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Full Length Research Paper

Development and use of microsatellites markers for genetic variation analysis, in the Namibian germplasm, both within and between populations of marama bean (*Tylosema esculentum*)

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***Tylosema esculentum* (marama) has long been identified as a candidate crop for arid and semi-arid environments due to its success in these environments and the high nutritional value of the seed. Molecular markers are essential for the assessment of the levels of genetic variation present within and between populations of marama as well for future marker-assisted breeding efforts. Microsatellites were isolated using a modified FIASCO enrichment technique. Eighty pairs of primers were designed to amplify across a selected set of perfect microsatellite repeats with greater than 5 repeat units. Of the 80 primer pairs screened, 76% were able to detect polymorphism and 21% gave monomorphic bands while the other 3% gave inconsistent results. Four of the polymorphic SSR's were used for genetic variation analysis and have proved to be useful and informative markers for assessing intra-specific and inter-specific variability of marama bean. Heterozygosity (H) within and between populations of marama bean in the Namibian germplasm ranged from 0.30 to 0.74. Some of the populations had low genetic variation while others had high genetic variation.**

Key words: *Tylosema esculentum*, microsatellites, FIASCO, polymorphism, genetic variation, heterozygosity.

INTRODUCTION

The marama bean, *Tylosema esculentum* (Burchell.) A. Schreiber, belong to the Fabaceae (Legumionosae) family, sub family Caesalpinioideae (Dubois et al., 1995). It is presently known only in the wild state and is not cultivated. Marama bean is found in South Africa in the western and Northwestern Gauteng and in the Northern part of the Cape Province, Botswana and North-east of Namibia (Bower et al., 1988; Naomab, 2004). Its primary agronomic potential is based upon the high nutritional value of the seeds. The seeds and tubers are edible after roasting and cooking respectively. There is an increasing interest in its cultivation, due to its potential as a cash crop and food source especially in the face of climate change since it occurs in a very harsh environment.

Plant microsatellites, simple sequence repeats (SSRs), have been developed for germplasm conservation, cultivar identification and for assessing genetic diversity not only in crops such as tomato (He et al., 2003), sweet orange (Novelli et al., 2006), soybean (Rongwen et al., 1995; Akkaya et al., 1992), peanut (Fergurson et al., 2004) and common bean (Gaitán-Solis et al., 2002), but also in perennial plants such as the *Melaleuca* (the tea tree) (Rossetto et al., 1999). Until recently developing SSR markers for new species was a laborious and costly exercise thus limiting their potential application to novel plant species. The development of SSR enrichment techniques, in which selective genomic libraries con-

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Table 1. Location, number of accessions of marama individuals collected and region where the locations are within the Namibian germplasm used for genetic variation analysis.

Location	Number of accession	Region
Omitara	19	Khomas
Otjovanatje	20	Omaheke
Sanveld	21	Omaheke
Harnas	25	Omaheke
Ozondema	26	Otjozondjupa
Epukiro/Post 3	30	Omaheke
Omipanda	31	Omaheke
Osire	40	Otjozondjupa
Ombujondjou	40	Otjozondjupa
Otjiwarongo	40	Otjozondjupa
Okomumbonde	40	Omaheke

taining pre-screened inserts are prepared, has increased the efficiency of SSR characterization in new species (Zane et al., 2002). The availability of such technology opens new opportunities for large scale SSR characterization of species with no previous knowledge such as marama bean. The fast isolation by AFLP of sequences containing repeats (FIASCO) technique is one of such method and a variation of it was used in this study.

This paper describes the isolation of an enriched microsatellite fraction from marama, the sequencing of this enriched fraction using 454 sequencing, the identification of SSR's and the use of Polymerase Chain Reaction (PCR) with primers designed in the flanking sequences to characterize the level of polymorphism within the marama collection assembled (Santana et al., 2009). In this study four of these markers were used to characterize the 332 individual marama plants in the germplasm.

MATERIALS AND METHODS

Distribution of marama bean and sample collection

A total of 332 *T. esculentum* individuals representing 11 populations described in Nepolo et al. (2009) were sampled in the Namibian germplasm (Table 1). The 11 populations were from several localities in the Omaheke, Otjozondjupa and Khomas regions of Namibia. Leaf materials were collected from randomly selected marama plants at each location. Leaf material was stored at -4℃ in the laboratory following field collections.

DNA extraction

DNA was extracted from leaves of each of the plant samples collected from the 11 sampling sites using the manufacturer's protocol for the DNeasy (Qiagen, Valencia, CA, USA) mini-kit for purification of genomic DNA from plant tissue. The DNA concentration was determined on a 1% agarose gel stained with ethidium bromide using known molecular weight standards and stored in clearly labeled microcentrifuge tubes at -20℃. DNA with a concentration of 25 – 250 µg/µl. DNA samples were then diluted to 10 ng/µl for PCR amplification.

Microsatellite isolation using a modified FIASCO technique

Marama DNA was enriched for microsatellites by a modified Fast Isolation by AFLP of Sequences Containing microsatellite (FIASCO) technique (Zane et al., 2002) as described below. Nine marama bean microsatellite libraries enriched for different SSR motifs using biotinylated $(AAG)_7$, $(GTT)_7$, $(AGG)_7$, $(GAG)_7$, $(CA)_{10}$, $(CT)_{10}$,$(TCC)_7$,$(CA)_{15}$ and $(CAC)_7$ were created. The steps in the protocol followed are described below:

Restriction enzyme digests and purification

One microgram of DNA was digested with the restriction enzymes, *Msp* 1, H1, *Csp* 6I and *Sau 3A*, as per the supplier's instructions. The digest was then cleaned using the Qiagen PCR purification kit.

Adaptor ligation and amplicon preparation

Adaptors that were used were those designed for representational difference analysis (Oh et al., 2007) so that a wide range of restriction enzymes could be used to fragment the genome. The ligation was performed with 500 ng of the restriction-digested DNA, 1 µl of 12 mer adapter, 1 µl 24 mer adapter and 3 µl ligation buffer in a final volume of 28 µl. The reaction mixture was heated to 72℃ for 3 min then cooled by 1°/min to 4℃. 2 µl ligase was added and the reaction was incubated at 4℃ for 16 h. The 12 bp adaptors were removed by heating to 72℃ for 5 min to melt off the 12 mer followed by purification using the QIAquick PCR purification kit. Next, the ligated DNA was amplified with PCR by combining 5 µl PCR buffer, 5 µl (20 mM) MgCl2, 4 µl (10 mM) dNTPs, 2 µl adapter (100 µM), 34.75 µl water, and 1 µl ligated DNA. The reaction was heated at 72℃ for 5 min, 5 units of *Taq* polymerase enzyme was added and incubated for 5 min at 72℃. The DNA was amplified for 20 cycles of 95℃ for 30 s and 72℃ for 90 s with a final hold at 72℃ for 5 min. Following the PCR, another QIAquick PCR cleanup was performed.

SSR enrichment

The SSR enrichments were repeated four times (with each enzyme

of the preparation steps) with $(AAG)_7$, $(GTT)_7$, $(AGG)_7$, $(GAG)_7$, $(CA)_{10}$, $(CT)_{10}$, $(TCC)_7$, $(CA)_{15}$ and $(CAC)_7$ biotinylated primers. First the amplified digest was denatured and annealed to the biotinylated primer by combining 20 µl PCR product (200 µg) and 1 µl primer (10 µM) and heating at 95°C for 5 min, followed by incubation at room temperature for 30 min. Before combining the primed DNA with streptavidin beads, 10 µl of unrelated DNA (sheared herring sperm at 1 mg/ml) was added to minimize non-specific binding. The annealed DNA mixture was then added to 1 mg of magnetic beads and incubated for 30 min at room temperature, allowing the streptavidin beads to bind to the biotinlyated primers. Five washes with TEN100 (Tris/EDTA/NaCl) followed by 5 washes with 0.2X SSC, 0.1% SDS were performed to remove non-specific DNA binding. Next, two denaturation steps were performed to separate DNA containing SSRs from the beads. The first denaturation was done by adding 50 µl of TE (Tris-HCl 10 mM, EDTA 1 mM) and heating to 95°C for 5 min. The remaining solution was separated magnetically and stored. The second denaturation used 12 µl 0.5 N NaOH, which was neutralized with 12 µl 0.5 N HCl and separated magnetically. Each denaturation product (2 per enzyme) was amplified separately with PCR by adding 5 µl PCR buffer, 3 µl $MgCl_2$ (25 mM), 4 µl dNTPs (each 2.5 mM), 2 µl adapter (10 µM, 34.75 µl water, 0.25 µl r*Taq* (2 units) and 1 µl DNA into a PCR tube. The mixture was cycled 20 times from 95 to 72°C. Each of the amplified denaturation products were separated on a 1.5% agarose gel.

Sequencing

All the microsatellite-enriched genomic DNA pools were combined and a total of 5 µg of the extracted microsatellites were sequenced and analyzed with the Roche 454 GS-FLX platform at Inqaba Biotech (Pretoria, Gauteng, South Africa). Sample preparation and analytical processing such as base calling, were performed at Inqaba Biotech using the manufacturer's protocol as described previously (Santana et al., 2009). The sequence information gathered was sorted into contig and single read files that were used for SSR identification in the sequences and subsequently in primer design.

SSR identification and primer design

The Simple Sequence Repeat Identification Tool (SSRIT) software SSRIT, (http://www.gramene.org/db/markers/ssrtool) was used to identify the microsatellites in the contig files obtained from 454 sequence data. The search parameters were for SSRs from dimers to pentameters with a minimum of 5 exact repeats required for each type of microsatellite. Primers were then developed in the flanking region around the SSR sites using Primer 3 (http://frodo.wi.mit.edu/primer3) and the primers were synthesized by Inqaba Biotech. Eighty microsatellite primers pairs were then used for amplification of marama DNA from the 11 different Namibian sites as well as 1 location in South Africa (Pretoria). Each microsatellite primer marker was given a name consisting of the prefix "MARA" followed by a number (001 - 080).

SSR primer screening

Each of the primer sets was initially used to screen individual marama DNAs from 19 individuals from the Omitara population. PCR amplifications were performed in 25 µl reaction volumes, with a 2X PCR master mix from Fermentas. Each PCR reaction contained 1 µl template genomic DNA, 1 µl (1 µM) of SSR forward primer, 1 µl of SSR reverse primer, 12.5 µl of the 2X PCR master mix and 9.5 µl nuclease free water. The PCR reaction profile used involved an initial denaturation step of 95°C for 4 min, followed by 35 cycles of denaturation at 95°C for 30 s, an annealing at between 55 and 65°C (primer sequence dependent) for 60 s and an extension at 72°C for 2 min, a final extension at 72°C for 5 min and then held at 4°C. Agarose gel (2%) visualization of PCR products was then used to determine if a primer pair was polymorphic or monomorphic based on its separation of amplification products and banding patterns generated on the agarose gels in the different DNA templates. A total of 80 microsatellite primers were screened and described as polymorphic, monomorphic or unable to amplify. Four of the 80 microsatellites were then used for diversity analysis of the 332 individual marama bean plants from the Namibian germplasm.

Genetic diversity analysis

Polymorphic primers MARA 001, MARA 065, MARA 068 and MARA 077 were used to determine genetic variation in each of the 12 ecotypes using the same reaction profile and conditions described in the screening for polymorphic microsatellites. Agarose gels (2.5%) were used to visualize amplification products. The DNA bands obtained were scored with 1 denoting presence of a band and 0 absence of a band, generating a binary data matrix and analysed using Primer 5 (Version 5.2.0) software.

RESULTS

Characterization of the 80 SSR primers

Microsatellite loci were isolated from the marama bean germplasm using the modified FIASCO enrichment technique and used to design 80 microsatellite primers based on perfect microsatellites. The primers (Table 2) were screened using the Omitara sub-population. Microsatellite loci were described (Table 3) as: Group1-Monomorphic (for example *MARA 004);* Group 2-Polymorphic in typical SSR type pattern (for example *MARA 001)*; Group 3-Polymorphic but not SSR type pattern (for example *MARA 045)*; Group 4-Not amplifying some individuals or poor quality amplification products (for example *MARA 067).*

Of the 80 primers screened, 76% were able to detect polymorphism (Group 2 and Group 3) yet 21% of them gave monomorphic bands (Group 1). The remaining 2 primers out of the 80 primers did not give clearly scorable amplifications or they gave no product at all. Four primer pairs (MARA 001, MARA 065, MARA 068 and MARA 077) out of the 80 primer pairs screened that gave reproducible polymorphic patterns were used for analysis of genetic variation of marama bean.

Characterization of individuals from 11 populations using 4 primer pairs

Primer pairs: MARA 001, MARA 065, MARA 068 and MARA 077 were used to amplify and detect polymer-phism in all 11 populations of the Namibian germplasm. The properties of the four primer pairs used are given in Table 4. Genetic variation in each of the populations was

Table 2. The 80 microsatellite loci and primers designed to screen for polymorphic microsatellite loci in this study.

Marama microsatellites designed and screened for polymorphism (80)						
Primer	**Left primer (L)**	**Right primer (R)**	**SSR**	**Repeats**	**Annealing (L/R)**	**Expected product (bp)**
MARA001	GCACAACCAATTTCCTGCTT	TCCCTCACTGGCCTATATCC	gag	5	60.12/58.96	137
MARA002	CTCCCTCCTCCTCCTCGTAG	CGGGAGCAAATAGACCCTTT	acc	8	60.34/60.44	106
MARA003	TCTCACCGACCGGGTCTC	CCTCTATCCCGCTCCCTATC	ctc	5	62.30/60.02	160
MARA004	TGCAGGCTTCACCAGAGTAA	TCTAAGACTGCGCACACAGC	ga	5	59.59/60.36	170
MARA005	GCTATCCGAGGGAGGATCA	GTGTCTATGTGTGCGCGTGT	ag	6	60.13/60.82	128
MARA006	GCTATCCGAGGGAGGATCA	TCCCATTCAGCCATTTTAGG	tg	7	60.13/59.89	171
MARA007	TATCCGAGGGAGGATCATGT	TCACATCCTAAGACTCGAACTTCA	ac	6	59.32/60.29	150
MARA008	GCTGGTCCATGGCTTCAT	TTTGTAATCGGTTGACACTTTGA	tg	5	59.59/59.54	185
MARA009	GGGAGGATCAACCTCAACAA	TGTACAAAAAGCAGGCTCCA	gaa	5	59.90/59.46	216
MARA010	TGTGCTATCCGAGGGAGGAT	ACGTCGCGATTAAACAAACC	aag	7	61.92/60.00	152
MARA011	TGTCAACGCTTACGTTGGTC	TCATTTGAAACCCTTGTACTGC	tc	8	59.76/59.13	169
MARA012	ATATGGTGGCTCGTCGATGT	GCACATAATTCGAACAGAACACA	ag	5	60.37/60.05	163
MARA013	GCTTCTCGTACATGGGCTTT	GCATTTATCGCGAATACAGCA	tc	5	59.34/61.10	154
MARA014	GGTGGTGGTGTAGGAGGAGA	GACTTGAGTGCATGCCATTT	agg	5	59.96/58.73	167
MARA015	ACTCATCCCGCTCCTAAGGT	AAACAGGCTCGTATTTTATCTTCG	tg	5	60.10/60.05	204
MARA016	TTCAATTTTCTTCACCACAAACTC	ACAGGAAGGTCTTCCACAGC	ca	7	59.56/59.30	102
MARA017	ACCCTTGAATTGTGGTAGGG	ACTGTGCTATCCGAGGGAGA	ct	6	58.76/59.83	105
MARA018	ATTTTGGCTTTACCGCACAC	AGCACTCTCCAGCCTCTCAC	cttga	3	60.00/59.74	158
MARA019	CCGGAACAGGAGAAGCTATG	TCAACTTTTGCAATGAACGAA	ctt	4	59.83/59.33	161
MARA020	TGTCTTCCCCTCCTCTTCCT	TTGACACTTTGGGACTGCTG	cag	4	60.19/59.87	175
MARA021	GAGGGAGGATCACCACTCAG	TGGCCATCAATCATGTTACG	tgt	4	59.64/60.34	166
MARA022	CCCCTGTACCCAAGACTCTG	TCCATGAAGTCAGGAGAAGGA	tagc	3	59.57/59.79	171
MARA023	ATGGGGATACTCCCGAAACT	AATGGGAGCAAGAATTTCCA	aaag	3	59.65/59.50	250
MARA024	CCAAGAGTGGGGATGAAAGA	TTGGAATAGTTCCCCCTTCC	aga	4	60.04/60.12	227
MARA025	CACGTGGGTTGTACTTATCTGC	TAATGTGTTGAGCGCCGTAG	tcttc	3	59.56/59.90	160
MARA026	GCTGTTGGGAACCGTAGAAA	CCTATTGTCAGTGTAAGCAACCA	tc	4	60.11/59.21	208
MARA027	TTGTTCCAAACCACAGGTCA	TGGCCATCTCCCAATTTTAC	ca	4	59.98/59.76	194
MARA028	CTCCGCATCTGACTTCAAAA	CCTCCTCTCCCTGATTTTCC	aga	3	59.00/60.01	150
MARA 029	CCGAGGGAGTAGTGCTTCAT	CGCCACTTAGCATTTGCTTT	tg	4	59.31/60.40	155
MARA030	GAGCCAAAGCCATGATCCTA	CCCATGTTGTATATTCGTGGAA	caa	3	60.18/59.59	178
MARA031	CTCAGCACTCTCCAGCCTCT	CCGAGGGAGGATCATTAACA	gga	7	59.88/59.89	126
MARA032	AGACGCACTCCCTCTCACC	GCTATCCGAGGGAGGATCA	aca	5	60.41/60.13	151
MARA033	GCACTCAGGCAACTGTGCTA	AGCACTCTCCAGCCTCTCAC	aac	7	60.21/59.74	132
MARA034	CTCAGCACTCTCCAGCCTCT	AGGGAGGATCACCTCCAAAC	gag	8	59.88/60.31	168

Table 2. Contd.

MARA035	GACGCACTCAGCAACTCTCC	TCCAGCCTCTCACCGATTAC	acc	8	61.17/60.22	152
MARA036	GACGCACTCAGGCAACTGT	CCGAGGGAGGATCAAAGAAT	gga	11	60.05/60.40	180
MARA037	GGGAGGATCAATCTTCACCA	TCCGAGAGAAGAGGAGGAAA	gagt	5	59.86/59.09	165
MARA038	TGTTGATGAACTAGTGCTAGTGGT	AGCACTCTCCAGCCTCTCA	tgg	7	58.02/58.79	126
MARA039	TCATTAAAGGGCTCCATTGC	ATGCCCAAAATCACCAACAT	aga	7	60.04/60.06	176
MARA040	GACGCACTCAGGCAACTGT	CTGGCCTATATCCCCTCCTC	gga	8	60.05/59.88	192
MARA041	AGACGCACTCAGCACTCTCC	GCTATCCGAGGGAGGATCAC	gga	7	60.77/60.96	150
MARA042	CAAATAGCCAAAGCCCGTTA	ACTCTCAAACCGTGGCACAT	agg	7	60.09/60.58	184
MARA043	TGTTGATGAACTAGTGCTAGTGGT	AGCACTCTCCAGCCTCTCA	tgg	7	58.02/58.79	126
MARA044	AGACGCACTCAGCATTCTCC	GGTCTCGTCTTCCCCTTCAT	gag	8	60.56/60.46	156
MARA045	GACGCACTCAGGCAACTGT	CTGGCCTATATCCCCTCCTC	gga	8	60.05/59.88	192
MARA046	GCACTCAGGCAACTGTGCTA	TGGCTGGCACTCTGATTAAG	cta	10	60.21/59.02	169
MARA047	GCACTCAGGCAACTGTGCTA	TGACTAGTCCCCGTGATGGT	caa	7	60.21/60.39	188
MARA048	AGACGCACTCCACCACTGTA	TGCTGAAACCGTGAGAGAGA	ct	12	59.34/59.70	212
MARA049	GCACTCAGGCAACTGTGCTA	GGCGAACTAGTGCTATCGAG	ct	13	60.21/57.75	183
MARA050	AGACGCACTCAGCACTCTCC	TGTGCTATCCGAGGGAGGAT	cac	7	60.77/61.92	116
MARA051	GCACTCAGGCAACTGTGCTA	AGCCTCTCACCGATTACTGC	ca	15	60.21/59.46	139
MARA052	GCACTCAGGCAACTGTGCTA	CACGCCTCTCACAAGAAACA	ct	14	60.21/60.02	249
MARA053	CTCAGCACTCTCCAGCCTCT	CCCTCATCTCCCTTTCCTTC	gga	9	59.88/60.01	238
MARA054	GCACTCAGGCAACTGTGCTA	AGCACTCTCCAGCCTCTCAC	gtt	8	60.21/59.74	159
MARA055	GACGCACTCAGCAACTCTCC	TCCAGCCTCTCACCGATTAC	acc	8	61.17/60.22	150
MARA056	AGACGCACTCAGGCAACTGT	ATCCGAGGGAGGATCATTAAA	gga	8	61.07/59.75	150
MARA057	GACGCACTCAGGCAACTGT	TGAAGATCCTCCCTCGGATA	gga	11	60.05/59.58	171
MARA058	GCACTCAGGCAACTGTGCTA	ACGACGAACGTAGTCGTCTC	ca	20	60.21/57.96	246
MARA059	GACGCACTCCTGTGCTATCC	ACGTCGCGATTAAACAAACC	aag	7	60.83/60.00	162
MARA060	CTCAGCACTCTCCAGCCTCT	CCTTCGTGTTTTACAGTTGTCG	gtg	11	59.88/59.71	179
MARA061	GAGGGAGGATCAAGGGACAC	AGCACTCTCCAGCCTCTCAC	ctc	7	60.86/59.74	183
MARA062	GACGCACTCCTGTGCTATCC	TGCGCAAGGACAATGATTAC	aag	7	60.83/59.69	202
MARA063	GTGCAAGACCCGTTTAGGAA	AGGACGAACACGTGCGTATC	ct	9	60.11/61.13	185
MARA064	GGAGGAGGAGGAGGAGTTTG	GAGGATCCACTCCCTCACTG	gag	5	60.19/59.64	192
MARA065	TGGTGGTAGGGTGGTGGTAT	CCACTTTTCACAGGCAAACA	ttc	6	59.97/59.73	191
MARA066	GCACTCAGGCAACTGTGCTA	GCTATCCGAGGGAGAGAGGA	cct	5	60.21/60.83	161
MARA067	AGACGCACTCAGCCTCTCAC	CCTCTATCCCGCTCCCTATC	ctc	5	60.77/60.02	174
MARA068	GGAGGAGGAGGAGGAGTTTG	GAGGATCCACTCCCTCACTG	gag	5	60.19/59.64	192
MARA069	GGGAGGATCAACCTCAACAA	TGTACAAAAAGCAGGCTCCA	gaa	5	59.90/59.46	216
MARA070	GACGCACTCAGGCAACTGT	GGGAGGATCACTTCCACTCTC	gga	5	60.05/60.07	168

Table 2. Contd.

MARA071	CTCAGCACTCTCCAGCCTCT	CCTGTTGGGGAGTTGTTGTT	cac	6	59.88/59.86	202
MARA072	CTCAGCACTCTCCAGCCTCT	GATTGCTGTTGTTGGCAGTG	caa	6	59.88/60.31	225
MARA073	AGCACTCTCCAGCCTCTCAC	ATGTTGAGGCAGAGGAGGAA	cat	5	59.74/59.80	161
MARA074	CCAGCCTCTCAACCGATTAC	AGGCACAGCCCTAGACTCCT	aag	6	59.69/60.41	250
MARA075	GGTGGTGGTGTAGGAGGAGA	TCCAGCCTCTCACCGATTAC	agg	5	59.96/60.22	202
MARA076	ATTTTTGCATCAGCAACAGC	ATCCGAGGGAGGATCCTATG	aca	5	58.92/60.25	193
MARA077	CTCAGCACTCTCCAGCCTCT	GGGTTGGTTGAAGAGGGAGT	aag	5	59.88/60.35	197
MARA078	GCACTCAGGCAACTGTGCTA	GTTCACCATCCCCTCTCTGA	gct	5	60.21/60.05	223
MARA079	GACGCACTCCAACTGTGCTA	TCATTTGAAACCCTTGTACTGC	tc	8	60.06/59.13	238
MARA080	ACCGACCGAGAGAATGAAGA	GAGTCCTCAACAGGGAGCTG	atc	6	59.80/59.99	153

calculated as heterozygosity for the 2 microsatellite loci MARA 001 and MARA 068 as these 2 loci gave the characteristic diploid type profile enabling calculation of heterozygosity (Figures 1 and 2). Out of 20 individuals from Otjovanatje amplified with primer pair MARA001, ten (50%) were heterozygous (Figure 1). Comparatively, eleven individuals (55%) out of 20 individuals from Otjovanatje amplified using primer pair MARA068 were heterozygous (Figure 2). These two primer pairs gave between one and two bands (alleles) with dominant upper bands in all 11 populations. The calculation of heterozygosity for the two microsatellite loci is tabulated in Table 5.

Analysis of SSR amplicons of primer pair (MARA065)

Primer pair "MARA065" gave many separation. This profile pattern was observed in Harnas as well as the other 10 populations amplified using the same primer pair. Individual 1,2,10 and 19 from Harnas gave three distinctive bands, while individual 20, 22 and bands with minimum 25, two bands each. Other individuals such as 4, 6 and 12 gave bands that cannot be clearly distinguished (Figure 3).

Analysis of SSR amplicons of primer pair "MARA077"

Amplification of all 11 populations using primer pair "MARA077" gave polymorphic bands (alleles) which ranged from one to four bands among individuals of marama bean. The amplicons obtained with primer pair MARA 077 shows smears together with bands for certain individuals. For instance, in individuals 1, 4 6, 9, 15 and 21 smears together with associated bands were observed (Figure 4).

DISCUSSION AND CONCLUSION

The isolation and use of microsatellite markers for genetic variation analysis in *T. esculentum* in this study was successful. Polymorphisms for both length band intensities were observed. From the 80 microsatellites primers developed, 5% of the SSR's were used for genetic variation analysis and these SSR primers have proved that microsatellites are useful and informative markers for assessing intra-specific and inter-specific variability of marama bean populations as in other legumes like soybean (Rongwen et al., 1995). The information reported here is the first for the use of perfect microsatellites whose primers were designed for genetic variation analysis.

Microsatellite markers have been used to assess genetic diversity in large numbers of cultivars in legumes like soybean (Rongwen et al., 1995) and the number of alleles amplified was 11 to 26 (Akkaya et al., 1992). In the present study one to six alleles per primer pair were amplified from the 332 marama bean individuals at the four polymorphic loci. Heterozygosity was used as an estimate of variability within and between populations of marama bean in the Namibian germplasm and ranged from 0.30 to 0.74. It is a good measure of variation because it estimates the probability that two alleles taken at random from a population are different (Mason et al., 2005). Some of the populations had low genetic variation while others had high genetic variation. This suggests genetic variation within as well as between populations of marama bean is high.

Table 3. Characterization of 80 microsatellite primers screened.

Number of primers screened	Number of Group 1 primers	Number of Group 2 primers
80	17	17
	Number of group 3 primers	**Number of group 4 primers**
	44	2

In the group 2 type of loci, usually 2 bands for any SSR were observed suggesting a single copy region with 2 alternative forms (Figure 1). The distribution of alleles within populations was not as expected in random outcrossing populations that started out with equal numbers of the 2 forms of the alleles at each locus. The founding population in such cases could have been skewed towards the presence of a particular allele that led

Table 4. List of selected primer pairs used to detect polymorphism and for the analysis of genetic variation in 11 marama populations of the Namibian germplasm.

Primer	Sequence 5'→ 3'	Repeat	Expected PCR product size (bp)
MARA001	L - GCACAACCAATTTCCTGCTT R – TCCCTCACTGGCCTATATCC	$(gag)_5$	137
MARA065	L - TGGTGGTAGGGTGGTGGTAT R – CCACTTTTCACAGGCAAACA	$(ttc)_6$	191
MARA068	L - GGAGGAGGAGGAGGATTTG R – GAGGATCCACTCCCTCACTG	$(gag)_5$	192
MARA077	L- CTCAGCACTCTCCAGCCTCT R - GGGTTGGTTGAAGAGGGAGT	$(aag)_5$	197

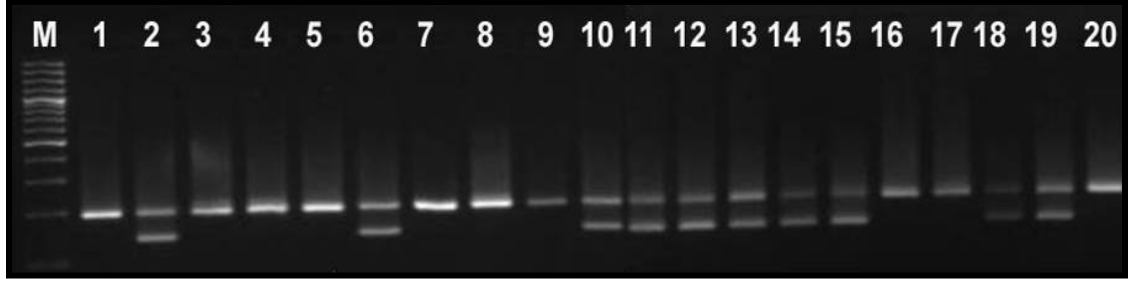

Figure 1. A 2.5 % agarose gel electrophoresis of amplification products 20 marama bean individuals from Otjovanatje using primer pair MARA001, M indicates the DNA molecular size marker (O'gene ruler 100 bp).

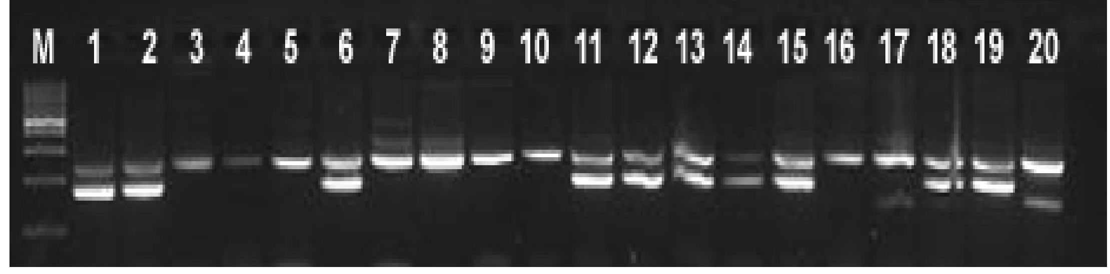

Figure 2. A 2.5% agarose gel electrophoresis of amplification products 20 marama bean individuals from Otjovanatje using primer pair MARA068 , M indicates the DNA molecular size marker (O'gene ruler 100 bp).

to a founder effect or these observed dominant type bands could be linked to a trait that is advantageous in the environment it is occurring in so the allele type has an increased frequency. Furthermore, in some cases observed bands were found to be either more than 30 bp above or below the expected SSR band size. It is not yet known whether such observed patterns were caused by SSRs or by insertions that have nothing to do with SSRs. Experiments to investigate this question through sequencing amplicons need to be initiated.

Microsatellite primers are now available for use as markers in the breeding of marama bean and in conservation efforts. It is desirable to isolate and characterize more DNA markers in marama bean for, more productive genetic studies such as genetic mapping, molecular marker assisted selection and gene discovery. Therefore the development of microsatellite markers for marama bean holds a promise for such studies. The primers can also be tested and used across species amplification of closely related legume species.

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Table 5. Heterozygosity and average heterozygosity at two loci (*MARA 001* and *MARA 068*) in populations of the Namibian marama bean germplasm as determined by electrophoresis.

Population	Number of Individuals			Heterozygosity		Average heterozygosity at two loci (MARA 001 and MARA 068)
	Heterozygotes (MARA 001)	Heterozygotes(MARA 068)	Total	Locus MARA 001	Locus MARA 068	
Omitara	8	11	19	0.42	0.58	0.50
Otjovanatje	10	11	20	0.50	0.55	0.53
Sandveld	9	7	21	0.43	0.33	0.38
Harnas	10	12	25	0.40	0.48	0.44
Ozondema	12	12	26	0.46	0.46	0.46
Epukiro/Post 3	13	16	30	0.43	0.53	0.48
Omipanda	19	27	31	0.61	0.87	0.74
Osire	14	20	40	0.35	0.50	0.43
Ombujondjou	17	37	40	0.43	0.93	0.68
Otjiwarongo	16	37	40	0.40	0.93	0.67
Okomumbonde	10	14	40	0.25	0.35	0.30
	Average			0.43	0.51	0.51

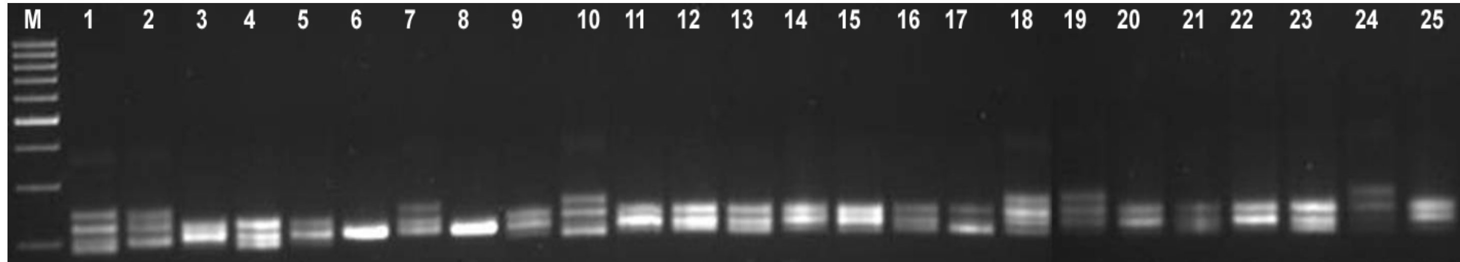

Figure 3. Amplification of individuals from Harnas obtained by the primer pair (MARA065) and visualized on a 2% agarose gel, M indicates the DNA molecular size marker (O'gene ruler 100 bp).

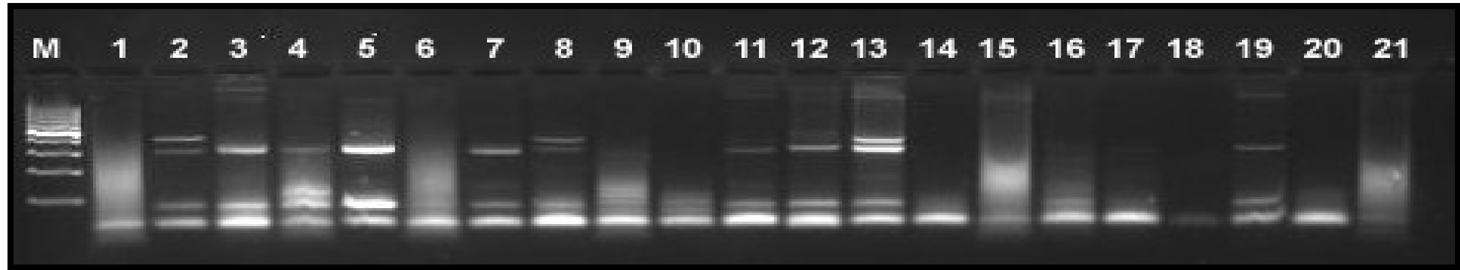

Figure 4. Amplification of individuals from sandveld obtained by the primer pair (MARA077) and visualized on a 2% agarose gel, M indicates the DNA molecular size marker (O'gene ruler 100 bp).

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