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Gilda Santos Mühlen
Universidade Federal de Rondônia, Rolim de Moura, RO, Brazil

Alessandro Alves-Pereira
Programa de Pós-Graduação em Genética e Melhoramento de Plantas Escola Superior de Agricultura "Luiz de Queiroz", Piracicaba, SP, Brazil Laboratório de Evolução Aplicada, Universidade Federal do Amazonas, Manaus, Brazil

Charles R. Clement
Laboratório de Evolução Aplicada, Universidade Federal do Amazonas, Manaus, Brazil Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil, cclement@inpa.gov.br

Teresa Losada Valle
Instituto Agronômico de Campinas, Campinas, SP, Brazil

ARTICLE

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Gilda Santos Mühlen
Universidade Federal de Rondônia, Rolim de Moura, RO, Brazil

Alessandro Alves-Pereira
Programa de Pós-Graduação em Genética e Melhoramento de Plantas
Escola Superior de Agricultura "Luiz de Queiroz", Piracicaba, SP, Brazil
Laboratório de Evolução Aplicada, Universidade Federal do Amazonas, Manaus, Brazil

Charles R. Clement
Laboratório de Evolução Aplicada, Universidade Federal do Amazonas, Manaus, Brazil
Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil

Teresa Losada Valle
Instituto Agronômico de Campinas, Campinas, SP, Brazil

Abstract

Manioc (*Manihot esculenta* Crantz) originated in Amazonia and is the main staple for more than 800 million people worldwide; it also had a fundamental role as a source of calories for many pre-Columbian peoples, especially in Amazonia, where it was domesticated. There are two major groups of manioc varieties: sweet varieties have low amounts of toxic substances (cyanogenic glycosides) and may be consumed with minimum processing, while bitter varieties have a high degree of toxicity and must be detoxified to be safe before consumption. These groups are outcomes of divergent selective pressures. Natural selection probably maintains large amounts of cyanogenic glycosides to serve as a plant defense when in cultivation. Human selection may reduce the toxicity of the plants when roots are directly consumed, but may be neutral when the roots are consumed after some kind of processing. Although farmers recognize the distinction of the two groups of varieties, the variation of cyanogenic glycosides is continuous among different varieties. Genetic differentiation between sweet and bitter varieties was detected with molecular markers, as well as different patterns of groupings of varieties from different regions of Brazil. The genetic distinctions suggest that the sweet varieties originated during the initial domestication in southwestern Amazonia and bitter varieties arose later during cultivation in Amazonia, as hypothesized by Arroyo-Kalin in a recent paper. They also suggest that these groups of varieties were dispersed independently, even though they are cultivated complementarily today, with sweet varieties in home-gardens and bitter varieties in swiddens.

Keywords: genetic diversity, population genetics, population structure, domestication

Introduction

Manioc (*Manihot esculenta* Crantz ssp. *esculenta*) was domesticated in Southwestern Amazonia (Olsen and Schaal 1999, Olsen 2004), and there are currently hundreds of cultivated varieties throughout the Tropics. It is the staple food crop for more than 800 million people (Lebot 2009), and was a fundamental energy source for various pre-Columbian Amazonian peoples. Its cultivation, processing, and use have been studied by archaeologists and anthropologists (Rival and McKey 2008), as well as agronomists, geneticists, and food technologists (Lebot 2009).

Cultivated manioc is commonly divided into two major groups: sweet and bitter varieties (McKey and Beckerman 1993). Bitter varieties have large amounts of cyanogenic glycosides (CG) and require significant processing to detoxify them for safe consumption, while sweet varieties have low CG amounts and may be consumed after basic processing (peeling and boiling). Although this distinction is recognized by farmers, the CG content varies continuously and there are no morphological characters that differentiate the two groups (McKey and Beckerman 1993). However, molecular marker-based studies support the existence of genetic divergence between the sweet and bitter manioc varieties (Mühlen et al. 2000, Elias et al. 2004, Peroni et al. 2007).

Many studies used microsatellite (or simple sequence repeat - SSR) variation to investigate the genetic diversity and population structure of bitter and sweet varieties from Brazil (Mühlen et al. 2000, Emperaire et al. 2003; Elias et al. 2004, Peroni et al. 2007, Siqueira et al. 2009). However, those studies targeted different locations, used different sets of SSR markers and were of small to medium geographical scale. In this study we examined the genetic diversity of a much wider sampling of sweet and bitter manioc with nine SSR markers. The distribution and organization of the genetic diversity was evaluated across the two major groups of varieties and across Brazil's ecogeographic regions.

Material and Methods

A total of 494 manioc varieties were sampled (1 individual/variety). Although the sampling was not systematic, the number of individuals analyzed is much larger than any of the previous studies. Sample collections were carried out between 1990 and 2001 by different people using different methodologies and with different objectives. Some varieties are from germplasm collections of the Agronomic Institute of Campinas (IAC), EMBRAPA's Cerrado Center, and the Genetics Department of the Luiz de Queiroz College of Agriculture, University of São Paulo. The collection of the upper Negro River varieties was authorized by the Federação das Organizações Indígenas do Rio Negro – FOIRN, in the context of a bilateral research project (CNPq-ISA/IRD). Other varieties were sampled in non-indigenous traditional communities with prior informed consent. The individuals were classified into sweet or bitter varieties.

The varieties from Amazonia were collected along major rivers: upper Negro River (135 varieties); middle and lower Negro River (31); middle and lower Amazon River (35); upper Juruá River (18); upper Xingu River (7). Other regions were also represented: Cerrado (107 varieties); Cerrado-Pantanal ecotone (24; all sweet); Cerrado-Atlantic Forest ecotone (97); southeastern Atlantic Forest (27); northeastern Atlantic Forest (5); Semi-Arid northeastern Brazil (8).

Microsatellite amplification and detection of polymorphism

DNA was extracted, quantified and processed with standard methods (Chavarriaga-Aguirre et al. 1998). Six SSR loci developed by Chavarriaga-Aguirre et al. (1998) and three developed by Mba et al. (2001) were selected (Table 1). Detection of SSR polymorphisms was done in a semi-automatic DNA sequencer (ABI Prism 377), as described by Chavarriaga-Aguirre et al. (1998). Sizes of the SSR loci were determined with the aid of a molecular weight marker (TAMRA 500; Perkin-Elmer) with the Genotyper program (Perkin-Elmer).

Locus	range (bp)	*A*	H_O	H_E	*f*
GA21[a]	105-119	6	0.443	0.462	0.041
GA126[a]	180-222	7	0.728	0.728	0.000
GA131[a]	97-139	11	0.635	0.716	0.114
GA134[a]	301-329	13	0.547	0.524	-0.043
GA136[a]	144-158	6	0.677	0.701	0.034
GA140[a]	144-170	11	0.708	0.829	0.145
SSRY9[b]	250-281	12	0.743	0.829	0.104
SSRY13[b]	179-235	18	0.662	0.842	0.213
SSRY89[b]	105-119	5	0.209	0.218	0.039
Mean		**9.9**	**0.595**	**0.650**	**0.072**

Table 1 – Diversity indices, including number of alleles (*A*), observed (H_O) and expected (H_E) heterozygosities and inbreeding coefficient (*f*), for 494 cassava varieties using nine SSR loci. bp = base pairs. SSR were developed by [a]Chavar riaga-Aguirre et al. (1998) and [b]Mba et al. (2001).

Estimation of genetic diversity and statistical analyses

Genetic diversity estimates, including the total (*A*) and mean ($\bar{A}$) number of alleles, observed (H_O) and expected (H_E) heterozygosities, and the inbreeding coefficient (*f*), were estimated for each SSR marker, for the groups of sweet and bitter manioc varieties, and for the groups of varieties from different regions with *GenAlEx v.6* (Peakall and Smouse 2006). The dispersion of the different varieties, based on the genetic variation revealed by SSR markers, was evaluated in a Principal Coordinates analysis (PCoA) done with *GenAlEx v.6* (Peakall and Smouse 2006). To evaluate the relationships among individuals, a Neighbor-Joining dendrogram was constructed with *MEGA v.4* (Tamura et al. 2007), based on Nei et al.'s (1983) genetic distances, which were estimated with *POPULATION v.1.2.28* (Langella et al. 1999). The genetic structure of the manioc varieties was evaluated with Bayesian analyses implemented with *STRUCTURE v.2.2* (Pritchard et al. 2000), following the scheme described by Evanno et al. (2005) for the selection of the number of clusters (*K*) that best explains the genetic data. Ten independent simulations were performed for each *K* (with *K* varying from 1 to 20 clusters) with no prior population information, under the admixture model, correlated allele frequencies, with 500,000 iterations of the Monte Carlo Markov Chain after a burn in period of 200,000 iterations for each simulation. Results were interpreted according to the origin of the varieties and the dichotomy of sweet and bitter varieties.

Results and Discussion

The SSR markers used in this study revealed high indices of genetic diversity (Tables 1 and 2). The mean number of alleles per locus was 9.9, varying from 5 (SSRY89) to 18 (SSRY13), and sweet and bitter manioc varieties showed similar mean numbers of alleles (8.1 and 9.0, respectively). When the varieties' regions of origin are considered, the upper Negro River showed the highest mean number of alleles ($\bar{A}$= 6.7), while the northeastern Atlantic Forest showed the lowest ($\bar{A}$= 3.1), partially due to sample sizes. The mean heterozygosities were high (H_O= 0.59 and H_E= 0.65), and the groups of sweet and bitter manioc varieties showed similar values of observed heterozygosity (0.598 and 0.590, respectively). The northeastern Atlantic forest showed the highest observed heterozygosity (H_O= 0.706), while the upper Xingu river showed the lowest (H_O= 0.491). The mean inbreeding coefficient (*f*) was 0.072, varying from -0.043 (GA134) to 0.213 (SSRY13), and only the GA134 locus showed negative inbreeding coefficients (excess of observed heterozygotes). The bitter manioc varieties presented an inbreeding coefficient (*f*=0.069) more than five times greater than the sweet varieties (*f*= 0.012). The varieties from the upper Xingu River showed the highest in-

breeding coefficient (*f*= 0.097), while the varieties from the northeastern Atlantic Forest showed the lowest (*f*= -0.411). In general our study found more genetic diversity than Elias et al. (2004), Mühlen et al. (2000) and Peroni et al. (2007), who also used sweet and bitter manioc from Brazil, with greater mean number of alleles (3.3, 4.5, and 2.9 for sweet, and 5.4, 4.2, and 4.3 for bitter manioc, respectively), and higher observed heterozygosities (0.678, 0.725, and 0.730 for sweet, and 0.465, 0.611, and 0.49 for bitter manioc, respectively).

Groups	N	$\bar{A}$	H_O	H_E	f
Bitter varieties	224	9.0	0.590	0.632	0.069
Sweet varieties	270	8.1	0.598	0.615	0.012
Upper Negro River	135	6.7	0.587	0.612	0.046
Mid-Lower Negro River	31	6.2	0.611	0.611	-0.012
Mid-Lower Amazonas River	35	5.1	0.584	0.571	-0.011
Upper Juruá River	18	5.1	0.624	0.600	-0.050
Upper Xingu River	7	4.0	0.491	0.574	0.097
Cerrado	107	5.9	0.577	0.588	0.018
Cerrado-Pantanal	24	4.0	0.537	0.509	-0.039
Cerrado-Atlantic Forest	97	5.2	0.608	0.588	-0.046
SE Atlantic Forest	27	5.2	0.643	0.629	-0.044
NE Atlantic Forest	5	3.1	0.706	0.507	-0.411
Semi-Arid	8	4.4	0.663	0.614	-0.073

Table 2 – Diversity indices, including number of sampled varieties (N), mean number of alleles ($\bar{A}$), observed (H_O) and expected (H_E) heterozygosities, and inbreeding coefficients (f), for the sweet and bitter groups of varieties and for the regions sampled, based on nine SSR loci used in this study.

The dispersion of genetic variability analyzed by the Principal Coordinates Analysis (PCoA) suggests that sweet manioc varieties form a somewhat distinct set from the bitter manioc varieties, although there is a reasonable overlap between these two groups (Figure 1). Cluster analysis, based on the Neighbor-Joining algorithm and Nei et al.'s (1983) genetic distance, corroborated the pattern observed in the PCoA by showing that almost all individuals were grouped according to the two major groups of varieties (Figure 2). These results corroborate previous genetic studies (Mühlen et al. 2000, Emperaire et al. 2003, Elias et al. 2004, Peroni et al. 2007) that clearly indicate that there is a genetic base for the traditionally recognized distinction between sweet and bitter manioc (McKey et al. 2010). Additionally, the results suggest that genetic differentiation between sweet and bitter manioc varieties is consistent in South America, as Bradbury et al. (2013) showed recently that sweet and bitter manioc varieties from Ecuador and French Guiana are genetically differentiated from each other, although the same pattern was not observed in varieties from Africa. The occurrence of hybridization, incorrect passport data and even farmer error in identifying a sweet or a bitter variety may be the explanation for the overlapping and the mixing of individuals from sweet and bitter manioc varieties detected in PCoA and the dendrogram.

Bayesian analyses implemented in *STRUCTURE* showed that the best number of clusters was *K*= 2, with considerable sub-structure at *K*= 3, and also some sub-structure at *K*= 4 (figures not shown). The two clusters at *K*= 2 correspond well with the major groups of sweet and bitter manioc varieties, which was an expected result given the other results found in this and in previous studies. At *K*= 3 there is one group which corresponds well with the bitter varieties, and two groups of sweet varieties. The first one is composed almost exclusively of sweet varieties from the Brazilian Central Plateau (including individuals from the Cerrado, the Cerrado-Pantanal and the Cerrado-Atlantic Forest transitions), while the other is composed of sweet varieties from the other regions, including some varieties from the Brazilian Central Plateau. At *K*= 4 there are two groups

of sweet varieties (which are very similar to those found at *K*=3) and two groups of bitter varieties, one of which is composed almost exclusively of varieties from the upper Negro River region, and the other is composed of varieties from all other regions. The distinction of part of the sweet manioc varieties from the Brazilian Central Plateau found at *K*= 3, and the distinction of bitter manioc varieties from the upper Negro River found at *K*= 4, may be related to selection of different desirable traits in these regions.

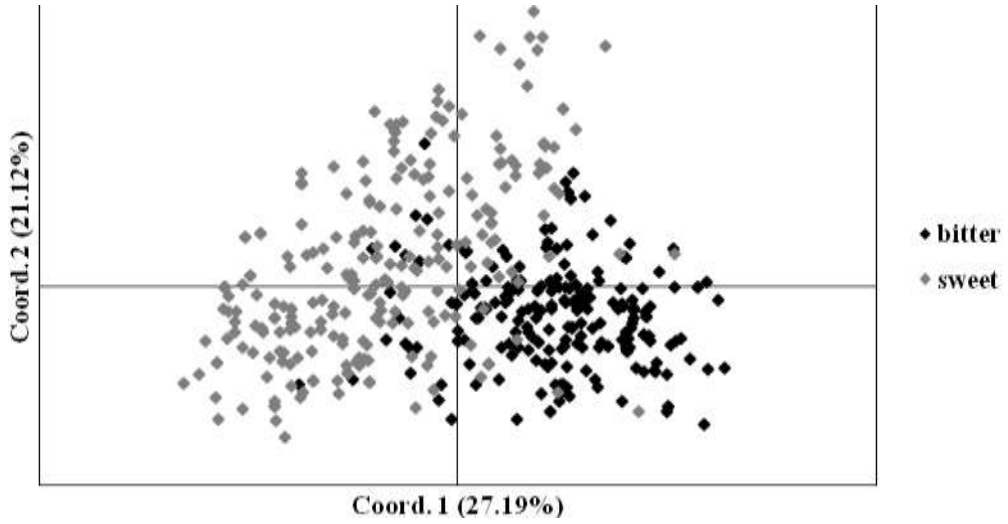

Figure 1 – Principal Coordinate Analysis showing the dispersion of the 494 varieties sampled, based on the genetic diversity revealed by nine SSR loci. Bitter manioc varieties are represented in black, while sweet manioc varieties are represented in gray.

The patterns of distribution of the genetic diversity of manioc varieties revealed by *STRUCTURE* may be used to make inferences about the process of diffusion of the crop, although our sampling lacks varieties from southwestern Amazonia, especially Rondônia state, where manioc was domesticated (Olsen and Schaal 1999, Olsen 2004). From manioc's center of domestication, the sweet varieties seem to have been dispersed in all directions, with a remarkable differentiation throughout the Brazilian Central Plateau. The bitter varieties may have arisen in Amazonia (Arroyo-Kalin 2010) and then were distributed along the Brazilian coast as far as southeastern Brazil. The patterns of distribution of manioc varieties observed in this study may be related with the diasporas of indigenous peoples of the Tupi linguistic group, following the hypothesis of Rodrigues (1964, 2000, cited by Macario et al. 2009). Based on linguistic studies, Rodrigues located the center of origin and dispersal of Tupi speakers in southwestern Amazonia. From this region, which overlaps with that of manioc's domestication, the Guarani speakers dispersed southwards, passing through the Brazilian Central Plateau. On the other hand, the Tupinambá speakers dispersed towards Brazilian coast, following the major rivers of Amazonia. This dispersal might be related to the development of bitter manioc.

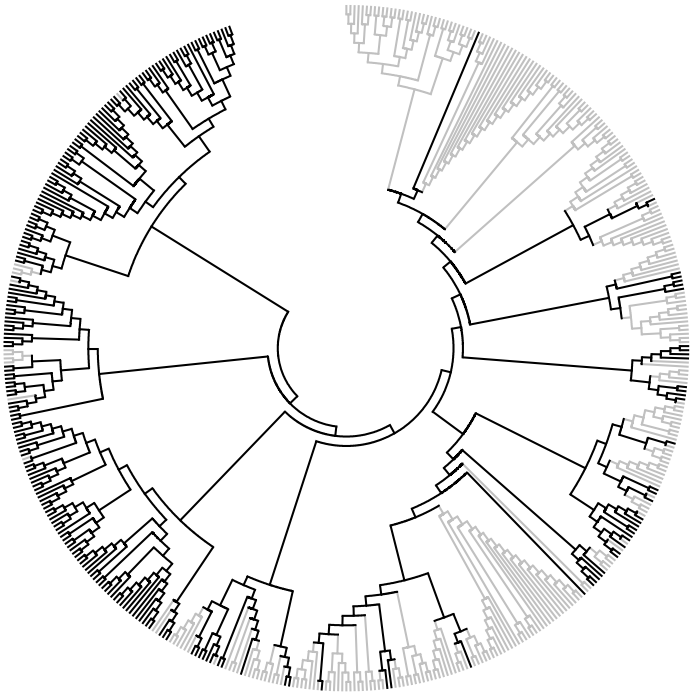

Figure 2 – Relationships among the 494 individuals of manioc varieties represented in a Neighbor-Joining dendrogram based on Nei et al.'s (1983) genetic distance. Each individual is represented by a branch, and the closer the individuals are clustered the more genetically similar they are. Individuals from bitter and sweet manioc varieties are represented by black and gray branches, respectively.

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