



Characterization of microsatellite markers in cassava based on microsatellite-AFLP technique

S. Whankaew¹, S. Sraphet¹, R. Thaikert¹, D.R. Smith¹ and K. Triwitayakorn^{1,2}

¹Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom, Thailand

²Center for Cassava Molecular Biotechnology, Faculty of Science, Mahidol University, Bangkok, Thailand

Corresponding author: K. Triwitayakorn

E-mail: mbktw@mahidol.ac.th

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ABSTRACT. We developed molecular markers for cassava based on the microsatellite-amplified fragment length polymorphism (M-AFLP) technique. Twenty primer pairs were developed and used for the analysis of 48 samples of *Manihot* species, consisting of *M. esculenta* (33), *M. esculenta* ssp *flabellifolia* (3), *M. chlorosticta* (3), *M. carthaginensis* (3), *M. filamentosa* (3), and *M. tristis* (3). Nine microsatellite loci that were polymorphic among these *Manihot* species were identified, giving 32 polymorphic alleles and from two to seven alleles per locus. Unbiased and direct count heterozygosity varied from 0.0233 to 0.7924 and 0.0000 to 0.7083, respectively. There was significant deviation ($P < 0.05$) from Hardy-Weinberg equilibrium at five loci. Genotypic data from the *Manihot* species were subjected to genetic diversity analysis. We found that *M. chlorosticta* and *M. esculenta* ssp *flabellifolia* were the closest populations, while *M. filamentosa* and *M. esculenta* ssp *flabellifolia* were the most divergent. Considering within *M. esculenta*, the samples from Nigeria and Fiji were the most closely related, while those from Venezuela and of unknown origin were the most divergent. We conclude that the M-AFLP technique is an effective method for

generating microsatellite markers that are useful for genetic diversity analysis in *Manihot* species.

Key words: Cassava; M-AFLP; *Manihot* species; Microsatellites

INTRODUCTION

Cassava (*Manihot esculenta*), a shrubby root crop, is one of the leading crops of the world and is important both as a food source and in industrial usage. Cassava ranks fourth among all crops in worldwide production (FAO, 2007), and as such has been the focus of significant research aimed at improving both the quality and the yield. In the past decade, molecular markers have become an important tool for the study of genetic diversity of germplasm, which have immediate value in selection as well as for improving the breeding programs called marker assisted selection (MAS) (Charcosset and Gallais, 2003). Molecular markers are therefore being developed in many plants, including cassava.

Microsatellites, also known as simple sequence repeats (SSRs), are one of the most favorable molecular markers because of their high degree of polymorphism, co-dominant inheritance and the presence of abundant sequences dispersed throughout most of the genome (Morgante and Olivieri, 1993; Zane et al., 2002). These features have made them powerful markers for both genetic diversity studies and MAS (Varshney et al., 2005), and they have been used in many plant species (Roubos et al., 2010; Turkoglu et al., 2010; Iqbal et al., 2011). The major disadvantage in using microsatellite markers is that they require *de novo* development in most species (Zane et al., 2002). Traditionally, microsatellite marker development has involved genomic library construction and screening. This is both time-consuming and expensive (Fisher et al., 1996; Hayden and Sharp, 2001), and moreover, the recovery rate of useful microsatellite markers is generally low. In addition, predetermination of the copy number of microsatellite loci is not possible (Hayden and Sharp, 2001). An alternative approach to overcome these limitations is the microsatellite-based amplified fragment length polymorphism (M-AFLP) technique, a PCR-based approach that combines the concept of AFLP with the microsatellite anchor primer technique (Van Eijk et al., 2001; Yang et al., 2001; Acquadro et al., 2005) through a two-step “primer extension”, which offers the rapid development of SSR markers at a lower cost (Van Eijk et al., 2001).

For cassava, previous studies have developed microsatellite markers based on genomic DNA libraries (Mba et al., 2001) and expressed sequence tag databases (Tangphatsornruang et al., 2008; Raji et al., 2009). In this study, we report a new set of microsatellite markers for cassava and other *Manihot* species, developed based on the M-AFLP technique and their application in genetic diversity analysis.

MATERIAL AND METHODS

Plant materials

Genomic DNA of 33 accessions of *M. esculenta* from 17 different countries, namely Thailand (2), Argentina (3), Brazil (2), Peru (2), Venezuela (1), Columbia (2), Mexico (4), Cuba (2), Paraguay (2), Ecuador (2), Guatemala (2), Malaysia (2), Nigeria (2), China (1), Fiji

(1), Panama (1), Bolivia (1), and an unknown country (1), and 3 accessions each of *M. esculenta* ssp *flabellifolia*, *M. chlorosticta*, *M. carthaginensis*, *M. filamentosa*, and *M. tristis*, which were provided by the International Center for Tropical Agriculture (CIAT), was isolated from young leaf tissue using the DNeasy Plant Mini kit (QIAGEN, Hilden Germany).

M-AFLP development

Genomic DNA of the cassava variety “Hanatee” was digested with *EcoRI* and *MseI* enzymes, and ligated with *EcoRI* and *MseI* adapters, followed by pre-amplification reactions according to Vos et al. (1995). To generate the forward primer sequences, selective amplifications were carried out using nine primer combinations of three *EcoRI* adapter primers with three selective nucleotides (ACC, ACG and ACA) and three 5'-anchored microsatellite primers [GTCG(AG)₇, GACG(TG)₇, and CAGC(TC)₇], as described by Albertini et al. (2003). The reactions were set up in a total volume of 20 µL containing 2 µL of a 1:50 dilution of the amplified PCR products, 0.2 mM of each dNTP, 1.5 mM MgCl₂, 10 pmol of each primer and 1 U *Taq* polymerase (Promega) in the manufacturer-supplied buffer. Amplified fragments were separated on a 5% denaturing polyacrylamide gel (Sambrook and Russell, 2001) and visualized by silver staining (Benbouza et al., 2006). Selected M-AFLP fragments were excised from the gel and incubated at 37°C for 2 h in 20 µL sterile distilled water, and re-amplified. The purified PCR products were cloned into pGEM-T easy vector (Promega), then transformed to *Escherichia coli* DH5α cells by the heat shock method (Sambrook and Russell, 2001). Plasmid DNA-containing M-AFLP fragments were sequenced, and forward primers were designed from the derived sequences using the Primer 3 program (<http://frodo.wi.mit.edu/primer3/>).

To generate the reverse primer sequences, the genomic DNA ligated to *EcoRI* and *MseI* adapters from above was used as a template for amplification with the newly designed forward primers (also from above) in combination with *MseI* adapter-directed primers with no selective nucleotide in order to amplify the opposite side of the microsatellite flanking sequence using the approach described by Acquadro et al. (2005). PCR was carried out in a total volume of 20 µL containing 10 µL DNA template, 0.2 mM of each dNTP, 1.5 mM MgCl₂, 10 pmol of each primer and 1 U *Taq* polymerase (Promega) in the manufacturer-supplied buffer. PCR cycles were performed as follows, 94°C for 2 min, then 35 cycles of 94°C for 30 s, 55°C for 45 s and 72°C for 1 min, and ending with 72°C for 5 min. Amplified products were separated on 5% denaturing polyacrylamide gels and specific fragments were excised from the gel before cloning and transformation to *E. coli* DH5α, as described above. Nucleotide sequences of the fragment were analyzed and used to design the reverse primers.

Analysis of M-AFLP markers

All developed primers were tested for amplification with the donor species and tested for polymorphism within *M. esculenta* and among *Manihot* species. The analysis was performed in a total volume of 20 µL containing 25 ng genomic DNA, 10 pmol of each forward and reverse primer, 0.2 mM of each dNTP, 1.5 mM MgCl₂, 1 U *Taq* polymerase (Promega) in the manufacturer-supplied buffer. The PCR conditions consisted of 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 45 s and 72°C for 1 min, and ending with 72°C for 5

min. The PCR products were separated on 5% denaturing polyacrylamide gels (Sambrook and Russell, 2001) and visualized by silver staining (Benbouza et al., 2006). A 100-bp + 1.5-kb DNA ladder (SibEnzyme) was used to define allele sizes.

Data analysis

Genotypes were scored manually according to country of origin and species. Allele frequencies were calculated followed by calculations of unbiased and direct count heterozygosities, Hardy-Weinberg equilibrium, the percentage of polymorphic loci (using the 95% criterion) and genetic distance using TFPGA 1.3 (Miller, 1997). The Mantel test was used to assess the association between Nei's unbiased (1972, 1978) genetic distance matrix and the Euclidian distance matrix using the same program. In addition, GENEPOP 4.0 (Rousset, 2008) was used for analysis of linkage disequilibrium.

RESULTS AND DISCUSSION

DNA of the cassava variety Hanatee that had been double-digested with *EcoRI* and *MseI* restriction enzymes was amplified by PCR with nine primer combinations of *EcoRI* plus three additional nucleotides and three 5'-anchored microsatellite primers. Selected M-AFLP fragments were isolated, cloned and sequenced in order to design specific microsatellite forward primers. These specific forward primers were then used together with *MseI* adapter primers to generate fragments containing the corresponding reverse sequences. These PCR products were cloned and sequenced and used to design specific microsatellite reverse primers.

In total, 20 primer pairs were developed, which successfully amplified genomic DNA of cassava variety Hanatee. From testing with *Manihot* species consisting of 33 accessions from *M. esculenta* and each of three accessions from *M. esculenta* ssp *flabellifolia*, *M. chlorosticta*, *M. carthagenensis*, *M. filamentosa*, and *M. tristis*, the results showed that 9 microsatellites displayed polymorphisms between *Manihot* species, with a total of 32 polymorphic alleles and allelic variation ranging from two to seven alleles per locus (Table 1). The heterozygosity of unbiased and directed count varied from 0.0233 to 0.7924 with an average of 0.3127 and from 0.0000 to 0.7083 with an average of 0.2143, respectively. The analysis revealed that significant deviations ($P < 0.05$) from Hardy-Weinberg equilibrium were observed at 5 loci (MAF222, MAF421, MAF512, MAF522, and MAF532). Significant linkage disequilibrium ($P < 0.05$) was found between MAF413-MAF512, MAF512-MAF222, MAF512-MAF522, MAF513-MAF522, and MAF421-MAF522. Nei's genetic distance ranged from 0.0291 to 0.7724. In support of this analysis, the data with UPGMA are shown in Figure 1. The results of genetic distance indicated that *M. chlorosticta* and *M. esculenta* ssp *flabellifolia* were the closest populations, while *M. filamentosa* and *M. esculenta* ssp *flabellifolia* were the most divergent.

Within a population of *M. esculenta*, seven polymorphic microsatellites were generated with a total of 24 alleles and allelic variation ranging from two to six alleles per locus. The genetic distances ranged from 0.0000 to 0.4895 (Figure 2). The closest relationship was found between *M. esculenta* from Nigeria and Fiji, while the samples from Venezuela and unknown origin were the most divergent.

Table 1. Characteristics of nine polymorphic microsatellites derived from *M. esculenta* based on M-AFLP technique.

Locus	Primer sequence (5'-3')	Motif	Size range (bp)	Heterozygosities		P _{HWE}	No. of alleles	
				Unbiased	Direct count		Me	Others
MAF211	F: GATTAGCTCGACCAGAACAG R: ATTTGTTTTTATCTGAGAG	(CT) ₄ (CT) ₄	110-120	0.0412	0.0417	1.0000	1	2
MAF222	F: GAATCAGGTTCTGAGTGAGC R: TTCATCCTCGGTGTTCTCT	(CT) ₁ (CAGA) ₂ (TC) ₂ (TA) ₁ (AG) ₁ (TA) ₁ (GGC) ₂ (CA) ₂ (CA) ₂ (AAG) ₂	160-190	0.7924	0.3235	0.0000	6	7
MAF411	F: ATAAACACACAGCTCCCTACA R: GCTAACAAITTTGCCCTTCTTGA	(TC) ₃ (GGT) ₂	225-235	0.5236	0.5333	0.7477	3	3
MAF413	F: CCTTGACGAGGAGAAATCTA R: CTAAGTAAGAATGTTTTCAG	(GC) ₁ (CCA) ₂ (CG) ₂ (CTC) ₁ (CA) ₁	190-195	0.0233	0.0233	1.0000	2	2
MAF421	F: GCACGCCAAITTCATTCAIC R: GAGTAAGCGCGGTCCACGT	(CT) ₁₂ (GAT) ₂ (AT) ₂ (GA) ₂ (TA) ₂ (ATTT) ₂ (TC) ₃ (TC) ₃ (CA) ₈ (CAG) ₂	210-220	0.0460	0.0000	0.0118	1	2
MAF512	F: AATCTCCGATTACTCAAGCA R: CTCCTGAAAGCCTGTTAIG	(CA) ₂ (CT) ₂ (CA) ₆ (GC) ₁ (TA) ₂ (CA) ₂ (CT) ₁ (TA) ₂	360-370	0.5331	0.7083	0.0169	4	4
MAF513	F: GCCACTCACCTAATCACACT R: ACAAAGCAAAGCTAAACTA	(AG) ₁₁ (GA) ₂ (GA) ₂ (CG) ₂ (TTA)TC ₂	240-245	0.0412	0.0417	1.0000	2	2
MAF522	F: CATTAGTTCACCAATGCACAG R: TTGCTAATGTTGGCTATA	(CA) ₂ (CT) ₂ (CA) ₆ (GC) ₁ (TA) ₂ (CA) ₂ (CT) ₁ (TA) ₂	280-285	0.2352	0.0426	0.0000	2	4
MAF532	F: GGCAATCAAATATCGAGTT R: GGAATAAGAAATAGATCGT	(AG) ₁₁ (GA) ₂ (GA) ₂ (CG) ₂ (TTA)TC ₂	180-210	0.5780	0.2143	0.0000	6	6

The number of alleles and heterozygosities (unbiased and direct count) of each locus and significant deviations from Hardy-Weinberg equilibrium (P_{HWE}) were calculated for 33 from *M. esculenta* and 15 from *M. esculenta* ssp *flabellifolia*, *M. chlorosticta*, *M. carthaginensis*, *M. filamentosa*, and *M. tristic* (others).

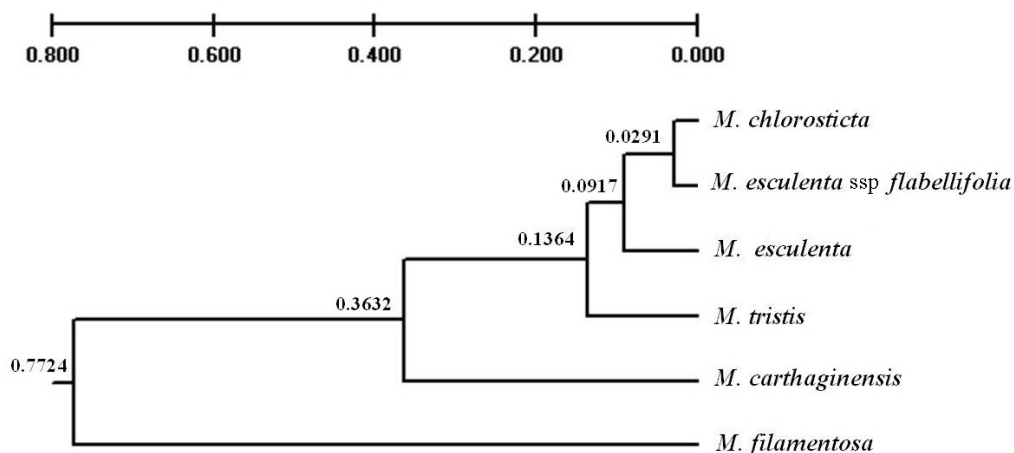


Figure 1. UPGMA dendrogram showing the genetic relationship between cassava accessions (*Manihot esculenta*) based on their landraces.

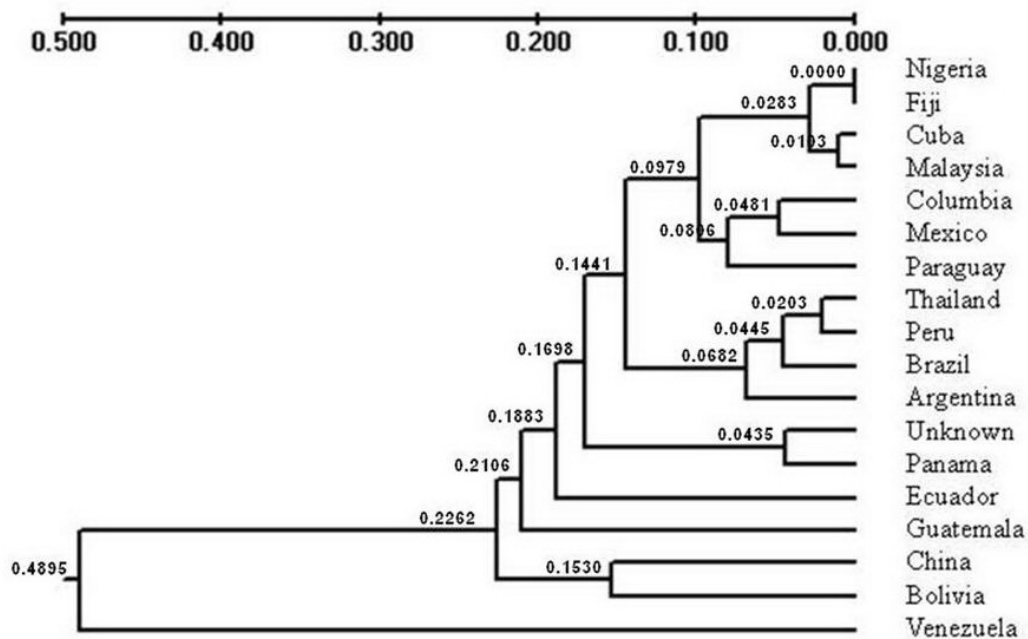


Figure 2. UPGMA dendrogram showing the genetic relationship between *Manihot* species.

In this project, new microsatellites were identified based on the M-AFLP technique. Nine of 20 microsatellite primer pairs (45%) showed polymorphic patterns and were successfully applied to a genetic diversity analysis of *Manihot* species. In addition, seven microsatel-

lite loci were useful for genetic diversity study of cassava. These microsatellites will be potentially applied in linkage mapping as well as comparative genome studies. The results of this study also suggest that M-AFLP technique provides a fast, inexpensive and effective method to develop microsatellites without the need for the construction of microsatellite-enriched libraries. This technique could be an alternative choice for marker development for cassava and other species.

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