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An Assessment of Genetic Diversity Within a Collection of Cassava (*Manihot esculenta* Crantz) Germplasm Using Molecular Markers

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Cassava (*Manihot esculenta* Crantz) cDNA clones were used to detect restriction fragment length polymorphisms in a collection of *Manihot* germplasm maintained as *in vitro* plants at ORSTOM, Montpellier. The collection consisted of mostly African cultivars of *M. esculenta*, together with a few *M. glaziovii* Mueller von Argau and *M. caerulescens* Pohl, and some interspecific hybrids between *M. esculenta* and *M. glaziovii*. The clones revealed significant levels of polymorphism both within and between the species; sufficient to construct dendrograms indicating the genetic diversity within the collection.

Key words: Cassava, *Manihot esculenta* (Crantz), restriction fragment length polymorphisms, RFLPs, genetic diversity.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is grown throughout the humid tropics where its storage roots are an important source of food (Cock, 1985). The genus *Manihot* originates in South and Central America, where there are two centres of diversity, in Brazil and Mexico. Apart from *M. esculenta* there is little geographical overlap between these two groups (Rogers and Appan, 1973). Cassava was possibly first domesticated in America between 5000 and 7000 BC (Lathrap, 1970) and is normally propagated vegetatively through cuttings, though sexual propagation via seed can occur. The plant is essentially perennial, but the roots are harvested on either an annual or biennial basis. Wild *Manihot* species are normally propagated by seed and only produce roots of minor economic importance (Rogers and Appan, 1973). After the conquest of America, cassava was dispersed throughout the tropical regions of the old world. For example, it was first introduced into west Africa by the Portuguese in the sixteenth century, from where it spread across sub-Saharan Africa (Jones, 1959).

Although cassava is an important crop it has largely been ignored as a target for genetic improvement until recently. This was predominantly due to its cultivation by subsistence farmers. However it is now considered a priority tropical crop for improvement as indicated by the formation of the international Cassava Biotechnology Network. Included amongst the areas for research on cassava are improved nutritional quality, disease and pest resistance, cyanogenesis and genome characterization. It is envisaged that the plant will be improved both through classical breeding techniques and through genetic engineering.

A prerequisite for any programme of genetic improvement of cassava is knowledge of the extent of genetic variation present between cultivars, and the genetic distance between cassava and closely related species with which hybrids could be produced. In a crop where cultivars have generally been selected and distributed by the farmers themselves and where different cultivars can often possess the same name and where the same variety may bear more than one name (J. Mabanza, pers. comm.), some objective method of identification, classification and measurement of genetic diversity is required. A second need for information on genetic diversity is in germplasm conservation. While cultivation is largely in the hands of subsistance farmers a broad range of genetically varied cultivars will be used. However if elite varieties of cassava come to be increasingly grown this broad genetic base may become threatened. For this reason, in addition to the need of genetic improvement, germplasm collections are vitally important. To be economically manageable and agronomically useful germplasm collections should avoid over duplication yet maintain the greatest genetic diversity possible. Tools to aid this process are therefore essential.

The classic taxonomic studies of Rogers and Appan (1973) and Rogers and Fleming (1973) on the genus *Manihot* and on *M. esculenta*, respectively, rely on morphological characteristics. While this taxonomic method is important, it has, in many plant groups, been extended by genetic, biochemical or molecular approaches. Unfortunately few marker genes have been identified so far in cassava (Hershey and Ocampo, 1990). In cassava, as with other species, isoenzymes have been used as a rapid and relatively cheap technique for cultivar identification (Hussain *et al.*, 1987;

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Ramirez *et al.*, 1987; Lefèvre, 1988). However, isoenzymes often show low levels of polymorphism and problems of reproducibility arise due to tissue type and condition. On the other hand, DNA sequences show greater variation than amino acid changes in enzymes. The composition of the genome (DNA) is also consistent between tissues and is not affected by environmental changes. Therefore DNA is a far better and potentially greater source of polymorphism than isoenzymes. For these reasons restriction fragment length polymorphisms (RFLPs) are recommended as the technique for evaluating germplasm in collections (Flavell, 1991). RFLPs have been successfully used to evaluate genetic diversity in *Solanum* species, the genus *Lycopersicum*, *Oryza sativa*, *Brassica* and *Lens* species (Havey and Muehlbauer, 1989; Debener, Salamini and Gebhardt, 1990; Miller and Tanksley, 1990; Song, Osborn and Williams, 1990; Zhang *et al.*, 1992).

Though cassava originates from Latin America, Africa produces and consumes 37% of the world's cassava crop (Cock, 1985). It is probable that there has been selection for adaptation to African conditions during the centuries since the first introduction of cassava to that continent. Therefore an assessment of genetic diversity within collections of African cultivars of cassava is of interest and importance in its own right. In this paper we demonstrate the use of RFLPs to detect and measure genetic diversity in cassava taking, as an example, the collection of predominantly African cassavas maintained *in vitro* at ORSTOM, Montpellier.

MATERIALS AND METHODS

Plant material

The ORSTOM, Montpellier, collection of cassavas consists of some 80 accessions, mostly of *Manihot esculenta*, together with some representatives of *M. glaziovii* Mueller von Argau, *M. caerulescens* Pohl and interspecific hybrids between *M. esculenta* and *M. glaziovii* (Table 1). Most of these accessions came from Africa and originate from collections maintained there or from collecting expeditions. The collection was maintained axenically *in vitro* (Brizard, Noirot and Engelmann, 1991).

DNA methods

Manihot DNA was extracted from 0·5–1·0 g of fresh leaves by the method of Dellaporta, Wood and Hicks (1983). Five micrograms of genomic DNA were digested with 2·5-fold excess of *Eco*RI or *Hin*dIII (Boehringer Mannheim, Germany), loaded into two tiers of 36 wells on 20 × 25 cm 0·8% agarose gels in Tris-borate buffer and electrophoresed at 0·6 V cm^{-1} for 16 h. Molecular weight markers and internal standards were loaded per gel and per lane, respectively, as suggested by Hoisington and González de Leon (1990). The DNA was transferred to Hybond-N+ membranes (Amersham International, Slough, UK) by alkaline blotting according to the manufacturer's instructions. The probes were from a cDNA library constructed in lambda gt10 from mRNA purified from 10-d-old germinating cassava cotyledons (Hughes *et al.*, 1992). These included CAS5, a cDNA clone for the cyanogenic β-glucosidase (Hughes *et al.*, 1992), and clones for glucosyltransferase. The seven glucosyltransferase clones were isolated by Dr J. Hughes (University of Newcastle upon Tyne, UK) from the gt10 cDNA and subcloned into the

TABLE 1. *Cassava varieties and relatives in the collection at ORSTOM analysed in this study*

Lab. no.	Identification	Species*	Origin	Source†
02	7902	M.e.	Ivory Coast	IDESSA
04	7905	M.e.	Ivory Coast	IDESSA
06	Kataoli	M.e.	Togo	CIRAD
07	5543/33	M.e.	Kenya	Storey-Jennings
08	4762	M.e.	South America	N/D
10	H43	M.e.	Madagascar	N/D
11	Chorokote	M.e.	East Kenya	N/D
12	Kibandameno	M.e.	East Kenya	N/D
13	5318/34	M.e.	Kenya	Storey-Jennings
14	Mwakasanga	M.e.	East Kenya	N/D
15	4748	M.e.	South America	N/D
17	46106/34	M.e.	Kenya	Storey-Jennings
18	Kasimbidgi	M.e.	East Kenya	N/D
19	50284/33	M.e.	Kenya	Storey-Jennings
20	Arpin Valenca	M.e.	Brazil	N/D
21	Garimoshi	M.e.	India	N/D
22	Nusu Rupia	M.e.	India	N/D
23	Kasimbidgi Red	M.e.	East Kenya	N/D
25	Mpira	M.e.	India	N/D
25R	25R	M.e.	N/D	N/D
26	4760	M.e.	South America	N/D
27	Viro3	M.e.	South America	N/D
29	Viro9	M.e.	Ivory Coast	N/D
33	30211	M.e.	Nigeria	IITA
34	30337	M.e.	Nigeria	IITA
36	No. 36	M.e.	N/D	N/D
37	30395	M.e.	Nigeria	IITA
38	30555	M.e.	Nigeria	IITA
39	B32	M.e.	R.C.A.	N/D
40	A13	M.e.	Ivory Coast	CIRAD Bouaké
43	30040	M.e.	Nigeria	IITA
44	30785	M.e.	Nigeria	IITA
45	Toumoudi	M.e.	Ivory Coast	N/D
46	No. 46	M.e.	N/D	N/D
50	Bounoua Rouge 1	M.e.	Ivory Coast	CIRAD Bouaké
52	TA49	M.e.	Ivory Coast	CIRAD Bouaké
53	57	M.e.	Madagascar	CIRAD Bouaké
54	H58	M.e.	Madagascar	CIRAD Bouaké
65	31	M.e.	Guyana	N/D
67	Abyssinica	M.e.	Ethiopia	P. Fiorino
68	M. Col. 1505	M.e.	Colombia	CIAT
96	TMS4(2)0267	M.e.	Nigeria	IITA
55	127G	M.g.	Ivory Coast	N/D
56	167	M.g.	Ivory Coast	N/D
61	Glaziovii	M.g.	Ivory Coast	N/D
80	127-I	M.g.	Ivory Coast	ORSAY Paris
70	MC1	M.c.	South America	ORSAY Paris
71	MC3	M.c.	South America	ORSAY Paris
58	GE3-5	g.e.	Ivory Coast	ORSAY Paris
59	GE3-3	g.e.	Ivory Coast	ORSAY Paris
60	GE31-2	g.e.	Ivory Coast	ORSAY Paris
76	GE33H	g.e.	Ivory Coast	ORSAY Paris
77	GE3-6	g.e.	Ivory Coast	ORSAY Paris

* M.e., *Manihot esculenta*; M.g., *M. glaziovii*; M.c., *M. caerulescens*; g.e., *M. glaziovii*/*M. esculenta* hybrids; N/D—no data.

† IDESSA, Institut des Savanes, Bouaké, Ivory Coast; R.C.A., Central African Republic; IITA, International Institute of Tropical Agriculture, Nigeria; P. Fiorino, IPSL-CNR, Florence; CIAT, Centro Internacional de Agricultura Tropical, Colombia.

*Not*I site of pBluescript KS +/− (Stratagene Ltd., Cambridge, UK) or pGEM5 (Promega, Southampton, UK). None of the clones cross hybridise with each other at the high stringencies used in this study. In addition preliminary studies showed that all the clones hybridised with distinct regions of the genome since different *Hin*dIII fragments are identified. The size of the clones used varied from 0·9 to 1·7 kilobase pairs. The cDNA inserts were isolated from the plasmids by digestion with *Not*I and the freeze/squeeze method (Sambrook, Fritsch and Maniatis, 1989), labelled with Amersham's Multiprime kit and hybridised as in Beeching *et al.* (1989). The stringency of the final wash was 0·1 × SSC at 65 °C. The blots were exposed to Hyperfilm (Amersham) using intensifying screens at −80 °C for 3–10 d.

Data analysis

The autoradiographs were scored in terms of the presence or absence of individual bands. The resulting data was analysed using the RESTSITE (version 1.2) package of programs (Miller, 1992) which is based on the equations of Nei and Miller (1990). Presence or absence band data was converted to distance matrices based on the similarity indices of Jaccard (1901) and Simple Matching (Sokal and Michener, 1958) using a computer program written in BASIC; these matrices served as input to the distance program NEIGHBOR within the PHYLIP (version 3.4) package (Felsenstein, 1989). NEIGHBOR uses distance matrix data to construct dendrograms via the unweighted pair-group method of averaging (UPGMA) of Sneath and Sokal (1973).

RESULTS AND DISCUSSION

Quality of the data

Fifteen probe–enzyme combinations (PECs) were successfully tried and full data sets were obtained for 53 different plants. With the exception of one of the glucosyltransferase probes, which was only used with *Hin*dIII-digested DNA, genomic digests with both *Eco*RI and *Hin*dIII were used with all probes. Each PEC gave between two and 21 scorable band levels on the autoradiographs, with an average of just over ten bands per PEC. For the overall combination of plants and PECs used there was a total of 155 scored bands; of these nine bands (5·8 %) were common to all plants, 28 (18 %) were unique to individual plants, and the remainder, 118 bands, were polymorphic, in the sense that they were shared by at least two, but not all, plants. It is interesting that half (14) of the unique bands were detected in one individual plant, *M. esculenta* 31, from Guyana. All 155 bands were used in the analyses.

All the PECs detected some polymorphism in the plants examined, though some were more informative than others. This informativeness was neither probe nor enzyme dependent. For example, probe pCGT3 detected eleven band levels with *Eco*RI including eleven different band patterns amongst *M. esculenta*, while with *Hin*dIII, only three band levels were detected and all plants except one had the same band pattern. A similar, but opposite, effect was detected with probe pM2, *Hin*dIII revealing a significantly higher level of polymorphism than *Eco*RI.

Data analysis

All three methods, RESTSITE, Jaccard and Simple-Matching produce distance matrices which can then be used to construct dendrograms via UPGMA which show degrees of relatedness between individual plants. However, the approaches are based on different principles. RESTSITE (Miller, 1992) estimates a genetic distance based on the number of nucleotide substitutions per restriction site (Nei and Miller, 1990). This distance is essentially the same as that obtained by the method of Nei and Li (1979). The Jaccard and Simple-Matching indices bear no connection to the biological origin of the data and are numerical tools for classification where characters can be scored in terms of their presence or absence, in this case of restriction fragments. The two indices differ in the weighting that they give to matches of presences or absences. The similarity index of Jaccard between plants i and j is given by:

$$S_{ij} = a/(a+b+c)$$

The simple-Matching similarity index is given by:

$$S_{ij} = (a+d)/(a+b+c+d)$$

Where a is the number of characters present in both i and j, b is the number of characters present in i but not in j, c the number of characters absent in i but present in j and d is the number of characters absent in both i and j.

While these two indices are symmetrical in b and c, Simple-Matching gives equal weight to the absence of characters in both individuals, d, as it does to the presence in both, a. Jaccard only takes into consideration the presence of characters in both individuals, a. The dissimilarity ($D_{ij} = 1 - S_{ij}$) is used to calculate the distance matrix between individuals from which dendrograms can be constructed.

The RFLP data was analysed by all three methods in order to detect common clusters of plants and to avoid the possibilities of bias that might be inherent in using only one approach. In all cases the dendrograms were very similar showing the same broad clusterings within which the finer detail was virtually the same (Fig. 1). The similar dendrograms produced by these different analytical methods enable the clusterings to be accepted with confidence. For reasons of space, dendrograms produced from the Jaccard index are presented here when comparing the individual plants. The Jaccard, or closely related indices, have also been used for similar studies (Wetton *et al.*, 1987; Debener *et al.*, 1990; Packer *et al.*, 1991). The most noticeable difference between the methods was in the positioning of isolated individuals which did not seem to fit into any natural grouping. With the exception of one *M. esculenta*, 31, the three species formed three distinct clusters with all three methods of analysis. The *M. esculenta*/*M. glaziovii* hybrids clustered between these two species.

M. esculenta 31 and, to a lesser extent, Abyssinica were very interesting cassava cultivars in terms of their clustering positions. *M. esculenta* 31 was always positioned on its own at a very considerable distance from the other *M. esculenta* cultivars and the other species (Fig. 1). This cultivar possessed 14 unique restriction fragments not shared with any of the other plants examined. In terms of morphology,

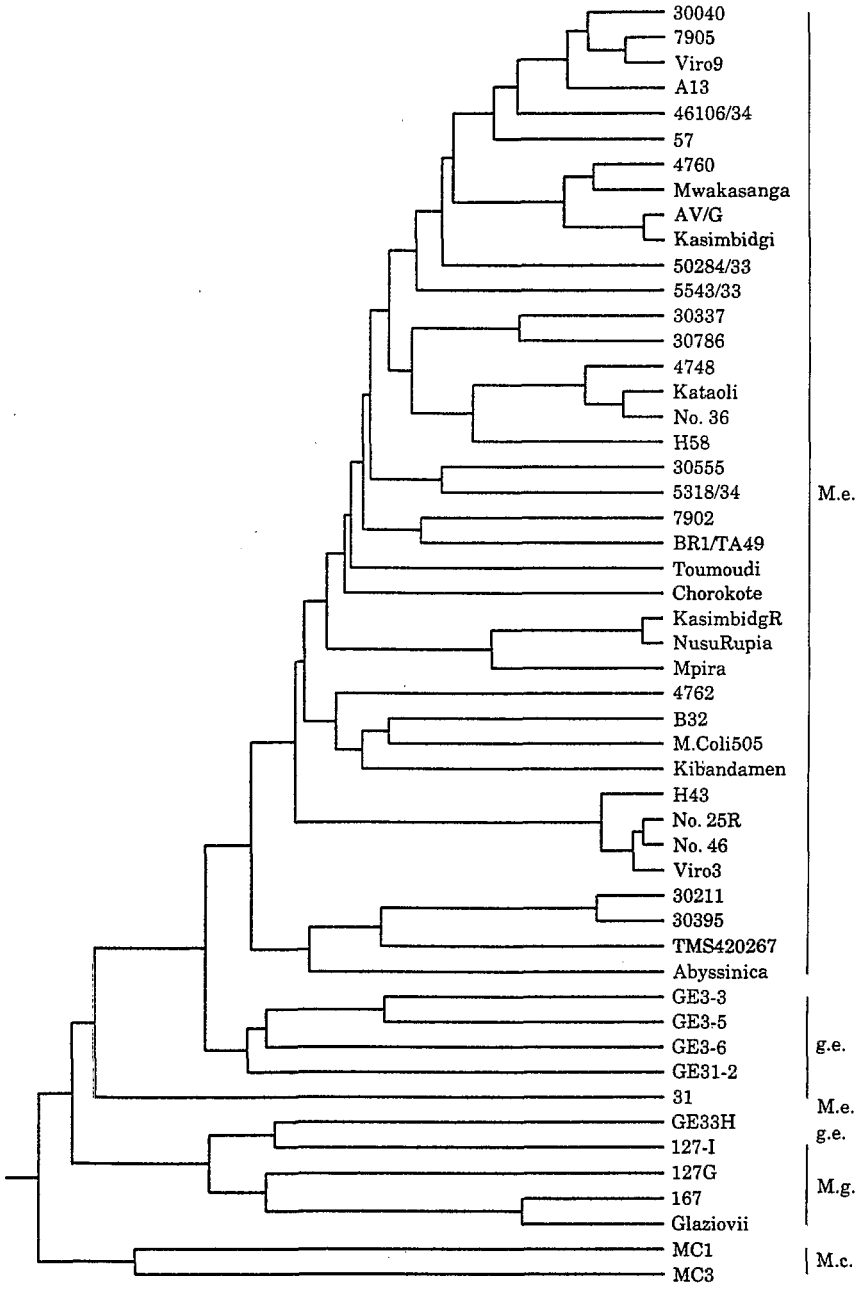

FIG. 1. Dendrogram showing diversity of the individual *Manihot esculenta* (M.e.), *M. glasiovii* (M.g.), *M. caerulescens* (M.c.) and *M. esculenta*/*M. glaziovii* interspecific hybrid (g.e.) accessions produced with the Jaccard index and UPGMA. The cultivars which were found to be identical in this study are indicated by the following abbreviations: AV/G, Arpin Valenca and Garimoshi; BR1/TA49, Bonoua Rouge 1 and TA49.

as observable in *in vitro* grown plants, it did not stand out from the other *M. esculenta* accessions. However, the data presented here suggest that it is genetically very distinct from the other *M. esculenta* members of the collection, in fact it is sufficiently different to place it in another *Manihot* species altogether. In the ORSTOM collection it is listed as a cassava from Guyana in South America, unfortunately no other details are available. The possibility exists, therefore, that 31 is either a mis-classified member of another *Manihot* species, or that it is an interspecific hybrid between *M. esculenta* and a distant *Manihot* species not represented in this study.

Abyssinica, another isolated *M. esculenta*, in this case from Ethiopia, was clustered as a remote member of a *M. esculenta* grouping (Fig. 1). However, it shares a few restriction fragments with *M. glaziovii* which are not shared with any other *M. esculenta*, suggesting, perhaps, that its origin includes hybridization with *M. glaziovii*.

Within the ORSTOM collection only two pairs of *M. esculenta* were found to be identical, TA49 and Bonoua Rouge 1, and Arpin Valenca and Garimoshi. The former two accessions come from the same collection in the Ivory Coast, the possibility must therefore exist that they represent different names for the same local variety, a not unusual phenomenon (J. Mabanza, pers. comm.). With the latter pair, this possibility does not arise as Arpin Valenca comes from Brazil, while Garimoshi is an Indian cultivar. While the genetic identity of the two cultivars cannot be dismissed,

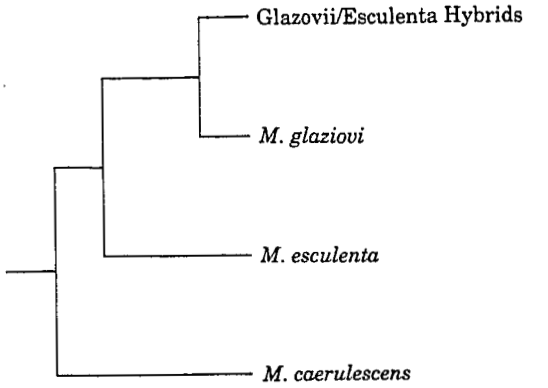

FIG. 2. Dendrogram showing the interspecific diversity between *Manihot esculenta*, *M. glasiovii*, *M. caerulescens* and the *M. esculenta*/*M. glaziovii* interspecific hybrids. The data for each were pooled, the distance calculated by RESTSITE and the dendrogram constructed by UPGMA.

cassava was after all taken from Brazil to India in the eighteenth century (Cock, 1985), it is possible that the methods employed here were not sensitive enough to differentiate between them.

The restriction fragment data for the two enzymes, *Eco*RI and *Hin*dIII, were analysed separately using the RESTSITE program, to examine whether the enzymes gave any noticeable bias to the resultant dendrograms. There were no major differences between the dendrograms from the single enzyme data and that from using all the PECs together, apart from the placing of distant individual plants in other clusters and some plants appearing as identical which were normally close but distinct (data not shown). Minor changes of this nature were expected due to the reduced number of characters used in these sub-analyses.

The data for the three species and the interspecific hybrids were pooled in order to estimate the average genetic distance between these four populations using the RESTSITE program (Fig. 2). This dendrogram is in agreement with Rogers and Appan (1973) who also positioned *M. glaziovii* closer to *M. esculenta* than *M. caerulescens*. The *M. esculenta*/*M. glaziovii* hybrids were located at an intermediate position between the parental species. It is interesting that greater diversity was found within *M. glaziovii* and *M. caerulescens* than within *M. esculenta* (Fig. 1). This is perhaps to be expected as *M. esculenta* is the cultivated species. However, the wild species were not well represented, so it was not possible to tell whether this apparent difference in intraspecific diversity was significant.

CONCLUSIONS

The data presented and analysed here bear out the recommendation for the use of RFLPs in the assessment of genetic diversity within collections of plants (Flavell, 1991). Certainly the dendrograms produced a cluster of the three *Manihot* species examined separately, while the interspecific hybrids are clustered in an intermediate position between the parent species. These species differentiations provide strong support for the acceptance of the sub-clusterings within the *M. esculenta* cluster as genuine. The positioning of *M. esculenta* 31, and, to a lesser extent, Abyssinica, outside the *M. esculenta* cluster, suggests that there are some real differences between these individual plants and the species into which they have been classified.

It has been argued, principally on theoretical grounds, that RFLPs are not a good tool for phylogenetic reconstruction because they violate the assumption of independence among characters (Swofford and Olsen, 1990). These authors recommend instead the considerably more laborious and costly restriction site analysis. Bremer (1991) empirically compared real restriction data in four different ways, including restriction sites and RFLPs. She concludes, that while the resultant cladograms show some differences, none of the methods significantly bias the result. Therefore, while RFLP may not be of the same quality as site data, the former is more rapid to accumulate. The choice between the two is dependent on a trade-off between quality and resources. Certainly RFLP data has been successfully applied to studies of genetic diversity in other important crop plants (Havey and Muehlbaner, 1989; Debener *et al.*, 1990; Miller and Tanksley, 1990; Zhang *et al.*, 1992; Song *et al.*, 1990).

Three methods, RESTSITE, and the Jaccard and Simple-Matching indices were used to analyse the data; despite being based on different principles they gave very similar results. This confirms the usefulness of these analytical methods for this kind of work, and at the same time enhances the confidence which can be placed on the clusterings obtained. This is reassuring as there exist a wide range of methods and different schools of thought in taxonomic and phylogenetic analysis (Jackson, Somers and Harvey, 1989; Swofford and Olson, 1990). It is interesting that the two numerical methods, which differ in the weighting given to matches of absences, gave broadly similar results. This suggests that the shared absence of bands may be as significant as the shared presence, certainly they do not appear to bias or distort the resultant dendrograms. However, most studies using data generated from RFLPs or DNA fingerprinting, have used either the Jaccard index or derivatives of it, which do not take into account matches of absences (Wetton *et al.*, 1987; Debener *et al.*, 1990; Packer *et al.*, 1991). For reasons of comparability with these studies, results derived from the Jaccard index are presented here when comparing individual plants. However, RESTSITE provides the opportunity to pool data from different individuals, which is convenient when comparing populations from different species.

Two accessions classified as *M. esculenta* were identified as being sufficiently different from the major cluster of *M. esculenta* accessions for one, 31, to be considered potentially a separate species, and the second, Abyssinica, to have hybridized with *M. glaziovii* in its ancestry. *Manihot* species have a ploidy of $2n = 36$ and there appear to be no genetic barriers to hybridization within the genus (Rogers and Appan, 1973). Naturally occurring hybrids between *M. esculenta* and *M. glaziovii* have been detected where the two species grow together (Lefèvre, 1988). Rogers and Appan (1973) suggest that there is putative evidence that where cassava occurs, or has been transported by man, in America, there has been hybridization with locally growing wild *Manihot* species, thereby producing cultivars which are unique to particular regions. It is possible that 31, from

Guyana, is just such a unique cultivar. This potential for interspecific hybridization should not be ignored in the genetic improvement of cassava (Chàvez, 1990).

The accessions of *Manihot* species in the ORSTOM collections at Montpellier were made for other purposes than the analysis of genetic diversity. However, this study has demonstrated considerable diversity within the African *M. esculenta* accessions, certainly not less than within the, admittedly few, American accessions examined. This suggests that within the several collections of African cultivars of cassava maintained in that continent, there already exists some of the variation necessary for a programme of genetic improvement of cultivars specifically adapted to African conditions. Prior to such a programme a quantitative assessment of this genetic diversity using the methods described in this paper is required. The largest African collection, that of the International Institute of Tropical Agriculture (IITA) in Nigeria, has 2000 cassava varieties. While it would not be impossible to apply these techniques to that number, a selection would be more practicable. The selection should be on an agro-ecological basis to include agronomic, pest-resistance and geographical factors which may significantly contribute to the distribution of genetic diversity in cassava. The study should also include reference cultivars of cassava, perhaps from the Centro Internacional de Agricultura Tropical (CIAT), Colombia, and some other *Manihot* species.

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