Genetic relatedness between cassava (Manihot esculenta Crantz) and M. flabellifolia and M. peruviana based on both RAPD and AFLP markers

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Genetic relatedness between cassava (*Manihot esculenta* Crantz) and *M. flabellifolia* and *M. peruviana* based on both RAPD and AFLP markers

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Abstract

The taxonomy of the genus *Manihot* is still uncertain and the genetic origin of cassava (*M. esculenta* Crantz) continues to be controversial. We studied the degree of genetic relatedness between cassava and two naturally occurring species (*M. flabellifolia* and *M. peruviana*) which are probably involved in the evolution of cassava, using RAPD and AFLP molecular markers. Thirty-three clonal accessions of cassava of known genetic diversity and 15 accessions of the wild species *M. flabellifolia* and *M. peruviana* were analyzed using 92 polymorphic RAPD bands and 73 polymorphic AFLP bands. The genetic markers were unable to differentiate the two wild species, which confirms their botanical similarity. Half of the total number of amplified bands were monomorphic in all of the genotypes evaluated. The mean genetic similarity (Jaccard) between cassava and the species *M. flabellifolia/M. peruviana* was 0.59. A grouping analysis (neighbor-joining method) with RAPD markers of cultivated cassava, *M. flabellifolia/M. peruviana* and the other wild species located the genotypes of cassava and *M. flabellifolia/M. peruviana* at one extremity and the three Mexican species (*M. aesculifolia, M. michaelis* and *M. chlorostica*) at the other. An intermediate position between these groups was occupied by two wild species (*M. glaziovii* and *M. reptans*) native to central and northeastern Brazil. These results are consistent with the hypothesis that the species *M. flabellifolia* and *M. peruviana* gave rise to the cultivated species.

INTRODUCTION

Natural occurrence of the genus *Manihot* is limited to the tropical regions of the American continent, between latitudes 33ºN (southern part of the USA) and 33ºS (central part of northern Argentina). According to Rogers and Appan (1973), the genus *Manihot* contains 98 species, one fifth of which are native to North America, while the remaining four fifths occur in South America. Harlan (1971) and Nassar (1978) identified the central region of Brazil as the main center of diversity for *Manihot* species, followed by two other centers in southern Mexico and northeastern Brazil. *Manihot* species are classified as sporadically distributed colonizing perennials commonly found in semi-arid regions or disturbed zones of humid regions (Rogers and Appan, 1973). However, natural interspecific hybridization makes it difficult to determine the true taxonomic limits of the genus (Rogers and Appan, 1973).

The genetic origin of cassava is controversial. Comparative studies of reproductive characteristics, botanic origin and phylogenetic relatedness of cassava with other *Manihot* species, as well as the history of the domestication of cassava are still in the early stages. While some researchers believe cassava to be a cultigen that resulted from hybridization between several natural species (Rogers and Appan, 1973), others (Allem, 1994) consider cassava as a true natural species with two subspecies, *M. esculenta flabellifolia* and *M. esculenta peruviana.*

Only a relatively small number of wild species of the *Manihot* have been systematically collected for study. According to Hershey (1987), related species with specific characteristics of interest could be used to produce improved forms of manioc. For such studies, Allem (1994) proposed the use of wild species belonging to the primary genic pool (GP I), as defined by Harlan and Wet (1971). Determination of the reproductive barriers between related species of cassava is a highly complex and difficult task. Several techniques using molecular markers have been developed recently and are increasingly applied to the study of plant genetics. Gepts (1993) suggested that molecular markers provide more useful information than morphological markers for studies on the domestication and evolution of plants. Random amplified polymorphic DNA markers (RAPD) were used in studies conducted by Cisneros and Quiros (1995) on triploid potatoes (*Solanun chaucha*), by Demeke and Adams (1994) on mustard and radish, by Sharma *et al*. (1996) on lentils, by Reamon-Büttner *et al*. (1996) on beets and by Kaga *et al*. (1996) on the genus *Vigna*. Amplified fragment length polymorphism markers (AFLP) have been used in studies of genetic relatedness performed by Hill *et al*. (1996), Cervera *et al*. (1996) and Sharma *et al*. (1996).

We used RAPD and AFLP molecular markers to investigate the genetic relatedness between cultivated cassava and two naturally occurring species, *M. flabellifolia* and *M. peruviana*.

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MATERIAL AND METHODS

Material

One plant of each thirty-three clonal accessions of *M. esculenta* (cultivated cassava), nine genotypes of *M. flabellifolia* and six genotypes of *M. peruviana,* as well as one genotype of each of the naturally occurring species *M. glaziovii, M. reptans, M. chlorostica, M. aesculifolia* and *M. michaelis* were studied (Table I). The cultivated cassavas were divided into two groups. The first group consisted of 13 local cultivars (landraces) collected from the small village of Santa Isabel (located in the central portion of the Rio Negro River in the Amazon region), while the second group contained 20 genotypes of different sources, selected to provide the largest possible genetic diversity (Colombo, 1997).

DNA isolation and genetic analysis

DNA was isolated from leaves dried for 20 h at 48°C. The dry leaves (0.5 g) were ground in liquid nitrogen and transferred to a 20-ml plastic tube to which 10 ml of extraction buffer (0.1 M Tris HCl, pH 8.0, containing 1.25 M NaCl, 0.02 M EDTA, 4% MATAB (mixed alkyltrimethylammonium bromide) and 1% β-mercaptoethanol (added just before use)) was added. After a 90-min incubation at 65°C, with slow stirring, an equal volume of chloroform/isoamylalcohol (24:1) was added twice and the resulting supernatant transferred to a clean plastic tube. RNAse (100 µl of a 10 mg/ml solution) was added immediately after these extractions and the solution subsequently incubated at 37°C for 30 min. DNA pellets were obtained by addition of 0.8 v of isopropanol. After washing with 70% ethanol, the DNA pellet was vacuum dried and dissolved in 200 µl of TE buffer (10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA). The quality and concentration of the DNA fragments were evaluated by electrophoresis in 0.8% agarose gels.

The RAPD amplification reactions were done in 25 µl as described by Williams *et al*. (1990) and Welsh and McClelland (1990). The amplification products were separated by electrophoresis in 1.8% agarose gels, stained with ethidium bromide and photographed under UV light using Polaroid film. AFLP analysis was done as described by Vos *et al*. (1995). The DNA fragments produced by digestion with the enzymes *Eco*R1 and *Mse*1 were amplified in the presence of radioactive nucleotides (^{33}P) and separated on 5% acrylamide gels under denaturing conditions followed by autoradiography.

The RAPD and AFLP fragments were scored as present (1) or absent (0) for each plant sample. Genetic similarity matrices for these sequence differences were calculated for each sample markers (Jaccard, 1908), and the correlation between the two similarity matrices (RAPD and AFLP) was then obtained as described by Mantel (1967). The genetic diversity of the genotypes was analyzed by principal coordinate analysis (PCA) (Gower, 1996) and by the means of the hierarchic classification (UPMGA aggregation model). The degree of relatedness between cultivated cassava and the other five naturally occurring species was assessed using only RAPD markers. Dendrograms showing the genetic relatedness between these species were constructed using the neighbor-joining method.

RESULTS

One hundred and ninety-two amplified RAPD bands (average of 9.2 per primer) were analyzed. Of these, 48% were polymorphic (average of 5.2 polymorphic bands per primer). For AFLPs, the two different enzyme-primer combinations (PK) used produced 73 polymorphic bands (31 bands for the first and 42 bands for the second combination).

The relationship between *M. flabellifolia* and *M. peruviana* was studied using data generated with the RAPD and AFLP markers (91 RAPD and 70 AFLP bands). The mean value of similarity was 0.61 for the 15 individuals (0.57 and 0.63 for *M. flabellifolia* and *M. peruviana*, respectively). *M. flabellifolia* and *M. peruviana* were classified by the UPGMA method (Figure 1). The genotypes of these two species are in the two main branches of the dendrogram (A1 and A2), showing that it was not possible to differentiate these species with the markers used and they are therefore considered here as a single species (Figure 1). Fifty percent of the bands were monomorphic for the genotypes studied. The mean genetic similarity between the cassavas and *M. flabellifolia/M. peruviana* was 0.59 (Figure 1). *M. flabellifolia/M. peruviana* were grouped in branch A, while the cassavas of the World Collection and those from Santa Isabel (Amazon region) were located in branch B. The two groups were separated from each other by a distance of approximately 0.44. A distance of about 0.38 (B1 and B2) differentiated the two groups of cassava (Santa Isabel vs. World Collection) which were more closely related to each other than to the *M. flabellifolia/M. peruviana* species.

The global genetic diversity of the cultivated cassavas and the *M. flabellifolia/M. peruviana* group was determined (Figure 2). The cassavas were separated from the *M. flabellifolia/M. peruviana* accessions by axis 1 (9.4% of the total inertia) while axis 2 (7.1% of the contribution) distinguished the cassavas pertaining to the World Collection from those found in Santa Isabel.

An unrooted tree was constructed to show the genetic relatedness between the species *M. esculenta* and the wild species of *Manihot* (Figure 3). This relationship was based on the RAPD markers of two representative samples of the cultivated species, one representative sample of the *M. flabellifolia/M. peruviana* group and one representative sample of each of the five naturally occurring species of *Manihot*. The two varieties of cassava (SRT1276 and E8220) and the *flabellifolia* genotype (Fla2-10) were lo-

Table I - Plant samples used to investigate the genetic relatedness among *Manihot* species.

	Code	*Manihot*	Section*	Origin*	Source
1	E 8228	*esculenta*	*Manihot*	-	Santa Isabel/Amazon State
2	E 8220	"	"	-	"
3	E8216-1	"	"	-	"
4	E 8214-1	"	"	-	"
5	E 8215-1	"	"	-	"
6	E 8209-1	"	"	-	"
7	E 8207-1	"	"	-	"
8	E 8203-1	"	"	-	"
9	E 8201-1	"	"	-	"
10	E 8196-1	"	"	-	"
11	E 8192-2	"	"	-	"
12	E 8191-1	"	"	-	"
13	E 8189-2	"	"	-	"
14	VEN 25	"	"	-	Venezuela
15	THAI 1	"	"	-	Thailand
16	MEX 59	"	"	-	Mexico
17	MAL 2	"	"	-	Malaysia
18	ECU 82	"	"	-	Equador
19	CUB 51	"	"	-	Cuba
20	COL 2066	"	"	-	Colombia
21	COL 1522	"	"	-	Colombia
22	COL 1438	"	"	-	Colombia
23	COL 22	"	"	-	Colombia
24	BOL 3	"	"	-	Bolivia
25	ARG 11	"	"	-	Argentina
26	BGM 81	"	"	-	Brazil (Northeastern region)
27	BGM 1269	"	"	-	Brazil (Northeastern region)
28	BGM 5	"	"	-	Brazil (Southeastern region)
29	BGM 243	"	"	-	Brazil (Northeastern region)
30	SRT 1316	"	"	-	Brazil (Northeastern region)
31	SRT 1276	"	"	-	Brazil (Northeastern region)
32	SRT 454	"	"	-	Brazil (Southeastern region)
33	F 4113	"	"	-	Brazil (Southeastern region)
34	Fla 10-6	*flabellifolia*	*Heterophyllae*	South America	Brazil (Porto Franco, MA)
35	Fla 8-7	"	"	"	Brazil (Porto Franco, MA)
36	Fla 4-14	"	"	"	Brazil (Goiânia, GO)
37	Fla 2-8	"	"	"	Brazil (Comodoro, MT)
38	Fla 3-7	"	"	"	Brazil (Rio Branco, AC)
39	Fla 1-8	"	"	"	Brazil (Jaru, RO)
40	Fla 2-10	"	"	"	Brazil (Vilhena, RO)
41	Per 3-4	*peruviana*	"	"	Brazil (Porto Velho, RO)
42	Per 1-7	"	"	"	Brazil (Porto Velho, RO)
43	Per 1-3	"	"	"	Brazil (Guajara-Mirim, RO)
44	Fla 1-10	*flabellifolia*	"	"	Brazil (Lambari, MT)
45	Per 2-6	*peruviana*	"	"	Brazil (Lambari, MT)
46	Per 1-1	"	"	"	Brazil (Pontes e Lacerda, MT)
47	Fla 2-11	*flabellifolia*	"	"	Brazil (Pontes e Lacerda, MT)
48	Fla 1-9	"	"	"	Brazil (Vilhena, RO)
49	Per 2-4	*peruviana*	"	"	Brazil (Cacoal, RO)
50	-	*glaziovii*	*Glaziovianae*	Brazil central and northeast	Brazil (Northeast region)
51	-	*reptans*	*Crotalariaeformes*	Brazil central	Brazil (Central region)
52	-	*chlorostica*	*Parvibracteatae*	Mexico	Mexico
53	-	*aesculifolia*	"	"	"
54	-	*michaelis*	*Foetidae*	"	"

*From Rogers and Appan (1973).

cated at the center of gravity of each of the three groups in the principal coordinate analysis (Figure 2). Ninety-one polymorphic RAPD markers with an average size of 993 pb were used in this study. An unrooted tree best represented the grouping of the two cassava genotypes and the three naturally occurring Mexican species (*M. aesculifolia*, *M. michaelis* and *M. chlorostica*) as the outgroup. The wild Brazilian species *M. glaziovii* and *M. reptans* were more closely related to cassava and *M. flabellifolia/M. peruviana* than the naturally occurring species from Mexico.

The two molecular marker techniques were compared by determining their maximum, minimum and mean Jaccard index values. Genetic diversity within each group was similar for both types of markers. A comparison of the mean

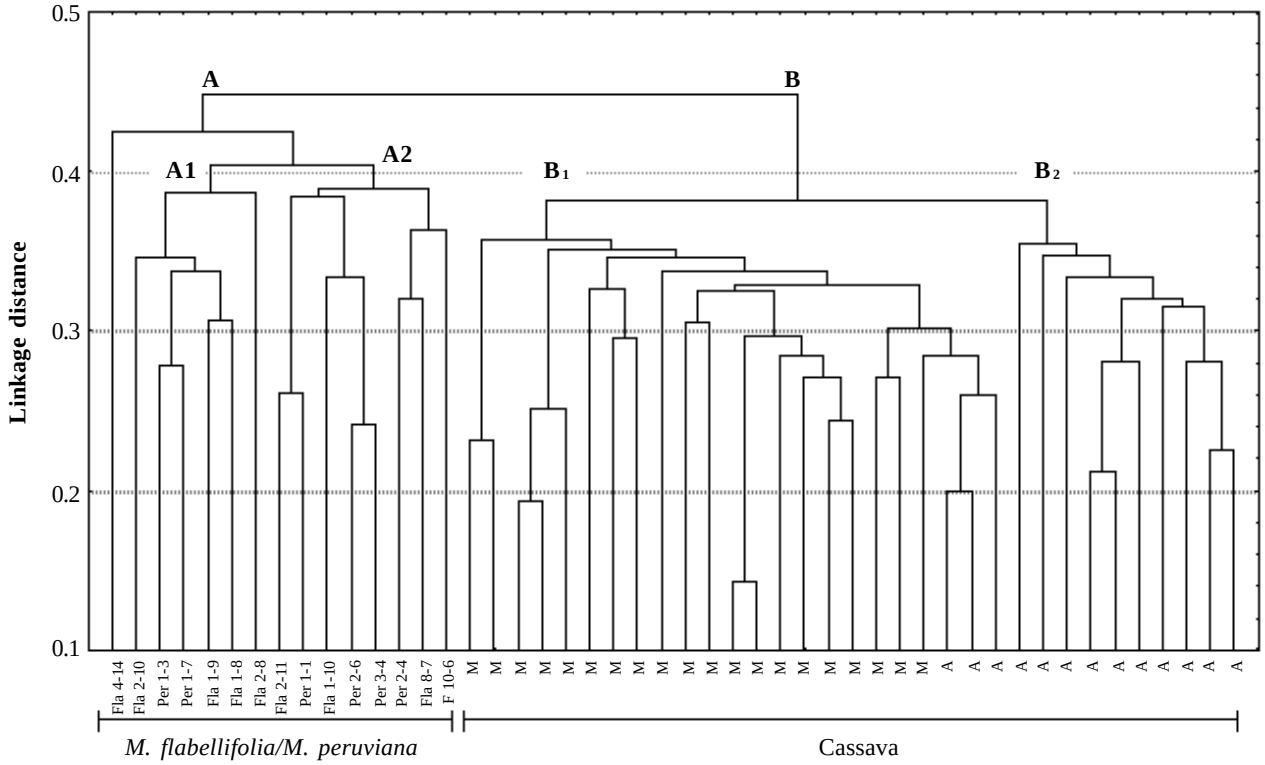

Figure 1 - Classification of *M. esculenta* (two groups, M for the samples of a world collection and A for samples from the Amazon region), *M. flabellifolia* and *M. peruviana* based on the UPGMA grouping method. The distances (similarity index of Jaccard (1908)) were calculated using the combined RAPD and AFLP data (92 and 73 markers, respectively).

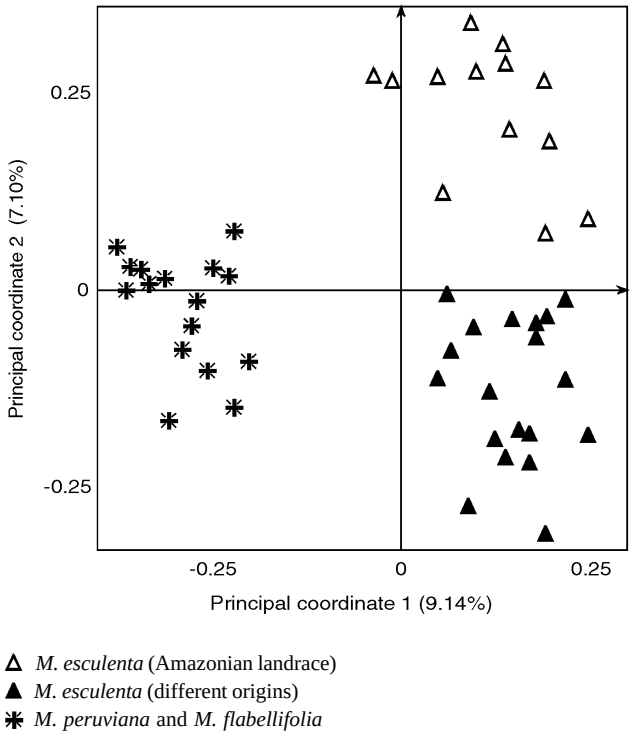

Figure 2 - Principal coordinate analysis calculated using the genetic similarities (Jaccard index) of 33 clonal accessions of cultivated cassava (*Manihot esculenta*) and 15 genotypes of the naturally occurring species *M. flabellifolia* and *M. peruviana*.

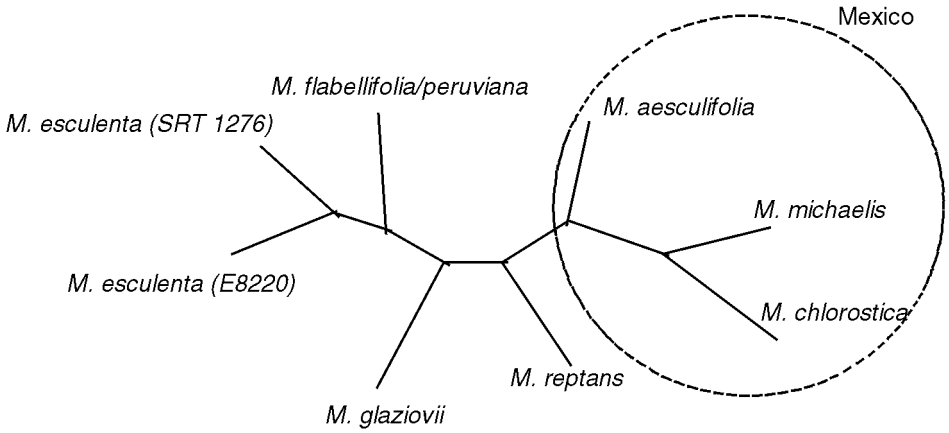

Figure 3 - Unrooted tree classification of cassava and other wild species of *Manihot* elaborated by the neighbor-joining method using the similarity coefficients of Jaccard derived from RAPD data.

values of similarity using the *t*-test showed that they were not significantly different. Another comparison between the two types of markers used here was done using Mantel's correlation test (1967). This calculation, which takes into account the similarities between all comparisons of the individuals available, i.e., those associated with RAPD markers on the one hand and those relative to AFLP markers on the other, showed a significant correlation ($r = 0.75$) between the two molecular markers. According to Lapointe and Legendre (1992), the *r* value is significant with a 1% probability if > 0.5 for more than 12 OTU (operational taxonomic units). With the 2,000 permutations done for this test, the *Z*-value obtained was 0.0020.

DISCUSSION

The genetic relatedness among *Manihot* species revealed by our results demonstrates that both RAPD and RFLP markers are equally suited as tools to study them and the choice of markers for investigating genetic relatedness must take into account practical factors such as ease of use and costs. Technically, AFLPs are more difficult to handle, although they reveal a higher degree of polymorphism. Thus, Mackill *et al*. (1996) used AFLP and obtained 147 polymorphic bands with eighteen primer-enzyme combinations in 12 japonica and two indica rice cultivars compared with 43 polymorphic bands obtained for RAPD markers (21 random primers tested). Lin *et al*. (1996) compared the polymorphism generated by RAPD (two bands per primer) with that generated by AFLP (12 polymorphic bands for one PK combination) in soybeans. The principle of both RAPD and AFLP markers is the amplification of anonymous DNA sequences of unknown size. One of the main criticisms of the use of RAPD markers for studying genetic relatedness, especially among different species, is that there is no information on the homology of the nucleotide sequence of the same band in different individuals. Sequence homologies were not determined in this study, although others (Williams *et al*., 1990; Thormann and Osborn, 1992) have reported high levels of sequence homology for RAPD in other genera. For AFLP markers, the problem of homology is less important (Vos *et al*. 1995). Since the two types of molecular marker equally differentiated the species, the diversity between the *Manihot* species studied was evaluated using a combination of the AFLP and RAPD test data. According to Loarce *et al*. (1996), the larger the number of parameters used to compare two individuals genetically, the more accurate the estimate of similarity between them, as illustrated by their study on the genetic relatedness of rice varieties using both RAPD and AFLP markers.

This is the first study to examine the relatedness between cassava and *M. flabellifolia* and *M. peruviana* species using RAPD and AFLP markers simultaneously. *M. flabellifolia* and *M. peruviana* species proved to be so closely related that the markers used were unable to group them separately. From a botanical standpoint, these species are extremely similar. According to Allem (1994), the only morphological difference is the absence in *M. flabellifolia* or presence in *M. peruviana* of short, thin soft hairs on the surface of the reproductive organs. Classifying these two taxa as different species on this basis would not be justified. Furthermore, the geographical region where *M. peruviana* occurs naturally overlaps considerably with that of *M. flabellifolia*, although the range of the first is much smaller. The genetic similarities, the geographical distribution and the botanical characteristics all indicate that *M. peruviana* originated from *M. flabellifolia.*

Our results provide evidence to support the hypothesis of Allem (1994), who identified *M. flabellifolia/M. peruviana* as being the wild or naturally occurring forms of cassava. Thirty-four bands (20% of the polymorphic bands) were monomorphic in all of the *M. flabellifolia*/*M. peruviana* and *M. esculenta* genotypes studied. The dis-

tinction between the wild species and cassava was attributable primarily to variations in the frequencies of the polymorphic bands of each of the species. No band whatsoever was found to be specific (100% occurrence) to either *M. flabellifolia/M. peruviana* or *M. esculenta*.

Other studies have examined the genetic proximity of species belonging to the genus *Manihot*. Second *et al.* (1997) reported a high level of similarity between *M. flabellifolia* and *M. peruviana* based on AFLP markers. Schaal *et al.* (1997) reached the same conclusion using RAPD markers and two ITS regions of nuclear ribosomal DNA. Based on the analysis of DNA extracted from the chloroplasts of the two species, Fregene *et al*. (1994) classified *M. flabellifolia* as being very closely related to cassava. These results suggest that *M. flabellifolia/M. peruviana* and *M. esculenta* share the same origin. *M. esculenta* may have been domesticated to produce cultivated cassava (*M. esculenta* ssp. *esculenta*), while other specimens of the same species, represented by *M. esculenta* ssp. *flabellifolia/peruviana*, continued in their natural state. Contrary to and, at the same time, partly supporting the theory of Rogers and Appan (1973), who claimed that there were no wild forms of cassava, the presence of 20 bands in naturally occurring species but absent from cultivated cassava may indicate the existence of a third species which, when crossed with *M. flabellifolia/M. peruviana*, would have given rise to cassava, or which, when crossed with cassava, would have given rise to *M. flabellifolia/M. peruviana*. This uncertainty will only be clarified by finding the third species mentioned above.

Second *et al*. (1997) studied the taxonomy of a large number of *Manihot* species, some of which are little known. Their results, based on 93 AFLP markers, show that other naturally occurring species, such as *flabellifolia*, were genetically very closely related to the varieties of cultivated cassava: *M. procumbens, M. fruticulosa, M. pentaphylla* and *M. pruinosa.* On the basis of field observations, Nassar (1978) found that the fertility rate of crosses between *M. oligantha* (Pax) Nassar and cassava is about 90%, indicating that the former species can be classified in the primary genic pool, as proposed by Harlan and Wet (1971).

The high degree of relatedness between cassava and *M. glaziovii* and *M. reptans* emphasizes the tenuous reproductive barriers that exist between these two species and cassava. Nassar *et al.* (1985) described a population of *M. reptans* in central Brazil with traces of introgression from cassava. Similarly, spontaneous hybrids of *M. esculenta* and *M. glaziovii* were described in Africa by Nichols (1947), Cours (1951), INEAC (1952) and Lefèvre (1989). Contrary to our results, Fregene *et al*. (1994) found no difference between *M. glaziovii* and *M. michaelis*, based on an analysis of DNA extracted from chloroplasts. Likewise, Haysom *et al*. (1994) used RFLP markers to classify the Mexican species *M. chlorostica* as the species most closely related to *M. flabellifolia*, followed by *M. esculenta* and *M. glaziovii*. The results of these authors corroborate the hypothesis of Rogers and Appan (1973) and refute the idea that *M. flabellifolia* is a native species of South America.

Using only molecular markers, we were able to demonstrate in this work the genetic relatedness between the subspecies *M. esculenta* and five other naturally occurring species in the genus, each represented by one genotype. There was a much greater similarity between *M. flabellifolia/M. peruviana* and cultivated cassava than between *M. esculenta* and the other wild species. Among the five wild species investigated, *M. glaziovii* and *M. reptans* proved to be more closely related to *M. esculenta* than the remaining species (*M. aesculifolia*, *M. michaelis* and *M. chlorostica*). These results are consistent with the geographical distribution of these species. The three Mexican species are quite different from the Brazilian species. *M. glaziovii* occurs in the arid zone of northeastern Brazil, an important center of genetic diversity for the genus *Manihot* (Nassar, 1978). This species has been widely used to introduce genes of specific agricultural importance into cultivated cassava, especially because of the ease in producing desired crosses and the possibility of restoring the fertility of infertile hybrids (Léfèvre, 1989). *M. reptans*, a species much less used than *M. glaziovii* in genetic studies, is native to the humid regions of central Brazil, the most important center of diversity for *Manihot* (Nassar, 1978). The three species most distantly related to cultivated cassava are those native of Mexico, the second most important center of diversity for *Manihot* (Nassar, 1978).

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RESUMO

A taxonomia do gênero *Manihot* em grande parte não está resolvida e a origem genética da mandioca (*M. esculenta* Crantz) continua controvertida. Na tentativa de contribuir para elucidar sua história evolutiva, as relações de proximidade genética da mandioca com duas espécies selvagens que provavelmente participaram da sua história evolutiva, *M. flabellifolia* e *M. peruviana*, foram estudadas através de dois tipos de marcadores moleculares, os RAPDs e os AFLPs. Para tanto, foram empregados 33 acessos clonais de mandioca de reconhecida diversidade genética e 15 acessos das espécies selvagens *M. flabellifolia* e *M. peruviana* das regiões central e norte do Brasil, importantes centros de ocorrência natural destas espécies. Noventa e duas bandas polimórficas RAPD e 73 AFLP foram utilizadas para análise dos resultados. Ambos marcadores foram incapazes de diferenciar as duas espécies selvagens utilizadas, confirmando a grande semelhança botânica entre elas. Em relação à mandioca cultivada, os resultados revelaram grande proximidade genética entre estas e as espécies selvagens. Metade do total de bandas amplificadas apresentaram-se monomórficas entre todos os genótipos avaliados. O valor médio de similaridade genética (Jaccard) entre a mandioca e as espécies *M. flabellifolia/M. peruviana* é de 0.59. As relações de proxi-

midade genética entre a mandioca e *M. flabellifolia/M. peruviana* foram confirmadas quando outras cinco espécies selvagens foram também incorporadas em relação ao polimorfismo gerado pelos RAPDs. A análise de agrupamento (neighbor-joining) realizada com genótipos de mandioca, de *M. flabellifolia/M. peruviana* e das demais espécies selvagens reuniu numa extremidade os genótipos de mandioca e *M. flabellifolia/M. peruviana* e na outra extremidade três espécies *mexicanas (M. aesculifolia, M. michaelis e M. chlorostica*). Entre estes dois grupos se posicionaram outras duas espécies selvagens cuja ocorrência natural é na região central e no nordeste brasileiro (*M. glaziovii* e *M. reptans*). Embora não conclusivos, os resultados apresentados são coerentes com a hipótese de que as espécies *M. flabellifolia* e *M. peruviana* poderiam ter originado a espécie cultivada. No entanto, outras espécies pouco estudadas (*M. procumbens*, *M. fruticulosa*, *M. pentaphylla* e *M. pruinosa*) foram recentemente citadas como geneticamente muito próximas da mandioca. Assim, um estudo abordando maior número de espécies e com marcadores mais apropriados, a exemplo dos microsatélites, merece ser feito.

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