional collaboration in Africa. He outlined proposals to a) transfer existing technology to farmers and b) develop new varieties. For the technology transfer, it was suggested that 10 clones from each country be assessed in trials for release, as clones, to farmers. Objectives for the breeding work would be to produce improved varieties with a broader genetic base which incorporate resistance to diseases not yet present in the region, with associated activities focusing on the management and utilisation of genetic resources, new techniques to accelerate breeding

and improved agronomic and disease control practices. A suggestion was made by Dr. Weise that the STCP could facilitate the coordination by organising a meeting for 3-4 representatives from each institute for further discussions.

Asia

Dr. Yoel Efron gave an introduction to the problems facing cocoa farmers in South-East Asia and the need for a long-term coordinated breeding programme to address their needs. He suggested that although a regional programme based on links between existing institutes might have advantages in that it would strengthen these institutes and be relatively inexpensive to manage, thus appealing to both donors and national governments, it would be dependent on the existence of strong and capable institutions with adequate facilities and resources and might lack the independence, flexibility and freedom from conflict of interest of a newly created regional centre. He suggested that such a centre might offer the best technical solution for high quality long-term research and could be established for a relatively modest investment, the main needs being the right staff, land and transport, especially since the outputs from the fields could cover much of the ongoing costs. He proposed the establishment of a combined regional breeding centre in partnership with the region's cocoa producing countries, and their existing institutions. The activities of the centre would be to collect and conserve the region's germplasm, develop heterotic populations, develop improved screening and breeding methodologies and place major emphasis on population improvement. The centre would be responsible for distributing germplasm, organising and co-ordinating breeding trials, resource allocation, training and cooperation with other regional efforts. He suggested that the centre should be located in an area such as Sulawesi, Indonesia, where the cocoa would be exposed to all the region's pests and diseases, and where conditions of security, stability and infrastructure would not limit progress.

Closing Summary

The presentations were followed by discussions on the relative merits of the creation of new regional centres compared to linking to existing institutes and/or virtual centres, with many participants concerned that a new centre would compete for funding with existing national programmes/ institutes. However, the Chairman summed up by saying that the meeting gave general support for the concept of regionalisation of breeding activities and that differences in the views on how this could best be achieved might be driven by the specific situations in the regions. It was possible that different approaches might be taken in each region, with Africa and Latin America currently favouring a collaborative approach between existing institutes, whereas Asia might base its collaboration around a new breeding site in Sulawesi. He suggested that an evolutionary approach should be taken to build on existing infrastructure, starting small but with potential to grow. The chairman noted that some exploration of self-funding of activities (through cocoa sales) or a broader role than just breeding (to include training, farmer participation, agronomy) would be appropriate but may come as a future refinement of the concept. The regional approaches should be linked to other stakeholders, including farmers, and this could be addressed by links to other organisations, for example STCP. He concluded that the regionalisation of this work might make it more attractive to donors.

Next Steps

It was agreed that the next steps should be:

1) Focus on team structure and operating principles
2) Formalise who constitutes the core of each regional team (Dr. Adu-Ampomah, Dr. Efron and Dr. Motamayor to lead)
3) Meet as regional groups, including stakeholders, and seek opportunities to report activities into bodies such as World Cocoa Foundation (meetings in April and October 2004)
4) Africa group to meet facilitated by Stephan Weise of STCP.
5) Each regional head to investigate and summarise the existing framework of restrictions concerning germplasm and intellectual property in their region.

Obituary

Hille Toxopeus
11 December 1932 to 5 July 2003

Bertus Eskes and Kolawole Badaru

Hille embodied and transmitted, through his enthusiasm, the typical cocoa breeder "genotype", *i.e.* passion for the plant that has been a precursor for its second nature of "once a cocoa breeder, always a cocoa breeder"! We express our gratitude to Hille for the important contributions he made to cocoa breeding and for the characteristic enthusiasm with which he constructively participated in the international cocoa research community activities, including those of INGENIC. Although necessarily incomplete, we wish to use this note to highlight some of his contributions to cocoa breeding.

Born on December 11, 1932 in Buitenzorg, Indonesia, he was kept in a camp under the Japanese occupation during the second world-war, separate from his parents, brother and sister, who were kept in two other camps. The family came to the Netherlands in 1946. Hille's father, who was a well-known potato breeder in Wageningen, may have stimulated Hille to

study plant breeding at the Agricultural University of Wageningen. Hille met his wife during his practical stage, working on citrus, in South Africa. He graduated in 1961 and left soon afterwards for Nigeria, working first with the West African Cocoa Research Institute (WACRI), and later with the Cocoa Research Institute of Nigeria (CRIN), created in 1964 after the independence of Nigeria, till 1969. He was part of a very active team of researchers, including Nigerians (*e.g.* Drs. L.K. Opeke and L. Are and J.A. Williams), Dr. V.J. Jacobs, and Dutch researchers (*e.g.* Drs. M. Wessels and G. Westeijn), dedicated to cocoa breeding, agronomy, disease resistance and pest control. During this period, Hille developed a comprehensive view of cocoa breeding, starting with the management of genetic resources, the origins of cocoa varieties, and on to how to conduct long-term breeding programmes using multi-trait selection. After his "Nigerian experience", he worked for 26 years until his retirement as a plant breeder on several crops, mainly Brassicas, at the former "Foundation for Plant Breeding (SvP)", presently part of "Plant Research International", in Wageningen, the Netherlands. In between, he returned to the tropics for three years (1987-90) on an assignment with DGIS in Indonesia.

Together with his Nigerian colleagues, he laid the foundations of and actively executed the "Second Nigerian Cocoa Breeding Programme" during the 1960's, which had as its main objectives the creation of cocoa varieties with good yield potential, establishment ability and resistance to Black Pod. He identified varieties with better establishment ability, which is an increasing problem for the cocoa belt in West Africa, hit by reduced rainfall and frequent dry periods. He was also particularly concerned about resistance to *Phytophthora* pod rot, and helped to identify genotypes possessing "disease escape", *i.e.* varieties producing later in the season, outside the main epidemic period, as well as genotypes with "true resistance", expressing low infection levels in the field during the epidemic season. The results of this work were the basis of new breeding trials carried out by his successors. Several of the Toxopeus/Westeijn selections are now being re-used by CRIN, with partial funding of the CFC/ICCO/IPGRI project, launching new breeding activities from 1998 onward.

Hille's published work substantiated the positive effect on yield potential and precocity of the out-crossing of genotypes, compared to the negative effect of inbreeding in cocoa (observed in Trinitario genotypes). He expressed his views on cocoa breeding in 1972 as "The effect of mating type, heterosis and population structure" in an FAO publication. This view has been a commonly used guideline behind cocoa breeding programmes around the world from the 1960's till the 1990's, *i.e.* the use of bi-parental crosses between local selections and introduced genotypes from other genetic groups. This method has been generally successful, as far as yield potential and precocity are concerned. However, Hille's view on long-term cocoa breeding also involved proper management and selection of cocoa populations for several traits simultaneously over successive cycles (recurrent selection), including disease resistance. In Nigeria, he enhanced the genetic basis of the Amazon cocoa genepool by introducing a large number of crosses within the Nanay and Parinari Upper Amazon groups from Trinidad in 1967. This material served as the basis for the "Third Nigerian Cocoa Breeding Programme". Studies on this material later indicated superiority of between-group crosses (NA x PA) compared to within-group crosses (NA x NA and PA x PA), which provides support for improvement of cocoa based on "reciprocal recurrent selection", using separate "heterotic" populations (such as presently being carried out in Côte d'Ivoire).

Although Hille worked for only a relatively short period of nine years as a practical breeder, he never lost his keen interest in cocoa breeding. He voluntarily supervised cocoa breeding research carried out in the 1970's by a series of Dutch students at CEPLAC in Brazil (including that of the first author), continued to carry out consultancy missions for the FAO, the World Bank and NGO's, as well as to actively publish many review papers on the origin and history of cocoa populations, as well as on cocoa breeding. He was always a very keen advocate for enhanced international collaboration in cocoa breeding and genetics, and has been a strong supporter of INGENIC and its objectives. Among his latest contributions to INGENIC, was a review on the search for *Phytophthora* pod rot resistance and escape carried out in Nigeria during the 1960's (Proceedings of the Second INGENIC Workshop), and Hille emphasised his views on the use of "populations" in cocoa germplasm management and utilisation in INGENIC Newsletter Issue 2.

We owe a lot to Hille's enthusiasm and to his well-enunciated views on cocoa breeding. We feel a bit as orphans, without the possibility anymore of visiting him to discuss, in his usual very friendly manner, any cocoa breeding matter. We are sure that his direct contributions to cocoa breeding science, as well as his indirect contributions to the cocoa breeders' community, will continue to deliver their fruits over the years to come.

Hille left his wife Ans, his two daughters and his first two grand children when he passed away on the fifth of July 2003. To them we offer our condolences.

Obituary

Oil Palm and Cocoa Breeder in Malaysia

E. Rosenquist

Rob Lockwood

Eric, who died last year, was a modest man, who might have been surprised to find his passing recorded in our Newsletter. He will be remembered more for his outstanding contribution to the breeding of oil palms than for his early work on cocoa. Having known Eric for nearly twenty years, I have often wondered how cocoa breeding would have developed had he remained with the crop.

Eric's career began with the then Colonial Department of Agriculture in Malaya where he arrived as Botanist in January 1949. He described his early work in "Cocoa selection and breeding in Malaya" Malaya Agricultural Journal (1950) 33: 181-193. The emphasis was on the comparison of populations and the development of clones, an approach that remains appropriate today. The paper is well worth reading as an excellent account of the early history of cocoa improvement in Malaysia.

I got to know Eric in 1985 when I began to learn about oil palms. He was a generous and patient teacher. At that time, he was plant breeding advisor at Bah Lias Research Station in Sumatra. Although oil palm was and remains the main crop, Eric put in place a series of cocoa progeny and clone selection trials. I well remember his disappointment to learn that the higher number Parinaris, like PA 310, were no more highly selected than those with lower numbers! By contrast some of the palms he was showing to me were at their third cycle of recurrent selection. Recently, Ang Boon Beng, who selected PBC 123, which is an outstandingly good clone in Malaysia, suggested to me that it originated from Serdang, so it passed through Eric's hands. Would Eric have developed a recurrent selection programme with cocoa had he remained with the crop? I rather think he would.

We regret to inform the cocoa community of the death of **Emeritus Professor F. W. Cope**, which occurred on February 23, 2004. Professor Cope headed the Department of Botany and Plant Pathology, The Imperial College of Tropical Agriculture, Trinidad, upon Professor Purseglove's retirement, and was the first Head of the Department of Biological Science (a union of the former departments of Botany and Plant Pathology and Zoology and Entomology). He was also former Editor-in-Chief of the journal of the Faculty of Agriculture (*Tropical Agriculture*) published in the United Kingdom, a post he held until the journal was repatriated to Trinidad. Prof. Cope's sterling contribution to cocoa research, highlighted in his seminal publications in the journals *Nature* (181) and *Heredity* (17) on the mechanisms of self-incompatibility in cocoa, has been of enormous benefit to cocoa researchers. His funeral took place on March 1, 2004 in Doncaster, South Yorkshire where he lived. Professor Cope's widow, Joyce, and three children (Betty, Ian and Margaret) survive him.

NOTICE

The Cocoa Research Unit, Trinidad is pleased to announce the launch of its official website *CRUWeb*

(http://sta.uwi.edu/cru)

hosted by

The University of the West Indies.

CRUWeb features information on research projects at CRU, the International Cocoa Genebank, the Save-a-tree program and more.

INGENIC Committee

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