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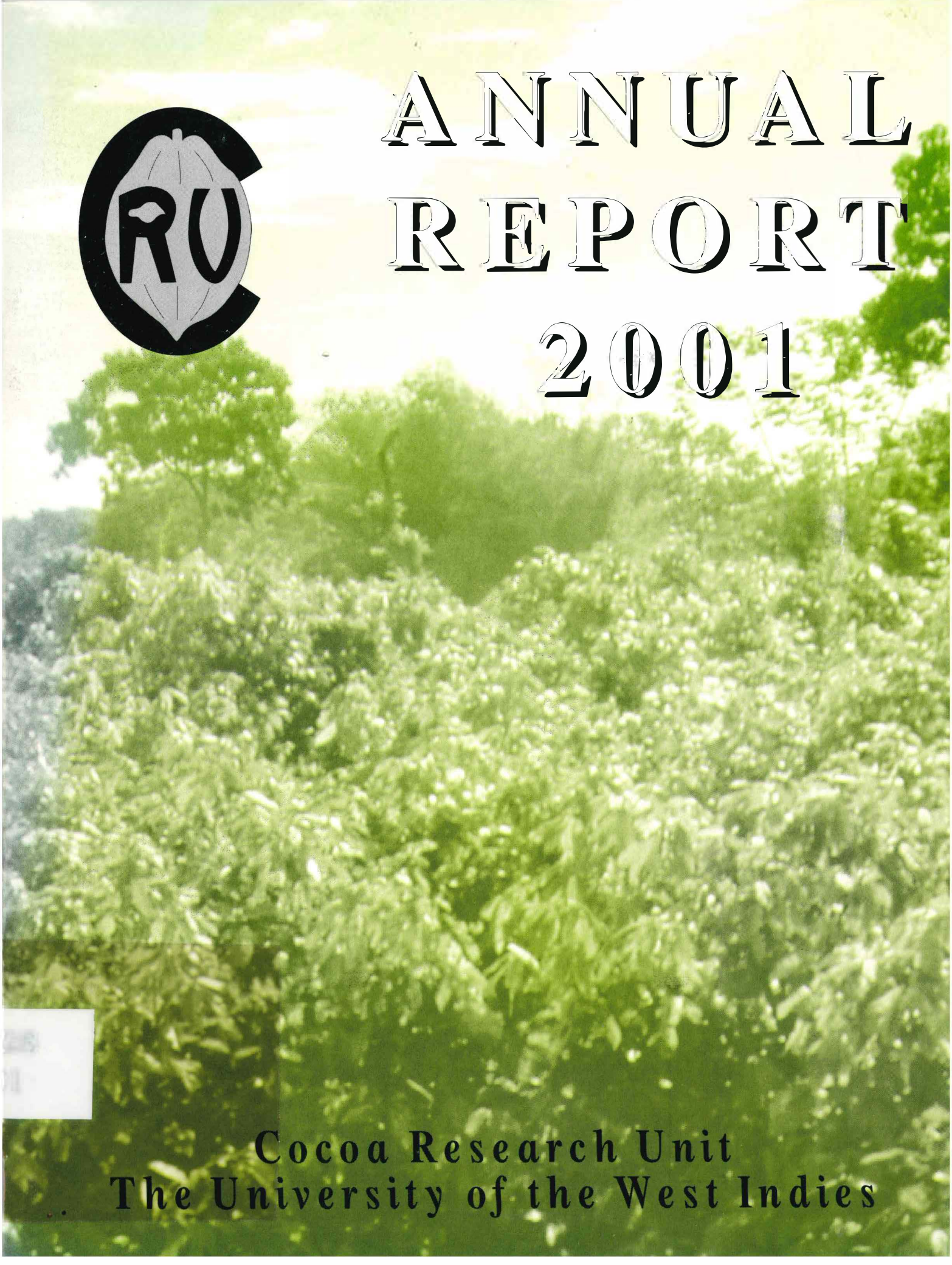

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Cover photograph. Canopy of closely planted trees on a commerical cocoa estate in east Trinidad.

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Sampling strategies for DNA extraction in the USDA fingerprinting project

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Introduction

CRU is taking part in the USDA fingerprinting project for identification of *T. cacao* accessions in the Americas. Approximately 2,300 of the estimated 8,000-10,000 accessions held in collections in the American continent are in the ICG,T, so the participation by CRU in the project is crucial. CRU has agreed to provide a DNA sample from each accession held in the ICG,T, taken from the most original tree available. Many of the original trees were planted in Marper Farm in the 1940s, so they are now about 60 years old. At the time of establishment, they were propagated as budded plants and, in many cases, chupons from the rootstocks have grown into mature trunks, which need to be distinguished from the scion. In addition, some of the original scion trunks have fallen and new uprights have grown up in different locations to the original tree. Identifying these old trees from existing maps and obtaining a leaf sample from the correct trunk can therefore be a significant challenge.

The most original trees from the majority of the ICS accessions are in the Cheesman Field, planted in the 1950s in the San Juan Estate. This is a commercial estate, so supply trees have been planted over the years to replace many of the original ICS accessions. Fortunately, five blocks (replicates) of the ICS collection were planted when it was established, and there are surviving trees in at least one of the blocks of more than half of the accessions (ICS 1 – 100).

Other accessions in the ICG,T at UCRS, were propagated from trees established on the UWI campus at St. Augustine. Many of these "original" trees in campus fields are individually labelled, but there is continuous need to update field maps and to confirm the location of trees in the field.

For other accessions in the ICG,T there is no reference tree available, since the only specimens still existing in Trinidad are in UCRS. Many of these trees, but not all, are recent introductions to the genebank, both from Trinidad from other countries, introduced through the BCQS.

Tasks and actions

Accuracy of the work is a key to the success of the project and every step of the procedure outlined below has to be carried out with great care.

Identification of the trees to be sampled

Field maps for all the locations (Marper Farm, Cheesman Field, Campus Fields and UCRS) have been updated in recent years. Individual tree labels have been fixed on the majority of trees, each with a unique number to identify the location of every tree within plots.

The most recent fields checked in 2001 were those in Marper Farm, so we now have up-to-date information on original trees still living in Blocks C and D.

Collection of samples

Once the tree identification has been ascertained, a leaf from the most original tree of each accession is sampled. Where present, samples are taken in order of priority from Marper Farm, the Cheesman Field, San Juan Estate, the UWI Campus and UCRS. Samples are only being taken from the genebank, UCRS, when the accession is not present anywhere else.

Mature leaves in a clean condition, were collected for samples used with the Kobayashi DNA extraction method. When using the D^2 Biotechnologies DNA extraction method, flush leaves or newly expanded mature leaves (when flush leaves are not available) were collected.

At the time of collection, a blue embossed label indicating the accession name, the plot number and tree number where appropriate, was attached to the branch of the tree from where the sample was taken. When available, pods were collected from the same trunk as the leaf sample, wrapped carefully to avoid bruising and carried back to CRU to be photographed.

DNA extraction

The Kobayashi extraction method (Kobayashi *et al.*, 1998) was chosen after preliminary tests were carried out on the quantity and quality of DNA. Later, the D^2 Biotechnologies extraction kit was adopted in accordance with recommendations by USDA, Beltsville.

Accomplishments

Field maps and tree identities

Approximately 2,300 cacao accessions are present in the various fields of the ICG,T. When the genebank at UCRS was established in the 1980s, 1,471 accessions were recorded as being present in Marper Farm, Blocks C and D. Of these, 155 trees had lost their identity, and had been assigned new codes from CRU 1 to CRU 155 at the time of propagation of germplasm for the ICG,T (in the 1980s). Recent checks in Marper Farm however, indicate that only 1,154 accessions are still alive. Since the 1980s, the identities of 45 more trees have been lost, and these have been assigned new codes from MARPER 1 to MARPER 45. This project is providing a good opportunity to update the records of Marper Farm.

The presence of each tree in Blocks C and D of Marper field has been checked systematically, using pod colour and other morphological traits to verify the identity of each accession. The survival of the scion and the re-growth of the rootstock have been a major concern, and temporary labels have been attached to the appropriate trunk on every checked tree.

Maps of the campus fields at UWI were revised again in 2001, and a metal label with x, y co-ordinates within the field attached to each tree. The updated map of the Cheesman field has also been rechecked, using pod descriptions given by Pound (1934, 1935 and 1936) to verify the tree identification. Checking field maps, plot labels and tree labels in UCRS is a day by day practice to maintain up-to-date records.

Collection of leaf samples

Samples have been collected using guidelines from USDA; either young or fully expanded mature, healthy leaves were taken from well-identified trees.

Collection and extraction of samples are well on their way. A summary of completed samples and those remaining is given in Table 1. In addition to the accessions sampled in Table 1, 105 leaf samples have been taken from nursery material in the greenhouse in UWI and in the BCQS.

Table 1. Number accessions sampled for DNA extraction and pod photographs.

Location	Field	Leaf samples		Pod photographs
		Completed	Remaining	
Marper Farm	Block C	453	252	251
Marper Farm	Block D	441	3	257
San Juan Estate	Cheesman	59	Finished	58
UCRS	4A	230	36	-
UCRS	5A	53	62	-
UCRS	5B	76	70	-
UCRS	6A	8	3	-
UCRS	6B	126	70	-
UWI	Campus	47	203	-

Pod photographs

Pods were collected from 566 accessions in the Cheesman Field, and Marper Farm (Table 1). When available, two undamaged pods were taken from each accession, one was fully-grown but immature and the other was mature (ripe). The pods were wrapped in bubble wrap bags (large bubbles) to avoid bruising, and transported to CRU. They were arranged on a black background, with a label, a standard colour chart (Kodak) and a centimetre scale. They were photographed using a Nikon 950 Digital Camera mounted on a tripod, under uniform fluorescent light with the automatic camera setting. Digital images were downloaded onto a computer and cropped (Plate 1).

DNA extraction

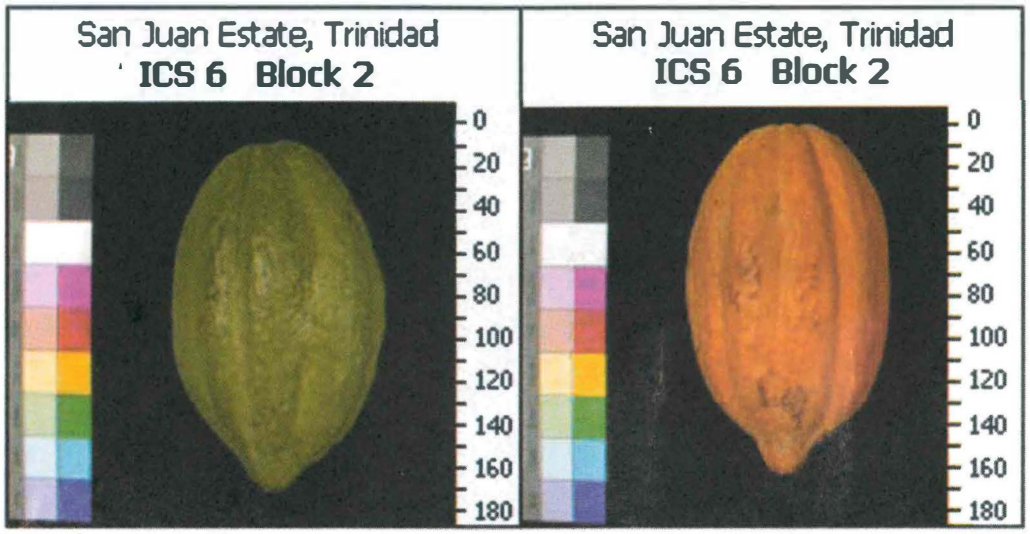
DNA was extracted from 1,200 accessions as described by Kobayashi *et al.*, 1998. Up to 100 mg of fresh mature leaf tissue was used for DNA extraction with the recommended volume of extraction buffer I (600 µL). The effect of leaf age on extract quality was not tested with this protocol.

Supernatants obtained after the organic extraction step tend to be dark and discoloured with particulate debris (presumably from excess polyvinylpolypyrrolidone). A second organic extraction step generally does not appear to 'clean up' the supernatant, however additional centrifugation of the supernatant proved useful in removing some of the insoluble particulate matter.

Because of the discoloured supernatants, many of the resulting DNA pellets tend to be dark brown to almost black though some do not retain as much pigment. DNA stock solutions are usually viscous and difficult to pipette, possibly due to the presence of glycoproteins. Diluted DNA extracts (1: 150) were however, easily amplified with SSR primers.

DNA was extracted from 400 accessions using the D^2 DNA X-Tract™ Plus Solution Kit and protocols (D^2 Biotechnologies Inc., Atlanta, GA). Up to 50 mg of cacao leaf tissue can be used in 2 mL H-tubes (Qbiogene, Vista, CA). However, when the leaf tissue has lost most of its moisture

Plate 1. Digital images of fully-grown immature and mature pods from the San Juan Estate, showing the morphological characteristics of ICS 6. The scale is in cm.



content, as much as 900 µL of Solution 1 from the D^2 kit is required instead of the recommended 600-800 µL. Leaf age and/or density can also severely reduce the amount of Supernatant I (obtained after centrifugation following the addition of Solution 2 and incubation at room temperature).

DNA extracts re-suspended easily in water or low TrisEDTA buffer. Most of the DNA stock solutions were not viscous and were easy to pipette as they lack glycoproteins and mucilage commonly obtained when other protocols are used to extract DNA from cacao leaf tissue. Dilutions of the order of 1:50 to 1:150 were successfully amplified with SSR primers. Fragments were separated on 5% polyacrylamide gels and visualised by silver staining.

Conclusion

Leaf samples from about 70% of the accessions in the ICG,T have been collected and DNA extracted. Checks at USDA, Beltsville indicate that the quality of the DNA is good for analysis with their automatic sequencer. The first DNA samples are ready for transfer to Beltsville for analysis.

Dr Jim Saunders is co-ordinating this collaborative project and will analyse each DNA sample using 15 SSR primers developed by CIRAD.

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