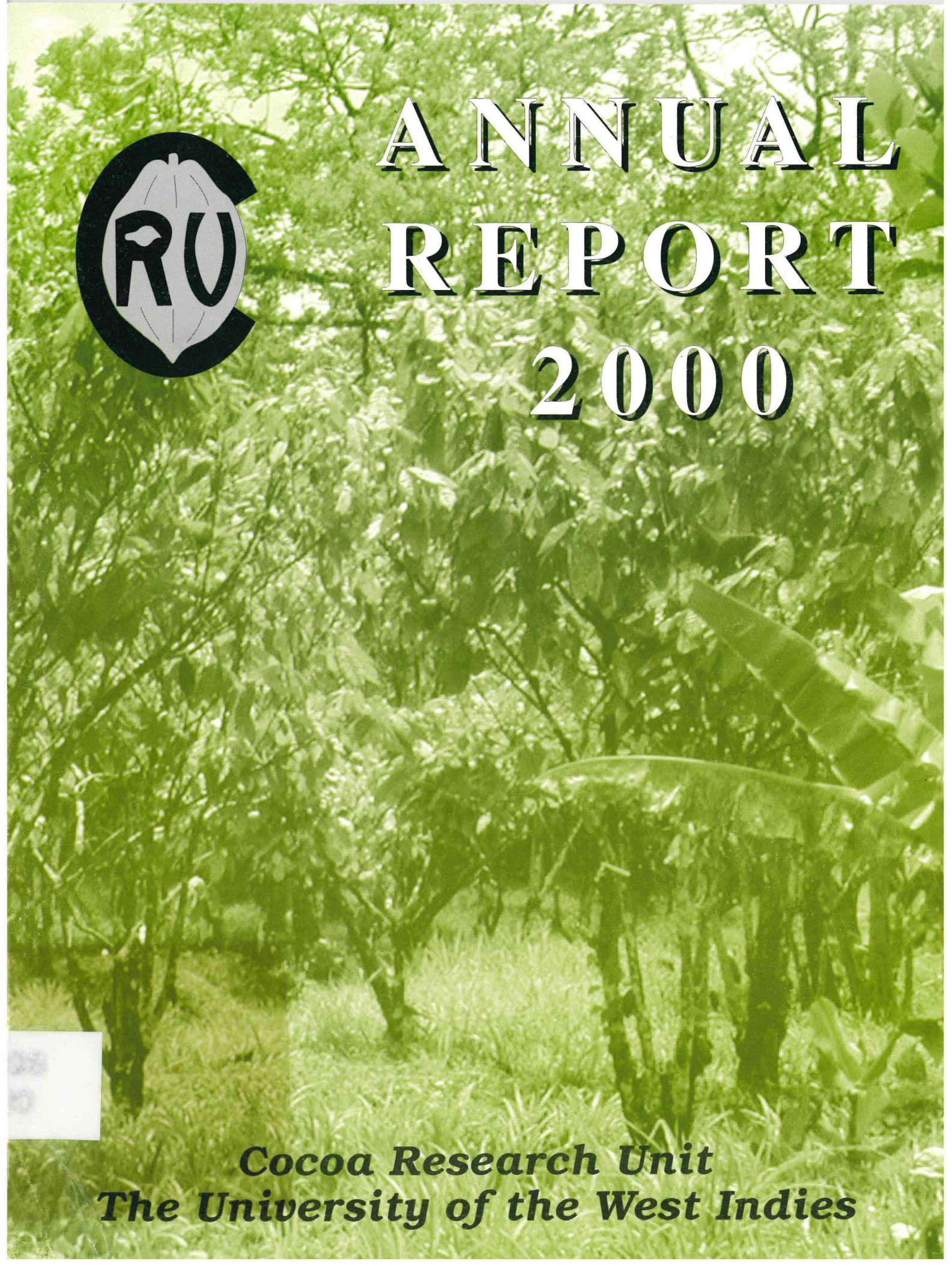

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Cover photograph. Progeny from the cross of ICS 1 × SCA 6 planted in the 1950s.

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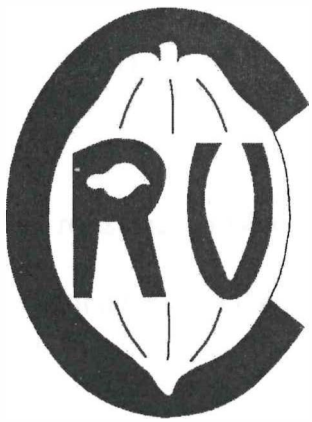

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Verification of clonal accessions in the ICG,T using RAPD and SSR analyses

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Introduction

The presence of misidentified plant material is commonly encountered in genebanks. This problem has been acknowledged for cocoa; some authors have pointed out dissimilarities between trees representing the same accession in different genebanks (Figueira, 1998) as well as in the same genebank (Sounigo *et al.*, 2001). In the case of the ICG,T, maintained by CRU, it is important to identify and correct these mistakes because we provide clonal material and information to cocoa researchers worldwide.

A preliminary programme was started in 1997, to detect the presence of mislabelled trees. Up to 1999, trees from 132 cocoa accessions in the ICG,T were verified using RAPD markers and the results were used to put tags on the analysed trees to indicate whether or not they are true to type.

Since 1999, we have introduced some modifications to the strategy and methodology. Priority for verification is being given to material being used in active research projects: these include accessions used as controls for phytopathology tests, accessions studied for the evaluation of the inheritance of the flavour traits, and accessions pre-selected for the "CFC/ICCO/IPGRI project collection". In addition, the use of RAPD was discontinued because of the problems of repeatability sometimes observed with this technique. Instead we initiated the use of SSR (Simple Sequence Repeats) markers.

Material and Methods

The trees selected for analysis are located in the ICG,T and on the UWI campus fields (St. Augustine). When possible, the molecular profiles of the trees representing an accession were compared to the one obtained from the tree representing the same accession in Marper Farm. The trees in Marper Farm were planted in the 1940's and were propagated by grafting budwood collected from plants which had been quarantined in Barbados. These plants were grown from seed collected by Pound (1938) on spontaneous trees (in the case of Upper Amazon Forastero accessions from Peru) and on cultivated trees (in the case of Refractario accessions from Ecuador) during his collecting expeditions. For this reason, these trees are considered to be the reference material for verification.

During the preliminary programme, molecular analyses were conducted using 14 RAPD primers, allowing the scoring of 39 reproducible markers. For the new programme, we used nine pairs of SSR-primers. Both RAPD and SSR products were separated on agarose gels at a concentration of 1.5% for RAPD products and 3% for SSR products.

When comparison with the reference tree from Marper was possible, the trees sharing its molecular profile were given a distinct label (orange coloured) with their original accession name. Trees showing a molecular profile differing at one or more fragments from the tree at the

Marper farm were given a label with a "CRU" prefix and a number suffix. In the absence of a reference tree, the analysed trees were given a label with the original accession name if they all shared the same molecular profile, otherwise they were given a label with the original accession name followed by the letter "V" (for "verified") and a number.

Results and discussion

The use of RAPD allowed us to detect differences between trees of the same designation, not only between the reference tree and the ones planted in the ICG,T, but also within a plot in the ICG,T. In some cases, every tree analysed presented a distinct profile, however for many of these, differences were found at the level of only one RAPD marker and could be attributed to problems of reproducibility of the technique.

Table 1 shows results obtained using SSR markers on selected accessions. In six accessions, all the analysed trees were found different to the reference tree. In two accessions, PA 165 and PA 200, all the trees were found to be identical to the reference tree, and in one accession, NA 26, all but one of the six trees analysed were found to be identical to the reference tree. In the case of two accessions, NA 26 and JA 5/25, differences were found between trees in the same plot in the ICG,T.

Table 1. Results of the comparison of SSR profiles of different trees representing the same accessions.

Accession	Number of trees in the genebank or on campus			Number of different molecular profiles
	Analysed	Identical to the Reference tree	Different from the reference tree	
AM 2/38	2	0	2	1
B 5/7	3	0	3	2
JA 5/25	6	0	6	2(5+1)
NA 26	6	5	1	2(5+1)
NA 90	3	0	3	1
NA 246	2	0	2	1
PA 165	3	3	0	1
PA 200	2	2	0	1
PA 300	2	0	2	1

N.B: the numbers in brackets indicate the number of trees falling in the different types of molecular profiles. These numbers are presented only when the distribution of different profiles is not obvious.

Table 2 shows that in the case of eight out of thirteen accessions, all the trees tested shared the same profile, while differences were found in the remaining five accessions. The worst case was that of POUND 18, where all three trees were found different from one another.

Our data show that the 20 trees representing AMELONADO are identical, which confirms the validity of this accession for its current use as a susceptible control when performing inoculation tests to screen for resistance to WB.

Unfortunately, some misidentification problems appear to exist for SCA 6, which is currently used as a resistant control for inoculation tests in screening to assess resistance to WB and to

Phytophthora. Similar problems were found for UF 11, which is used as a susceptible control in inoculation tests to screen for resistance to WB. In these cases it is essential to know which trees are true to type, and which are off-types.

Table 2. Results of the comparison of SSR profiles of different trees representing the same accessions.

Accession	Number of trees analysed	Number of different RAPD profiles observed
AMELONADO	20	1
GU 243/H	2	1
ICS 1	2	1
IMC 67	2	1
PA 175	3	2
PA 191	3	1
PA 218	3	1
POUND 7/A	3	1
POUND 18	3	3
SCA 6	3	2
SLA 8	3	2
UF 11	3	2

These results indicate that the problem of misidentified germplasm material may be quite frequent in the ICG,T. This could have resulted from mistakes at several steps in the process of establishing clonal accessions in the ICG,T. Steps in which errors may have arisen include:

- collection of budwood for cuttings during clonal propagation of trees from Marper Farm, prior to their planting in the ICG,T and on the campus. The grafted trees in Marper Farm were already old when the multiplication process started, in the 1980s. It is therefore possible that budwood for cuttings could have been collected from the rootstock instead of the scion. In addition, for a large number of trees, there are several trunks, the origin of which is not always easy to determine. One possible origin could be the germination of seeds from the original tree or from a neighbouring tree.
- mislabelling of plants in the greenhouse after clonal propagation, eg. when rooted cuttings are moved from the propagation bin to harden off, or from the hardening-off area to another part of the greenhouse.

Mistakes could also occur during the verification study, resulting in the over-estimation of the percentage of mislabelled trees. Errors could have taken place in collecting leaves for DNA extraction, especially in Marper Farm. The trees are even older now than they were when budwood was collected to establish the ICG,T, so the problem is more severe now.

Conclusion

These studies indicate that the problem of misidentified accessions existing in the ICG,T may be more serious than had been suspected from morphological observations. In order to avoid the

risk of detecting "false differences" between clonal trees, the RAPD analysis was replaced by the SSR technique, but the lack of necessary equipment has prevented us from making the best use of these markers. Indeed, differences between SSR alleles are often at the level of a very small number of pairs of nucleotides, which require sequencing gels to detect them. In the absence of this equipment, we separated our PCR products on agarose gels, and therefore lost much of the separating power of the technique. This means that our results are probably "optimistic", since it is likely that we were unable to detect some of the real difference between trees of the same accession.

Nevertheless the results of this study emphasise the importance of knowing precisely which particular tree was studied. This is now possible thanks to the completion of the labelling of each tree in the ICG,T with unique identification numbers.

Future direction

This programme of work on verification must continue to discern the magnitude of the mislabelling problem and to solve it. Thanks to support from the United States Department of Agriculture (USDA), CIRAD and BCCCA, equipment will soon be acquired by CRU which will allow us to reveal the SSR products on sequencing gels instead of agarose gels. This technical modification will considerably increase the discriminating power of these markers and allow us to get much more precise and reliable information on the extent of the mislabelling problem in our genebank.

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