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DEVELOPMENT AND USE OF MICROSATELLITE MARKERS IN MARAMA BEAN

M. TAKUNDWA, E. NEPOLO, KHOLA MOGOTSI[1], M.A. KANDAWA-SCHULZ[2], A.C. CULLIS[3], K. KUNERT[4], J.J. JACKSON-MALETE[5], L. CHIWONA-KARLTUN[6] and P.M. CHIMWAMUROMBE
Department of Biological Sciences, University of Namibia, P. Bag 13301, Windhoek, Namibia
[1]Department of Plant Production, Botswana College of Agriculture, Gaborone, Botswana
[2]Department of Chemistry and BioChemistry, University of Namibia, P. Bag 13301, Windhoek, Namibia
[3]Case Western Reserve University, Department of Biology, Cleveland, Ohio, USA
[4]Department of Plant Science, University of Pretoria, 0001 South Africa
[5]Centre for Scientific Research, Indigenous Knowledge and Innovation (CESRIKI), University of Botswana, Gaborone, Botswana
[6]Department of Rural and Urban Development, Swedish University of Agricultural Sciences, Uppsala, Sweden
Corresponding author's email address: pchimwa@unam.na

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ABSTRACT

Marama bean [*Tylosema esculentum* (Burchell) Schreiber] occurs naturally in arid parts of southern Africa. Due to the high nutrient value of the seeds and tubers; richness in protein, oil and starch; it is a potential crop for arid areas where few conventional crops can survive. Microsatellites are becoming the molecular marker system of choice because they are multiallelic and generally more informative. Recently, the development of SSR enrichment techniques has increased the efficiency of SSR characterisation in new species. The aim of the study was to develop SSR's for detection of polymorphisms in Marama bean. The microsatellite regions of the genome were the main focus for potential to be used in Marama bean genetic diversity studies. Microsatellite loci were isolated from the Marama bean germplasm using a modified FIASCO enrichment technique. Nine Marama bean microsatellite libraries, enriched for $(AAG)_7$, $(GTT)_7$, $(AGG)_7$, $(GAG)_7$, $(CA)_{10}$, $(CT)_{10}$, $(TCC)_7$, $(CA)_{15}$ and $(CAC)_7$, were created. Of the 80 primers designed, 76% were able to detect polymorphism. Four of the SSR's were used for a genetic variation analysis and have proved to be useful and informative for genetic diversity studies.

Key Words: FIASCO, polymorphism, SSR, *Tylosema esculentum*

RÉSUMÉ

Le haricot Marama [*Tylosema esculentum* (Burchell) Schreiber] est produit naturellement en milieux arides de l'Afrique du Sud. Etant donné la valeur nutritionnelle des ses graines, carottes, sa richesse en protein, en huiles et amidon, le haricot Marama est considéré comme une culture potentielle pour des milieux arides où peu de cultures conventionnelles peuvent survivre. Dans ces conditions, les microsatellites deviennent de plus en plus un système moléculaire de choix parce que elles sont multialléliques et généralement plus informatives. Récemment, le développement des techniques d'enrichissement SSR a augmenté l'efficacité de la caractérisation de SSR dans de nouvelles espèces. L'objet de cette étude était de développer SSR pour la détection du polymorphisme dans le haricot Marama. Les régions microsatellitaires du génome étaient le principal point d'intérêt pour des études de la diversité génétique du haricot Marama. Les microsatellites loci étaient isolés du germoplasme du haricot Marama en en utilisant une technique d'enrichissement FIASCO modifié. Neuf librairies microsatellitaires du haricot Marama, enrichies pour $(AAG)_7$, $(GTT)_7$, $(AGG)_7$, $(GAG)_7$, $(CA)_{10}$, $(CT)_{10}$, $(TCC)_7$, $(CA)_{15}$ et $(CAC)_7$, étaient créées. Parmi les 80 désignées, 76% étaient capables de détecter le polymorphisme. Quatre des SSR

étaient utilisées pour une analyse de la variation génétique et ont prouvé être plus utiles et informatives pour des études de la diversité génétique.

Mots Clés: FIASCO, polymorphisme, SSR, *Tylosema esculentum*

INTRODUCTION

Marama bean (*Tylosema esculentum)* is widespread, with large populations in Botswana (around the central Kgalagadi), eastern parts of Namibia, and smaller populations in the provinces of Limpopo, north-west and Gauteng of South Africa (Castro *et al.*, 2005). It grows at altitudes of between 1000 and 1500 m above sea level, with 300 to 700 mm rainfall. It prefers minimum temperature above 15 ^{o}C and a maximum of approximately 33°C (Müseler and Schönfeldt, 2006). The plant is adapted to the harsh conditions of Botswana and Namibia, which are characterised by low rainfall and nutritionally poor soils (Hartley *et al*., 2002).

The seeds of Marama have traditionally been an important source of protein for the inhabitants of the Kalahari region; however, the plant has never been cultivated by these people but it is harvested from extensive populations which are scattered throughout the natural range (Halloran and Monaghan, 1996; Amarteifio and Moholo, 1998). Due to the potential of this plant as an arid agricultural crop, there is increasing interest in its possible domestication. Despite its traditional use as a food source in Botswana and Namibia, little is known about the germplasm diversity, genomic variability and relationships between the different ecotypes (National Academy of Sciences, 1979).

Marama beans' good quality protein compares well with other protein foods, including soybean (Bower *et al*., 1988). The Marama beans have a protein content of about 36% and like other legumes, are rich in lysine, but low in methionine. They also contain some proteins with pharmaceutical potential such as protease inhibitors (Elfant *et al*., 1985). Its oil is rich in mono- and di-unsaturated fatty acids, and contains no cholesterol (Mmnonatau, 2005). It is also a good source of calcium, iron, zinc, phosphate, magnesium, the B complex vitamins and folate (Hartley *et al.,* 2002).

Information on genetic variation is a prerequisite for the genetic improvement of any plant species for effective use of germplasm in breeding and for conservation. The natural populations of Marama bean are under pressure from both grazing and human exploitation. Therefore, a detailed knowledge of the genetic structure of these populations is important for developing a strategy for conserving and developing the remaining wild germplasm (Naomab, 2004).

Microsatellites or simple sequence repeats (SSR's) consists of direct tandem repeats of short (2-6) nucleotide motifs according to Tautz *et al.* (1986). For a wide range of genetic and population studies, SSR markers are a suitable choice based on cost, labour and genetic informativeness. Microsatellites are commonly identified through: (i) Screening of small-insert or microsatellite enriched genomic libraries by hybridisation, with oligonucleotide primers followed by sequencing and/or (ii) Searching DNA sequence databases. Database searching is only suitable for the development of SSR markers in plant species well represented in databases (Sharopova *et al*., 2002).

Microsatellites have become one of the most widely used molecular techniques for genetic studies in recent years (Sharopova *et al*., 2002). Enriching the AFLP or specific adaptor-amplified DNA fragments is a simple and efficient approach for SSR isolation and has been successfully applied to a number of plant genomes. Microsatellites are typically neutral, co-dominant and are used as molecular markers which have wide ranging applications in the field of genetics, including kinship and population studies (Brown, 1998).

Several alternative strategies have been devised in order to reduce the time invested in microsatellite isolation and to significantly increase yield. The most recently proposed Fast Isolation by AFLP of Sequences Containing Repeats (FIASCO) technique (Zane *et al*., 2002) was used in this study with some modifications.

The method is fast and simple and many unnecessary steps in previously proposed methods have been eliminated. The protocol relies on the extremely efficient digestion ligation reaction of the AFLP procedure (Zane *et al*., 2002).

The objective of this study was to identify and isolate microsatellites from Marama bean and design primers based on the microsatellites that can be used as a possible molecular tool for germplasm characterisation of the Marama populations.

MATERIALS AND METHODS

Sampling sites and strategy. The sampling area covered a wide geographic range of about 166,250 Km^2 in the north-eastern regions of Namibia. The sampling sites were near or around villages or farming settlements.

Leaf material was collected from the Omaheke, Khomas and Otjozondjupa regions of Namibia where *Tylosema esculentum* is known to occur. The sites sampled were Otjozondjupa: Ozondema, Ombujondjou, Osire, Otjiwarongo; Khomas: Omitara; and Omaheke: Sandveld, Otjovanatje, Omipanda, Post 3, Harnas, Okomumbonde. At least 20 individual plants within each sub-population were sampled for leaf material. Plants that were at least 10 metres apart were sampled to avoid multiple sampling, and plants with as many phenotypic differences visible to the eye were selected. The sampling areas at each locality were about 1 Km^2. The phenotypic differences included were, overall size of the plant, length of the creepers, leaf size, internode length and number of seeds per pod. A total of 361 *T. esculentum* individuals, representing 11 populations described in Nepolo *et al.* (2009), were sampled in the Namibian germplasm (Table 1) and a twelfth site, Pretoria, was also included. Leaf material was stored at -20 °C in the laboratory following field collections.

DNA extraction. DNA was extracted from each plant samples using the DNeasy mini-protocol for purification of genomic DNA from plant tissue. The manufacturer's protocol was followed to obtain DNA from the leaves (Qiagen, 2006). The DNA was stored in clearly labelled microcentrifuge tubes at -20 °C. DNA with a concentration of 25- 250 $\mu g\ \mu l^{-1}$ was collected. The concentration was determined on a 1% agarose gel stained with ethidium bromide using known molecular weight standards and also using a spectrophotometer. DNA samples were then diluted accordingly to get equal concentrations of 10 ng μl^{-1}.

Microsatellite isolation. The genomic DNA extracted was enriched for microsatellites by a modified FIASCO technique (Zane *et al*., 2002) without cloning as described below. Nine Marama bean microsatellite libraries enriched for $(AAG)_7$, $(GTT)_7$, $(AGG)_7$, $(GAG)_7$, $(CA)_{10}$, $(CT)_{10}$, $(TCC)_7$,

TABLE 1. Accessions used in this study and their places of origin

Site	GPS	Number of accessions	Country
Omipanda (OMP)	S21 19.355 E20 04.553	31	Namibia
Ozondema (OZO)	S20 15.921 E18 02.490	26	Namibia
Omitara (OMI)	S22 21.596 E18 02.476	19	Namibia
Osire (OSI)	S21 02.031 E17 21.244	60	Namibia
Harnas (HAR)	S21 47.705 E19 19.921	25	Namibia
Sandveld (SAN)	S22 01.751 E19 08.009	21	Namibia
Otjovanatje (OTJ)	S20 27.393 E16 39.443	20	Namibia
Otjiwarongo (OTR)	S20 46.092 E16 65.123	40	Namibia
Ombujondjou (OMB)	S20 18.600 E17 58.525	40	Namibia
Okomumbonde (OKO)	S20 57.000 E18 55.000	44	Namibia
Epukiro (EPK)	S21 39.642 E19 25.092	30	Namibia
Pretoria farm (UP) , (PTA)	S25 45.490 E28 11.368	5	South Africa

GPS = Geo-positioning system

$(CA)_{15}$ and $(CAC)_7$ were created using the modified FIASCO technique described in the following section.

Restriction enzyme digests and purification. One microgram of DNA was digested with the enzymes, *Msp* 1, *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. The ligation was performed with 500 ng of the restriction-digested DNA, 1 µl of 12 mer adapter, 1µl 24 mer adapter and 3 µl ligation buffer in a final volume of 28 µl. The reaction mixture was heated to 72 °C for 3 min, then cooled by one degree per minute to 4 °C. Two micro-litres of µl ligase were added and the reaction incubated at 4 °C for 16 hours. The 12 bp adaptors were removed by heating to 72 °C for five minutes to melt off the 12 mer, followed by purification using the QIAquick PCR purification kit. The ligated DNA was amplified with PCR by combining 5 µl PCR buffer, 5 µl (20 mM) $MgCl_2$, 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 °C for 5 min; 5 units of *Taq* polymerase enzyme was added and further incubated for 5 min at 72 °C. The DNA was amplified for 20 cycles of 95 °C for 30 seconds, and 72 °C for 90 seconds, with a final hold at 72 °C for five minutes. 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. This was 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^{-1}) was added to minimise non-specific binding. The annealed DNA mixture was then added to 1 mg of magnetic beads and incubated for 30 minutes at room temperature, allowing the streptavidin beads to join with the biotinlyated primers. Five washes with TEN100 (Tris/EDTA/NaCl) and 5 washes with SSC 0.2X SDS 0.1% were performed to remove non-specific DNA. Then, two denaturation steps were performed to separate DNA containing SSRs from the beads. The first was done by adding 50 ìl of TE (Tris-HCl 10 mM, EDTA 1 mM) and heating to 95 °C for 5 minutes. The remaining solution was separated magnetically and stored. The second denaturation used 12 µl 0.5N NaOH, which was neutralised with 12 µl 0.5N 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. Electrophoresis was then performed on each of the denaturation products with a 1.5% agarose gel.

Sequencing. All the microsatellite-enriched genomic DNA pools were combined and 5 µg of the extracted microsatellites were sequenced and analysed with the Roche 454 GS-FLX platform at Inqaba Biotech (Pretoria, 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.

Microsatellite discovery and primer design. Primers were then developed around the SSR sites, identified with the Simple Sequence Repeat Identification Tool (SSRIT) software. Primer 3 was used to design the microsatellite primers. The primers were synthesised by Inqaba Biotech. SSRIT, an SSR finding programme (http://www.gramene.org/db/markers/ssrtool), was used to identify the microsatellites in the contig files obtained from 454 sequencing. The search was made to include SSR's up to pentamers with a minimum of 5 repeats required for each type of microsatellite picked out. Sequences containing perfect microsatellites were used to design PCR

primers complementary to the flanking region of the microsatellites. Eighty microsatellite primer pairs were designed from the sequence data of the libraries using Primer 3 software available online (http://frodo.wi.mit.edu/primer3). The primers were synthesised by Inqaba Biotechnology laboratory in South Africa and 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 used to screen individual Marama DNAs. Two sets of screening populations were used. The screening was accomplished by using pooled DNA from the 12 locations (11 Namibian (Table 1), plus Pretoria, South Africa) as template in one population. The 19 individual plants from the Omitara sub-population made up the second screening population used. 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 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 minutes, followed by 35 cycles of denaturation at 95 °C for 30 sec, an annealing at between 55 °C and 65 °C (primer sequence dependent) for 60 sec and an extension at 72 °C for 2 minutes, a final extension at 72 °C for 5 minutes and then held at 4 °C. Agarose gel (2.5%) visualisation 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 was screened and described as polymorphic, monomorphic or unable to amplify. The sequences from which the monomorphic primers were derived from were searched for using the BLAST tool at NCBI to see if there was any sequence homology with any genes with known functions.

SSR data analysis. The value discriminatory power (D_L) was used to compare the efficiency of the microsatellites to differentiate among individuals using the Omitara sub-population amplification profiles. The D_L value represents the probability that two randomly chosen individuals show different allelic patterns at the same microsatellite locus and, thus, are distinguishable from one another. That is, if p_i is the proportion of the population carrying the *i*th banding patterns at the *j*th primer and if p_i were calculated for each pattern generated by the primer (Tessier *et al.*, 1999), then $D_L = 1 - \Sigma p_i^2$. This is an extension of the polymorphism information content (PIC) (Anderson *et al.*, 1993), available from the frequencies of the different banding patterns (or genotypes) generated by a primer. The discriminatory power approach was applied here as was done for the most important grain legume for human consumption in the world, *Phaseolus vulgaris* (Gaitán-Solis *et al.*, 2002).

RESULTS

Microsatellite isolation and enrichment. The enrichment products obtained following the FIASCO protocol are shown in Figure 1. The products appeared as smears as they carried many different sized fragments enriched for the microsatellite repeats.

Microsatellite primer design. The output files from the primer design process are shown in Figure 2. Each of the contig or single read files that had microsatellites with sufficient flanking sequences for primer design on both the 5' and 3' ends were used to design primers.

Microsatellite primer screening (amplification profiles). A total of 80 primers were screened for polymorphism. The primers were then grouped according to the type of amplification patterns generated. The primers were screened using the panel of 12 ecotypes of Marama bean and the Omitara sub-population. Microsatellite loci were described (Figs. 3 - 6) as: Group1- Monomorphic (for example *MARA 004);* Group 2- Polymorphic in typical SSR type pattern (for example *MARA*

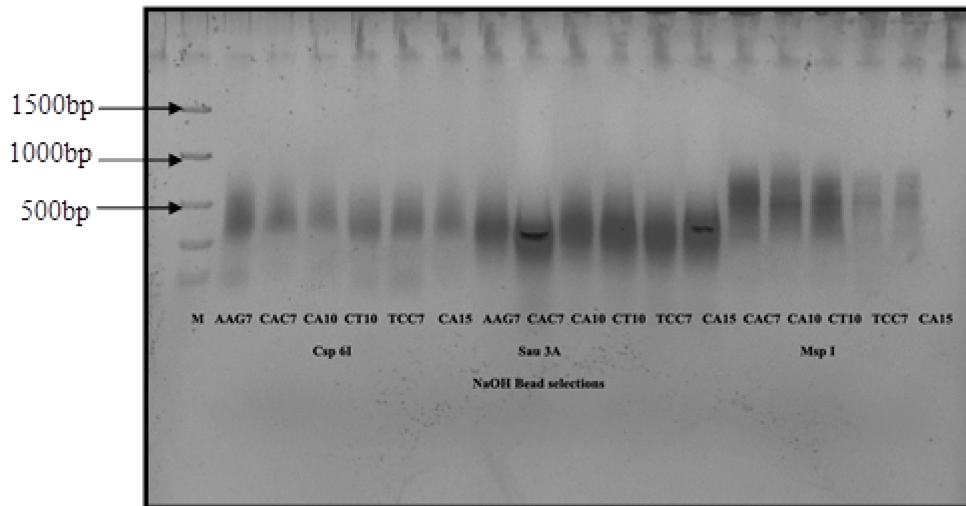

Figure 1. Agarose gel electrophoresis of microsatellite enriched DNA. A 100 bp ladder was used in lane M and the rest of the lanes contain enriched fragments carrying microsatellite DNA. The smears show the many different sized fragments present.

Figure 2. Primer 3 output for the design of primer pair MARA 011F and MARA011R to amplify the $(TC)_8$ microsatellite locus.

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)* (Figs. 3 - 6).

In the group of the 80 microsatellite primers screened, 16 belonged to Group 1, 17 were in Group 2, 44 were in Group 3 and 3 belonged to Group 4

SSR data analysis. Of the 80 primers screened (76%) were able to detect polymorphism (Groups 2 and 3) and 20% of them gave monomorphic bands (Group 1). The polymorphic primers can be useful in trait linkage for the Marama breeding program. The remaining 3 primers out of the 80 primers did not give clearly scorable amplifications or they gave no product at all.

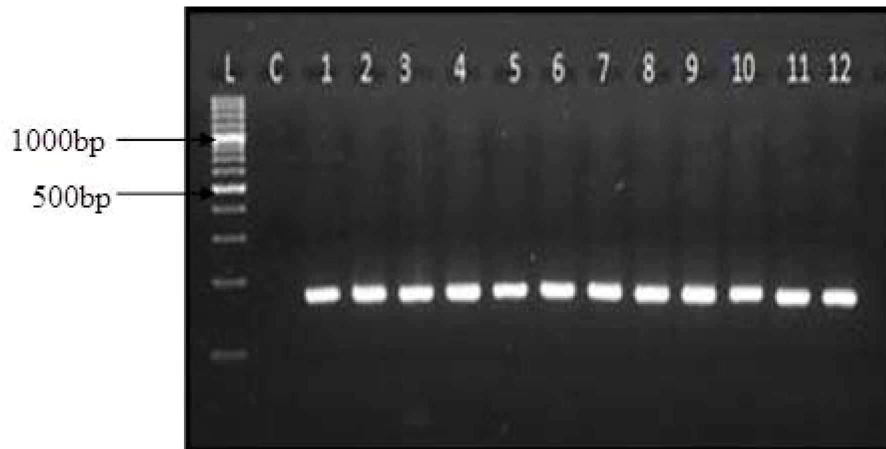

Figure 3. Primer MARA 004 a typical monomorphic (Group 1) microsatellite locus. Sample 1-12 are pooled DNA samples, lane L contains a 100 bp ladder and the gel was 2.5%. Electrophoresis was at 100 V for 1 hour.

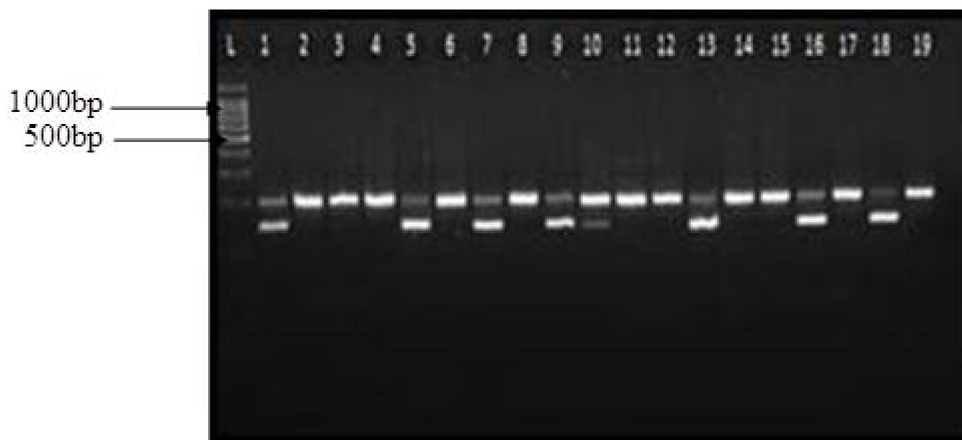

Figure 4. Primer MARA 001 a typical polymorphic (Group 2) microsatellite locus. Sample 1-19 are individuals from Omitara, lane L contains a 100 ladder and the gel was 2.5%. Electrophoresis was at 100 V for 1 hour.

The discriminatory power of each polymorphic primer D_L was calculated and ranged from 0.188 - 0.947 with an average D_L of 0.482. A primer with a D_L greater that 0.7 was considered to have a higher probability to be able to discriminate between two individuals. 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.

Some of the sequences contained microsatellite regions but the flanking sequences on either the right or left side or both were too short for primer design. All the primers designed were made to amplify perfect microsatellites that were 10 bases or longer. Of the microsatellites targeted, two were pentanucleotide repeats, three were tetranucleotide repeats and the remaining 75 microsatellites were dinucleotide and trinucleotide repeats. The longest microsatellite amplified was a $(CA)_{20}$ repeat amplified by primer MARA 058.

DISCUSSION

Microsatellites that can be used for germplasm characterisation in Marama been were isolated

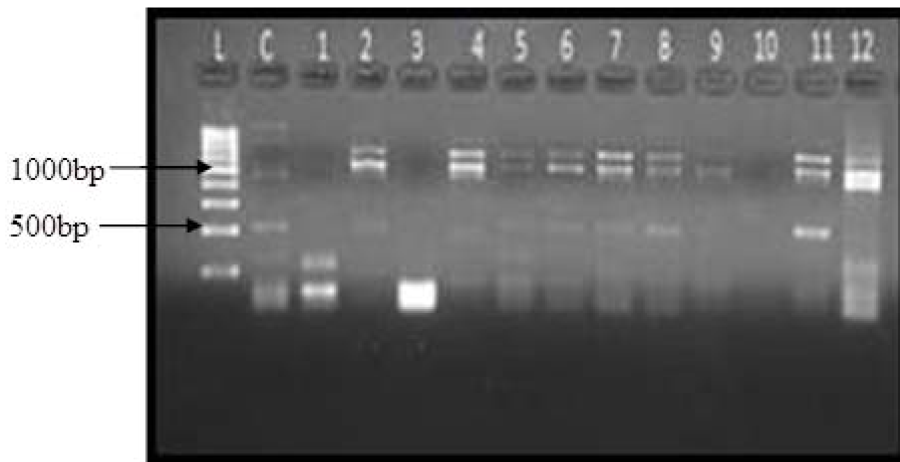

Figure 5. Primer MARA 045 a typical polymorphic (Group 3) microsatellite locus. Sample 1-12 are pooled DNA samples, lane L contains a 100 bp ladder and the gel was 2.5%. Electrophoresis was at 100 V for 1 hour.

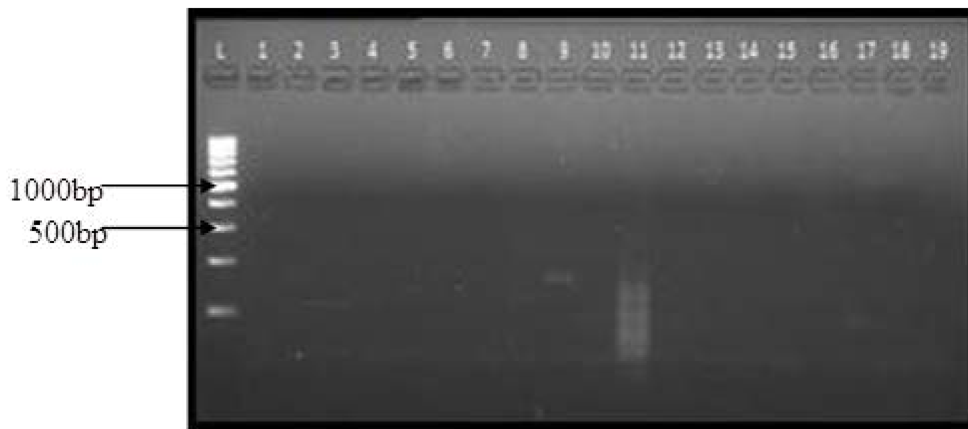

Figure 6. Primer MARA 067 a typical microsatellite locus that could not be scored or did not give any amplification. Sample 1-19 are individuals from Omitara, lane L contains a 100 bp ladder and the gel was 2.5%. Electrophoresis was at 100 V for 1 hour.

and described for the first time for the species. The products of amplification showed that a greater proportion of these microsatellites (76%) can be used as markers for genetic diversity studies as they were able to detect polymorphism in the germplasm collection used in this study. The microsatellites were also used for genetic variation analysis in *Tylosema esculentum* in a separate study. Out of the 80 microsatellites primers developed, 5% of the SSR's were used for genetic variation analysis (Nepolo, 2010) and these SSR primers have proved that microsatellites are useful and informative for assessing intra-specific and inter-specific variability of Marama bean. The microsatellites were able to detect between population variation better than the RAPD and AFLP markers used before.

Microsatellites have become one of the most widely used molecular markers for genetic studies in recent years. The FIASCO technique used was able to develop a number of polymorphic microsatellite primers. This further demonstrated that enriching the AFLP or specific adaptor-amplified DNA fragments is a simple and efficient approach to SSR isolation and has been successfully applied to a number of plant genomes. By employing modified procedures

from other plant species, corresponding isolating protocols were established in Marama bean for the development of microsatellites. The study also marked one of the first that employed Roche 454 technology for sequencing DNA libraries for microsatellites as was also done by Santana *et al.* (2009).

The discriminatory power of each polymorphic primer (D_L) was calculated in this study for *Tylosema esculentum* and ranged from 0.188 - 0.947 with an average D_L of 0.482. In the study of *Phaseolus vulgaris* microsatellites, this value ranged from 0.09 – 0.94 with an average of 0.73 (Gaitán-Solis *et al.*, 2002). The average in Marama was lower than that in common bean; however, 23 of the primers had a discriminatory power greater than 0.7. This was the first report of discriminatory power of microsatellite markers for Marama bean.

Compared to previous reports on peanut microsatellites (Hopkins *et al.*, 1999) for example, in which five polymorphic markers from 26 primers pairs (19%) were identified using library screening methods and a later study by He *et al.* (2003), where 19 of 56 primer pairs (34%) showed a polymorphism, the enrichment procedure used for Marama bean was more efficient in identifying SSR markers as 61 out of the 80 primer pairs (76%) showed polymorphism.

The molecular data gathered in this study based on microsatellite loci will subsequently need to be linked to phenotypic characters. The "phenotype", i.e. the organism defined by the characters made manifest, must be distinguished from the "genotype" or genetic constitution, which alone can transmit changes to the offspring. The best Marama bean would tentatively be one with a high number of seeds per pod or per plant, high oil content, high protein content, high tuber starch, early flowering and less shattering as these are the traits directly or indirectly linked to high yield and high nutritional value.

The polymorphic microsatellites identified through this study can potentially be applied to identify Marama microsatellite loci that affect phenotype in a selection and breeding programme. Some of the phenotypes observed in field collections were different internode lengths, germination times and leaf sizes which could directly or indirectly linked to yield traits in Marama bean. Plants showing differences in these traits would be selected in the field then screened with the microsatellite markers to see if for a particular trait a microsatellite marker could be linked with that trait by being able to distinguish individuals at the molecular level. Microsatellites have become one of the most widely used molecular markers for genetic studies in recent years (Rongwen *et al.*, 1995; George *et al.*, 2006) and they have proved useful in evaluating genetic variation in the Namibian Marama bean germplasm.

Microsatellites isolated from *Tylosema esculentum* and found to be polymorphic enabled genetic diversity studies to be carried out. The microsatellite primers developed through the use of the FIASCO technique were used to analyse the genetic variation of the selected populations of *Tylosema esculentum.* In addition several gaps in the knowledge of Marama bean germplasm in Namibia were filled. Before this study it was not known if microsatellites were abundant in the genome of Marama bean and if they could be able to detect any polymorphisms and be useful as molecular markers for Marama bean.

CONCLUSION

This is the first report of large scale molecular marker development for *Tylosema esculentum*. The molecular markers reported here are of particular value in the ongoing efforts to characterise the Marama bean germplasm. The development of the microsatellite markers is an important first step towards the development of a saturated genetic linkage map and the development of marker assisted selection programmes for traits of agronomic importance in Marama bean.

A high level of polymorphism exists in the Marama bean germplasm as demonstrated by 76% of the loci screened being polymorphic. The FIASCO technique is applicable to Marama bean and is efficient in producing many microsatellite enriched fragments that enabled the design of 80 microsatellite primers.

The microsatellite loci described will provide a pool of polymorphism useful for population studies, genetic mapping and possibly application in other *Tylosema* or *Fabaceae*. The

development of SSR markers linked to specific traits will facilitate the screening of Marama bean plants at early growth stages, thus accelerating selective breeding programmes.

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