Article title

Shotgun sequencing data and SSR mining data of aibika (*Abelmoschus manihot*, Malvaceae)

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Abstract

Aibika (*Abelmoschus manihot*) is a tropical leafy vegetable with great potential in the prevention of malnutrition. Its high fibre and micronutrient content makes it a valuable complement to staple foods such as rice and starchy crops consumed in South Asia and the Pacific. The present study is the first to report the development of a set of 21 nuclear single sequence repeat (SSR) markers of *A. manihot* from genomic sequence data obtained using NGS technology. The DNA library was prepared from a pool of four aibika accessions and sequenced using an Illumina MiSeq system. A total of 1,295,217 pair-end reads were generated. Raw data are available in the European Nucleotide Archive (accession number PRJEB88210; https://www.ebi.ac.uk/ena/browser/view/PRJEB88210). From the 8014 SSR motifs identified, using computational pipelines, we retained 91 candidate SSR loci, which were tested on 23 aibika accessions. Finally, we validated 21 high quality SSR loci by genotyping 45 accessions from three Pacific countries. The number of alleles per locus ranged from 3 to 21 with an average of 7.81 alleles/locus. The 21 selected SSRs were found to be useful in discriminating between accessions and revealing the diversity structure of *A. manihot*. They will help to optimize genebanks management and breeding programmes, and guide future collection activities.

Keywords

Bele; Genetic diversity; Illumina MiSeq; Island cabbage; Microsatellites; New Caledonia; Papua New Guinea; Vanuatu

BACKGROUND

Abelmoschus manihot (L.) Medik., commonly known as aibika, bele, or island cabbage, is a tropical leafy vegetable that has the potential to prevent malnutrition and micronutrient deficiencies [1–3]. The species is native to the Indomalaya biogeographical realm and has spread to New Guinea, Northern Australia, and Pacific Island countries. It is a popular crop in Melanesia where it is propagated mainly by stem cuttings, although seed propagation has been reported [4]. Research efforts to assess genetic diversity have focused primarily on its congener, *A. esculentus* (L.) Moench (okra) [5–7]. In contrast, our understanding of the genetic diversity of *A. manihot* is limited to a study conducted by Rubian-Yalambing et al. [8] who analyzed 23 aibika accessions of Papua New Guinea with RAPD and DAMD markers. The objective of our study was to develop a set of nuclear simple sequence repeat (SSR) markers for assessing the genetic diversity of *A. manihot*. To achieve this, we sequenced a DNA library with an Illumina MiSeq system. Raw sequencing data were processed using computational pipelines to find candidate SSR loci. Finally, we validated a set of 21 polymorphic SSR markers by genotyping 45 accessions from three Pacific Island countries.

DATA DESCRIPTION

Sequencing and genome assembly data

A pooled DNA extract of four *A. manihot* accessions from Vanuatu (VUT084, VUT290, VUT301, VUT313) was used to prepare the DNA library which was sequenced on an Illumina MiSeq system. A total of 1,295,217 pair-end reads were generated. The raw sequences have been deposited in the European Nucleotide Archive (ENA) at the European Bioinformatics Institute (EMBL-EBI) under accession number PRJEB88210.

The sequences were assembled using the ABySS software with a k-mer length of 64. In total 651,320 contigs were generated. The basic statistics of genome assembly are summarized in Table 1.

Table 1**.** Genome assembly statistics of *Abelmoschus manihot*

Parameter	Value
Number of contigs	651,320
Number of contigs >= 500 bp	812
Sum of contig lengths	819,639 bp
Largest contig	11,070 bp
Shortest contig	500 bp
N25	2909
N50	1110
N75	596
L50	157

SSRs data

Using MISA and Primer3, we identified 8014 microsatellite motifs, of which 4637 had a suitable primer design. We excluded complex motifs (4497 retained), then selected amplicon sizes ranging between 100 and 350 bp (384 loci retained). We blasted the selected primer pairs on the okra genome and we kept the primer pairs with only one hit (145 retained). After selection on motif design (from 2 to 6), 96 candidate primer pairs were tested for amplification by genotyping a subset of 23 *A. manihot* samples. Primer pairs were classified according to their polymorphism and the overall quality of the profile. Lastly, we selected 21 polymorphic markers (19 single-locus markers and one two-locus marker) with no ambiguity in allele size determination (Annex 1).

Genotyping data

Forty-five accessions from three Pacific countries were genotyped using the 21 selected SSR markers. The number of alleles observed per locus and per country is presented in Table 2. For the 21 loci, we found a total of 164 alleles and an average of 7.81 alleles per locus.

Table 2. Number of alleles, per locus and per country, observed by genotyping the 45 accessions of *Abelmoschus manihot*

	All countries (N=45)	Per country (n = 15)		
		NCL	PNG	VUT
AmCir0203	7	4	5	4
AmCir0518	6	4	5	2
AmCir0731	14	10	3	6
AmCir0868	5	5	4	3
AmCir1791	3	3	2	2
AmCir1965	4	3	2	3
AmCir2957	9	8	3	4
AmCir3417	7	5	5	5
AmCir3540	7	4	6	4
AmCir4539	6	4	4	4
AmCir4803	21	13	8	6
AmCir5172	6	6	3	3
AmCir5434	7	6	4	3
AmCir5534	8	5	4	4
AmCir5677	10	8	4	3
AmCir6454	9	7	4	5
AmCir8412	9	6	6	4
AmCir8690	6	4	3	3
AmCir9461	3	2	2	3
AmCir9548a	13	11	6	7
AmCir9548b	4	3	3	2
Total (A)	164	121	86	80
(A)/21 loci	7.81	5.76	4.10	3.81

The DARwin software was used to compute the dissimilarity matrix and to perform a factorial analysis. Using the set of 21 markers, we were able to distinguish the *A. manihot* accessions within each genebank and to show how genetic diversity is structured among three countries of the SW Pacific (Fig. 1).

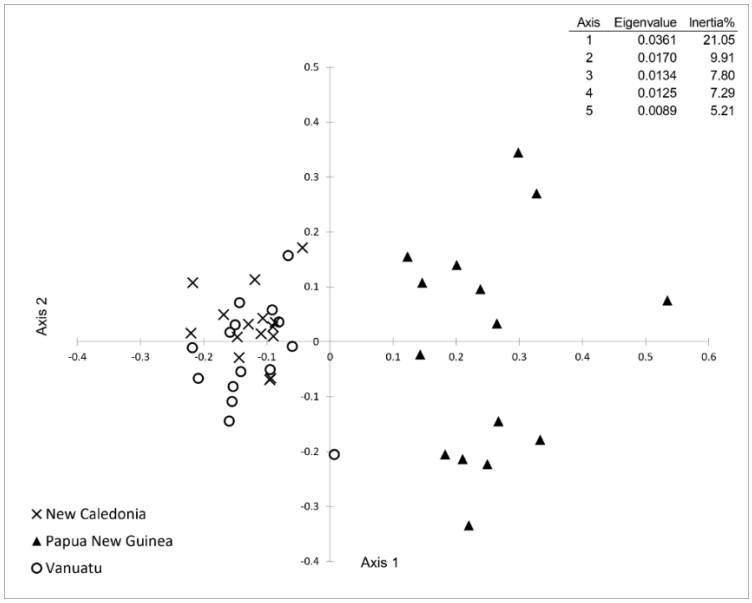

Fig. 1. Graphical representation of the factorial analysis (PCoA) of the distance matrix computed from the genotyping data of the 45 accessions of *Abelmoschus manihot*, using the 21 selected markers.

EXPERIMENTAL DESIGN, MATERIALS AND METHODS

Plant material sampling

The study screened 45 accessions, maintained by research institutions in three Pacific countries (Papua New Guinea, New Caledonia and Vanuatu), sourced from their respective field genebanks (Table 3). Among them, we selected four accessions from different islands of Vanuatu, exhibiting distinct morphological traits, to prepare the DNA library for sequencing. All of the 45 accessions were genotyped to validate the candidate SSR markers.

Table 3**.** List of the *Abelmoschus manihot* accessions used in the study

Provider	Country of origin	Accession numbers
Institut Agronomique néo-Calédonien (IAC) Port Laguerre station, Païta, New Caledonia	New Caledonia	NCL014; NCL024; NCL037; NCL045; NCL087; NC089; NCL100; NCL113; NCL132; NCL134; NCL136; NCL138; NCL144; NCL146; NCL151
National Agricultural Research Institute (NARI) Bubia and Laloki stations, Papua New Guinea	Papua New Guinea	BUBAm002; BUBAm003; BuBAm006; BUBAm009; BUBAm012; BUBAm013; LALAm039; LALAm200; LALAm206; LALAm223; LALAm239; LALAm253; LALAm254; LALAm257; LALAm260
Vanuatu Agricultural and Technical Center (VARTC), Santo, Vanuatu	Vanuatu	VUT006; VUT015; VUT062; VUT063; VUT084*; VUT262; VUT271; VUT273; VUT277; VUT290*; VUT301*; VUT313*; VUT315; VUT349; VUT371

*Accessions used for sequencing

DNA extraction

A piece of about 20 cm² of young, healthy, green leaf was taken from each sampled plant and dried in a ventilated oven at 40 °C for 48 hours. For each sample, 50 mg of dry leaves were ground 2 times at 1400 rpm during 1 min 30 sec with 2 steel beads of 3 mm on Genogrinder 2010 (Cole-Palmer®, Antylia Scientific, Vernon Hills, IL, USA), incubated at 56 °C for 45 min with 600 µl Lysis Buffer CF (Macherey-Nagel, #740946, Düren, Germany) and 200 ng Proteinase K (Macherey-Nagel, #740506). DNA was then purified on Thermo Scientific KingFisherTM Duo Prime System (Thermo Fisher Scientific, Watham, MA, USA) with NucleoMag® C-Beads (Macherey-Nagel, #744401). Genomic DNA was checked for quality by 1% agarose gel electrophoresis and quantified with a Fluoroskan Ascent FL fluorometer (Thermo Fisher Scientific).

Preparation of the DNA library and sequencing

The DNA library was prepared using DNA extracted from four *A. manihot* accessions. The NEBNext® Ultra™ II DNA Library Prep Kit for Illumina (New England Biolabs, #E7645) was used for DNA library construction according to the manufacturer's instructions. 500 ng of each DNA extract were sheared separately using mechanical fragmentation on a Bioruptor® (Hologic Diagenode, Belgium) to obtain an average fragment size of 300 bp. The sheared fragments were checked using a 4200 TapeStation with a D5000 screen tape (Agilent Technologies Inc., Santa Clara, CA, USA). After DNA quantification on a Qubit Flex fluorometer (Thermo Fisher Scientific, USA), pooling was performed to obtain at least 200 ng of fragmented DNA input. The quality of the DNA library was checked. Fragments were predominantly between 200 and 800 pb in size, with an average of 335 bp. The DNA library was quantified using the Takara kit (#638,324) on a qPCR machine (LightCycler® 480 Real-Time PCR System, Roche Life Science).

Finally, the library was sequenced using an Illumina MiSeq system (Illumina) provided by the CIRAD genotyping platform, Montpellier, France. A 500 cycle NANO V2 Illumina cartridge (2 x 250 pb) was used to sequence the library in paired-end. A total of 1,295,217 pair-end reads were generated from the DNA library.

Genome assembly, identification of candidate SSRs and primer design

For the bioinformatic analysis, we used the resources of the MESO@LR platform at the University of Montpellier (https://meso-lr.umontpellier.fr/).

Sequences were assembled using the ABySS software [9] with a k-mer length of 64 (Table 1). From the obtained assembly, SSRs were detected using the MISA Perl script [10] with following search parameters: at least six repeats for dinucleotide motifs, five repeats for trinucleotide motifs, and five repeats for tetra-, penta- and hexanucleotide motifs. Primers were designed using Primer3 software [11] with default settings. Finally, the primer pairs were blasted with the NCBI Basic Local Alignment Search Tool (BLAST) on the okra (*A. esculentus*) reference genome (GenBank accession number GCA_035048815.1) using the blastn-short parameter of the *blastn* suite [12]. The number of hits on the okra genome was obtained from the blast results. We selected the primers pairs showing only one hit. A final selection was made on motif design (from 2 to 6), which resulted in 96 microsatellite loci retained for screening.

Screening for polymorphic SSR markers

The 96 candidate primer pairs identified were tested for amplification on a subset of 23 *A. manihot* samples. Due to the presence of mucilaginous and sticky compounds, the DNA solution had to be

diluted at least 50-fold. Before the PCR reaction, we also had to purify a few recalcitrant samples with AMPure XP magnetic beads (Beckman Coulter, A63881) with a 0.8X bead/DNA ratio. PCR was performed separately for each primer pair in 10 µL final solution, containing 5-10 ng template DNA, 0.5 mM $MgCl_2$, 200 µM dNTPs, 0.2 µmol reverse primer, 0.16 µmol M13-tailed forward primer, 0.2 µmol M13 primer fluorescently labelled with FAM, VIC, PET, or NED (Applied Biosystems, Foster City, California, USA), 0.04 ng bovine serum albumin, 0.75X Q-Product (Qiagen, Hilden, Germany) and 0.06 U Taq DNA polymerase with 1X ThermoPol® reaction buffer (New England Biolabs, Ipswich, MA, USA). PCR was performed on a thermocycler Mastercycler® nexus, Eppendorf AG (Hamburg, Germany) with the following settings: initial denaturation at 95 °C for 5 min; 10 cycles of touchdown, starting at 95 °C for 30 s, then annealing temperature (T_a) plus 5 °C (0.5 °C decrease at each cycle) for 1 min, and 68 °C for 1 min; 23 cycles at 95 °C for 30 s, T_a for 1 min, and 68 °C for 1 min; and a final elongation step at 68 °C for 10 min. Allele size was determined after separation on an ABI 3500 xL Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Genemapper v.6.0 software (Applied Biosystems, Thermo Fisher Scientific) was used for data collection, calculation of allele size and visualization of alleles. Primer pairs were classified according to their polymorphism and the overall quality of the profile. Ten of them showed no amplification reaction, 30 loci were monomorphic, 25 loci showed low polymorphism, and 11 loci were difficult to score. Lastly, we selected 21 polymorphic markers (19 mono-locus and one with two different loci) with no ambiguity in allele size determination (Annex 1).

Validation of the 21 selected microsatellite markers

Genotyping was carried out on 45 accessions of *A. manihot* from three Pacific countries using the 21 SSR markers using the method described above. We used the R package *poppr* [13] to calculate the number of alleles per locus and per country (Table 2) , the R package *nikostourvas/PopGenUtils* to calculate the polymorphism information content (PIC) values (Annex 1), and the DARwin software [14] to compute the dissimilarity matrix with simple matching dissimilarity index and perform Principal Coordinate Analysis (Fig. 1).

CRediT AUTHOR STATEMENT

R. Rivallan: Investigation (sequencing, genotyping), Writing-original draft
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DECLARATION OF COMPETING INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES

[1] F. Luan, Q. Wu, Y. Yang, H. Lv, D. Liu, Z. Gan, N. Zeng, Traditional uses, chemical constituents, biological properties, clinical settings, and toxicities of *Abelmoschus manihot* L.: a comprehensive review, Front Pharmacol 11 (2020). https://doi.org/10.3389/fphar.2020.01068.

[2] J.J. Ohse, R.C. Bakhuizen Van den Brink, Vegetables of the Dutch East Indies (Edible Tubers, Bulbs, Rhizomes and spices included) - 1931, Reprint, Asher and Co., Amsterdam, 1977.

[3] S.R. Preston, ed., Aibika/Bele: *Abelmoschus manihot* (L.) Medik, IPGRI, Rome, 1998.

[4] A. Walter, V. Lebot, *Abelmoschus manihot*, in: Gardens of Oceania, ACIAR, Canberra (AUS), 2007: pp. 111–113. http://www.documentation.ird.fr/hor/fdi:010041802.

[5] S. Hamon, A. Charrier, Okra, in: A. Charrier, M. Jacquot, S. Hamon, D. Nicolas, M.K. Razdan (Eds.), Tropical Plant Breeding, CIRAD; Science Publ., Montpellier (FRA); Enfield (USA), 2001: pp. 361–380. https://www.documentation.ird.fr/hor/fdi:010027773.

[6] S. Kumar, M.J. Parekh, R.S. Fougat, S.K. Patel, C.B. Patel, M. Kumar, B.R. Patel, Assessment of genetic diversity among okra genotypes using SSR markers, J Plant Biochem Biot 26 (2017) 172–178. https://doi.org/10.1007/s13562-016-0378-2.

[7] R. Nieuwenhuis, T. Hesselink, H.C. Van Den Broeck, J. Cordewener, E. Schijlen, L. Bakker, S. Diaz Trivino, D. Struss, S. De Hoop, H. De Jong, S.A. Peters, Genome architecture and genetic diversity of allopolyploid okra (*Abelmoschus esculentus*), The Plant Journal 118 (2024) 225–241. https://doi.org/10.1111/tpj.16602.

[8] L. Rubiang-Yalambing, J. Arcot, H. Greenfield, P. Holford, Aibika (*Abelmoschus manihot* L.): Genetic variation, morphology and relationships to micronutrient composition, Food Chem 193 (2016) 62–68. https://doi.org/10.1016/j.foodchem.2014.08.058.

[9] J.T. Simpson, K. Wong, S.D. Jackman, J.E. Schein, S.J.M. Jones, İ. Birol, ABySS: A parallel assembler for short read sequence data, Genome Res 19 (2009) 1117–1123. https://doi.org/10.1101/gr.089532.108.

[10] T. Thiel, W. Michalek, R. Varshney, A. Graner, Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.), Theor Appl Genet 106 (2003) 411–422. https://doi.org/10.1007/s00122-002-1031-0.

[11] S. Rozen, H. Skaletsky, Primer3 on the WWW for General Users and for Biologist Programmers, in: Bioinformatics Methods and Protocols, Humana Press, Totowa, NJ, 1999: pp. 365–386. https://doi.org/10.1385/1592591922.

[12] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, J Mol Biol 215 (1990) 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

[13] Z.N. Kamvar, J.F. Tabima, N.J. Grünwald, *Poppr*: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction, PeerJ 2 (2014) e281. https://doi.org/10.7717/peerj.281.

[14] X. Perrier, J.-P. Jacquemoud-Collet, DARwin software. http://darwin.cirad.fr, (2006). http://darwin.cirad.fr.

Annex 1. Characteristics of the 21 selected markers of *Abelmoschus manihot.* The minimum and maximum sizes, and the polymorphism information content (PIC) of the markers were obtained from the genotyping results of the 45 accessions.

Locus	SSR.nr	Motif	Forward PRIMER (5'-3')	Reverse PRIMER (5'-3')	Tm(°C)	Min. size* (bp)	Max. size* (bp)	PIC
AmCir0203	p6	(CCATTA)10	CTGAGCAAACAACCTCCTT	ATGCCTATGCCTATGCCT	55	157	193	0.54
AmCir0518	p3	(ATA)11	TTGGCCTCGGAATGGAG	TCTGCTCAATGGTGGAAGA	56	182	197	0.41
AmCir0731	p4	(TTTC)7	CGCTCCTCCATGGACTT	TCCCACTTGCATCTTCATTC	56	216	268	0.71
AmCir0868	p2	(TA)8	ACTAGCGCAAATGAATCCA	AAGAGTTAACGAGATCCAATCA	55	177	185	0.58
AmCir1791	p2	(AT)8	GAAGGAAACTACTTGTCGGG	CGAGAGAAATCGTATTCCCG	56	213	217	0.40
AmCir1965	p6	(AGAAAC)5	TCAAGGTGGGGTGGAGA	CGAGCAGCCTATCGGTT	56	159	174	0.45
AmCir2957	p2	(TA)11	AGTCCTCGTGACCTTGA	AGATGTGTCGGTGTGTTT	54	123	139	0.51
AmCir3417	p2	(TA)10	AGTACTCGAATGGAAGCT	TGAACTTTGGTCTTGGTTT	52	204	218	0.58
AmCir3540	p3	(ATT)11	AGGGTTTTCATCTTATGCTT	AAAATCTATGCTGTCCAAGT	53	267	285	0.65
AmCir4539	p2	(AT)8	TCGCGTGTCATGTGTGA	TGGAATGTGGGTTGTGAATG	56	195	203	0.53
AmCir4803	p3	(TTA)16	GAAGCGCACCTACTTCG	AACTGACGTTCCAAAGCAT	55	129	213	0.69
AmCir5172	p2	(AT)7	GGGAAGATAAGCATCTGGG	CAATCCGTTCAGTACCCAA	55	210	221	0.57
AmCir5434	p2	(AT)11	TTGTAGGGCCACTTCAGT	CCCCACCCAAGTAAACTTATT	55	90	106	0.59
AmCir5534	p2	(AT)15	CCCTCACCTTTAGCTAGCT	AGCCGTTTAATCGTATGACAA	56	126	146	0.57
AmCir5677	p2	(AT)10	CGACATACTCGTTGAAAGAGA	CAGGAGTGGGGACATAGG	56	238	274	0.43
AmCir6454	p5	(GGATG)7	TACCGGGTACAGAGGTGA	CCTCCATGTACAGATGAAACAA	56	119	147	0.56
AmCir8412	p2	(AT)16	ACGCACAGGTATGTATGC	AGGACTCATGTATGGTCGA	55	103	125	0.65
AmCir8690	p2	(TA)11	ACTTGCGATCGACTTGC	CATTGCTTCGTTGGCATAAA	55	218	227	0.22
AmCir9461	p2	(AT)9	TGGTCACGGGGAATCTG	TCTTGGTTTTGCGTTCAAAC	56	272	275	0.49
AmCir9548a	p3	(AAT)16	GCATTCTTTGCGCAGAC	CAAGCTGGCTGTGTTGA	55	96	144	0.77
AmCir9548b	p3	(AAT)16	GCATTCTTTGCGCAGAC	CAAGCTGGCTGTGTTGA	55	162	171	0.42

Tail M13 added to forward primer: CACGACGTTGTAAAACGAC; * Sizes are given without tail M13 (19bp)