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A review of geographical distribution of marama bean [*Tylosema esculentum* (Burchell) Schreiber] and genetic diversity in the Namibian germplasm

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Marama bean [*Tylosema esculentum* (Burchell) Schreiber] occurs naturally in the drier areas of Southern Africa, including Botswana and Namibia. The implementation of molecular breeding is important to advance the process of securing the world's food supply. The development of molecular markers is vital for mapping important traits that can then be followed in subsequent breeding programs. This study assessed the distribution of marama bean in Namibia and isolated microsatellite regions for microsatellite primer design for the purpose of determining genetic diversity and construction of molecular genetic maps for marama. This will greatly enhance the process of domesticating marama bean, currently a wild plant that is still picked wild and unsustainably. The geographical distribution was geo-referenced using Geographic Positioning System (GPS) points and microsatellites were isolated from the germplasm using a modified FIASCO technique. The study revealed widespread, but patchy distribution of marama bean in Namibia. Five Marama bean microsatellite enriched libraries were created. The initial results provided vital information for the ongoing marama bean conservation function and improvement program.

Key words: Marama bean, genetic diversity, microsatellite, FIASCO.

INTRODUCTION

Marama bean

Marama bean, [*Tylosema esculentum* (Burchell) Schreiber] (Family Fabaceae) is an underutilized drought-tolerant wild perennial legume which has not been brought into conventional crop system because it is not well understood scientifically. Currently this plant is not cultivated but harvested from wild growing plants. Unlike many legume species, such as cowpea, soybean and white clover that are cultivated for forage or as dietary supplements (Amarteifio and Moholo, 1998), there have not been any systematic studies to characterise marama and start a domestication program for this plant. Marama bean is a long lived, perennial species which generates

annually from a large underground tuber which is used to store water and can also be a food source. The above ground vegetation consists of numerous prostate vines which can go up to 6 m in length. The seed is an excellent source of good quality protein and compares well with other protein foods including soybeans (Bower et al., 1988; Mmnonatau, 2005). Its oil is rich in mono- and di-unsaturated fatty acids. It is also a good source of calcium, iron, zinc, phosphate, magnesium, the B complex vitamins and folate (Hartley et al., 2002; Bower et al., 1988).

Marama bean distribution

Marama bean (*Tylosema esculentum*) occurs naturally in the drier areas of Southern Africa, including Botswana and Namibia, where it is to a small extent harvested as a

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wild plant for human consumption (Amarteifio and Moholo, 1998). It is widespread in these areas, with large populations in Botswana (around the central Kgalagadi), Eastern parts of Namibia, while smaller populations are found in the South Africa provinces of Limpopo, North-West and Gauteng (Castro et al., 2005).

Value of the marama bean

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). This makes it a potential crop for semi-arid and arid agriculture. The seeds are roasted and eaten as a snack by the native Ovaherero people who dominate the Otjozondjupa and Omaheke regions of Namibia where several sub-populations of *Tylosema esculentum* are found. In their language, they refer to the plant as “Ovirema” and the seed that they roast as “Ombanui”. Due to the potential of this plant as an arid agricultural crop, there is increasing interest in its possible cultivation. 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, National Research Council 2006; Keegan and Van Staden, 1981; Hartley, 1997).

The application of genetics in conservation and domestication of potential crop plants

The conservation of plant genetic resources has, in recent years, attracted growing public and scientific interest and political support (Callow et al., 1997). New molecular biology techniques, including DNA marker development and second generation genome sequencing techniques, are being applied to solve the intricate problems that lie at the heart of today's concerns for poverty, nutrition and the environment (Callow et al., 1997). These techniques allow a better understanding of the composition and functioning of genomes, and to transfer useful genes among widely different groups of organisms. By such means, it is possible to more accurately tailor food crops to meet new pest and disease challenges and produce crops that can better withstand the rigor of stressful environments (Stephen, 2002).

Information on genetic variation is a prerequisite for the improvement of any plant species by breeding programs. The natural populations of marama bean are under pressure from both grazing and human exploitation of the seeds; therefore knowledge of the genetic structure of these populations is important for developing a strategy for conserving and developing the remaining wild germplasm (Naomab, 2004). The work that has already been initiated on physiological and agronomic aspects of marama bean needs to be underpinned by an under-

standing of the genetic diversity and genetic constitution of the species and mechanisms by which the plant can be improved through selection and breeding (Travlos et al., 2007).

Marama bean breeding program

A marama bean breeding program has been initiated with an introductory study on the genetic variability and distribution of the Namibian marama bean germplasm. This preliminary assessment will progress into a more detailed investigation of the genetic and associated phenotypic variation present in the Namibian marama bean germplasm. Furthermore, information of the geographical distribution and diversity of marama bean within Namibia is vital for conservation purposes. Effective conservation and use of plant genetic resources for domestication depends on identifying the extent of genetic variation present (Halloran and Monaghan, 1996) which is most effectively achieved through the use of appropriate markers and molecular technologies. One of the most powerful marker systems is that based on microsatellite variation. However, these regions need to be identified *de novo* for each species. Therefore microsatellite markers are being developed for the Namibian marama bean collection for use in both the assessment of the extent of genetic variation in this marama bean germplasm as well as a basis for subsequent molecular breeding programs.

Molecular markers

Markers based on polymorphic DNA fragments that are independent of the growing environment and can be unambiguously scored are one class of molecular markers that are extensively used in plant diversity analysis and as an aid in plant breeding through Marker Assisted selection (MAS) (Karp et al., 1996; Karp et al., 1997). Molecular markers allow the selection of desired traits based on genotype and can therefore accelerate plant breeding programs (Kölliker et al., 2001). Different techniques for identifying DNA markers include the analysis of Restriction Fragment Length Polymorphisms (RFLPs), Random Amplified Polymorphic DNAs (RAPDs), Amplified Fragment Length Polymorphism (AFLPs), microsatellites or Simple Sequence Repeats (SSRs) and Single Nucleotide Polymorphisms (SNPs). These marker systems differ in a variety of ways including the amount of time, money and labour needed to develop them; the number of genetic markers that can be detected throughout the genome and the amount of genetic variation found at each marker in a given population (Karp et al., 1996; Karp et al., 1997). The information provided by the markers for the breeder will vary depending on the type of marker system used. Each

one has its advantages and disadvantages for the breeder.

The AFLP and RAPD techniques used in previous marama bean projects have been unable to differentiate populations, that is although variation exists within marama, population differences are obscured by the within population variation (Naomab, 2004; Halloran and Monaghan, 1996). It is possible that microsatellite markers will be more effective in discriminating among populations and these will be developed.

Microsatellites

A microsatellite 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. The project therefore aims to isolate microsatellites from *Tylosema esculentum* using individuals from the Namibian germplasm in selected sites of the Otjozondjupa, Khomas and Omaheke regions of Namibia.

Microsatellites have been enthusiastically adopted in the past decade for linkage and population genetic studies because their high polymorphism. They are easy to detect with the Polymerase Chain Reaction (PCR) and a typical microsatellite marker has more variants than those from other marker systems (Dreher et al., 2000). In this study microsatellites will be isolated from the marama bean germplasm and an initial test for polymorphisms will be made using 50 primer pairs. The polymorphic primers obtained for the isolated microsatellites will then be used in diversity studies, germplasm characterization and genotype identification. The method used was a modification of the fast isolation by AFLP of sequences containing repeats (FIASCO) (Zane et al., 2002) to increase the number of possible restriction enzymes that can be used to make the initial amplicons.

METHODOLOGY

The distribution of marama bean in Namibia has been assessed and a series of microsatellite enriched libraries has been created from marama bean. These libraries are being sequenced and primers designed for those with the longest SSR regions. The preliminary identification and testing of these microsatellites indicated that they will be substantially polymorphic in marama. The potential establishment of this species as a crop and the risk of its over-exploitation, with the attendant loss of biodiversity (seed is gathered from the wild and widely consumed by the local people and range animals) make it imperative that the level of biodiversity in marama is detailed in order to move forward with a logical marama bean improvement and conservation program.

Marama bean distribution

The locations where *T. esculentum* was growing were georeferenc-

ed using the hand held Global Positioning System receiver (GPS). The recorded points were transformed into a database program (Map Source) and marked on the map to show the distribution of *T. esculentum* in Namibia.

Locations recorded were: Ozondema, Ombujondjou, Osire, and Otjiwarongo in Otjozondjupa region, Omitara in Khomas region, Sandveld, Otjovanatje, Omipanda, Post 3, Harnas, and Okomombonde in Omaheke region (Figure 1). Accessions to the total of 391 were collected from the sampled areas (Table 1). Marama beans are widely spread, but patchily distributed in the sampled areas. The largest marama bean distribution patches were observed at Omipanda, Otjovanatje, Post 3 and surrounding areas of Sandveld. Sparse distributions were observed at Omitara and Ozondema.

DNA extraction

DNA was extracted from each of the plant samples collected using the DNeasy mini protocol for purification of total DNA from plant tissue. DNA extractions from the different individual plant samples took place at the Molecular Biology Laboratory at the University of Namibia. Plant tissue was ground to a fine powder under liquid nitrogen using a mortar and pestle. The plant tissue powder and liquid nitrogen were transferred to a microcentrifuge tube and the liquid nitrogen allowed to evaporate. The manufacturer's protocol was followed to obtain DNA from the plant tissue (Qiagen, 2006). The DNA for each sample for each of the sub-populations was stored in clearly labelled microcentrifuge tubes in storage boxes arranged by the name of the sub populations. The boxes were kept in a freezer at -20°C.

Microsatellite isolation using the modified FIASCO technique

DNA with a concentration of 25 – 250 µg/µl was collected and the concentration was determined on a 1% agarose gel stained with ethidium bromide using known molecular weight standards.

The DNA samples from Omipanda were used for the purposes of the trial FIASCO experiment. The Fast Isolation by AFLP of Sequences Containing microsatellite repeats (FIASCO) method has been used to isolate microsatellites in other plants successfully and a modified method was applied to marama bean (Zane et al., 2002). The following steps were performed (Figure 2):

The PCR products of the amplicons that were used for the biotinylated SSR selection for *Bam* H1 (B), *Hind* III (H) and *Sau* 3AI (S) are shown in Figure 3.

SSR enrichment with the biotinylated microsatellites (GAG)₇ and CAC)₇ for *Bam* H1 (B) and *Hind* III (H) followed by amplification of the enriched fractions gave libraries enriched for the particular microsatellites (Figure 4).

The microsatellite enriched DNA will be sent to Inqaba Biotechnology laboratory in South Africa for 454 sequencing. The bioinformatics analysis on the contigs from the 454 sequencing will include using the SSR finding program SSRIT to identify those contigs containing SSR regions. Primers will be designed using Primer 3 software from the 50 contigs that had the longest SSR regions. Those with high levels of polymorphism will be determined by amplifying from the DNAs of 10 individual plants representing all the regions sampled. The primer pairs identifying polymorphisms will then be used across the complete range of germplasm available and a diversity analysis performed.

RESULTS AND DISCUSSION

Marama bean distribution

The assessment of the geographical distribution of marama-

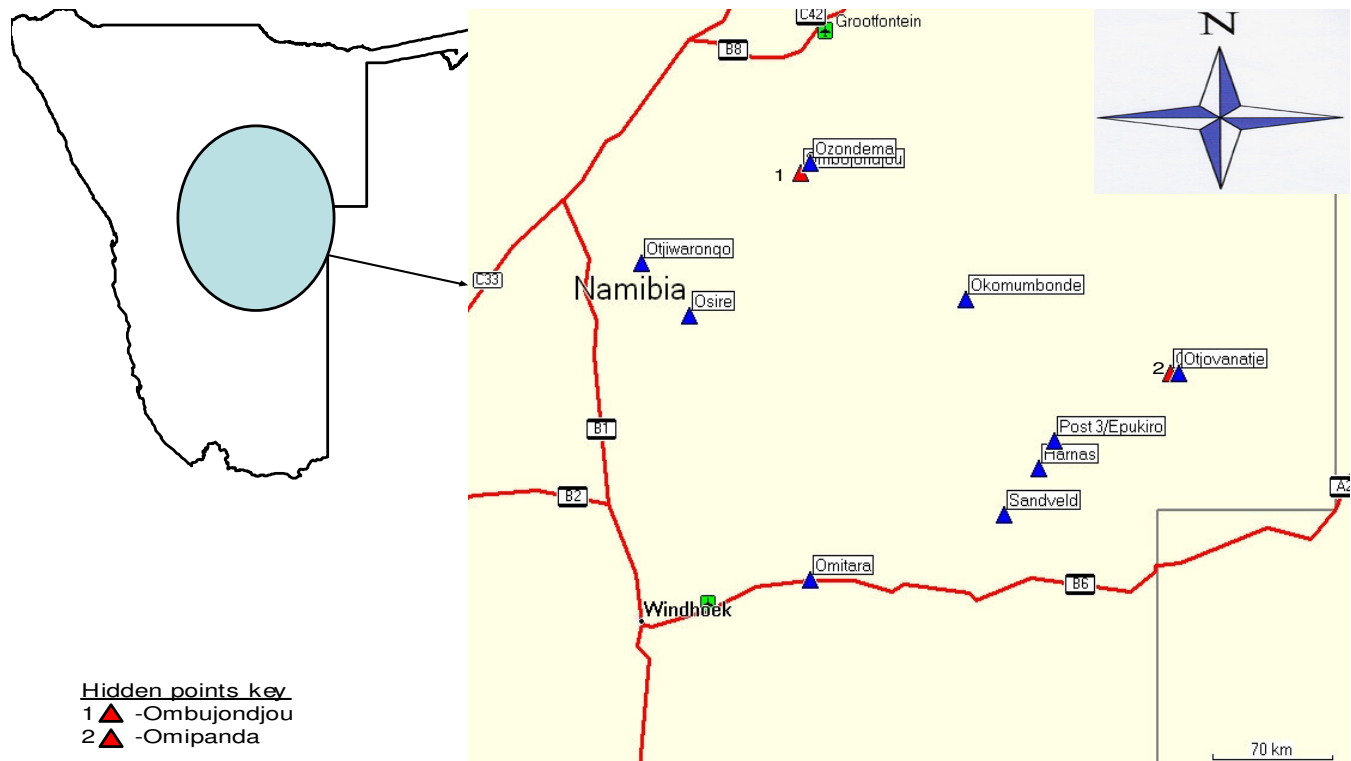


Figure 1. Geographical distribution of *Tylosema esculentum* in Namibia.

Table 1. Geographical positions of the sites of *Tylosema esculentum* in Namibia.

Location	Waypoint	Position	Number of accessions
Ozondema	1	S20 15.921 E18 02.490	25
Ombojondjou	2	S20 18.600 E17 58.525	26
Osire	3	S21 02.031 E17 21.244	96
Otjiwarongo	4	S20 46.092 E16 65.123	50
Omitara	5	S22 21.596 E18 02.476	19
Sandveld	6	S22 01.751 E19 08.009	25
Otjovantje	7	S20 27.39 31 E16 39.443	20
Ompanda	8	S21 19.355 E20 04.553	31
Post 3/Epukiro	9	S21 39.642 E19 25.092	30
Harnas	10	S21 47.705 E19 19.921	25
Okomumbonde	11	S20 57.000 E18 55.000	44
			391

ma bean using GPS has revealed a widespread, but patchy distribution of marama bean in Namibia. This finding fits that of Watts and Breyer-Brandwijk (1962). According to Watts and Breyer-Brandwijk (1962), marama bean is widespread, but restricted to clumps distributed in Southern Africa (Botswana, Namibia and South Africa). The bean has traditionally been collected for food by indigenous people, so the distribution of the genus may be associated with southern migration

(Schapera, 1937). Therefore it is possible that the widespread, but patchy distribution of marama in Namibia may be linked to the historical movements of traditional users of the plant.

Additionally, marama bean is confined to these areas because of differences in physical conditions of other habitats such as the amount of rainfall, evaporation of water from the soil surface, temperature and soil types which are not ideal for marama bean. Differences in phy-

Digestion of DNA with restriction enzymes (*Bam* H1/ *Hind* III/*Sau* 3A1)

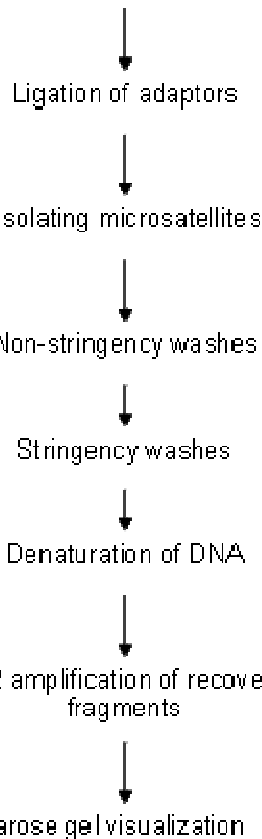


Figure 2. A flow chart depicting steps performed during microsatellite isolation using the FIASCO technique.

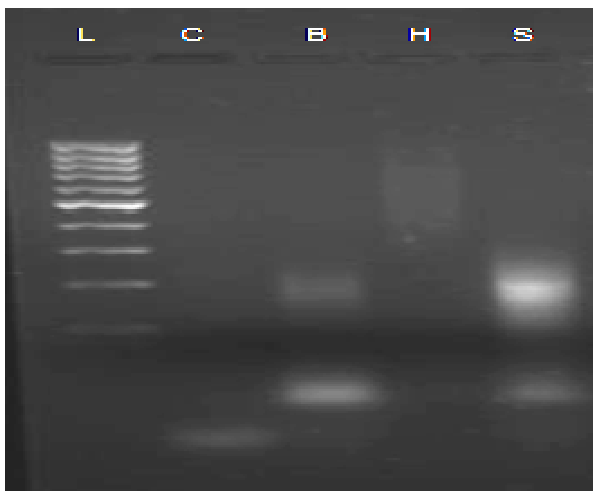


Figure 3. The PCR products of the biotinylated SSR selection for *Bam* H1 (B), *Hind* III (H) and *Sau* 3A1 (S). A 100 base pair ladder was used in the first lane (L).

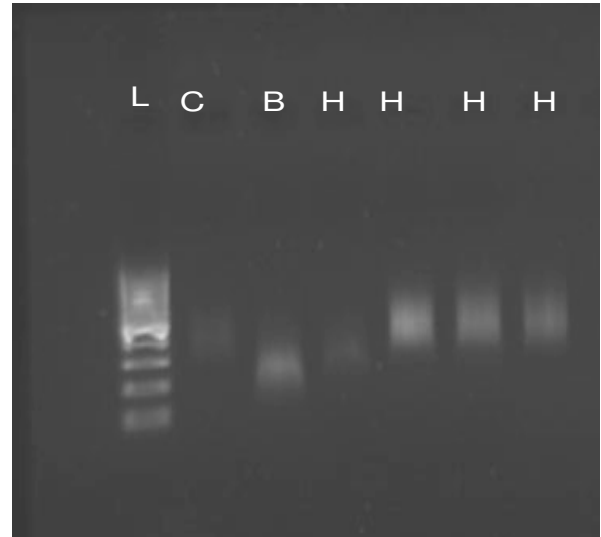


Figure 4. The PCR products for primer selection with (GAG)₇ and (CAC)₇ for *Bam* H1 and *Hind* III. A 100 base pair ladder was used in the first lane (L). Lane C is water control, B is *Bam* H1 enrichment PCR products and H represent *Hind* III enrichment PCR products.

sical conditions make it difficult for the species to maintain a population and can be viewed as barriers, which must be crossed by the species if it is to disperse to other favourable, but as yet uncolonized places. Therefore, any climatic or topographic factor, or combination of factors may provide barriers to the distribution of a species. However, the hostile factors of environment are not the ultimate barriers to the species distribution, but the species own physiology, which has become adapted to a limited range of environmental conditions.

The plant is well adapted to the low rainfall and infertile soils conditions due to its physiology. Our observation of the distribution in Namibia is that it is found growing in open savannah veld in competition with tall grasses, shrubs and small trees. The marama bean has been found to be associated with *Acacia mellifera* in some areas and the phenotype of the plant does appear to be dependent on whether or not it is associated with this species. Therefore, knowing the geographical distribution of marama bean within Namibia is vital for conservation purposes and for the establishment of a foundation for future *T. esculentum* studies.

Microsatellite isolation using the FIASCO technique

Five SSR enriched marama libraries for (AAG)₇, (CTT)₇, (ACC)₇, (GAG)₇ and (CAC)₇ from the Namibian germplasm have been isolated using the modified FIASCO technique. Initial sequencing from these libraries has confirmed the presence of SSR regions. Therefore SSR markers

will become increasingly available for marama diversity analysis and subsequent domestication and eventually be used in marker assisted selection as part of marama improvement program.

Conclusion

The preliminary study revealed a widespread, but patchy distribution of marama bean in Namibia. This widespread, but patchy distribution of marama in Namibia may be linked to the historical movements of traditional users of the plant. Marama bean was distributed in patches and it has been observed to be associated with certain shrubs and tree species. Different physical conditions are responsible for the distribution of species at either scales being geographic, habitat or microhabitat. This assessment provides vital information for marama bean conservation function and future studies. Furthermore it was confirmed that the modified FIASCO technique which has been applied to other plant genomes is applicable to marama bean as well. There is potential to use microsatellite primers as markers if polymorphisms are detected in the diversity analysis of the germplasm.

Future prospects

The primers that will be designed will be used to screen the Namibian population of marama bean for polymorphisms. The polymorphic SSR primers will first be used for phylogenetic studies on the population and then as potential markers for developing a molecular genetic map for use in a future breeding program.

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