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AFLP analysis of relationships among cassava and other *Manihot* species

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Abstract Despite the worldwide importance of cultivated cassava (*M. esculenta* Crantz) its origin and taxonomic relationships with other species in the genus have not been clearly established. We evaluated a representative sample of the crop's diversity and six wild taxa with AFLPs to estimate genetic relationships within the genus. Groupings of accessions of each species by data analysis corresponded largely with their previous taxonomic classifications. A mixed group, consisting of *Manihot esculenta* subsp. *flabellifolia* and *M. esculenta* subsp. *peruviana*, was most similar to cassava, while *M. aesculifolia*, *M. brachyloba*, and *M. carthaginensis* were more distant. Species-specific markers, which may be useful in germ-plasm classification or introgression studies, were suggested by the unique presence of AFLP products in samples of each of the three wild species. Heterogeneity of similarities among individuals of certain species suggested the existence of intraspecific gene pools, a hypothesis that was supported by morphological or ecogeographic evidence with varying degrees of success. Quantitative assessment of genetic diversity revealed greater homogeneity among cassava accessions than among its closest wild relatives. The demonstration of unique genetic diversity in the two *M. esculenta* subspecies and their genetic similarity to the crop supports the hypothesis that these materials may be the ancestors of cassava.

Key words Cassava · *Manihot* genus · AFLP · Genetic diversity · Wild relatives of cassava

Introduction

The genus *Manihot* (Euphorbiaceae) is native to the Neotropics, growing in diverse habitats between southern USA and Argentina (Rogers and Appan 1973). *Manihot* species are perennials and vary from acaulescent shrubs to trees of 10–12 m. Most produce tuberous roots, and some, such as cassava (*Manihot esculenta* Crantz), accumulate large quantities of starch. Cassava is a major tropical crop, produced mainly by small farmers for food and small-scale industrial use. It occupies fourth place, after rice, sugarcane and maize, as a source of calories in the human diet, feeding more than 500 million people in Africa, Asia, and Latin America (Cock 1985; Best and Henry 1994).

Despite its importance, aspects such as the origin and domestication of the cassava and phylogenetic relationships with other species of the genus have not been elucidated. Linguistic, ethnological, archeological, taxonomic and geographic evidence suggest that the crop had many points of initial cultivation. Rogers and Appan (1973) proposed that, of the 98 species they described, the Central American species *M. aesculifolia* (H.B.K.) Pohl is the closest wild relative. *M. esculenta* Crantz subsp. *flabellifolia* (Pohl) Ciferri was regarded as a synonym of cassava; the species *M. tristis* Muell. Arg. and *M. peruviana* Muell. Arg. were placed in different sections of the genus. Allem (1987) disagreed, suggesting *M. tristis* as the species most similar to cassava. He later reported (1994 a, b) finding wild forms of the cultivated species in north-eastern Brazil, regarding the glabrous form as *M. esculenta* subsp. *flabellifolia*; the pubescent variety as *M. esculenta* Crantz subsp. *peruviana* (Muell. Arg.) Allem; and *M. tristis* as

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a synonym, morphologically indistinguishable from *M. esculenta* subsp. *flabellifolia*.

Allem's propositions have since been challenged by suggestions that the re-classified wild forms may have been escapes from cassava crops (Renvoize 1972; Bertram 1993). The appearance of swollen nodes in these plants cast doubt on their nature as true wild relatives, as this character was considered to be an indirect result of artificial selection for efficient vegetative reproduction (Jennings 1976, cited by Bertram 1993). The question thus remains: is cassava the result of domestication of these wild populations, or are they escapes from cultivated material?

Molecular markers have recently been employed to help resolve some of these taxonomic and phylogenetic questions. Bertram (1993) analyzed 14 *Manihot* species from North and Central America, together with land races and improved varieties of cassava from different regions, performing a cladistic analysis of RFLP (restriction fragment length polymorphism) variation in chloroplast (cp) and ribosomal (r) DNA. He proposed that *M. aesculifolia* was most similar to cassava, together with *M. carthaginensis* (Jacquin) Muell. Arg. Fregene et al. (1994), analyzing RFLPs of cpDNA and rDNA of 29 accessions of wild species and cultivated varieties, showed that *M. tristis* and *M. esculenta* subsp. *flabellifolia* are also very close to, but divergent from, cassava. They suggested that cassava was more likely to have been domesticated from a complex of these species than from a hybridization event between them. But because molecular studies have rarely treated Central American species together with those of South America, a lack of consensus still exists on the origin of cassava, and the degree of its relationships with other *Manihot* species.

A new type of marker is now available to help address these questions. AFLP (amplified fragment length polymorphism) analysis is a technique through which selected restriction fragments from the digestion of total plant DNA are amplified by the polymerase chain reaction (Vos et al. 1995). The resulting DNA fingerprint provides a large number of genetic markers; and the multiplex ratio, defined as the number of information points analyzed per experiment, is much higher than for other types of markers, such as RFLP, RAPD (randomly amplified polymorphic DNA) or SSRP (simple sequence repeat polymorphism) (Powell et al. 1996).

We used the AFLP technique to analyze the five wild forms mentioned above as close relatives or ancestors of cassava, a distant species (*M. brachyloba* Mueller von Argau), and a representative sample of the genetic diversity available in the crop. Based on our results, we revised hypotheses on the ancestry and origin of cassava.

Materials and methods

Plant materials

A total of 105 genotypes were evaluated, comprising 35 landraces of cassava, three bred cassava lines, and 67 individuals from six additional taxa: *M. aesculifolia*, *M. carthaginensis*, *M. brachyloba*, *M. tristis*, *M. esculenta* subsp. *flabellifolia* and *M. esculenta* subsp. *peruviana* (Table 1). The landraces were selected from the larger core collection of cassava germ plasm conserved at CIAT, Cali, Colombia, using geographic, morphological and biochemical parameters (Hershey et al. 1994).

DNA extraction

Fresh young leaves were harvested from each genotype on liquid nitrogen and maintained at -80°C . DNA was extracted, using either the method of Dellaporta et al. (1983), with 3–4 g of leaf tissue, or the ORSTOM method, with freshly harvested and dried leaf tissue (at 48°C for 24 h) (C. Colombo, in preparation).

AFLP method

The AFLP protocol followed was that described by Vos et al. (1995), with slight modifications (Tohme et al. 1996). Primary template was prepared by the simultaneous digestion of DNA with *EcoRI* and *MseI* and subsequent ligation of asymmetric quantities of enzyme-specific adapters. The amount of information obtained by AFLP is a factor of the number of selective nucleotides employed, and the size and complexity of the genome being analyzed. Trials were conducted on two *Manihot* accessions, with 20 combinations of first- and second-round primers (see Table 2) to identify successful combinations of selective nucleotides, taking into account *Manihot*'s genome size of between 6.93 and 8.30×10^8 base pairs (Arumuganathan and Earle 1991) and recommendations made by Lin and Kuo (1995). DNA samples of eight genotypes were obtained independently, and in duplicate, with each of the two extraction methods. These samples were evaluated to ensure robustness and reproducibility of the AFLP protocol.

Following these preliminary trials, the entire set of plant materials was analyzed, using A and G as selective nucleotides (*EcoRI* primer +A, and *MseI* primer +G, respectively) for pre-amplification, and the two second-round primer combinations (*EcoRI* + AAC \times *MseI* +GTA, and *EcoRI* +ACA \times *MseI* +GTA). Radio active amplification products [from labelling the *EcoRI* primer in the second round of amplification with $[^32\text{P}]\gamma\text{dATP}$] were size-fractionated on 6% polyacrylamide denaturing gels on a Sequi-Gen (BioRad) sequencing apparatus. Electrophoresis was carried out for 2 h in $1 \times$ TBE at 40 V/cm and 45°C . The gels were covered with Saran Wrap, dried under vacuum for 1 h at 80°C , and exposed for 16 h on X-ray films (Kodak X-omat LS) for autoradiography.

Data analysis

AFLPs from the two primer combinations were registered in terms of the presence or absence of bands in each of the 105 accessions evaluated. The results were converted into a similarity matrix, based on the index of Nei and Li (1979).

The similarity matrix was analyzed, using the computer program NTSYS (version 1.8; Rohlf 1994). Dendrograms were constructed by employing the option TREE, and the UPGMA (unweighted pair grouping method of averages) method of Sneath and Sokal (1973). A correlation index was calculated between the similarity

Table 1 *Manihot* germ plasm selected for AFLP analysis

<i>Manihot</i> species	Accession code ^a	No. of individuals	Country of origin	<i>Manihot esculenta</i> accession code ^a	Country of origin	Common name
<i>M. aesculifolia</i>	AES 002	1	Mexico	M ARG 11	Argentina	Duro do Valle 30
	AES 404-004	1	Mexico	M BOL 3	Bolivia	Rosada de Bolivia
	WU 26 AES 2	1	Mexico	M BRA 12	Brazil	– ^c
	WU 25 AES 6	1	Mexico	M BRA 97	Brazil	Saracura II
	WU 24 AES 7	1	Mexico	M BRA 110	Brazil	Pangola
	WU 82 AES 8	1	Mexico	M BRA 383	Brazil	Vassourão
	WU 32 AES 4	1	Mexico	M BRA 881	Brazil	Branca de Sta. Catarina
	WU 27 AES 5	1	Mexico	M BRA 885	Brazil	Jabarú
<i>M. brachyloba</i>	BLO 001	1	–	M BRA 900	Brazil	Mandim Branca
	BLO 401	1	Colombia	M BRA 931	Brazil	Enganha Ladrão
	BLO 402	1	Colombia	M COL 1468	Brazil	Sip24-2 Mantiqueira
<i>M. carthaginensis</i>	CTH 005	1	Colombia	M COL 22	Colombia	Uvita
	CTH 011	1	Colombia	M COL 1438	Colombia	Llanera
	CTH 012	1	Colombia	M COL 1522	Colombia	Algodonera Amarilla
	CTH 106	1	Colombia	M COL 1684	Colombia	–
	CTH 121	1	Colombia	M COL 2061	Colombia	Regional Morada
	CTH 164	1	Colombia	M COL 2066	Colombia	Chiroza Gallinaza
	CTH 246	1	Colombia	M COL 2215	Colombia	Venezolana 1
	CTH 315	1	Colombia	HMC 1	Colombia	–
	CTH 409	2	Colombia	M CR 32	Costa Rica	Yuca Mangi
	CTH 411	1	Colombia	M CUB 51	Cuba	Pinera
	CTH 414	1	Colombia	M CUB 74	Cuba	Señorita
	CTH 415	5	Colombia	M ECU 41	Ecuador	De Tres Meses
	CTH 416	1	Colombia	M ECU 82	Ecuador	Blanca
	CTH 417	6	Colombia	M IND 33	Indonesia	No. 734-5
CTH Z	1	–	M MAL 2	Malaysia	Black Twig	
<i>M. esculenta</i> subsp. <i>flabellifolia</i>	ESC-FLA 423	2	Brazil	M MAL 48	Malaysia	Red Twig
	ESC-FLA 427	2	Brazil	M MEX 59	Mexico	–
<i>M. esculenta</i> subsp. <i>peruviana</i>	ESC-FLA 428	2	Brazil	M PAN 51	Panama	–
	ESC-FLA 430	1	Brazil	M PAR 110	Paraguay	Tacuara Sayyu
	ESC-FLA 438	2	Brazil	M PTR 19	Puerto Rico	No. 9588
<i>M. esculenta</i> subsp. <i>peruviana</i>	ESC-FLA 441	2	Brazil	M TAI 1	Thailand	Rayong 1
	ESC-PER 406	2	Brazil	M VEN 25	Venezuela	Querepa Amarga
	ESC-PER 407	2	Brazil	M VEN 45	Venezuela	–
	ESC-PER 411	2	Brazil	M COL 1505	Venezuela	–
	ESC-PER 412	2	Brazil	CM 2177-2	CIAT ^d	Hybrid
	ESC-PER 413	2	Brazil	CM 3306-9	CIAT ^d	Hybrid
	ESC-PER 414	2	Brazil	TMS 30572	IITA ^e	Hybrid
	ESC-PER 415	2	Brazil			
	ESC-PER 417	2	Brazil			
	<i>M. tristis</i>	TST-002	1	–		
TST-008		1	–			
TST-009		1	–			
TST-011		1	–			

^a *Manihot* species were collected as true-seed populations

^b Cassava accessions are maintained as vegetative clones

^c – = as used throughout field, indicates “no data”

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matrices resulting from the two different primer combinations to analyze the complementary or redundancy of the information.

The means of all pairwise similarities between the individuals within each of the principal clusters identified by UPGMA were compared, using Duncan's multiple range test. Similarities between individuals comprising all pairwise combinations of species were also calculated to determine the means and standard deviations of interspecific similarities. Multiple correspondence analysis (MCA) was conducted to evaluate the contribution of the specific active variables (AFLP products) to the variation observed among

genotypes, by employing the CORRESP option of SAS (version 6-11; 1989).

Morphological characterization

Except for several accessions of *M. aesculifolia*, which were available only as leaf samples, the germ plasm included in this study was characterized morphologically, using a set of descriptors comprised

Table 2 Informativeness of 20 combinations of selective primers used to detect AFLP between two *Manihot* genotypes (ESC-FLA 423-6 and ESC-PER 412-2). In all cases, pre-amplification was

carried out with *EcoRI* + A and *MseI* + G primers. Total number of bands and percentage of polymorphism are as observed for the two genotypes analyzed

EcoRI primer	MseI primer							
	GTA		GCG		GAC		GGT	
	Bands (no.)	Polymorphs (%)	Bands (no.)	Polymorphs (%)	Bands (no.)	Polymorphs (%)	Bands (no.)	Polymorphs (%)
AAC	45 ^a	44 ^a	37	38	35	51	44	36
ACG	42	74	– ^b	–	–	–	–	–
AGT	33	48	30	47	36	22	34	68
ACA	44 ^a	61 ^a	47	28	34	59	30	60
AT	87	40	24	29	56	41	84	49

^a Primer combinations selected for analysis of the *Manihot* germ-plasm set

^b – = “no data”

of 38 characters, both vegetative (stem, leaf, petiole and stipules) and reproductive (flower, fruit and seed).

Results

AFLP provides a high information content for *Manihot* species

A comparison of information content and the resolution of amplification products from 20 combinations of primers revealed between 24 and 87 amplification products per individual and 22% to 74% polymorphic bands between the two individuals analyzed (Table 2). Three primer pairs employing (*EcoRI*) + 2 and (*MseI*) + 3 selective nucleotides revealed a larger number of AFLP products in the germ plasm than the + 3/ + 3 combinations, but these were generally less polymorphic. Among the + 2/ + 3 combinations, the generation of the lowest number of AFLP products with *MseI* primer + GCG is consistent with expectations based on sequence context. The primer combinations *EcoRI* + AAC × *MseI* + GTA, and *EcoRI* + ACA × *MseI* + GTA were chosen as the most informative, and applied to the complete set of germ-plasm accessions.

Analysis of the 105 genotypes presented a total of 139 AFLP bands for the first primer combination, and 165 bands for the second, when bands from all individuals were considered. The number of bands obtained per individual ranged from 17 to 51, confirming the high multiplex ratio obtainable with this type of marker (Fig. 1). Only two bands revealed by analysis with each primer combination were monomorphic in the germ-plasm set, demonstrating a polymorphism of 98.6%, a higher rate than that obtained with other marker types, using different plant materials: isozyme analysis of cultivated and wild *Manihot* species showed 55%–58% polymorphism (Lefèvre and Charrier 1993), RAPD patterns 31% (Carvalho et al. 1994), and RFLPs 6%–98% (Bertram 1993; Haysom et al. 1994).

The matrices of genetic similarity estimates, based on AFLP patterns from the two separate primer combinations, were highly correlated ($r = 0.91$). The analysis permitted the unique identification of each individual analyzed, indicating that the collection does not contain genetic duplicates, and facilitated analysis of the distribution of genetic diversity among accessions and species.

Cluster analysis

Figure 2 depicts the clustering of *Manihot* accessions into eight groups of individuals that correspond largely to prior taxonomic classification. Discrete groups were formed for the species *M. aesculifolia*, *M. brachyloba* and *M. carthaginesis* (clusters 1, 2 and 3, respectively), and cassava (*M. esculenta*, cluster 8). Most accessions pertaining to *M. esculenta* subsp. *flabellifolia* and *peruviana* fell into a mixed cluster (7). Three smaller groups were formed by two pairs of individuals adapted to high altitudes, the *M. esculenta* subspecies (4 and 5) and *M. tristis* (6).

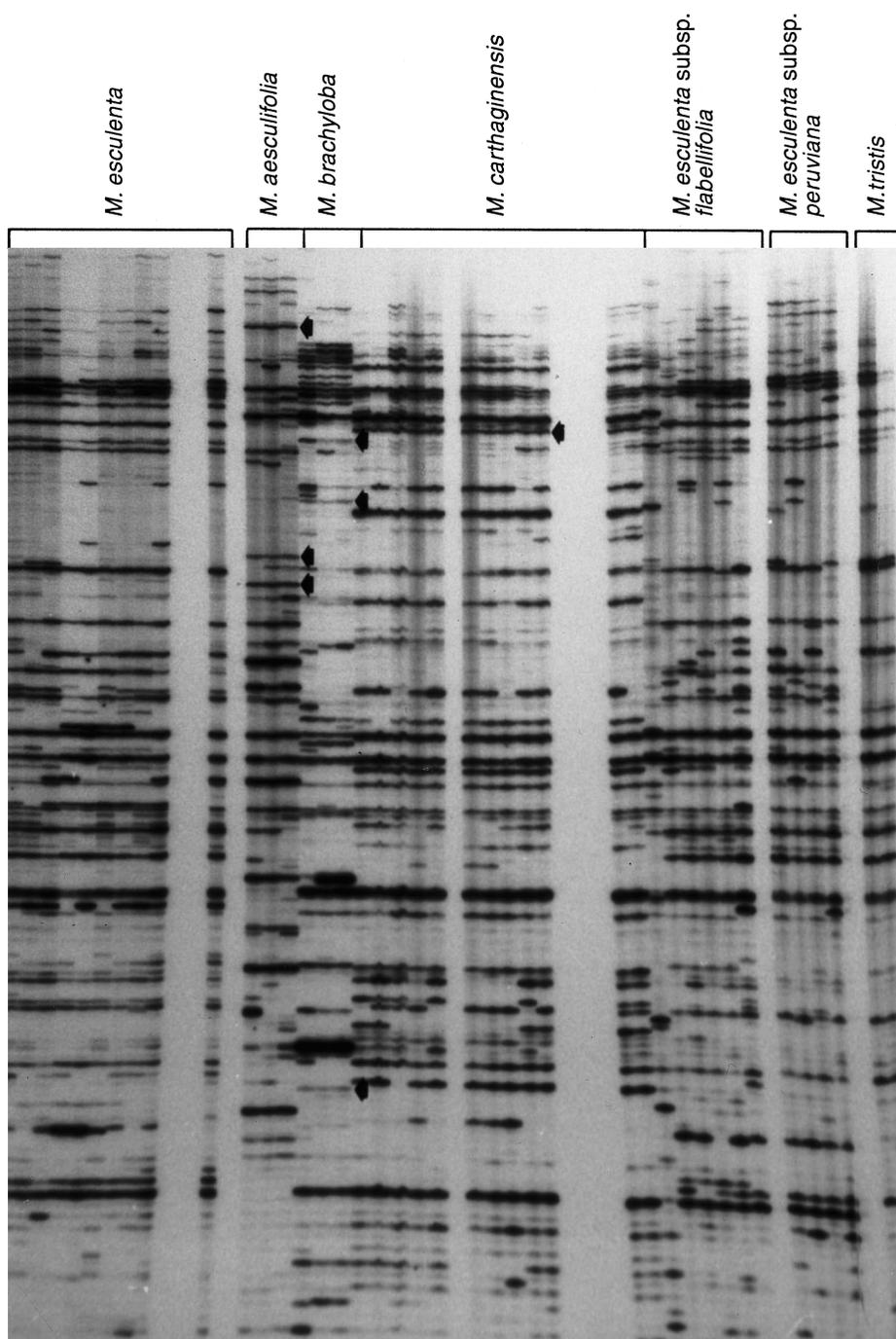
The accessions representing cassava appear as a compact group (8), with greater similarity among its members than in the next cluster (7). Because cluster 7 comprised a mixture of *M. esculenta* subsp. *flabellifolia* and *peruviana* accessions, these two subspecies were pooled into a single group for subsequent analyses.

Mean similarities between pairs of individuals within the six resulting groups were significantly different ($P < 0.05$), according to Duncan's multiple range test (Table 3). As expected, intraspecific similarity (Table 3, diagonal) was greater in all cases than interspecific (Table 3, upper triangle).

Multiple correspondence analysis

The multiple correspondence analysis (MCA; Fig. 3 a) portrayed *M. aesculifolia*, *M. carthaginesis* and

Fig. 1 Autoradiograph showing AFLP detected in a subset of *Manihot* germ plasm with the second-round selective primer combination *EcoRI* + AAC × *MseI* + GTA. Arrows indicate putative species-specific bands for *M. aesculifolia*, *M. brachyloba* and *M. carthaginensis*



M. brachyloba as discrete groups that are separate from the other accessions. *M. esculenta* appears as a compact group with accessions of *M. tristis* and the two *M. esculenta* subspecies nearby.

A separate multiple correspondence analysis was performed on the 69 genotypes representing the two *M. esculenta* subspecies, *M. tristis* and *M. esculenta*, to improve resolution of the structure of diversity within

these taxa (Fig. 3 b). The first dimension of MCA was effective in separating *M. esculenta*, as a single compact group, from the other taxa. The second dimension further distinguished *M. tristis* from the two *M. esculenta* subspecies. The third dimension separated a “pure” group of five accessions of the *M. esculenta* subsp. *peruviana* from the other individuals of the *M. esculenta* subspecific complex. The “pure *peruviana*”

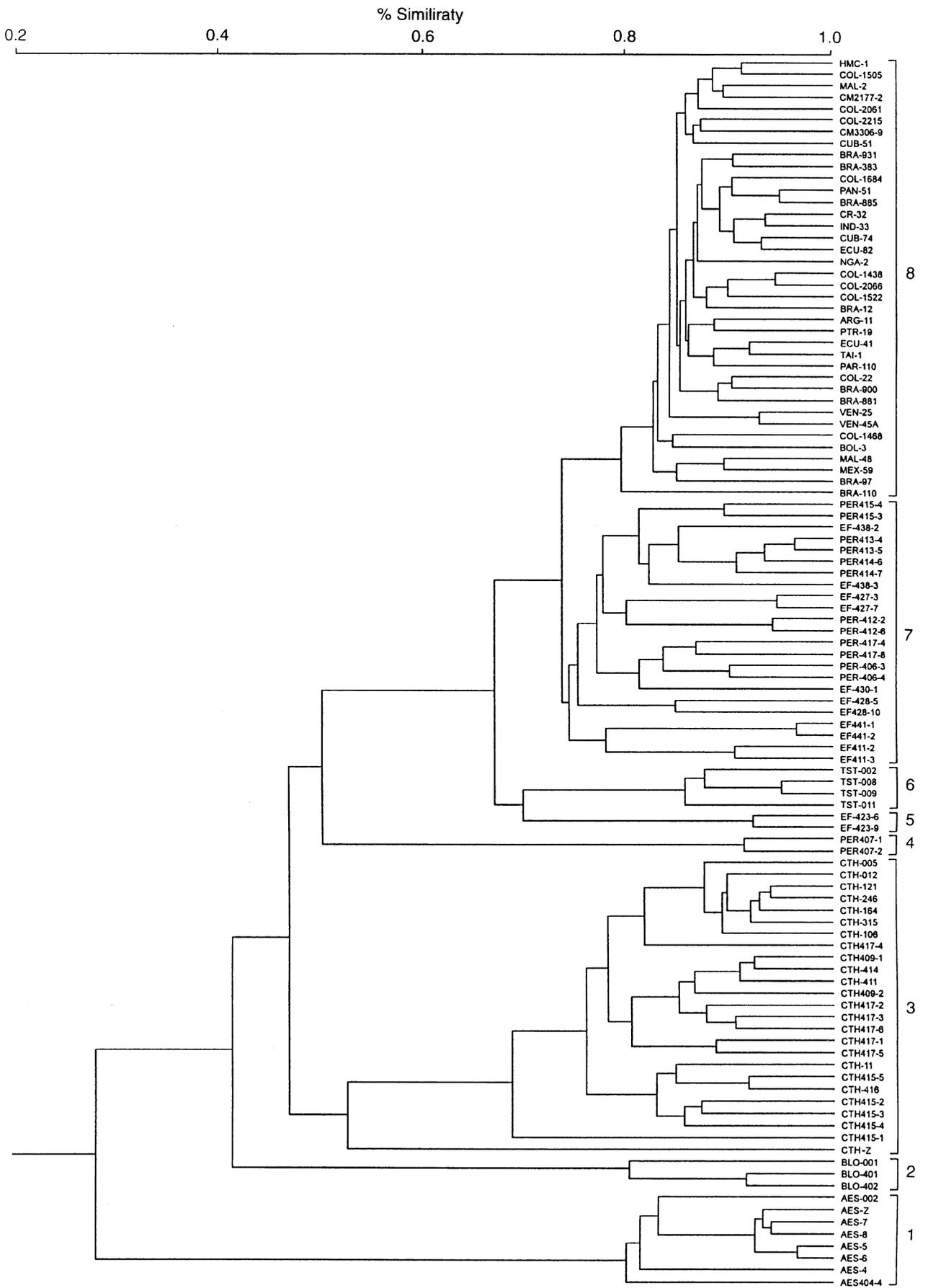


Table 3 Genetic similarity values (mean \pm SD) between (upper triangle) and within (diagonal) groups of *Manihot* germ plasm, based on all pairwise similarities between individuals. Different letters on

the diagonal indicate significant differences ($P < 0.05$) by Duncan's multiple range test

Group ^a	ESC (n = 38)	AES (n = 8)	BLO (n = 3)	CTH (n = 25)	TST (n = 4)	ESCFLA-PER (n = 14)
ESC	0.85 \pm 0.03ab	0.31 \pm 0.03	0.41 \pm 0.02	0.47 \pm 0.03	0.65 \pm 0.03	0.70 \pm 0.11
AES		0.86 \pm 0.06a	0.26 \pm 0.02	0.26 \pm 0.02	0.27 \pm 0.02	0.27 \pm 0.05
BLO			0.84 \pm 0.07ab	0.43 \pm 0.04	0.41 \pm 0.03	0.40 \pm 0.05
CTH				0.77 \pm 0.09cb	0.45 \pm 0.04	0.46 \pm 0.06
TST					0.88 \pm 0.04a	0.67 \pm 0.11
ESC FLA-PER						0.70 \pm 0.15c

^a ESC = *M. esculenta*; AES = *M. aesculifolia*; BLO = *M. brachyloba*; CTH = *M. carthaginensis*; TST = *M. tristis*; ESCFLA-PER = *M. esculenta* subsp. *flabellifolia* and *peruviana*

group came from disturbed Amazonian forest of Mato Grosso, Brazil. The remaining group of subspecific accessions was more dispersed, reflecting a structure of subgroups that corresponded with their respective habitats, geographical coordinates and altitudes.

The MCA also highlighted AFLPs that are specific to the accessions of each of the wild species (Fig. 1). With the first and second primers, 6 and 9, 4 and 6, and 1 and 0, putative species-specific bands were identified in *M. esculenta*, *M. brachyloba*, and *M. carthaginensis*, respectively. In addition, the latter two species were each distinguished by the exclusive absence of certain bands. MCA also facilitated the identification of AFLPs that reflect species heterogeneity by their exclusive presence in individuals of putative infraspecific gene pools, which should therefore be further sampled and considered in conservation and classification efforts.

Considering only the reduced set of germ plasms (Fig. 3 b), 52 bands were found in both *M. esculenta* subspecies, sometimes in *M. tristis* but absent from cassava. Furthermore, 21 AFLP bands were present in cassava while absent from the rest of the group, suggesting a larger pool of alleles for the two *M. esculenta* subspecies and *M. tristis* than for *M. esculenta*.

Supporting morphological evidence for inferences made from AFLP analysis

Morphologically, no significant differences were found between the two subspecific taxa. The presence of pubescence on ovaries and young fruits, used in Allem's

Fig. 2 Dendrogram drawn from the UPGMA analysis of Nei-Li estimates of genetic similarity based on AFLP analysis with two +1/ +3 primer combinations. The eight clusters shown are discussed in the text. 1 = (AES) *M. aesculifolia*; 2 = (BLO) *M. brachyloba*; 3 = (CTH) *M. carthaginensis*; 4 = (PER) *M. esculenta* subsp. *peruviana*, higher altitude; 5 = (EF) *M. esculenta* subsp. *flabellifolia* higher altitude; 6 = (TST) *M. tristis*; 7 = (EF and PER) *M. esculenta* subsp. *flabellifolia* and *peruviana*; 8 = *M. esculenta*, i.e. cassava

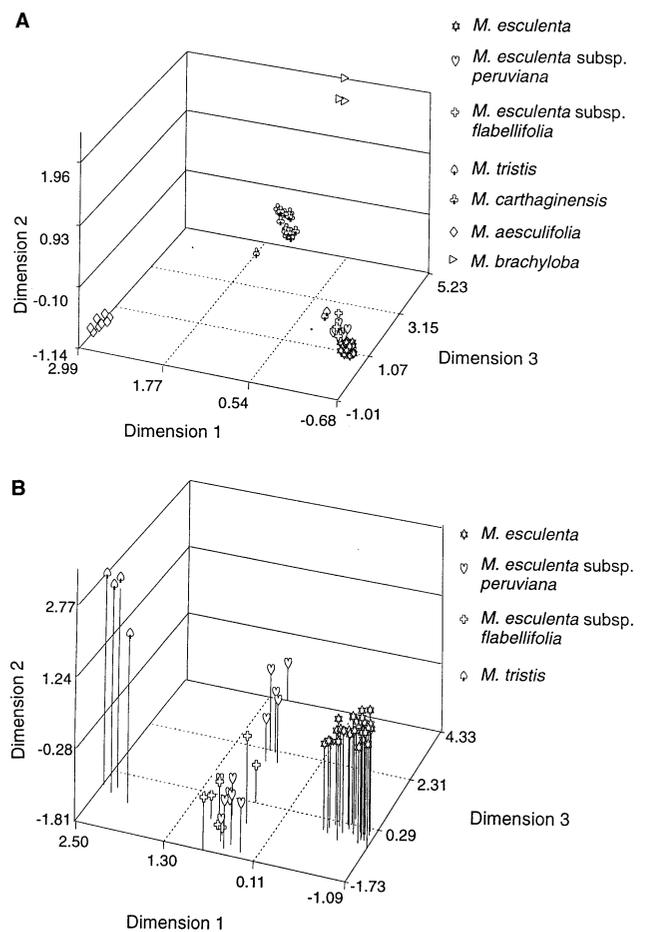


Fig. 3 Three principal axes of variation obtained by multiple correspondence analysis of AFLP among 105 samples of seven groups (A) of *Manihot* germ plasm resolved with two primer combinations; (B) shows a higher resolution of 69 samples of four groups

(1994 a, b) classification, could not consistently differentiate the subspecies. For example, four genotypes originating from accessions classified as *M. esculenta* subsp. *flabellifolia* presented abundantly pubescent ovaries; and, reciprocally, 15 plants from accessions of *M. esculenta* subsp. *peruviana* had glabrous ovaries. We

have also observed this character to be variable in *M. brachyloba*. Observations were repeated over time to account for the character's transitory nature, but it may be plastic in contrasting environments.

For the character of swollen nodes, morphological evaluation showed that, while leaf scars and the associated enlarged nodes along the plants' woody stems were prominent in all cassava accessions, they were not marked, or else only slightly prominent in the subspecific taxa. This character varies according to the degree of habitat disturbance and variation of the ecological factors (Rogers and Appan 1973; Allem 1994 a). Thus, we consider both "pubescent ovaries" and "swollen nodes" as descriptors of limited confidence, with little bearing on taxonomic status.

No significant morphological differences were found among the accessions of *M. tristis* and *M. esculenta* subsp. *flabellifolia* in the collection. Two characters – petiole attachment and pubescence of vegetative parts – have been used to distinguish among subspecies of *M. tristis* (Rogers and Appan 1973). The *M. tristis* accessions in this collection present non-peltate leaf-petiole junctures with pubescence only on leaf buds. In its turn, the subspecies *flabellifolia* was variable for peltate and non-peltate leaves, with the leaf juncture ranging from 0.5 to 2.0 cm from the leaf base. The subspecies also varied between totally glabrous and pubescent vegetative parts. These characters therefore do not seem useful for separating these taxa.

For those genotypes that differed from their conspecifics according to the AFLP analysis, morphological evaluation did not always support the distinction. For example, the accession PER 407, distinct from other accessions of the subspecies in the cluster analysis (Fig. 2), is itself variable for the principal character used to define the group: one of the two genotypes analyzed from this population had pubescent ovaries and the other did not. In contrast, the outlying accession of *M. carthaginensis*, CTH Z, showed a fruit surface character that differs from those described for the species. AFLP analysis confirms its distinction from the majority of Colombian accessions. As accurate passport data are not available for this accession, we assume that it represents either a subgene pool from northern Colombia or a natural hybrid.

Discussion and conclusions

We found AFLP analysis to be an effective and efficient means of providing quantitative estimates of genetic similarities related to the distribution of variability within and among previously recognized taxa of the relatively large and possibly taxonomically inflated genus *Manihot*. AFLPs also identified unique genetic diversity present in the wild relatives of the crop *M. esculenta*.

Interspecific similarities in *Manihot*

As shown in Fig. 2 and Table 3, *M. aesculifolia* was the most distant species from cassava in this study, having the lowest mean interspecific similarity (0.307), followed by *M. brachyloba* and *M. carthaginensis*. This outcome contradicts earlier reports that *M. aesculifolia* is the closest relative of cassava. *M. aesculifolia* was also the most distant species (mean interspecific similarities 0.258–0.274) from the remaining taxa.

Higher degrees of similarities (means 0.654–0.700) were observed between the taxa classified as *M. esculenta*, *M. tristis*, *M. esculenta* subsp. *flabellifolia*, and *M. esculenta* subsp. *peruviana*. The finding that the mixed group of subspecies is most similar to cassava (0.700) agrees with Allem's (1994 a, b) proposal that these con-specific taxa are the wild forms most closely related to the crop, and also with the classification by Fregene et al. (1994) into the same group by cpDNA and rDNA analysis.

The species relationships illustrated by AFLP are supported by preliminary data on crossability. CIAT recently hybridized some of these groups to test for compatibility and gene flow, and so generate possible benefits for cassava breeding. Literally thousands of seeds were obtained from 16 successful cross combinations – including reciprocal crosses – between cassava and *M. esculenta* subsp. *flabellifolia*, but only five seeds were obtained from two crosses between cassava and *M. aesculifolia*. Similar results were obtained with crosses between cassava and another Central American species, *M. chlorosticta* Standley and Goldman, which yielded between 1 and 148 seeds in 14 successful combinations with the wild species as the female parent, and with the seeds presenting substantial problems of dormancy.

Intraspecific variability in *Manihot*

As a whole, the subspecies complex showed the lowest intraspecific similarity value (0.700) of the taxa studied (Table 3). It forms a diverse set of germ plasms of considerable genetic heterogeneity corresponding to ecogeographic gene pools.

The intermixing of accessions of the two subspecies (Fig. 2) reflects their sympatric distribution and the tenuous morphological distinction used to separate them. For example, Allem (1994 b) proposed that the pubescence found on flowers, ovaries, and young fruits differentiated *M. esculenta* subsp. *peruviana* as a "wild pubescent form" of *M. esculenta*. But we found this character to be variable for each subspecies. The sympatric distribution of the wild forms of cassava (Allem 1994 b), and the predominance of allogamy and pollination by insects in the *Manihot* genus, also make the pubescent character unlikely to be constant and, therefore, unreliable for distinguishing taxa.

The two individuals of each *M. esculenta* subspecies that separated in the cluster analysis (groups 4 and 5) from the mixed group (7, Fig. 2) were from the upper extreme of the altitudinal ranges reported for the respective taxa in Brazil (Allem 1995). The two outlying representatives of the subspecies *flabellifolia* do not show morphological differences from the accessions in the main cluster, but originate from the higher areas of the Brazilian cerrados (dry semi-deciduous forest, mixed with pastures), which are characterized by a sub-humid to dry climate. Most of the other accessions are from disturbed Amazonian forest, where climates are humid to very humid.

Another taxon presenting a wide range of pairwise genetic similarities was *M. carthaginensis*. Heterogeneity was clearly related to distances between Colombian accessions and one accession (CTH Z) of unknown origin. Individual comparisons of similarities ranged from 0.95 among accessions from the same site to 0.53 between Colombian representatives and CTH Z, thus suggesting the existence of different gene pools within this species.

Despite the almost equal sample sizes analyzed for *M. brachyloba* and *M. tristis*, the range of similarities among individuals is notably greater in the former species. We have identified populations of *M. brachyloba* in distinct ecological habitats in Colombia, but we could not analyze their possible genetic differentiation because they survived poorly under ex situ conditions. Unfortunately, the more distant accession of *M. brachyloba* studied here lacks passport data.

Patchiness of species diversity and the existence of different gene pools within species have implications for future strategies of germ plasm sampling for conservation purposes. This is particularly relevant to the present assessment of *M. aesculifolia*, *M. carthaginensis* and *M. brachyloba*, which are variously distributed in several countries of Central and South America. *M. brachyloba* is the only species reported to be distributed in both South and Central America (Rogers and Appan 1973), and *M. carthaginensis* may be the only species distributed in Mexico and Brazil (Bertram 1993; Allem 1995). New introductions of *M. carthaginensis* from Brazil are now available, and additional collections are planned to expand the analysis.

The present work shows that the complex formed by the *M. esculenta* subspecies *flabellifolia* and *peruviana* possesses unique diversity relative to a broad sample of cassava germ plasm. This argues against these subspecies being escapes from cultivation of the crop. To strengthen this argument, the study should be complemented by more intensive sampling of landraces in the regions where the subspecies are found, as well as by complementary types of genetic analysis such as cladistic phylogeny.

The species-specific presence or absence of particular AFLP products may have implications of prac-

tical importance. Broader sampling is needed to confirm the extent of conservation of these bands in the respective species. It would then be useful to determine the genomic organization of these products to decide, for example, if they represent low copy number or repeated DNA sequences, and how they are arranged. Once the genomic or taxonomic distribution of these sequences is known, they may serve as markers for introgression or for identifying and classifying germ plasm.

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