

DNA profiling of F₁ Hybrid in Bambara Groundnut (*Vigna subterranea* L. Verdc.) using Simple Sequence Repeat Marker



Nariman S. Ahmad

Field Crops Department, Faculty of Agricultural Sciences, Sulaimani University, Kurdistan Region, Iraq. E-mail address: nariman.ahmad@univsul.net

Abstract

Bambara groundnut (*Vigna subterranea* L. Verdc.) is an indigenous crop of African origin. It is considered as a drought tolerant and the third most important leguminous crop in Sub-Saharan Africa due to its agronomic and nutritional potential. Screening of presumed hybrids and progenies with molecular marker proved to be more accurate and reliable compared to morphological characterizations. Testing the accuracy of presumed hybrids resulted from the cross of African and Indonesia landraces were performed using 12 SSR markers. Out of five crosses only one hybrid resulted from the cross BC x S19 was confirmed to be genuine with four polymorphic markers. The seeds of other crosses were found to come from the self-pollination of maternal parent. These results clearly demonstrate that SSR markers should be useful for rapid identification of true hybrid at the early stage of growth. Hence, the technique contributes to genotype the progenies more accurately to develop genetic mapping and QTL analysis, aiming towards more efficient utilization, conservation and development of bambara groundnut germplasm.

Keywords: Bambara groundnut, SSR marker, capillary electrophoresis, F₁ hybrid

I. Introduction:

Bambara groundnut (*Vigna subterranea* L. Verdc.) is underutilized indigenous African crop, belongs to the Fabaceae, subfamily Papilionoideae. Bambara groundnut is the most drought tolerant crop within the leguminosae family [1-3] and ranks the third most important legume after groundnut (*Arachis hypogea* L.) and cowpea (*Vigna unguiculata* [L]. Walp) in Semi arid Africa [4-5].

Bambara groundnut is a rich source of protein (19%) and along with other local sources of protein could help to alleviate nutritional problems in areas where staple

foods are predominantly carbohydrate sources [1, 6-7]. Worldwide production of bambara groundnut is estimated to be around 330,000 tons annually and the demand for this crop is greater than the production [8].

Despite the huge potentials of bambara groundnut, it has been a neglected crop within the research community [9], especially for its genetic improvement [1]. It has been suggested by Fernandez *et al.* [10] that morphological trait alone is not sufficient to evaluate the offspring precisely. This method is also time-consuming, costly, and requires extensive use of land [11].

Obtaining the hybrid seeds under controlled conditions is required to construct the mapping population for genetic mapping and gene transferring [12]. Hybrid characterization can be identified more rapidly and precisely with PCR-based genetic marker [13]. Identification of parental alleles using DNA markers will allow differentiation of true hybrids from self-pollinated parent and outcrossed lines in F₁ hybrids [14]. Detection of elite crop varieties and hybrids is also essential for protection and prevention of unauthorized commercial use. DNA markers allow the early identification of true hybrids, delivering substantial savings in time and resources for true population development [15].

Among the various molecular markers currently available, simple sequence repeats are widely accepted as reliable marker [16]. SSRs have proven to be abundant and well distributed throughout the genome of plants being found in both coding- and non-coding regions [17-19]. They possess co-dominant inheritance, and multi allelic, highly reproducible and are assayed efficiently by the PCR [16]. The objective of this study was DNA profiling of true hybrids for bambara groundnut with the accurate DNA marker, enabling the early disposal of non-hybrids and facilitate the validation of population to construct the linkage map.

II. Materials and methods:

A. Plant materials

Five African and Indonesian landraces were selected for hybridization. They were Uniswa Red (Swaziland), DipC (Botswana), S19 (Namibia), BC and GHC (Indonesia). Presumed F₁ seeds were collected from the crosses of DipC x GHC, BC x S19, GHC x Uniswa Red, GHC x S19

and Uniswa red x S19. They were grown along with their parents in the Growth room at Sutton Bonington Campus, the University of Nottingham, UK, in 2012.

B. DNA extraction

Genomic DNA of the parents and F₁s for above crosses were isolated from young fresh leaves (two weeks old) grown in the growth room. The extraction was carried out following Dellaporta procedure [20] with some modifications. The DNA of individual sample was quantified using 1% agarose gel alongside the standards of known DNA concentration - through agarose gels (1%) in TBE buffer, followed by spectrophotometer measurement. The gel was stained with ethidium bromide and visualized under UV light of electrophoresis (Fig. 1). The final DNA concentration of all samples was adjusted to 10 ng/μl for PCR reaction.

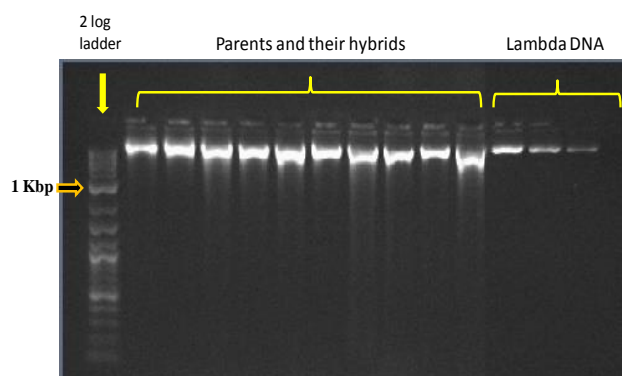


Fig. 1: Quantified DNA extracted from the fresh young leaf of bambara groundnut, produced the best results for quantity of DNA extraction.

C. PCR amplification

A total of 12 SSR Primer pairs derived from a 454-sequenced microsatellite-enriched library of bambara groundnut (UK) were designed according to web based system of Primer3 version 04.0 [21]. They were employed to screen the parents and their presumed F₁ hybrids for polymorphism with polymorphic loci.

Microsatellite Primers were synthesized using a three-Primer 'tagged' reaction. For the PCR, one part SSR-specific forward Primer with a 5' M13 (-21) tail (CACGACGTTGTAACGAC) to nine parts fluorescent M13 sequence Primer [fluorescently-labelled (WellRED dye D4) universal M13 (-21) Primer 5'-CACGACGTTGTAACGAC-3' (Sigma-Aldrich, St. Louis, MO, USA)] were used for the forward reaction [22]. Unmodified SSR-specific reverse Primer (Eurofins MWG Operon, Ebersberg, Germany) was used as a normal reverse reaction.

Individual PCR amplifications were performed in 20µl reaction volume containing 10ng of template DNA, 1x PCR buffer (including MgCl₂ to 1.5mM), 0.2mM dNTPs (Promega, Madison, WI, USA), 0.5 units *Taq* DNA polymerase (New England Biolabs, Ipswich, MA, USA) and 0.18 µM M13 fluorescently-labelled Primer, 0.02µM forward Primer and 0.2 µM reverse Primer. All microsatellite markers were optimized from 50°C to 65°C for the best annealing temperature. Thermo Hybaid Express PCR machine (Electron Corporation, Milford, MA, USA) was used and a mixture of all landraces was prepared as a DNA template. The optimal annealing temperature was used for the subsequent reactions.

PCR amplification was performed on an ABI PCR 9700 Thermocycler (ABI, Carlsbad, CA, USA) running the following profile: initial denaturation; 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 50-65°C for 1 min (at the best annealing temperature) and 72° C for 2 min, followed by a final extension at 72° C for 10 min.

D. Capillary electrophoresis

The PCR products were visualized under UV light and sized relative to the ladder. The products were mixed with size standard and loading buffer and then loaded onto a Beckman CEQ™ 8000 capillary electrophoresis machine (Beckman Coulter Inc., Fullerton, USA). All PCR products (SSR fragments) were sized on with a 400bp size standard. The sample loading solution (SLS; Beckman coulter Inc, Fullerton, USA) was mixed with the size standard (SS; Beckman Coulter Inc, Fullerton, USA) in the ratio of 1:100 (v/v) and 25µL of the mixture was loaded into the individual wells of a new PCR plate.

The amount of 2 - 4µl of the PCR products from each genotype was added into the mixture of SLS and SS solutions to be run in capillary sequencer. Each well was overlaid immediately with a drop of mineral oil (Beckman Coulter, Inc Fullerton, USA) before loading in to the Beckman CEQ machine.

E. Analysis of capillary result

Allele sizes were determined for each SSR locus using the most widely utilized capillary electrophoresis devices of Beckman CEQ™ 8000 fragment analyzer machine [23]. The CEQ™ 8000 (Genetic Analysis System, Beckman Coulter, USA) automatically fills the capillary array with a patented linear polyacrylamide (LPA) gel, denatures and loads the sample, applies the voltage program, and analyzes the data. The results were transferred to a Microsoft Office Word Document file. To maintain a consistent scoring for SSR markers the largest peak size from the electrophoretograms was recorded as the allele size for each sample.

III. Results and discussion:

A. Prime optimization

A Thermo Hybaid Express PCR machine was used for optimization. The best annealing temperature was identified

for the all primers (Table I), depended on the intensity of amplified bands which visualized in the gel electrophoresis.

Table.I: List of SSR Primers used for identification of true hybrid progeny in the crosses

Primer	Forward sequence (5'-3')	Length	Reverse sequence (3'-5')	Length	Optimal annealing temp. (°C)	Allele size (bp)
Primer1	AACTTGCCATACGTGGAAGG	20	ACACGCTGCATAATTCACCA	20	60	256
Primer2	CGTGGATACCCATACCGTCT	20	TAAGTCCATTTTGTCCGATTGA	22	51	171, 173, 175
Primer4	CCATTGTCTCTGCCACCATTTT	21	CAGACTGGGATTTGCATGTG	20	55	202
Primer10	TCAGTGCTTCAACCATCAGC	20	GACCAAACCATTGCCAAACT	20	54	234, 238
Primer16	CCGGAACAGAAAACAACAAC	20	CGTCGATGACAAAGAGCTTG	20	55	185, 189
Primer19	AGGCAAAAACGTTTCAGTTC	20	TTCATGAAGGTTGAGTTTGTC	22	57	234, 269, 273
Primer48	TACCTGCATTGCGGACAGTT	20	TCACTCTTTCTTGATCACATGC	23	60	224, 228, 242
Primer66	CGTTAGACTCTGAGACGCCATT	21	CATCCATCACCTGTCCACCAG	20	60	213, 225, 229
Primer81	CCCGGAACAGAAAACAACAAC	22	CGTCGATGACAAAGAGCTTG	21	60	184
Primer91	CATGGCTGTAGTGGCGTGAG	22	AGACCTGGGAACCTCCACCT	20	60	225, 237
Primer98	TTTTGTCAGTGTGGCCACAA	21	AGATTTATATCTGGATGAGAGAGAGAG	27	57	264, 286, 298
Primer104	CACGAGTGTGCGTGTGTGTTT	22	GGATCAGGCTCCACATAACC	20	57	142, 174

Twelve different temperatures were used for each primer in the range of gradient (50°C- 65°C). The fragment size of less than 300 bp was realized preliminary from the gel assay for all primers used (Fig. 2). The possibility of running these fragments in the capillary array was assured, as the fragment size in capillary electrophoresis adjusted with a higher number of base pair (400bp).

The genomic DNA of respective parents was screened for polymorphism. Twelve Primer pairs were used at their optimal annealing temperature and all of them were amplified. Agarose gel electrophoresis has relatively low resolution [24] and can only score the alleles with more than 20bp size differences, while the capillary array works with very high potential resolution to detect the sized differences up to one bp. A single allele was assign for Primer1, Primer4 and Primer81, being monomorphic for a particular microsatellite locus. While the others exhibited more than one alleles each, displaying the polymorphism (Table I). The high polymorphism of these markers might be referring to the origin of the primers designed. In the crop plants it has been reported that the SSR markers from genomic DNA are more polymorphic compared to the genic SSRs [25]. Also the high genotypic variations within bambara groundnut landraces is another reason for their highly polymorphism [1]. These polymorphic Primers were amplified with the presumed hybrids alongside their

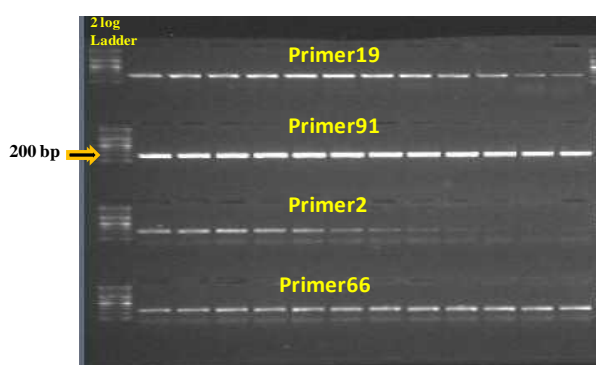


Fig. 2: Annealing gradient test of primers from the bambara groundnut microsatellite enriched-library, showing amplification across a wide range of temperatures (50-65°C) with different expected fragment sizes.

B. Polymorphism detection and hybrid validation

parents. Only the hybrid of cross ♀BC x ♂S19 was confirmed to be a genuine, showing both parental alleles in the

The verification of the true hybrid came from this cross was approved using 4 SSR markers of Primer19, Primer 48, Primer66 and Primer98. It was shown in Fig. 3 that the allele sizes of 273bp and 269bp for BC and S19 parents, respectively, were both displayed in their F₁ hybrid, when amplified with Primer19. Accessing the hybrid of ♀BC x ♂S19 amplified with Primer48 was also performed, displaying both parental alleles 224bp and 228bp, respectively (Fig. 4). Polymorphism of SSR markers allowed

electrophoretograms results of CEQ capillary sequencer.

other loci in BC and S19 to be present in the hybrid for other two markers (Primer66 and Primer98). The heterozygosity of other crossed tested with the same polymorphic markers were not affirmed because the presumed hybrids were developed from the self-pollination of maternal parents. In the cross of ♀Uniswa Red x ♂S19 the expected hybrid was came from the self-pollination of maternal parent (Uniswa Red), as the allele size of 235bp was only detected when amplified with Primer19 (Fig. 5).

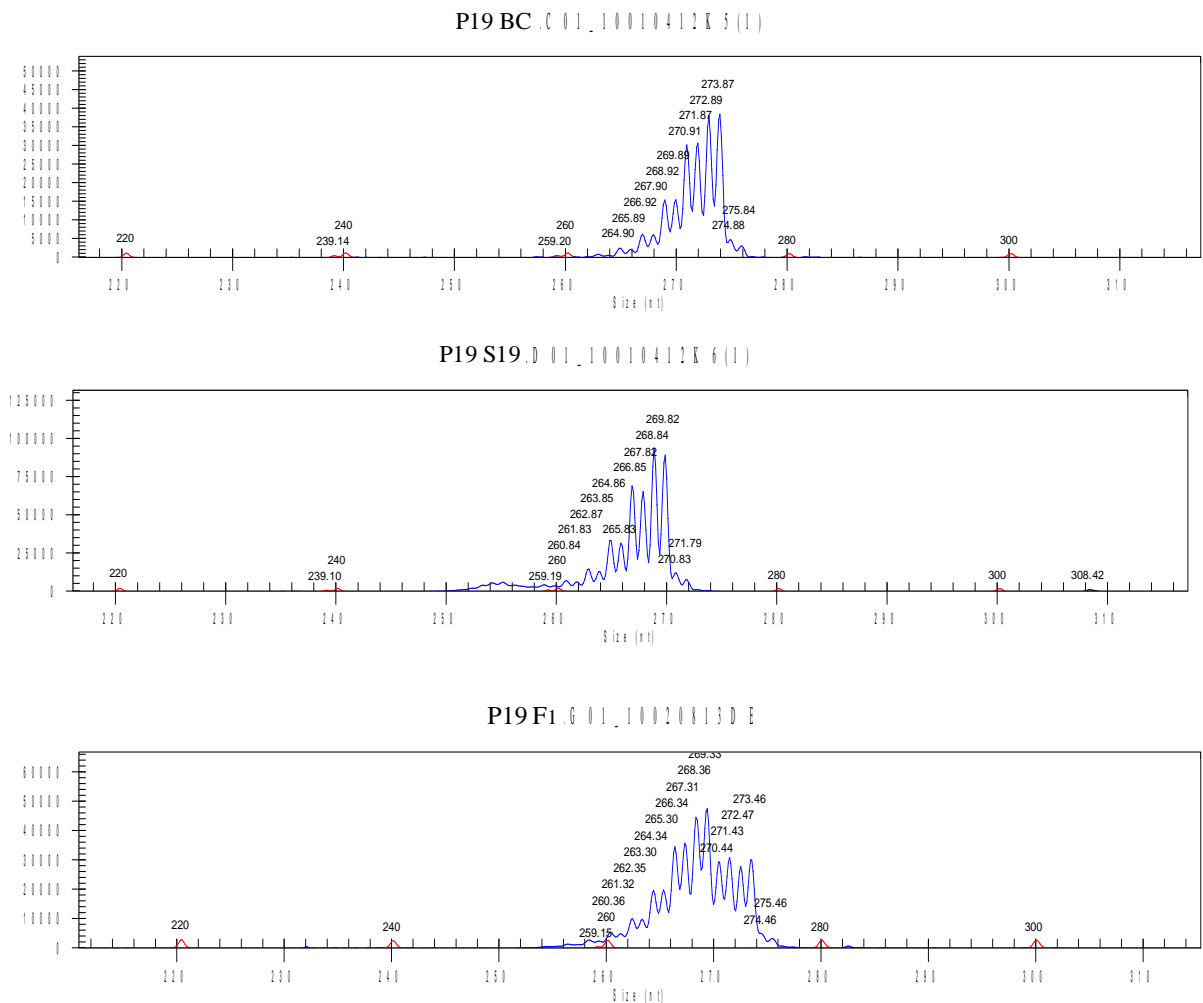


Fig. 3: Genuine hybrids of the cross (♀BC x ♂S19) in the electropherogram for polymorphic Primer19 using the Beckman CEQTM 8000 capillary electrophoresis machine. Different alleles of 273bp for BC and 269bp for S19 landraces are present in the F₁ hybrid.

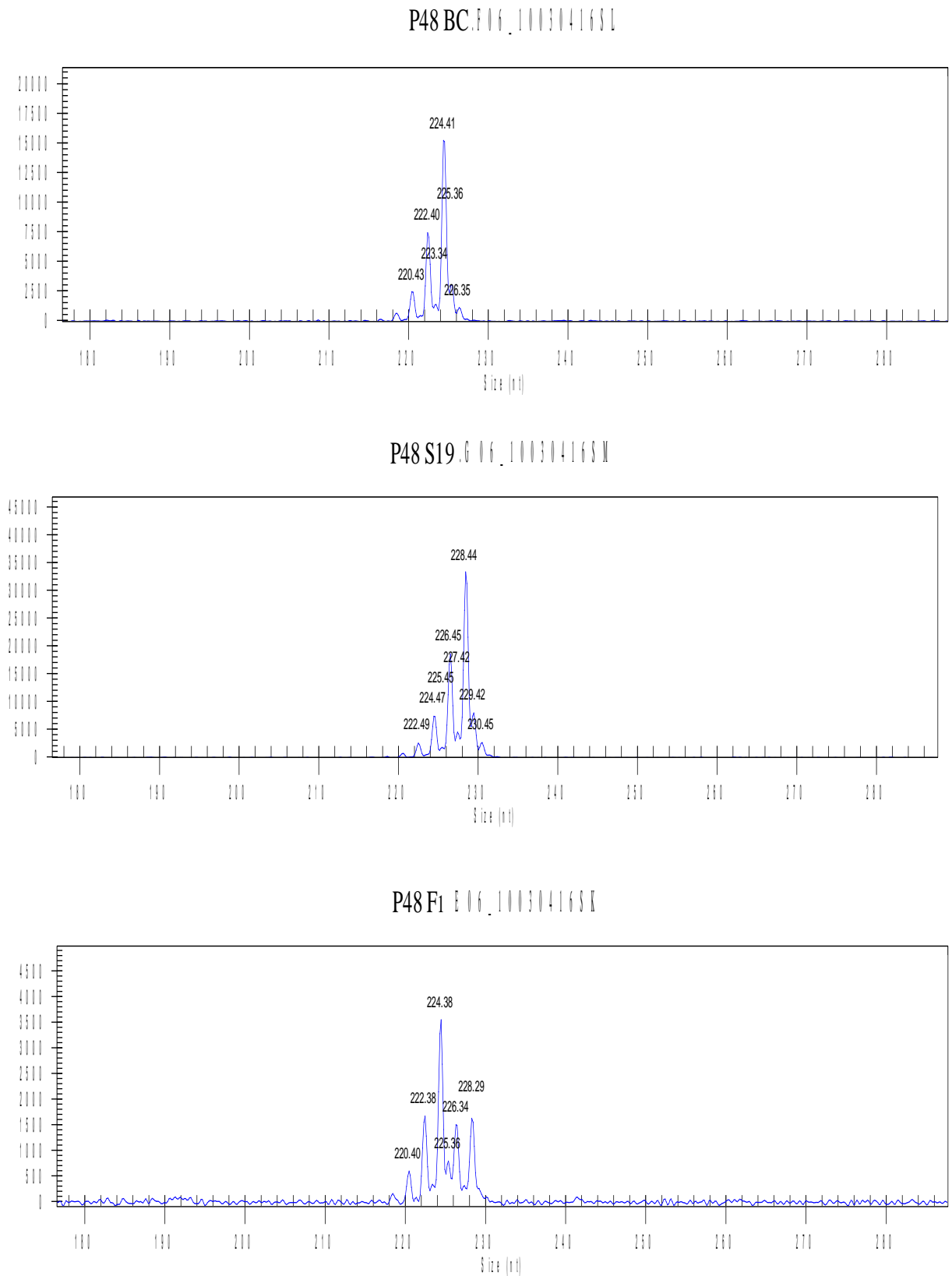


Fig. 4: Genuine hybrids of the cross ($\text{♀BC} \times \text{♂S19}$) in the electropherogram for polymorphic Primer48 using the Beckman CEQTM 8000 capillary electrophoresis machine. Different alleles of 224bp for BC and 228bp for S19 landraces are present in the F_1 hybrid.

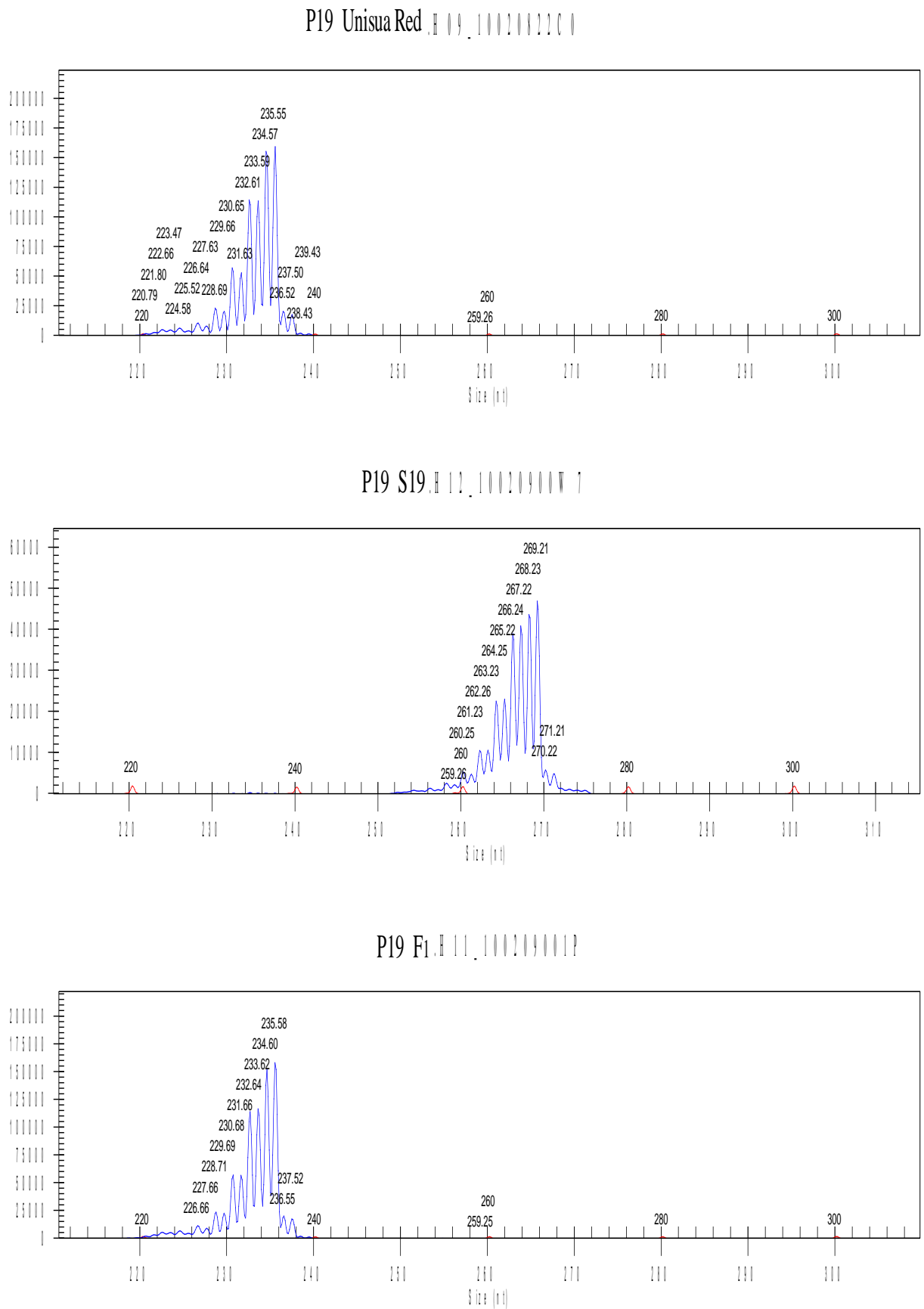


Fig. 5: Self pollination of maternal parent (Uniswa Red) from the cross (♀ Uniswa x ♂ S19) in the electropherogram for Primer19 using the Beckman CEQTM 8000 capillary electrophoresis machine. Only maternal allele of 235bp for S19 is displayed in the presumed F_1 hybrid.

Out of five attempted crosses only one (♀BC x ♂S19) was successful as the hybrid seed came from the right parents. This maybe refers to the difficulty of artificial crossing in bambara groundnut, especially the emasculation process, due to the small size and compaction of the flowers particularly in the bunch type. This will allows having self- pollination before the artificial pollination. Emasculation method and the time for practicing the process might be another reason to reduce the hybridization success [26].

IV. Conclusions:

SSR markers were applied to screen the presumed hybrids obtained from crosses between African and Indonesian landraces. A total of 12 SSR Primers were screened out of which 9 were polymorphic displaying 2-3 loci per primer when applied to the parents of crosses. Only the F₁ hybrid derived from the cross ♀BC x ♂S19 was estimated being a genuine. They

were approved, amplifying SSR loci with four Primer pairs (Primer19, Primer44, Primer66 and Primer98). Both parental alleles were displayed in the F₁ hybrid with all 4 polymorphic Primers. The present study revealed that utilizing DNA markers such as SSRs allows the early identification of true hybrids, saving time and resources, in any breeding programs of the hybrids and population development to follow the gene flow and to develop the genetic map of this crop.

Acknowledgements

The author would like to acknowledge European Union BAMLINK project for the financial support. Many thank to Dr. Sean Mayes at plant and crop sciences division, School of Biosciences, The University of Nottingham for his advices, technical support and his allowance for the lab facilities. Appreciation is extended to Dr. Endah Sri Redjeki for providing Indonesian landraces.

References

- [1] F. J. Massawe, S. S. Mwale, S. N. Azam-Ali, and J. A. Roberts, "Breeding in bambara groundnut (*Vigna subterranea* (L.) Verdc.): strategic considerations," *African Journal of Biotechnology*, 4 (6), pp. 463-471 (2005).
- [2] S. Basu, S. Mayes, M. Davey, J. A. Roberts, S. N. Azam-Ali, R. Mithen, R. S. Pasquet, "Inheritance of 'domestication' traits in bambara groundnut (*Vigna subterranea* (L.) Verdc.)," *Euphytica*, 157 (1-2), pp. 59-68 (2007).
- [3] S. Basu, J. A. Robert, S. N. Azam-Ali, and S. Mayes, "Bambara groundnut", in *Genomic mapping and molecular breeding in plants: Pulses, sugar and tuber crops*, edited by Kole C. (Springer, New York), Vol. 3, pp. 159-173, (2007).
- [4] M. O. Aremu, O. Olaofe, and E. T. Akintayo, "Chemical Composition and Physicochemical Characteristics of Two Varieties of Bambara Groundnut (*Vigna subterrenea*) " *Journal of Applied Sciences* 6 (9), pp. 1900-1903 (2006).
- [5] A. R. Linnemann, Bambara groundnut (*Vigna subterranea*) literature : *a revised and updated bibliography*. Wageningen, Netherlands: Wageningen Agricultural University, (1992).

- [6] J. Okpuzor, H. A. Ogbunugafor, U. Okafor, and M. O. Sofidiya, "Identification of protein types in Bambara nut seeds: Perspectives for dietary protein supply in developing countries," *EXCLI Journal* 9, pp. 17-28 (2010).
- [7] O. S. Ijarotimi and T. R. Esho, "Comparison of nutritional composition and anti-nutrient status of fermented, germinated and roasted bambara groundnut seeds (*Vigna subterranea*)," *Brit Food J* 111 (4-5), pp. 376-386 (2009).
- [8] M. Brink and G. Belay, *Plant Resources of Tropical Africa 1. Cereals and Pulses*. PROTA Foundation, Wageningen, Netherlands, (2006).
- [9] H. Jaenicke and I. Ho"schle-Zeledon, "Strategic framework for underutilized plant species research and development, with special reference to Asia and the Pacific, and to Sub-Saharan Africa. International Centre for Underutilised Crops, Colombo, Sri Lanka and global facilitation unit for underutilized species, Rome, Italy," (2006).
- [10] J. A. Fernandez, D. Migliaro, P. Crino, C. Egea-Gilabert, Identification of F1 hybrids of, and artichoke by ISSR markers and morphological analysis. *Mol. Breeding* 27:157-170., "Identification of F₁ hybrids of artichoke by ISSR markers and morphological analysis," *Molecular Breeding* 27 (2), pp. 157-170 (2011).
- [11] M. Wu, X. Jia, L. Tian, and B. Lv, "Rapid and reliable purity identification of F1 hybrids of maize (*Zea may* L.) using SSR markers," *Molecular Plant Breeding* 4 (3), pp. 381-384 (2010).
- [12] N. JONES, H. Ougham, and H. Thomas, "Markers and mapping: we are all geneticists now," *New Phytologist* 137 (1), pp. 165-177 (1997).
- [13] R. M. Sundaram, B. Naveenkumar, S. K. Biradar, S. M. Balachandran, B. Mishra, M. IlyasAhmed, B. C. Viraktamath, M. S. Ramesha, and N. P. Sarma, "Identification of informative SSR markers capable of distinguishing hybrid rice parental lines and their utilization in seed purity assessment," *Euphytica* 163 (2), pp. 215-224 (2008).
- [14] C. Mohan, P. Shanmugasundaram, and N. Senthil, "Identification of true hybrid progenies in cassava using simple sequence repeat (SSR) markers," *Bangladesh Journal of Botany* 42 (1), pp 155-159 (2013).
- [15] N. Nandakumar, A. K. Singh, R .K. Sharma, T. Mohapatra, K. V. Prabhu, and F. U. Zaman, "Molecular fingerprinting of hybrids and assessment of genetic purity of hybrid seeds in rice using microsatellite markers," *Euphytica* 136 (3), pp. 257-264 (2004).
- [16] L. Mondini, A. Noorani, and M. A. Pagnotta, "Assessing plant genetic diversity by molecular tools," *Diversity* 1 (1), pp. 19-35 (2009).
- [17] G. K. Chambers and E. S. MacAvoy, "Microsatellites: Consensus and controversy," *Comparative Biochemistry and Physiology Part B Biochemistry and Molecular Biology* 126B (4), pp. 455-476 (2000).
- [18] H. Ellegren, "Microsatellites: Simple sequences with complex evolution," *Nature Reviews Genetics* 5 (6), pp. 435-445 (2004).
- [19] Y. D. Kelkar, S. Tyekucheva, F. Chiaromonte, and K. D. Makova, "The genome-wide determinants of human and chimpanzee microsatellite evolution," *Genome Res* 18 (1), pp. 30-38 (2008).
- [20] S. L. Dellaporta, Wood J., and Hicks J. B., "A plant DNA miniprep preparation version II," *Plant Molecular Biology Reporter* 1, pp. 19-21 (1983).
- [21] S. Rozen and H. Skaletsky, "Primer3 on the WWW for general users and for biologist programmers", in *Bioinformatics Methods and Protocols: Methods in Molecular*

- Biology*, edited by S. Krawetz, Misener, S. (Humana Press, Totowa, NJ, 2000), pp. 365-386 (2000). Source code available at <http://fokker.wi.mit.edu/primer363/>.
- [22] M. Schuelke, "An economic method for the fluorescent labeling of PCR fragments," *Nat Biotechnol* 18 (2), pp. 233-234 (2000).
- [23] X. Wang, T.A. Rinehart, P. A. Wadl, J. M. Spiers, D. Hadziabdic, M. T. Windham, and R. N. Trigiano, "A new electrophoresis technique to separate microsatellite alleles," *African Journal of Biotechnology* 8 (11), pp. 2432-2436 (2009).
- [24] J. L. Wang, N. A. Barkley, and T. M. Jenkins, "Microsatellite markers in plants and insects Part I: Applications of biotechnology," *Genes, Genomes, and Genomics* 3 (1), pp. 54-67 (2009).
- [25] R. K. Varshney, A. Graner, and M. E. Sorrells, "Genic microsatellite markers in plants: features and applications," *Trends Biotechnol* 23 (1), pp. 48-55 (2005).
- [26] J. Suwanprasert, T. Toojinda, P. Srinives, and Sontichai Chanprame, "Hybridization technique for bambara groundnut," *Breeding Science* 56 (2), pp. 125-129 (2006).