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Full Length Research Paper

Determining genetic diversity based on ribosomal intergenic spacer length variation in Marama bean (*Tylosema esculentum*) from the Omipanda area, Eastern Namibia

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Marama bean [*Tylosema esculentum* (Burchell) L. Schreiber] is an under-utilized non- nodulating legume, native to the arid and semi-arid areas of Southern Africa, including Namibia, South Africa and Botswana. Its primary benefit to man is based upon the high nutritional value of the seeds. The protein content is slightly higher than that of soybeans and the oil content is twice that of soybeans and approaches that of peanuts. 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 changes. In this study, the intergenic spacers (IGS) region of the large ribosomal DNA gene was amplified to assess length variation in Marama bean. A pair of primers based in the conserved ribosomal DNA coding region, but both reading out the coding region, was used to amplify the IGS that separates the repeat units within the tandem arrays of the ribosomal DNA (rDNA) genes. A total of 79 scorable bands were generated by the primer pair used, of which 7 alleles were polymorphic. The rDNA genes proved to be a useful tool for identifying the genetic variation of Marama bean which will be the basis of its effective domestication.

Key words: Marama bean genetic diversity, rDNA gene, intergenic spacer, *Tylosema esculentum*.

INTRODUCTION

Marama bean

Marama bean [*Tylosema esculentum* (Burchell) L. Schreiber] is an under-utilized drought- tolerant legume, native to the arid and semi-arid areas of Southern Africa, including Namibia, South Africa and Botswana (Castro et al., 2005) . It is currently not cultivated and only grows in wild plant and consumed by local people as food source. Its primary benefit to man is based upon the high nutritional value of the seeds. The protein (43%) content is slightly higher than that of soybeans (40%), and the oil content (40 %) is almost twice that of soybeans (20%) and approaches that of peanuts (45%) (Mmonatau, 2005). There is an increasing interest on its cultivation due to its potential as a cash crop and food source, especially in the face of climate changes. Unfortunately, little is known about the plant and almost nothing is understood about its cultivation. Reliable estimates of variation and genetic relationship are the pre-requisites for effective utilization of genetic resources in crop improvements and domestication (Monaghan and Halloran, 1996). DNA markers are a reliable tool for the assessment of genetic relationships and diversity. Ribosomal RNA (rRNA) genes (present as tandem arrays, comprising the 18S-5.8S-28S rRNA *genes* and intergenic spacers (IGS), and the separate clusters of the 5S rDNA with the 5S rRNA gene and spacer) are found universally in plants, with multiple copies of the coding

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equences and intergenic spacers normally being located at a few discrete chromosomal sites (Kolchinski et al., 1991) . A variety of molecular techniques are in use to address questions on phylogeny, evolution, and population diversity (Kollipara et al., 1997). Analysis of 18S (small subunit) rRNA and, more recently, the internal transcribed spacer (ITS) have figured heavily in these studies, particularly those involving eukaryotic organisms (Chen and Nelson, 2004). The abundance, stage-independent expression and absence of influence of environmental factors on the expression make DNA markers invaluable tools for the assessment of variation and genetic relationship in plants (Callow et al., 1997).

This study seeks to assess the spacer length variation and rDNA polymorphisms in the Marama bean large rRNA genes, using 31 Marama bean individuals from the same geographical area, Omipanda in Eastern Namibia.

Molecular genetics for biodiversity conservation and genetic diversity studies

Biodiversity can be defined as the total variation found within all living organisms and their habitats. It can be accessed at three different levels: Communities (environment), species, and genes (Carvalho, 2004). When biodiversity is accessed at the species level, emphasis is on observation of differences among individuals or populations of that particular species. This can be referred to as the genetic diversity of the species (Chen and Nelson, 2004). Thus, genetic diversity is considered as a form of biodiversity. Genetic diversity is associated with the degree of differentiation among individuals in a population at their genetic material level.

The genetic material corresponds to the DNA, genic or cytoplasmic, called the genotype. Expression of genes contained in the DNA is the result of interaction between the environment and the genotype, resulting in the observed phenotype (Malacinski, 2003) . The genetic diversity of plants has been exploited in agriculture as they help in improving germplasms. Genetic diversity is important because it is the basis of evolution and adaptation of species to an ever changing environment and its recognition is necessary for the implementation of a breeding program for the improvement of any plant species (Carvalho, 2004).

Ribosomal DNA encoding genes in plants

Within their genomes, all organisms have DNA sequences that code for ribosomal RNA (rRNA), an essential component of cellular protein synthesis machinery (Kollipara et al., 1997). The basic organization of ribosomal DNA (rDNA), namely tandem arrayed repeats of the 18S, 5.8S and 28S coding regions separated by an intergenic spacer region have been maintained in most eukaryotic systems. One repeating unit consists of the 18S, 5.8S, and 25S rDNA-coding regions, the internal transcribed spacers (ITS) between these units and an intergenic spacer (IGS). In contrast to the conserved coding regions, the ITS and IGS regions are more variable within a species than the RNA coding regions.

Generally the intergenic spacer of the rDNA cluster evolves quickly and is highly polymorphic, providing a useful tool for assessing genetic variability, taxonomic and phylogenetic studies (Singh et al., 2008). The IGS frequently contains sufficient variation to allow for the examination of genetic relationships between closely related species or even populations or cultivated varieties (Penteado et al., 1996; Polanco and Pérez de la Vega, 1995; 1997; Nickrent and Patrick, 1998). The variation is normally the results of length variation in the sub-repeats in the IGS (Penteado et al., 1996; Polanco and Pérez de la Vega, 1997; Rogers and Benedich, 1987) . The variation in the length of IGS has been used as a taxonomic tool in many species including the Triticeae (Dvorak and Appels, 1982), maize (Zimmer et al., 1988), soybean (Doyle and Beachy, 1985), mugbean (Gerstner et al., 1988), and rice (Liu et al., 1996).

The ITS region comprising ITS1, 5.8S rRNA gene and ITS2 is also an area of particular importance for discrimination at the species level. The ITS sequences show more sequence divergence than their flanking coding regions (Kollipara et al., 1997) and they are often used to distinguish related plant species and to infer phylogenetic relationships from populations to families (Kolchinski et al., 1991).

Molecular markers based on selective genic (intergenic) amplification

A number of PCR-based DNA markers have been developed in recent years to evaluate genetic variation at the intra-specific and inter-specific levels (Wolfe and Liston, 1998). Molecular markers allow the selection of desired traits based on genotype and can therefore, accelerate plant breeding programs (Dolanská and urn, 2004). The rDNA genes have been shown to be particularly suitable for analyzing genetic variation and for phylogenetic analyses (Hamby and Zimmer, 1992). These rDNA *gene* families (18S-5.8S-28S, 45S and 5S rRNA) contain conserved (transcribed) and non-conserved (partly transcribed) regions (Hamby and Zimmer, 1992).

The variation in the latter regions makes the rDNA gene a useful tool for identifying genetic variation and

inferring evolutionary relationship in plants. Of the available molecular techniques, analysis of regions of the ribosomal gene repeat has been especially useful (Bruns et al., 1991). Therefore, the structural organization of ribosomal RNA genes (18S- 5.8S-28S) clusters originating from nuclear as well as organelle genomes genes had led to the design of many universal primers (Kirti, 2008) and development of techniques for rapid determination of the primary nucleotide sequence of rRNA molecules .

Marama bean breeding program

The increasing world population has taken a serious toll on the environment: Soils have been eroded, forests have been cut down, and biodiversity has been lost (Callow et al., 1997). The expected effects of climate changes will exacerbate the problems facing people that are living in harsh regions, where the -environments are marginal for agriculture, and so it will be important to increase awareness of and to improve under-utilized crops, which can thrive in these extreme environments, to improve food security.

One solution to tackle food shortage is through the application of genetics to utilized under-utilized crops and develop new varieties of the world's most important food crops that would be both high yielding and more responsive to inputs such as fertilizer and irrigation (Callow et al., 1997). The focus of this paper is on a Marama bean breeding program that has been initiated with an introductory study on the genetic variability of the Namibian Marama bean germplasm (Nepolo et al., 2009).

This study seeks to document the genetic variation of Marama bean to inform the selection of appropriate germplasm to include in a Marama bean breeding program. Within the framework of a breeding program, the detailed knowledge of the genetic and phenotypic diversity within Marama bean gene pool will facilitate a more efficient selection of parental genotypes and more rapidly yield improvement.

MATERIALS AND METHODS

Collection of samples

Thirty one *T. esculentum* individuals from the Namibian germplasm (Omipanda in Omaheke region, Eastern Namibia) were used in this study.

DNA extraction

Genomic DNA was extracted from each of the plant leaf samples collected using the DNeasy mini kit (Qiagen, 2006) for purification of total DNA from plant tissues. DNA extraction from different individual plant samples took place at the Molecular Biology Laboratory at the University of Namibia. The DNA for each sample was stored at -20°C.

PCR amplification of the IGS region

The pair of primers 18SL (5'-CTCAATGAGCCCGGTATTGT-3') and 28SR (5'- ACGAGAGGAACCGTTGATTC- 3') was used to amplify across the IGS region of the rDNA. A hot start PCR protocol with Takara SpeedStar Taq polymerase was used. The 50 µl reaction mixture containing 10X Fast Buffer I, 2.5 mM of each dNTP, 5 units of SpeedStar Taq DNA polymerase, 1 µM each of primers 18SL and 28SR, with 20 ng genomic DNA template. The PCR conditions were: 98°C for 45 s for initial genomic DNA denaturation; 30 cycles of 94°C (DNA denaturation) for 5 s, 58°C (primer annealing) for 20 s, 72°C (DNA amplification) for 1 min, and final extension at 72°C for 5 min. The PCR products were separated on a 1.5% agarose gel stained with ethidium bromide using 1X TBE buffer. The size of amplified fragments was determined using Bioline marker I.

Statistical analysis

The amplification products were scored as present (1) or absent (0) binary scores for all the primer pair combinations. Genetic diversity was estimated by Shannon information index (Lewontin, 1972):

$$H' = -\sum_{i=1}^{k} pi \log_e p_i$$

where *k* is the number of bands and *pi* is the frequency of the *i*th band in a given population. *H'* is the population diversity for the primer pair.

Percent polymorphism was computed by the formula given as:

% polymorphism = (Total number of polymorphic bands/Total number of bands) x 100

Molecular data were used to determine Bray-Curtis similarity coefficients using PRIMER 5 software. These similarity coefficients were then used to construct dendrogram depicting the genetic relationships among the Marama bean meta-population.

RESULTS

rDNA polymorphism and genetic relationship

The intra- population diversity using the Shannon information index for an individual population assessed was H' = 1.700 as revealed by the primer pair used. PCR amplicons *T. esculentum* obtained from the primer pair produced 79 scorable bands of which 7 alleles were polymorphic across the 31 individuals of Marama bean (Figure 1). Therefore, the primer pair used showed 8.9% polymorphism. The dendrogram (Figure 2) constructed for the pooled data indicated the existence of four clusters. The first (1) was formed by OMP15, OMP29,

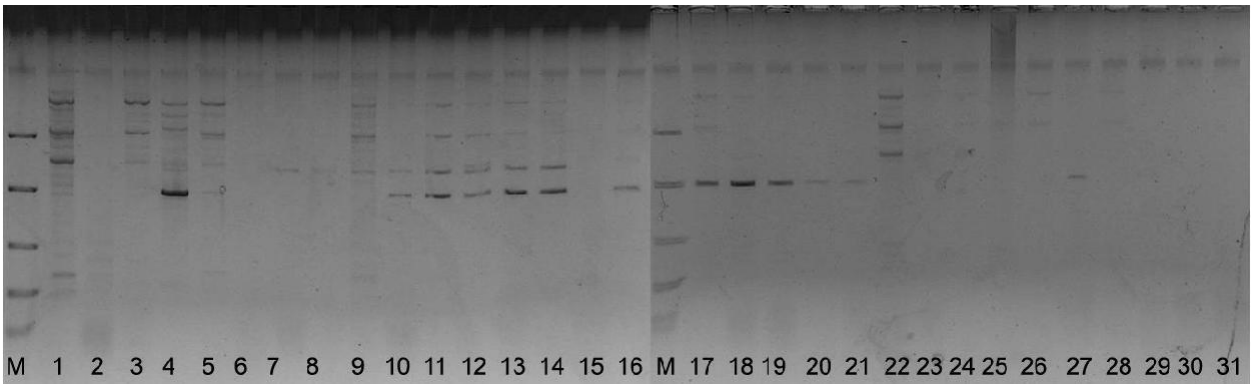

Figure 1. A 1.5 % Agarose gel electrophoresis of the IGS-PCR products for the large Marama bean ribosomal RNA genes, M indicates the DNA molecular size marker (Bioline marker I).

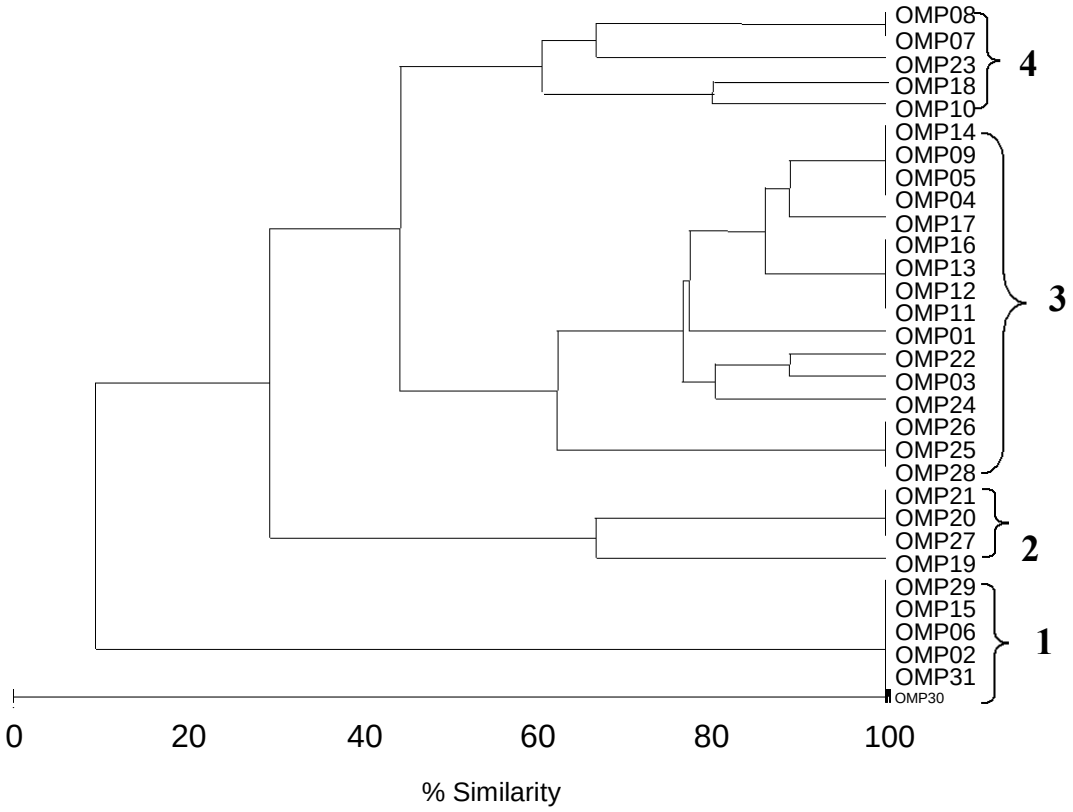

Figure 2. Dendrogram constructed from IGS polymorphism profile from the large ribosomal RNA genes of 31 individuals of *T. esculentum*. The scale at the bottom represents Bray-Curtis similarity coefficients (as percentage similarity).

OMP06, OMP02, OMP31 and OMP30 at a genetic similarity value of 0.1 with all individuals exhibiting 100% similarity (it this because no bands were seen and therefore, they may be considered as null set). The second (2) has two sub- clusters at a genetic similarity value of 0.3. OMP21 and OMP27, which formed the first sub-cluster, whereas, OMP19 formed the second sub-cluster. The third (3) cluster was formed by OMP14,

OMP17, OMP16, OMP01, OMP22, OMP24 and OMP26 at a genetic similarity of 0.63, which consisted of seven sub-clusters. OMP14, OMP09, OMP05 and OMP04 formed the first sub-cluster with the highest molecular variation of 100%. The second sub-cluster was formed by OMP17, while OMP16, OMP13, OMP12 and OMP11 formed the third sub-clusters. OMP1 formed the fourth sub-cluster and OMP22 and OMP03 formed the fifth sub-cluster.

The sixth sub-cluster was formed by OMP24 and OMP26, and OMP25 formed the seventh sub-cluster exhibiting a molecular variation of 100%. The fourth cluster (4) has three sub-clusters at a genetic similarity value of 0.45. Two individuals, OMP08 and OMP07 formed the first sub-cluster, whereas, OMP18 and OMP10 formed the third sub-cluster. Only one individual (OMP23) formed the second sub-cluster.

DISCUSSION

The assessment of length variation in Marama bean has disclosed a high intraspecific variation in the nuclear ribosomal DNA repeat units in Marama bean. Genetic diversity that was calculated using Shannon diversity index for genetic diversity, which was determined in this study (H' = 1.700), is an indication of a relatively high level of intra- specific genetic diversity in Marama bean populations. This finding supports the proposition from other studies that Marama bean is a predominantly out-crossing species (Monaghan and Halloran, 1996).

Genetic diversity calculated from the binary scores of primer pair revealed low percentage of polymorphism (0.089) within Marama bean rDNA unit length. This low variation may be ascribed to concerted evolution. Concerted evolution tends to reduce variability between gene copies by mechanisms such as gene conversion and unequal crossing over (Ohta and Dover, 1984). Some studies have invoked mechanisms such as natural selection (Rocheford, 1994), genetic drift accompanied by limited gene flow, small population size, and geographical isolation (Jellen et al., 1994) as factors associated with reduced variability of the rDNA spacer. Nothing is known about the cause of the Marama bean rDNA variation. Generally, it is in the IGS where most variation exists when comparing different isolates, species or genera of organisms. Variation assessed in this study maybe due to the presence of insertions or deletions as reported elsewhere (Kohn et al., 1988).

The Marama bean individuals assessed exhibited relatively high genetic similarity. Since, these individuals were collected from one geographic area, the lack of differentiation between the Marama bean individuals is not unexpected, given the relatively uniformity of the dry savannah environments in which they occur (Monaghan and Halloran, 1996). Therefore, the effective population size in Marama bean should be sufficiently large to reduce the effects of genetic drift which are associated with a loss of genetic variability especially in small populations. Additionally, lack of gene flow, which occurs when genes are transferred from one population to another, either through seed movements by grazing animals, insects and people moving from region to region, may have further retarded the process of differentiation within Marama bean population. Hence, gene flow can be a very important source of genetic variation when genes are transferred to a population where those genes did not previously exist.

Conclusion

The assessment of spacer length variation and rDNA polymorphisms in the RNA genes in Marama bean provides new insights in understanding the genetic variability among ecotypes and confirms that this is a useful region for genetic variability studies and phylogenetic relationships in Marama bean. Despite the fact that Marama bean is still a wild plant, which has not yet been cultivated, its nutritional composition alone makes it an important resource. Little is known about either the range of phenotypes available or what would be a good agronomic practice in its cultivation. Therefore, focused research and development efforts are needed if this wild species can be raised from obscurity and improved sufficiently to contribute to the food supply in both Namibia and Sub-Saharan Africa. The lack of knowledge is the major limitation to its cultivation with information needed on its adaptability to cultivation, genetic variation and on all aspects of its agronomy. The data reported here are the first molecular studies that begin to provide the necessary data that can be used to understand the extent and distribution of Marama bean genetic variation.

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