GENETIC DIVERSITY OF SORGHUM [*SORGHUM BICOLOR* (L.) MOENCH] AND ITS WILD RELATIVES IN NAMIBIA, USING SSR AND RAPD ANALYSIS

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ABSTRACT

Sorghum bicolor (L.) Moench is the fifth most important cereal crop in the world and the second most important in Namibia. The genetic resources of sorghum remain unexplored in Namibia. This could lead to a loss of genetic diversity, as wild relatives and foreign varieties could interbreed with landraces, resulting in an undesired alteration of its genetic make-up.

This study was conducted to evaluate the genetic diversity among the Namibian *Sorghum bicolor* accessions stored at the National Botanical Research Institute (NBRI), two relatives (*Sorghum halepense*) and four improved varieties (Macia, Pato, Larsvyt 46–85 and ZSV/30).

The objectives were:

- to determine the genetic diversity of *Sorghum bicolor* germplasm stored at the NBRI and
- to understand the germplasm genetic relationships among landraces, wild relatives and improved cultivars, using simple sequence repeat (SSRs) and random amplified polymorphic DNAs (RAPDs).

Unweighted Paired Group Method Using Arithmetic Average (UPGMA) -based results revealed a measure of similarity among genotypes (r) = 38 for RAPD analyses and at (r) =18 for SSRs respectively for 24 genotypes. The ordination method, non-metric multidimensional scaling (2D maps), confirmed the UPGMA results, with stress values ranging from 0,05 to 0,2. Two new primers (CD230935 and CD231028) designed have amplified nearly all the genotypes analysed in this study. It was also concluded that there is a genetic variation among *Sorghum bicolor* at the gene level in Namibia. In addition, the study revealed that RAPD and SSR techniques can be used in the evaluation of genetic diversity in sorghum species.

INTRODUCTION

Sorghum bicolor is the fifth most important grain crop in the world after wheat, maize, rice, and barley, and the second most important cereal crop, after maize, in sub-Saharan Africa (Zidenga, 2004). It consists of cultivated and wild species (Ayana *et al*., 2002). *Sorghum bicolor, bicolor* (2n = 20) is a sub-species, a taxon that includes agronomical important grain races, which are bicolor caudatum, durra, guinea and kafir, several hybrid races and working groups (Assar *et al*., 2005). According to Atokple (2004), sorghum, after millet (*Pennisetum glaucum*), is essential to diets of poor people in the semi-arid tropics, where droughts cause frequent failures of other crops.

In 1991, the Ministry of Agriculture, Water and Rural Development (Namibia), in joint collaboration with the International Crop Research Institute for the Semi Arid Tropics (ICRISAT) Center Patancheru (India) and SADC/ICRISAT in Bulawayo (Zimbabwe), launched a germplasm collection mission in northern Namibia (Rao *et al*., 1991), during which about 123 sorghum accessions were collected. The accessions were taken to the NBRI for preservation as seed in the gene bank (Matanyaire, 1998). Subsequent to the collection mission was the establishment of a national crop improvement programme, which focused on improving the major food crops, including sorghum. So far research has been concentrated on yield trials. In this study, two methods, namely RAPDs and SSRs, were applied.

MATERIALS AND METHODS

Genomic DNA extraction from *Sorghum bicolor* (L) Moench seeds

Two methods of DNA analysis, SSRs and RAPDs, were used to screen 24 accessions at Potsdam University Department of Genetics at the Institut für Biochemie und Biologie, and were repeated at the University of Namibia (UNAM). *Sorghum bicolor* seeds (six) from each accession were taken and ground to a fine powder in the mortar, using a pestle. Once the powder was in the Eppendorf tubes, an amount of 360 microlitres (µl) extraction buffer (EB) was added in order to neutralise the reactions. The tubes were vortexed for two seconds each time a reagent was added, in order to mix the contents well. An amount of 90 µl of 10 % SDS was added and vortexed again for two seconds. Proteinase K (2 µl of 20 mg/ml) was added to each sample, as this helps to degrade the protein. The samples were incubated in the oven at 65 °C for 20 minutes.

The RNAse (1 µl of 100 mg/ml) was added after incubation. To precipitate the DNA, acetate 3M (240 µl) of (CH_3COOK) pH: 4,8) 4 °C was added. The samples were then vortexed for two seconds before placing them on the ice cubes for 20 minutes to precipitate the DNA further. They were then centrifuged at 14 000 rpm in Avanti J-25 centrifuge from

Beckman Coulter for 20 minutes, to settle the residues and suspend the supernatant. Supernatant (400 µl) was transferred to new Eppendorf tubes, before adding two volumes ethanol 100 % (800 µl) to the supernatant in order to precipitate the DNA.

The tubes were placed on ice cubes again for 20 minutes for more DNA to be precipitated, after which the samples were centrifuged for 15 minutes at 14 000 rpm to collect the DNA pellet. Next the ethanol was poured out and the excess alcohol was dried off by placing the tubes face down on tissue paper. The pellet was then washed by centrifuge for five minutes at 14 000 rpm in 200 µl 70 % ethanol. After centrifuging, excess alcohol was again drained off by placing the tubes face down on tissue paper. To dissolve the DNA, an amount of 110 µl TE Buffer (pH 8) was added. The DNA was stored at -20 °C until used.

Generation of a Namibian Map based on the regions from where the accessions were collected

Arch view GIS 3.3 was used to generate the maps (Figure 1), using the coordinate points as obtained from the NBRI database system. The regions from which the accessions were collected are shown on the map of Namibia presented in Figure 1.

Electrophoresis of DNA and Polymerase Chain Reaction (PCR) products

Genomic DNA was quantified on 1 % agarose gel, while the PCR products were analysed on a 2 % agarose gel and polyacrylamide gel stained with 0,5 % ethidium bromide. Electrophoresis was run at 90 volts for 55 minutes. Genomic DNA pictures were kept on a gel documentation system (Bio zym at Potsdam and UVIdoc gel documentation system at UNAM). A protocol for direct purification of PCR products from NucleoSpin extract 2 was used to purify the DNA from the seeds.

RAPD Amplification

RAPD primers (four) used in this test are presented in Table 1; these were used to analyse 24 DNA samples. The following procedures were followed: (a mixture for one sample) master mix (MM) (10 µl) and 1 µl primer was added to a flat cap strip and DNA template (2 µl). The flat cap strips were sealed with thermowell sealers clear polyethylene to prevent the samples from spilling over and to protect them from evaporation during PCR. The samples were placed in the PCR machine for 2 hours and 30 minutes (see profile at Table 2). Different PCR machines were used (Eppendorf Mastercycler at UNAM and Gradient Cycler at Potsdam). After PCR analysis, DNA products were screened on an agarose gel stained with 0,5 % ethidium bromide. RAPD amplification was repeated twice.

SSR (Micro satellite) Amplification

Four SSR primer pairs were used (in Table 3 sequence written 5' to 3'). Two of these SSR primers (CD231028 and CD230935) were designed, using Primer 3 software www.cgi V0.2 free software online). The primers were ordered from EUROGENTEC S.A, Belgium. The instructions for use

Figure 1. Geographical distribution of the *Sorghum bicolor* accessions analysed.

were followed according to the manufacturer's guidelines. An amount of 20 µl was taken from the forward (F) and reverse (R) primers each and pipetted into one Eppendorf tube to make up 40 µl. Distilled water (160 µl) was added to the solution and then mixed by vortexing for three seconds. DNA (2 µl) was taken from each of the 24 samples of 10,0 ng/l into flat cap strips. An amount of 10 µl from the primer mixture was added to each sample.

Two pairs of SSR primers were used on 24 DNA samples at Potsdam University, Germany, where PCR products were analysed for 2 hours and 30 minutes in the Lycor machine (DNA) analyser on 6,5 % Polyacrylamide gel, while at UNAM four pairs of SSR markers were used and the PCR products were analysed on 2 % superfine agarose gel. Both tests were repeated twice to confirm the results obtained.

Data analysis

Cluster analysis

Data analysis to estimate the genetic distance among accessions was done by a multivariate method as described by Mohammadi and Prasanna (2003). Primer-E 5 for Windows (Plymouth Routines in Multivariate Ecological Research) software was used (PRIMER-E Ltd. 2001). Cluster analysis, which is an example of multivariate method as outlined by Mohammadi and Prasanna (2003) and Jongman *et al.* (1995), was used. Genetic diversity was estimated by scoring the DNA bands on the photograph obtained from electrophoresis, to generate binary data tables allocating a (1) point where the band is present and (0) point when the band is absent. The scores were then entered in Microsoft Office Excel (2003) and exported to the Primer-E 5 for Windows software (2001). The construction of dendrograms, objectively reflecting genetic relationships, depends mainly on the number of bands scored. Genetic similarities were computed by Bray-Curtis Similarity method using group average linkage, and subjected to clustering (UPGMA) and ordination Multi Dimensional Scaling (MDS) analysis in Primer-E 5.

CONCLUSIONS AND RECOMMENDATIONS

To the best of our knowledge, this is the first report on the genetic relationship among the Namibian sorghum genotypes at NBRI. This study provides a detailed analysis and quantification of the genetic diversity among Namibian sorghum germplasm at NBRI. Namibian-cultivated sorghums that included improved genotypes from SADC countries showed similarities indicating a low genetic diversity. Genotypes from the same place of collection and those from closely situated regions showed a closer genetic relationship.

The genetic analysis results also revealed that there were no genetic differences among the wild relatives, improved varieties and the cultivated varieties based on the Regional Governmental locations; however, the result indicated that Namibian sorghum genotypes are diverse at molecular level.

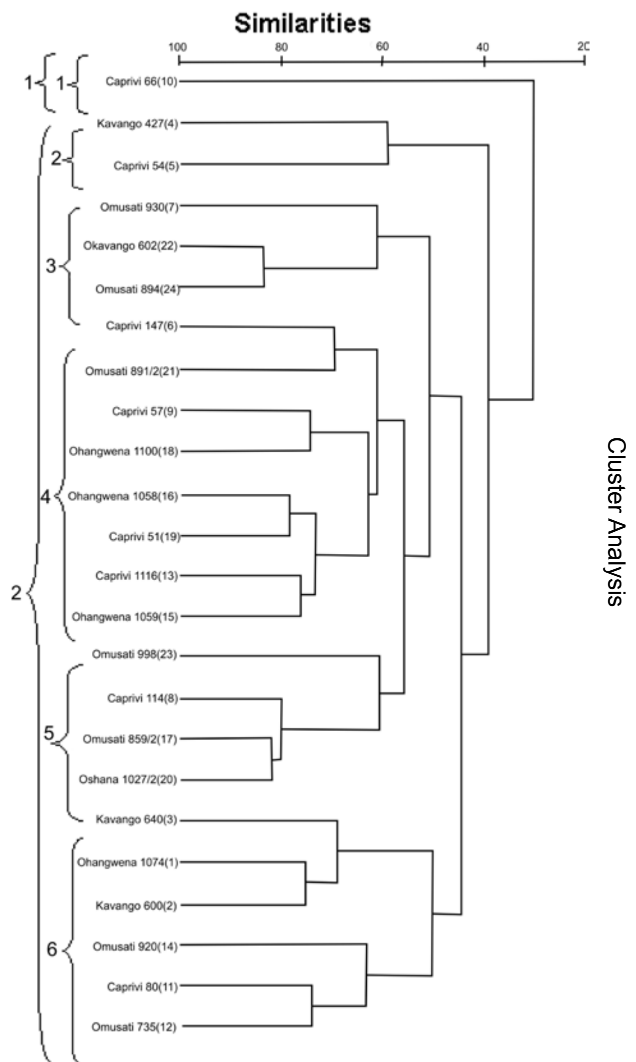

Figure 2. A dendrogram generated by UPGMA clustering, showing the similarities among 24 sorghum accessions based on Bray-Curtis similarities. These data are the combinations of the RAPD and SSR amplification results. Two major clusters and six sub clusters were formed. All accessions have been clustered in the second cluster, except accession Caprivi, which is clustered single in cluster 1. The similarities among all the accessions in cluster 2 are about 99% and 30% similar to accession Caprivi 66(10) in cluster 1.

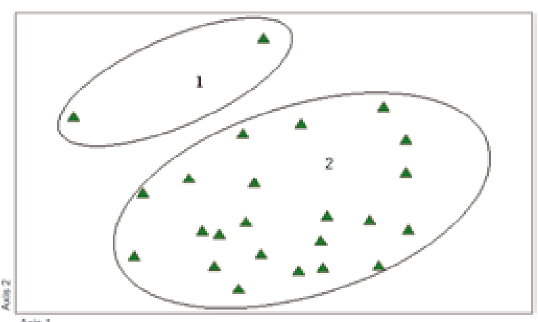

Figure 3. An ordination plot (non-metric multidimensional scaling) generated by UPGMA based on Bray-Curtis similarities for SSR and RAPD analyses. The triangular symbols in the figure represent the accessions. Two major clusters 1 and 2 have been formed with a stress value of 0,2. Cluster 1 represents accessions Caprivi 66 (ten), Kavango 427 (four) and Caprivi 54 (five). These accessions showed their uniqueness through population differentiation by appearing in different clusters.

This was also found in the study of genetic diversity among *Sorghum bicolor* from different regions, including southern Africa. The study revealed that the regional levels of genetic diversity in cultivars were greater in northern and central Africa than those in southern Africa (Aldrich *et al.*, 1992). Accessions from southern Africa were found to be closely related. It is concluded, therefore, that the germplasms screened in this study are different at the molecular level and therefore constitute different genetic make-up. However, the same accessions are interchangeably shared among farmers across the regions and thus caused the clustering based on regional background to be random.

The results have indicated that it is possible to use SSR and RAPD primers for studying genetic diversity in sorghum. Both SSR and RAPD primers can be used efficiently by researchers in developing countries where the technology is low, because neither requires radioactive isotopes. However, SSR primers were found to be more effective than RAPD primers for identifying genetic relationship among a diverse collection of accessions. This was concluded following the differences in banding patterns obtained by using SSRs and RAPDs. SSR bands were clearer and usually 1 to 5 in total, whereas RAPD bands were multiple and difficult to count. Despite the high number of bands obtained using RAPD primers, the advantage that SSRs have over RAPDs is not ruled out, SSRs being specific primers. In addition, the newly designed primers (CD230935 and CD231028) worked well and amplified nearly all accessions analysed.

It was also concluded that it is possible to improve the efficiency of using germplasm as a genetic resource, and that its use can be improved only if the information on the genetic diversity is available. It is concluded from the results that sorghum accessions at NBRI are closely related based on their geographical origins, but diverse at the molecular level. This suggests that sorghum genotypes introduced or collected from the same place tend to have a close genetic background. It is true, as supported by Anas (2004), that the same or limited genetic source in one's country or research can cause low genetic diversity among breeding lines. It could be seen clearly when the accessions were similar or differed, by simply comparing the levels at which the bands were detected.

In this case, it would be better to sequence the DNA to give the sequences of the bands obtained in order to distinguish these accessions. Therefore, it is concluded that SSR profiling is useful and works well in sorghum diversity studies. The SSR method of determining diversity could be better improved for the identification of sorghum inbred lines and hybrids in Namibia, and the improved method could be used in many applications for research in Namibia. This would eventually contribute to food security, conservation of Namibia's genetic resources and the alleviation of poverty amongst farmers, whose resources are in dire need of being enriched.

REFERENCES

ABU, A.A. H., UPTMOUR, R., ABDELMULA, A.A., SALIN, M., ORDON, F. & FRIEDT, W., 2005. Genetic variation in sorghum germplasm for Sudan, ICRISAT, and USA assessed by simple sequence repeat (SSRs). *Crop Science* 45: 1636–1644.

AGRAMA, H.A. & TUINSTRA, M.R., 2004. Phylogenetic diversity and relationships among sorghum accessions using SSRs and RAPDs. *African Journal of Biotechnology*. Department of Agronomy, Kansas State University, Throckmorton, Manhattan, KS 66506-5501, USA.

ALDRICH, P.R., DOEBLEY, J., SCHERTZ, K.F. & STEC, A., 1992. Patterns of allozyme variation in cultivated and wild *Sorghum bicolor. Theoretical and Applied Genetics* 85: 451–460.

ANAS, 2004. Studies on genetic diversity, Al tolerance selection method and effective of Al tolerance breeding program in sorghum (*Sorghum bicolor* (L) Moench). *Doctoral dissertation*. Tokyo University of Agriculture and Technology United Graduate School of Agriculture Science of Plant and Animal Production Course.

ANAS & YOSHINDA, T., 2003. Genetic diversity among Japanese cultivated sorghum assessed with simple sequence repeat markers. *Plant Production Science* 7 (2): 217–223.

ASSAR, A.A.H., UPTMOUR, R., ABDELMULA, A.A., SALIN, M., ORDON, F. & FRIEDT, W., 2005. Genetic variation in sorghum germplasm for Sudan, ICRISAT, and USA assessed by simple sequence repeat (SSRs). *Crop Science* 45: 1636–1644.

ATOKPLE, I.D.K., 2004. Sorghum and millet breeding in West-Africa in practice. *Journal of Science and Technology (Ghana)*. CSIR-Savanna Agricultural Research Institute, Tamale, Ghana.

AYANA, A., BECKELE, E. & BRYNGELSSON, T., 2002. Genetic variation in wild sorghum (*Sorghum bicolor*) Ssp. *Verticilliflorum* (L.) Moench) germplasm from Ethiopia assessed by Random Amplified Polymorphic DNA (RAPD). *Genetic Resources and Crop Evolution* 45: 499–510.

AYANA, A., BRYNGELSSON, T. & BEKELE, E., 2000. Genetic variation of Ethiopian and Eritrean sorghum (*Sorghum bicolor* (L.) Moench) germplasm assessed by random amplified polymorphic DNA (RAPD). *Genetic Resources and Crop Evolution* 47: 471–482.

BHATTRAMAKKI, D., DONG, J., CHABRA, A.K. & HART, G.G., 2000. An integrated SSR and RFLP linkage map of *Sorghum bicolor* (L.) Moench. *Genome* 43 (6): 988–1002.

BRAMEL-COX, P.J., LEE, M., PEREIRA, M.G., RATHAMANE, M., MENKIR, GREENE, S. & WILDE, G.E., 1993. RFLP map construction and its utilization for germplasm enhancement in sorghum. Use of molecular markers in sorghum and pearl millet breeding for developing countries. *Paper presented at Proceedings of an ODA plant science research programme conference 29th March–1st April 1993.* Norwich, UK.

CREGAN, P.B., JARVIK. I., BUSH, A.L., SHOEMAKER, R.C., LARK, K.G., KHLER, A.L., KAYA, N., VANTOAI, T.T., LOHNES, D.G., CHUNG, J. & SPECHT, J.E., 1999. An integrated genetic linkage map of the soybean genome. *Crop Science* 39: 1464–1490.

DJE, Y., HEUERTZ, M., ATER, M., LEFEBVRE, C., & VEKEMANS, X., 2004. *In situ* estimation of outcrossing rate in sorghum landraces using microsatellites markers. *Euphytica* 138: 205–212.

FOLKERTSMA, R.T., RATTUNDE, H.F., CHANDARA, S., RAJU, G.S. & HASH, C.T., 2005. The pattern of genetic diversity of guinea-race *Sorghum bicolor* (L.) Moench landraces as revealed with SSR markers. *Theoretical Applied Genetics* 111 (3): 399–409.

GHEBRU, B., SCHMIDT, R.J., BENNETZEN, J.L., 2002. Genetic diversity of Eritrean Sorghum landraces assessed with simple sequence repeat (SSR) markers. *Theoretical and Applied Genetics* 05: 229–236.

IQBAL, M.J., ASAD, S. & ZAFAR, Y., 1995. DNA polymorphisms in banana and sugar cane varieties revealed by RAPD analysis. *A paper presented at the proceedings of a symposium on induced mutations and molecular techniques for crop improvement*. Vienna, 19–23 June 1995.

JONGMAN, R.H.G., TER BRAAK, C..J.F. & VAN TONGEREN, O.F.R., 1995. *Data analysis in community and landscape ecology*. Cambridge University Press, United Kingdom.

KURTTO, A. & HELYNRANTA L., 1998. *The story of a city by its flora*. City of Helsinki environment centre and Helsinki University press, Finland.

MATANYAIRE, C.M., 1998. *Potential contribution of pearl millet (Pennisetum glaucum) variety Okashana-1 to household food security in northern communal areas of Namibia*. Ministry of Agriculture, Water, and Rural Development, Oshakati, Namibia.

MAWRD ANNUAL REPORT, 2004. *The seed situation among the communal farmers in the northern communal areas of Namibia*. Ministry of Agriculture Water and Rural Development. Windhoek, Namibia.

MOHAMMADI, S.A. & PRASANNA, B.M., 2003. Analysis of genetic diversity in crop plant-salient statistical tools and considerations. *Science Society of America* 43: 1235–1248.

NKONGOLO, K.K. & NSAPATO L., 2004. Genetic diversity in *Sorghum bicolor* (L.) Moench accessions from different ecogeographical regions in Malawi assessed with RAPDs. *Genetic Resources and Crop Evolution* 50 (2): 149–156.

PEJIC, I., AJMONE-MARSA, P., MORGANTE, M., KOZUMPLICK, V., CASTIGLIONI, P., TARAMINO, G., MORGANTE, M., 1998. Comparative analysis of genetic similarity among maize inbred lines detected by RFLPs, RAPDs, SSRs and AFLPs. *Theoretical and Applied Genetics* 97: 1248–1255.

PRIMER-E LTD., 2001. 6 Hedingham Gardens, Roborough, UK.

RAO, A.S., MONYO, L.R., HOUSE, M.H., MENGESHA & NEGUMBO, I., 1991. *Germplasm Collection Mission to Namibia*. Genetic Resources Unit, International Crops Research Institute for the Semi-Arid Tropics, Patancheru, A.P. 502 324, India.

SMITH, J.S.C., KRESOVICH, S., HOPKINS, M.S., MITCHELL, S.E., DEAN, R.E., WOODMAN, W.L., LEE M. & PORTER K., 2000. Genetic diversity among elite sorghum inbred lines assessed with simple sequence repeats. *Crop Science* 40: 226–232.

SZAB, T.A., 1999. Genetic erosion, human environment and ethnobiodiversity studies. *Germplasm BTN* 766: 1–16.

TANTO, T., & DEMISSIE, A., 2005. *A comparative genetic diversity study for four major crops managed under Ethiopian conditions*. Addis Ababa, Ethiopia.

TAO, Y., MANNERS, J.M., LUDLOW M.M. & HENZELL, R.G., 1993. DNA polymorphisms in grain sorghum (*Sorghum bicolor* (L) Moench). *Theoretical and Applied Genetics* 86 (6): 679–688.

TARAMINO, G., TARHINI, R., FERRARIO, S., LEEM, M. & PE, M.E., 1997. Characterization and mapping of simple sequence repeat (SSRs) in Sorghum bicolor. *Theoretical and Applied Genetics* 95 (1–2): 66–72.

UPTMOOR, R., WENZEL, W., FRIEDT, W., DONALDSON, G., AYISI, K. & ORDON, F., 2003. Comparative analysis on the genetic relatedness of *Sorghum bicolor* accessions from Southern Africa by RAPDs, AFLPs and SSRs. *Theoretical Applied Genetics* 106 (7): 1316–1325.

ZHANG, X.Y., LI, C.W., WANG, L.F.H.M., YOU AND DONG, Y.S., 2002. An estimation of minimum number of SSR alleles needed to reveal genetic relationships in wheat varieties. Information from large-scale planted varieties and cornerstone breeding parents in Chinese wheat improvement and production. *Theoretical and Applied Genetics* 106: 112–117.

ZIDENGA, T., 2004. *DNA-Based methods in sorghum diversity studies and improvement*. Plant Biotechnology Center, Ohio State University.