THE UNIVERSITY OF THE WEST INDIES

COCOA RESEARCH UNIT

REPORT FOR 1995

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Evaluation of the Reproducibility of RAPD Markers on Cacao

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RAPDs are commonly used for genetic diversity asessment and genome mapping in a large number of plants , including on cocoa. On the other hand, the generation of unstable bands lead researchers to be more dubious about the use of this technique for characterization of genotypes, which requires the obtention of stable markers, not affected by the place where DNA extraction and DNA amplification are performed. This experiment has been performed in order to know if such markers exist.

Material and methods.

1. Laboratories involved

The experiment has been performed at the biochemistry laboratory of the C.R.U, in Trinidad, and at AGETROP laboratory of CIRAD, in France.

2. Levels of repetition

a. Amplification experiment

Each DNA extract has been amplified in two or three separated experiments. The type of repetition consisting in several amplifications of the same extract in a same experiment has not been done, because it has been well established from our previous results that this type of repetition always allows a very high reproducibility of the banding patterns in our conditions .

The two or three experiments have been performed whether in the same laboratory or in the two different laboratories.

b. DNA extract.

In the case of some accessions, several DNA extracts have been amplified during the same experiment. These DNA extracts can originate from:

- the same plant, after two extractions performed in the two laboratories, according to the same extraction procedure.
- different plants, after extractions performed whether in the same laboratory or in the two laboratories, according to the same extraction procedure.
- different plants, after two extraction experiments, according to two different extraction procedures, which allow the obtention of very different levels of DNA purity and integrity.

3. Vegetal material

The choice of the 15 accessions used in this experiment has been done according to :

- the polymorphism observed among this sample

- the presence of these accessions in the two laboratories where the experiment has been performed.

4. Protocol

a. DNA extraction

In most of the cases, the protocol adopted has been a disc-leaf extraction procedure, perfected by Johnson *et al.* (1992), which is actually the protocol routinely used at C.R.U. This protocol is very simple but allows the obtention of degraded DNA.

In few cases, the extracts have been obtained through a more complicated protocol (Laurent 1993), including a separation on a CsCl gradient performed through an ultracentrifugation step. This protocol allows the obtention of a very pure and non degraded DNA.

b. PCR experiment

In all the cases, exactly the same protocol has been followed, using the same amplification program (described below) and conditions (described below) and the same model of thermal cycler (DNA thermalcycler , purchased from Perkin Elmer).

In all cases, electrophoresis has been applied on the amplification products on a 1.6% agarose gel with a 100V electric field, applied for 4 hours and the gels have been stained in a 2% EtBr solution before visualization under U.V.

Amplification program:

First step: 40 cycles, consisted of the three following segments:
5 seconds at 94°C
30 seconds at 36°C
1 minute at 72°C

Second step: 1 cycle, composed of the following segment:
5 minutes at 72°C

Amplification reaction:

dNTP (2.5mM of each)	2.5 µl
Klen Taq I (AB Peptides, USA)	0.25 µl = 6.25 units
Klen Taq I buffer	2.5 µl
primer (OpO15 or opP14)	5µl = 15ng
H_20	11.75 µl
template DNA	3 µl = 0.45 ng

Results

1. Comparison of the complete banding patterns

a. Legends of the figures

Some of the banding patterns obtained are shown in figures 1, 2, 3 and 4.

T, T1 and T2 : DNA extracted from a tree planted in the genebank or on the campus of the University, in Trinidad.

M: DNA extracted from a tree planted in Marper field, in Trinidad, which is the original place where accessions were planted.

C: DNA extracted from a plant in the CIRAD quarantine, in Montpellier

All the DNA extracts noted T, T1, T2 , M and C have been obtained following the simple leaf-disc protocol, routinely used at C.R.U.

U: DNA extracted from a plant in the CIRAD quarantine, in Montpellier, using the protocol involving an ultracentrifugation step.

The figures show different types of differences between the amplification products.

b. Types of differences between the patterns

α) *At the level of the whole banding patterns*

Sometimes the patterns obtained are completely different, without almost no matching band. In these cases, one of the patterns usually presents a lower number of bands, these bands corresponding to lower molecular weight fragments. These aberrant patterns result from completely inefficient amplifications and are indicated by a x in figures 1 and 2. These cases are observed with the same extracts with both primers, except for two of them, properly amplified only with opP14, indicating that the poor quality of these amplifications is caused by the poor quality of these extracts. It also indicates that amplifications obtained with this primer are less affected by the quality of the template DNA.

β) *At the level of high molecular weight bands*

It can happen that high molecular weight major bands can be present or absent, when the same extract is amplified in two distinct experiments, as in the case of OpO15 (figure 3). These changes can occur in the same laboratory and are caused by a change in the quality of the amplifications, usually due to changes in the quality of the DNA extracts. These bands disappear most of the time when the extracts become too old (more than one year). However, this change can hardly be a source of error, since all the high molecular weight components disappear in this case, even the completely monomorphic ones, making it clear that the absence of these bands do not result from genetic polymorphism.

γ) *At the level of major bands*

In most of the cases, differences at the level of major bands occur when different extracts of the same accession, but obtained, from different plants, are compared. A typical example of

PRIMER OpO15

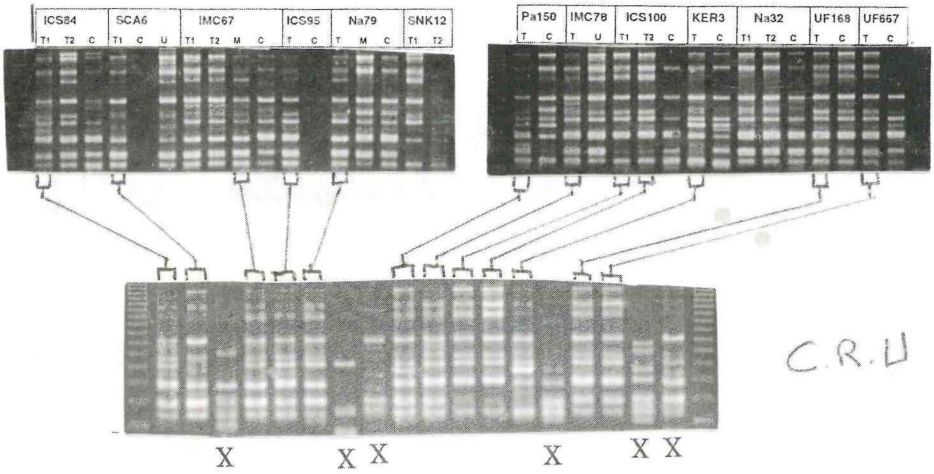

Figure 1. Comparison of banding patterns obtained after two amplifications experiments using primer OPO15. X: missed amplification : difference between the banding patterns.

PRIMER OpP14

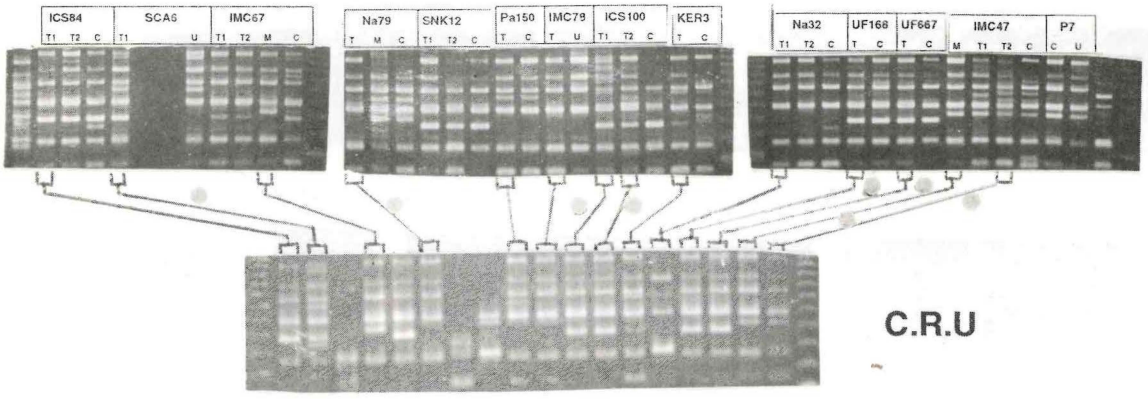

Figure 2. Comparison of banding pattern obtained after two amplification experiments using primer OpP14. : difference between the banding patterns.

PRIMER OpO15

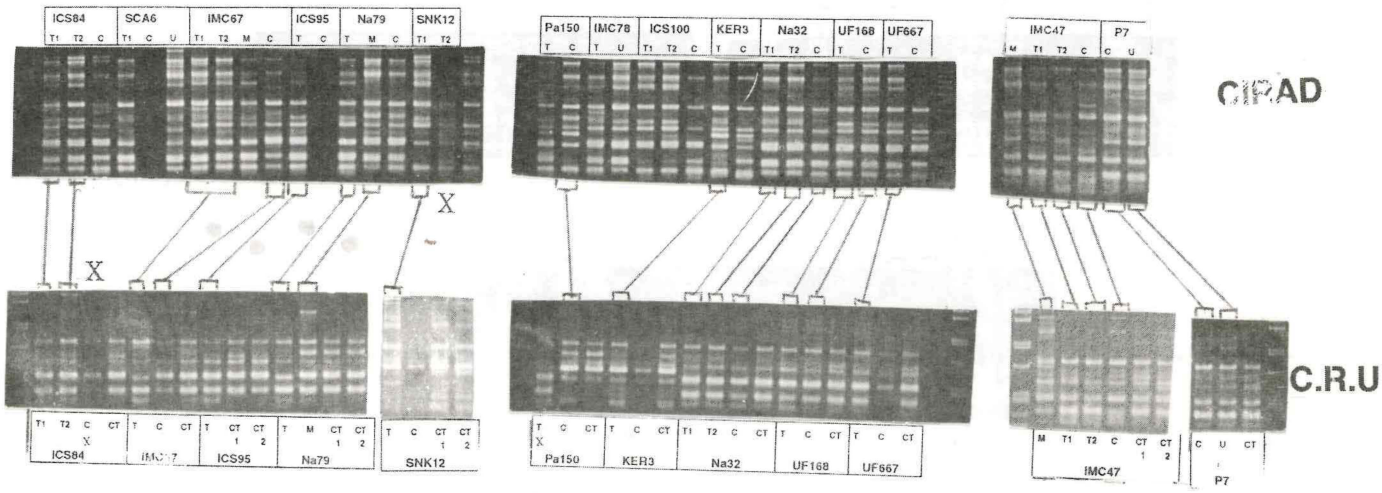

Figure 3. Comparison of banding patterns obtained after two amplifications experiments using primer oPO15. X: missed amplification : difference between the banding patterns.

PRIMER OpP14

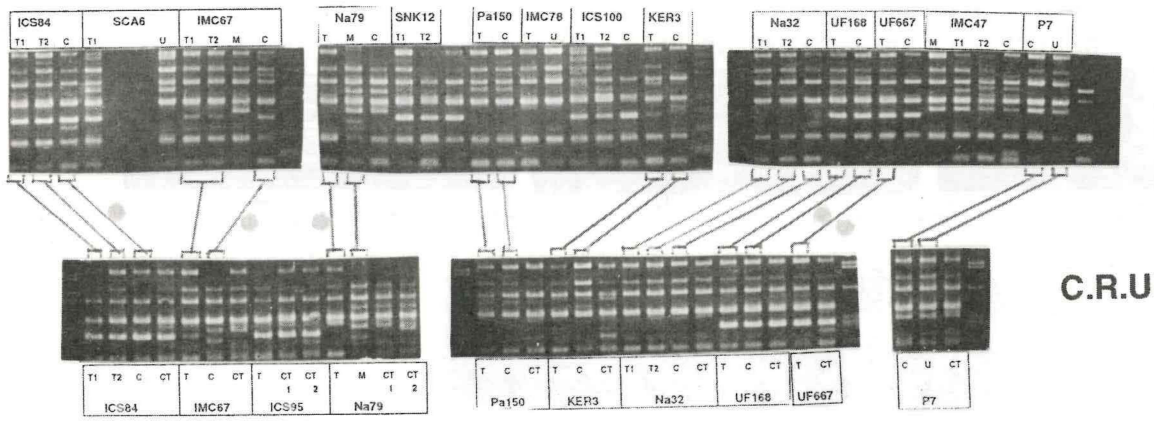

Figure 4. Comparison of banding patterns obtained after two amplification experiments using primer OpP14. : difference between the banding patterns.

this is the case shown in figures 2 and 3, where three extracts from Na79 are compared, from three different trees: the extract noted M comes from Marper field, and is for this reason supposed to be the correct one. The one noted T comes from a tree planted in the genebank. The one noted C comes from a plant of Montpellier quarantine. The results obtained with both primers clearly show two types of banding patterns: one for the T extract and the other for both M and C extract. These differences also appear for other clones, such as Sca6, IMC67, SNK12 and IMC47. The differences at the level of these major bands are reproducible from one experiment to the other. It is then strongly suspected that these differences are most of the time caused by off-type problems. Consequently, only the comparison of banding patterns from the same plant will be considered to evaluate the reproducibility of the technique.

δ) At the level of minor bands

This type of difference is the most common in this experiment. This can be found at the level of every type of comparison. These bands are usually not reproducible from one experiment to the other. Different explanations can be proposed for this lack of reproducibility:

* some uncontrolled factors can affect the general quality of all the amplification reactions of the same experiment. In this case, the fainter bands will be visible in one experiment and not in the other.

* some uncontrolled factors can affect the quality of the electrophoregrams, allowing or not the observation of the fainter bands.

* standard conditions for staining the gels are not always respected ("age" of the EtBr solution, time spent for staining and soaking the gel..). This situation can lead to differences in the quality of the pictures obtained, which will allow or not to visualize the fainter bands. This problem can be solved by increasing the rigour of people performing RAPD.

* These faint bands originate from imperfect annealing of the primers, caused by an incomplete homology. The annealing is in this case very fragile and unstable and the occurence of amplifications is also very unstable.

b. Level of reproducibility

The levels of reproducibility (i.e the % of banding patterns perfectly matching) are indicated in Tables 1 and 2 for the two primers, when all the bands are considered. These levels of reproducibility are low.

Table 1. Matching of the banding patterns considering all the bands obtained with oPO15.

	same extract		different extracts from the same plant	
	different laboratories	same laboratory	different laboratories	same laboratory
Number of pairwise comparisons	32	24	12	14
Number of clearly unmatching cases	24	6	2	4
Number of ambiguous cases	0	0	0	2
Number of matching cases	8	18	10	8
% of clear matching	25	75	83	57

Table 2. Matching of the banding patterns considering all the bands obtained with opP14.

	same extract		different extracts from the same plant	
	different laboratories	same laboratory	different laboratories	same laboratory
Number of pairwise comparisons	35	19	13	8
Number of clearly unmatching cases	20	11	8	4
Number of ambiguous cases	1	0	1	0
Number of matching cases	14	8	4	4
% of clear matching	40	42	30	50

Table 3. **Matching of the banding patterns obtained with oPO15, considering only the bands assumed to be reproducible from previous studies.**

	same extract		different extracts from the same plant	
	different laboratories	same laboratory	different laboratories	same laboratory
Number of pairwise comparisons	36	24	14	16
Number of clearly unmatching cases	18	5	0	0
Number of ambiguous cases	0	0	0	2
Number of matching cases	18	19	14	14
% of clear matching	50	79	100	87
Number of tested accessions	15	12	6	4
Number of accessions with only clear matching	5	7	5	3
Number of accessions with ambiguous cases	0	0	1	1
Number of accessions with clear unmatching	10	5	0	0

Table 4. **Matching of the banding patterns obtained with opP14, considering only the bands assumed as being reproducible .**

	same extract		different extracts from the same plant	
	different laboratories	same laboratory	different laboratories	same laboratory
Number of pairwise comparisons	36	25	22	14
Number of clearly unmatching cases	1	0	1	0
Number of ambiguous cases	0	2	2	0
Number of matching cases	35	23	19	14
% of clear matching	97	92	86	100
Number of tested accessions	15	12	9	4
Number of accessions with only clear matching	14	10	7	4
Number of accessions with ambiguous cases	0	2	1	0
Number of accessions with clear unmatching	1	0	1	0

Table 5. **Matching of each band considered as reproducible, obtained with oPO15.**

		band	Number of pairwise comparisons	Number of clearly unmatching cases	Number of ambiguous cases	Number of matching cases	% of clear matching
same extract	different labs	1,9	19	0	0	19	100
		0,8	36	5	0	31	86
		0,55	36	13	0	23	63
		0,5	36	0	0	36	100
	same lab	1,9	1	0	0	1	
		0,8	24	2	0	22	91
		0,55	24	4	0	20	80
		0,5	24	0	2	22	91
different extracts from the same plant	different labs	1,9	2	0	0	2	
		0,8	14	0	0	14	100
		0,55	14	0	0	14	100
		0,5	14	0	4	14	71
	same labs	1,9	2	0	0	2	
		0,8	14	0	0	14	100
		0,55	14	0	0	14	100
		0,5	14	0	4	10	81

Table 6. **Matching of each band considered as reproducible, obtained with opP14.**

		band	Number of pairwise comparisons	Number of clearly unmatching cases	Number of ambiguous cases	Number of matching cases	% of clear matching
same extract	different labs	1,1	36	1	0	35	97
		0,75	36	0	0	36	100
		0,5	36	0	0	36	100
		0,45	36	0	0	36	100
	same lab	1,1	25	0	0	25	100
		0,75	25	0	0	25	100
		0,5	25	0	0	25	100
		0,45	25	0	2	23	92
different extracts from the same plant	different labs	1,1	49	2	0	47	95
		0,75	49	2	0	47	95
		0,5	49	2	0	47	95
		0,45	49	2	4	43	87
	same labs	1,1	14	0	0	14	100
		0,75	14	0	0	14	100
		0,5	14	0	0	14	100
		0,45	14	0	0	14	100

2. Comparison based on the observation of bands considered as reproducible from experiments conducted at C.R.U.

Since the two primers chosen for this experiment have already been used to analyse a large number of accessions at the C.R.U., we have chosen the most reproducible bands obtained from each primer. For each primer, there are four such bands, which are indicated in figure 1 and 2. Most of these bands were major bands, but some others were not very bright, despite their apparent reproducibility.

In this case, the % of matching obtained when comparing the banding patterns observed with these four bands was much higher with opP14, but still not very high with opO15, as shown in the Tables 3 and 4.

In both cases, the results are more different when the amplifications have been performed in two different laboratories. It is true that the patterns obtained at CIRAD presented a much higher quality, with clearer and brighter bands, and with more minor bands appearing.

From the results obtained with opO15, the amplifications seem to be less affected by different extractions, even if these extractions are performed in two different laboratories. Considering the impact of the technique of DNA extraction, it is difficult to conclude, because the extracts have been obtained from different plants, and hinder us from distinguishing differences due to the technique from differences due to the genotype.

3. Comparison based on observations on individual bands

Table 5 shows that, in the case of opO15, only the 0,5kb band can be considered as reproducible enough for characterization. Too few comparisons were possible for the 1,9kb band.

Table 6 shows that, in the case of opP14, the four bands can be considered as consistent enough for characterization, with more caution for the 0,45kb band.

Conclusions

From this experiment, some conclusions can be done:

- In our conditions, it does not seem possible to use the whole banding patterns to fingerprint clones.

- However, RAPD can generate markers allowing a high reproducibility (>95%), which can be used for characterization purpose. Such markers need to be severely screened, on the basis of their intensity and their reproducibility. One difficulty for this screening can be difference of intensity of a marker, according to the genotypes under analysis (e.g: 0,5kb and 0,45kb bands are very clear in most of the accessions, but not in the French Guiana accessions).

- The severe screening of the potential markers increases the number of primers to use and consequently the number of amplifications to perform. A promising technique, suitable for finger printing, could be AFLP, which is also based on PCR. This technique is presently being evaluated at CIRAD and has shown a good level of reproducibility. If the use of radiolabelled nucleotide becomes possible at UWI this technique could be tried at CRU.

- One way to score RAPD results in the database could be the scoring of the intense and reproducible bands, even if they are monomorphic. One note could be given to each band, according to its discriminatory power.

Other potentially interesting markers are microsatellites amplified by PCR, which are much more reproducible than RAPD. Presently, six polymorphic loci have been obtained with these markers at CIRAD, and efforts continue to find other ones. These primers are made available to C.R.U.

References.

Johnson E., Russell J.R., Hosein F., Powell W., Waugh R. 1992: A laboratory manual for RAPD analyses in cocoa. 17 pp.

Laurent V. 1993: Etude de la diversité génétique du cacaoyer (*Theobroma cacao* L.) basée sur le polymorphisme de la longueur des fragments de restriction (RFLP). Thèse de l'Université Paris XI.