Short Communication

Phylogeography and the origin of cassava: New insights from the northern rim of the Amazonian basin

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1. Introduction

The origin of cassava (*Manihot esculenta* Crantz) is still unclear, although several recent studies have addressed this issue (Fregene et al., 1994; Roa et al., 1997, 2000; Olsen and Schaal, 1999, 2001; Elias et al., 2000; Olsen, 2004). Rogers and Appan (1973) postulated that cassava was a "compilo-species", *i.e.*, the result of hybridization events between several species, among them *Manihot aesculifolia* (Kunth) Pohl, a species endemic to Central America. On the other hand, Allem hypothesized the crop to be issued from a single species, *Manihot esculenta*, with two subspecies found only in the wild: *Manihot esculenta* ssp. *flabellifolia* (Pohl) Ciferri and *Manihot esculenta* ssp. *peruviana* (Muell. Arg.) Allem (Allem, 1994; Allem et al., 2001). These wild taxa together have a broad ecological range, from southwestern Amazonia to the savannas of the Guianas.

Molecular studies have favored the latter scenario, generally showing that cassava was domesticated only once, in South America, with no contribution from the Mesoamerican species pool, or at least not from *M. aesculifolia* (Roa et al., 1997, 2000; Olsen and Schaal, 1999, 2001; Olsen, 2004). Study of this and seven other Mesoamerican species (A. Duputié, unpublished data) show that all are only distantly related to cassava, excluding the possibility that

cassava was domesticated in Central America. These studies, however, suffer several limitations. First, they have considered only a limited sample of domesticated cassava accessions. Second, they overlooked a part of the range of *Manihot esculenta* ssp. *flabellifolia*: while the taxon is distributed on an arc partially circling the Amazon basin, from eastern Bolivia westwards to central Brazil eastwards, and in the Guianas and eastern Venezuela northwards, these studies considered only samples from Brazil, thereby excluding any possibility of testing the hypothesis that cassava could have more than one center of domestication. Yet, several other crops have been shown to have been domesticated twice (*e.g.*, the common bean, Gepts et al., 1986).

The present study aims at filling some of these gaps. Olsen and Schaal (1999) sampled accessions of *M. esculenta* ssp. *flabellifolia* and the closely related species *M. pruinosa* Pohl from Brazil and 20 accessions of cultivated cassava from the CIAT core collection, a collection constituted with the goal of representing cassava's genetic and morphological diversity worldwide (Hershey et al., 1994). We combined their sample of wild *Manihot* with samples of *Manihot esculenta* ssp. *flabellifolia* from the northern rim of the Amazonian basin, to cover most of the range of this taxon. Furthermore, to test the hypothesis that cassava could have more than one center of domestication, we also broadened the sample of domesticated cassava to include landraces cultivated in the Guianas, thus filling an acknowledged gap in earlier studies (Olsen and Schaal, 2001). Even though these samples all come from the same region,

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it has been shown that cassava genetic diversity in a single village in Guyana can be comparable to that in the CIAT (International Center for Tropical Agriculture) core collection (Elias et al., 2000). Finally, we included samples of two additional wild species from South America and two outgroups. Specifically, we addressed the following questions: (i) Was cassava domesticated only once? (ii) Did post-domestication gene flow play a role in shaping the crop's diversity in French Guiana?

2. Material and methods

2.1. Plant material

Six wild *Manihot* species have been described in the Guianas (Rogers and Appan, 1973; Allem, 1999). They include rainforest vines, and shrubs of savanna and savanna-forest ecotone. Within the latter, several taxa have been named, some of doubtful distinctness. One of them, *M. surinamensis* Rogers and Appan, was synonymized with *M. esculenta* ssp. *flabellifolia* by Allem (1994). We sampled populations of this taxon (i) in coastal regions of French Guiana, where human activity is relatively high, and (ii) in rock-savanna islands on inselbergs located in the densely forested south of French Guiana, where recent gene flow with the domesticate or presence of escapees from cultivation are both highly unlikely. Sampling locations are presented in Fig. 1 and detailed in Supplementary Table 1.

One population (SM) was formerly in sympatry with domesticated cassava, which is no longer cultivated in the site. Natural hybridization has been shown to occur between the two taxa (Duputié et al., 2007), and we sampled individuals of domesticated, wild and intermediate phenotype (4, 4 and 3 individuals, respectively). Only the three individuals of wild phenotype which were found to be purely

wild individuals in another study (Duputié et al., 2007) were included in this analysis. Individuals of domesticated or intermediate phenotype were analyzed as supplementary individuals.

Our sample of domesticated cassava includes 37 plants (belonging to 31 landraces) from French Guiana and 49 plants (16 landraces and 33 seedlings) from the Amerindian village of Rewa, Guyana. Sampling details are given in Supplementary Table 2.

Two individuals of *Manihot glaziovii* Muell. Arg. and three of *M. aff. quinquepartita* Huber ex Rogers and Appan were included, together with three individuals found in the Monts d'Arawa, initially determined as two different species (*M. aff. quinquepartita* and *M. brachyloba* Muell. Arg.). These species are more distantly related to cassava (Chacón et al., 2008).

Two outgroup specimens were collected in French Guiana: *Cnidoscolus urens* (L.) Arthur, and *Jatropha gossypiifolia* L.

2.2. DNA sequences

We sequenced a 962-bp long portion of the nuclear gene G3pdh, encompassing four exons and three introns, one of which contains a minisatellite region, as described by Olsen and Schaal (1999). The sequences of the 28 G3pdh haplotypes already identified by Olsen and Schaal (1999) were obtained from GenBank (Accession Nos. AF136119–AF136149).

DNA was extracted from dried leaves using DNeasy Plant kit (Qiagen GmbH). PCR amplification of the G3pdh region was performed as described in Olsen and Schaal (1999) and sequencing reactions were performed using classical protocols with products from Applied Biosystems, on an ABI 310 monocapillary sequencer. We sequenced the two haplotypes of heterozygotes at the G3pdh locus together, using the "haplotype subtraction" approach (Clark, 1990), as did Olsen and Schaal (1999). Alignment was done using
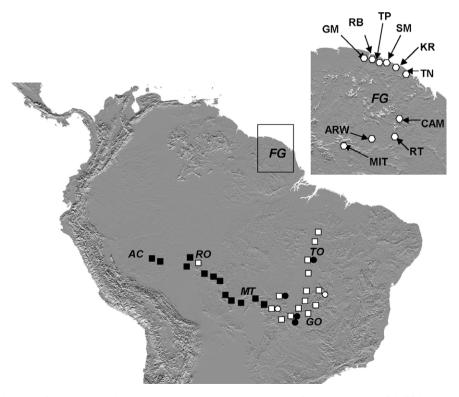

Fig. 1. Populations of the wild relative of cassava sampled in the Guianas (hexagons), and populations of *M. esculenta* subsp. *flabellifolia* (squares) and of *M. pruinosa* (circles) studied by Olsen and Schaal (1999). Shades of grey indicate elevational contours. Abbreviations: Brazilian states: Acre (AC), Goiás (GO), Mato Grosso (MT), Rondônia (RO), Tocantins (TO); French Guiana (FG); populations in French Guiana: Grand Macoua (GM), Roche Blanche (RB), Trou Poissons (TP), Savane Manuel (SM), Kourou (KR), Tonate (TN), Camopi (CAM), Roche Touatou (RT), Monts d'Arawa (ARW), Petit Mitaraka (MIT). Filled symbols represent populations containing G3pdh haplotypes shared with domesticated cassava.

Clustal X (Thompson et al., 1997) and manually corrected using BioEdit (Hall, 1999).

2.3. Phylogenetic analysis and analysis of molecular diversity

The sequenced region contained 89 variable sites within the genus *Manihot*. Translation of the exon sequences revealed a single amino acid substitution. One phylogenetically informative character, a substitution in position 309, was not used in the analysis, due to amplification ambiguities. The indel corresponding to the mini-satellite region, comprising up to five repeats of a 25-bp motif, has already been shown to present strong homoplasy (Olsen, 1999), and was therefore not taken into account when defining haplotypes, nor in drawing the haplotype network. The haplotype network was obtained by manual addition of the new sequences to the network presented in Olsen and Schaal (1999).

Phylogenetic reconstruction using maximum likelihood was conducted using the program PhyML version 2.4.4 (Guindon and Gascuel, 2003), using the GTR + Γ + I substitution model. Indels were coded as missing characters. Estimates of within-population diversity (genetic diversity and nucleotide diversity, Nei, 1987) were calculated using the program Arlequin (Excoffier et al., 2005).

3. Results

We identified 12 new haplotypes in the genus *Manihot* (Accession Nos.: AM901263–AM901274): three in domesticated cassava (haplotypes D1–D3), four in *Manihot esculenta* ssp. *flabellifolia* (haplotypes G1–G4) two in *M. glaziovii* (GL1 and GL2) and three in *M.* aff. *quinquepartita* (BA1–BA3).

Forty-two sites were phylogenetically informative. Monophyly of the genus *Manihot* is strongly supported by a bootstrap value of 100%, but G3pdh sequences do not sort species within the genus *Manihot* (Supplementary Fig. 1). The haplotype network (modified from Olsen and Schaal, 1999) is shown in Fig. 2.

3.1. M. esculenta ssp. flabellifolia populations from French Guiana

Only four haplotypes, all of them never previously documented, were encountered in the 46 accessions from French Guiana. These haplotypes differed by at most four substitutions from alleles already documented in *Manihot esculenta* ssp. *flabellifolia* (Fig. 2). Allele counts are given in Table 1. Strong differences in haplotype frequencies between populations of coastal savannas (G1: 82%, G2: 11%, G3: 5%) and those of rock-savannas on inland inselbergs (G1: 37%, G2: 35%, G3: 28%) suggest geographic structuring.

Genetic diversity in *Manihot esculenta* ssp. *flabellifolia* from coastal populations was lower than in inland sites, this diversity being in turn lower than that found in Brazilian populations of the taxon (except those from central Brazil; Table 1). Nucleotide diversity was much lower among French Guianan populations than among Brazilian accessions, as the four Guianan haplotypes differed from each other by at most seven substitutions, as compared to up to 24 substitutions between Brazilian haplotypes.

3.2. Domesticated cassava

We detected eight haplotypes of G3pdh in 86 cassava accessions from the Guianas. Combined with earlier data (Olsen and Schaal, 1999), ten G3pdh haplotypes have been documented so far in
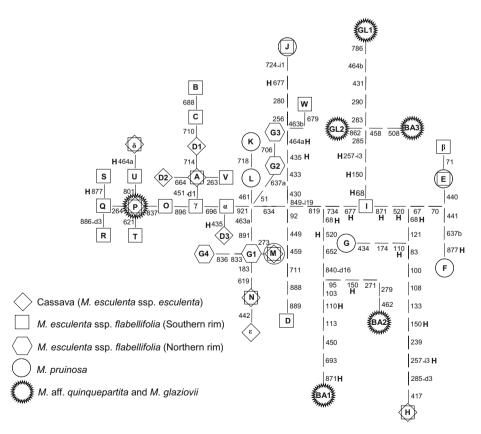

Fig. 2. G3pdh haplotype network (modified from Olsen and Schaal, 1999). Haplotype names are as in Table 1, and the shapes around them indicate the taxa in which the haplotypes were present (diamonds: domesticated cassava; squares: *M. esculenta* ssp. *flabellifolia* from Brazil; hexagons: *M. esculenta* ssp. *flabellifolia* from French Guiana; circles: *M. pruinosa*; spiny circles: more distant species of *Manihot*). Each dash represents a single mutational step. The numbers on the lines indicate the position of the substitution with reference to the alignment; i and d followed by numbers indicate an insertion or deletion and its length. For branches with more than one mutational step, the order of the mutations is arbitrary. Multiple substitution events are followed by letters a and b. Homoplasic mutations are indicated by H.

Table 1

Distribution of G3*pdh* haplotypes in the studied populations of *M. esculenta* ssp. *flabellifolia*, *M. pruinosa* and domesticated cassava. Haplotypes in light grey correspond to haplotypes found in domesticated cassava, and haplotypes in darker grey correspond to those found in wild *Manihot* in the Guianas. *n* represents the number of haplotypes (*i.e.*, twice the number of samples).

Population	n	I	H	G	F	β	E	J	W	D	L	K	G3	G2	G4	G1	M	N	ε	D3	α	V	γ	B	C	D1	D2	A	P	δ	U	T	R	Q	S	O
Guianan *M. esculenta* ssp. *flabellifolia* (this study)																																				
Touatou RT [FG]	4												2			2																				
Camopi CAM [FG]	14												5			9																				
Monts d'Arawa ARW [FG]	12													12																						
Petit Mitaraka MIT [FG]	8												4			4																				
Inselbergs, total	38												11	12		15																				
Tonate TN [FG]	10															10																				
Kourou KR [FG]	14														1	13																				
Savane Manuel SM [FG]	6													2		4																				
Trou Poisson TP [FG]	8													3		5																				
Roche Blanche RB [FG]	4												1	1		2																				
Grand Macoua GM [FG]	12												1			11																				
Coast, total	54												2	6	1	45																				
Guianas, total	92												13	18	1	60																				
Brazilian *M. esculenta* ssp. *flabellifolia* (Olsen and Schaal, 1999)																																				
Rondônia and Acre	96		10									7					5	12							3			4	17	3	21	4	6	4		
Mato Grosso	70	8	3					11										1						10	15			2	6				6	5		1
Goiás	96						48	1																	47											
Tocantins	52					4	25	6	1											3						11	2									
M. pruinosa (Olsen and Schaal, 1999)																																				
Mato Grosso	24			9	12		1	1			2						1																			
Goiás	46			9			1	1				18					17																			
Cultivated cassava																																				
Local landraces [FG; GU] (this study)	172																1	5	2	5						6	1	140	12							
World Core Collection (Olsen and Schaal, 1999)	40	1																1	2									19	15	2						
Domesticated cassava, total	212	1															1	6	4	5						6	1	159	27	2						

Table 2
Gene diversity and nucleotide diversity of *Manihot esculenta* ssp. *esculenta*, *M. esculenta* ssp. *flabellifolia* and *M. pruinosa* at the regional level.

	Sample size	Gene diversity (± s.d.)	Nucleotide diversity ($\times 10^3$) (± s.d.)
Manihot esculenta ssp. *esculenta* (cultivated cassava)			
Local landraces (FG and GU)	86	0.331 ± 0.046	1.5 ± 1.0
World core collection (data from Olsen and Schaal, 1999)	20	0.644 ± 0.049	5.5 ± 3.1
Manihot esculenta ssp. *flabellifolia*			
Acre-Rondônia	48	0.884 ± 0.015	14.5 ± 7.3
Mato Grosso	35	0.884 ± 0.015	16.3 ± 8.2
Goiás	48	0.516 ± 0.013	19.6 ± 9.7
Tocantins	26	0.713 ± 0.051	20.0 ± 10.0
French Guiana – inland	19	0.679 ± 0.023	2.8 ± 1.7
French Guiana – coast	27	0.297 ± 0.076	1.4 ± 1.0
Manihot pruinosa			
Mato Grosso	12	0.627 ± 0.062	13.2 ± 6.9
Tocantins	23	0.686 ± 0.032	14.8 ± 7.5

cassava, of which six are shared with populations of *M. esculenta* ssp. *flabellifolia* from the southern Amazonian rim (from Mato Grosso to Acre), and one is shared both with these populations and with populations of *M. pruinosa* from Goiás (Table 1, Fig. 1). All three new cassava G3*pdh* haplotypes documented in this study differ from already documented haplotypes by only one mutation.

The two haplotypes that dominated in Olsen and Schaal's (1999) sample also predominated in ours: haplotypes A and P had a cumulative frequency of 88% in Guianan landraces, as compared to 85% in Olsen and Schaal's sample from the CIAT world core collection (Table 1). Although we scored more alleles than did Olsen and Schaal, gene diversity and nucleotide diversity were much lower in our sample than in the 20 accessions of diverse origins included in their study (Table 2).

3.3. Interspecific relationships

In Savane Manuel, the individuals of domesticated phenotype were all homozygous for haplotype A, while three of the four individuals of wild phenotype were homozygous for G1 or G2. The fourth one and all three individuals of intermediate phenotype were heterozygous, with one copy of haplotype A and one of either G1, G2 or G3, consistent with their being hybrids or introgressed individuals.

The two samples of *M. glaziovii* were heterozygous, with one copy of haplotype P and one of either GL1 (individual from French Guiana) or GL2 (individual from Indonesia).

Finally, the three individuals from the Monts d'Arawa, initially determined as two different species, were all homozygous for haplotype BA1. Two other haplotypes (BA2 and BA3) were detected in *M.* aff. *quinquepartita* from other localities.

4. Discussion

4.1. Site of domestication

Our sample, combined with Olsen and Schaal's (1999), now covers most of the range of *Manihot esculenta* ssp. *flabellifolia*, as defined by Allem (1994). Our data do not support a second center of domestication for cassava on the northern rim of the Amazonian basin, and reinforce Olsen and Schaal's (1999) conclusions, *i.e.*, that cassava was domesticated only once, on the south-western Amazonian rim. In addition to haplotypes A, H, N, P, δ and ε already observed by Olsen and Schaal (1999) in domesticated cassava, we found another haplotype (M) shared between Guianan cassava accessions and a population of *M. esculenta* ssp. *flabellifolia* from

Jaru (Rondônia, south-western Amazonian rim), and three new haplotypes (D1, D2 and D3) directly derived from haplotypes A and α of Olsen and Schaal (1999; Fig. 2), further supporting a single domestication event for the crop.

4.2. Genetic diversity in cassava and its closest wild relatives

The same alleles predominated in our samples of cultivated cassava and those of Olsen and Schaal (1999), showing that traditional landraces from the Guianas share the same genetic basis as do cultivars from other locations.

Haplotype diversity of *Manihot esculenta* ssp. *flabellifolia* is geographically structured, and is highest in southwestern Amazonia, decreasing towards the east and north. This may indicate a population expansion from southwestern Amazonia eastwards and northwards, through a dry corridor that may have existed repeatedly during glacial times (van der Hammen and Hooghiemstra, 2000). The weak gene diversity and low differentiation in Guianan *M. esculenta* ssp. *flabellifolia* suggest a strong bottleneck effect followed by limited subsequent differentiation by nucleotide substitution.

4.3. Species boundaries, interspecific hybridization and Manihot taxonomy

Species delimitations are far from clear in *Manihot*. Distinctions on purely morphological grounds can lead to mistakes, as some traits are highly variable within species, owing either to genetically based variation or to environmental or developmental plasticity. For example, two leaf morphs are present in populations of Guianan *M. esculenta* ssp. *flabellifolia*. The form with narrow lobes was distinguished by Rogers and Appan (1973) as *M. surinamensis*, but neither G3*pdh* nor microsatellite data (unpublished data) suggest any genetic differentiation between them. Similarly, what first appeared, from herbarium vouchers, to be three separate species (*M.* aff. *quinquepartita* with five-lobed leaves, *M. brachyloba* with trilobate leaves and an undetermined species with deeply dissected leaf lobes) in the Monts d'Arawa were fixed for a single G3*pdh* haplotype (BA1). Subsequent field work showed that leaf morphology of *M.* aff. *quinquepartita* varies over the life cycle in a predictable way (D. McKey, unpublished data) and the re-examination of the three voucher specimens after field work suggested that all three individuals were indeed *M.* aff. *quinquepartita*.

Do gene sequences enable us to sort species? G3*pdh* sequences showed that the genus *Manihot* was monophyletic with regard to *Jatropha* and *Cnidoscolus*, but did not sort species. Some species (*e.g.*, cassava), show astonishing genetic diversity. Some cassava haplotypes (*e.g.*, haplotypes M and P) are also shared with closely related species, suggesting either shared ancestral polymorphism or hybridization. Sequencing of a few other genes will help sort out this issue.

In another case, hybridization seems clearly the more likely explanation for patterns observed. Both *M. glaziovii* individuals studied bore one copy of haplotype P, frequent in cassava but very different from the other haplotype borne by these individuals (GL1 and GL2, differing by only six substitutions). *Manihot glaziovii* has been widely introduced by humans, as a source of latex (Para rubber) and as a shade tree, and has been subjected to crossing with domesticated cassava in programs aimed at increasing disease resistance and productivity of cassava (Jennings, 1976).

Does hybridization occur between more closely related species, and can it be detected using this marker? As already stated by Olsen and Schaal (1999), there is little evidence of hybridization between *Manihot pruinosa* and *Manihot esculenta*, because only two alleles (out of 70: haplotypes E and J, each found once) scored in this taxon were shared with a sympatric species (*M. esculenta* ssp. *flabellifolia*).

Within the *M. esculenta* group, hybridization is possible, as shown in Savane Manuel (Duputié et al., 2007). However, no haplotype of *M. esculenta* ssp. *flabellifolia* from the Guianas was found in cultivated cassava there, suggesting that hybridization between these two taxa is only a recent and local event. In French Guiana, cassava is traditionally cultivated in forest openings, under slash-and-burn systems, while its wild relatives are savanna shrubs. Only recently has cassava begun to be marginally cultivated in small savanna areas in this region.

We may surmise that ongoing deforestation in other regions where cassava and its wild relatives co-occur may create similar situations of secondary contact, hybridization and introgression. Further insight could be gained from studies in areas of contact between wild and domesticated populations where Amerindian farmers incorporate volunteer seedlings and hence individuals of possibly hybrid origin (Elias et al., 2000).

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.05.003.

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