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Microsatellite analyses of the cowpeas (*Vigna unguiculata*) accessions in Namibia reveal low genetic diversity.

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ABSTRACT

Cowpea (*Vigna unguiculata*) is an important nitrogen-fixing grain legume crop grown for its protein rich grains and leaves that can be used as a vegetable delicacy. In times of drought, there are always severe cowpea losses. The major reason for the low cowpea yield in the dry areas of Namibia is poor choice of cultivars that are drought-tolerant. Current and previous research activities have aimed at improving yield by performing cultivar suitability trials. In this study, 28 domesticated cowpeas accession and one wild cowpeas accession were included and 17 microsatellite primer pairs were used with the objective of determining genetic diversity of wild and domesticated cowpeas of Namibia. One to three alleles per primer were detected. All data analysis was performed using the PRIMER5 program. From this study, we concluded that cowpea has low genetic diversity in Namibia and that geneflow exists between the wild and domesticated cowpeas in Namibia. This geneflow may have implications to wild cowpeas if GMO-domesticated cowpea were to be introduced into Namibia.

Key words: Microsatellite analyses, *Vigna unguiculata*, Namibia, genetic diversity.

INTRODUCTION

Cowpea (*Vigna unguiculata*) is an important legume in Africa, where it is grown over 6 million hectares of land. Cowpea is an important component of the predominantly cereal/legume production systems in the African continent; it is ranked second to field beans (*Phaseoulus vulgaris*) as a food legume in Africa. The most important cereals are sorghum and pearl millet and cowpea is often intercropped with these cereals[1]. It is estimated that cowpea is now cultivated globally on at least 12.5 million hectares, with an annual production of over three million tons[2].

In Namibia, cowpea is cultivated in the northern regions since most of country is arid making crop husbandry difficult to practice. Cowpea forms one of the basic staple foods because of its rich protein content. Cowpea has a good drought tolerance mainly because it has a strong tap root. This makes it better adapted to lighter and sandier soils that are predominant in Namibia than other legumes. However, in times of drought, there are always severe crop losses resulting from use of drought –sensitive cultivars. Lack of information on cowpea genetic variability together with in-bred lines hinders local cowpea breeding activities. Farmers are usually left with no choice but to plant even those varieties that are poorly adapted to the dry and hot agro–climate of Northern Namibia. Knowledge of genetic variation in cowpeas could help to maintain genetic diversity of cowpea and sustain long-term selection gain by providing right information to the breeders so that they can determine whether they have a good gene pool to use for breeding or not.

Polymorphisms in cowpea can be determined by microsatellites or simple sequence

repeats (SSRs). Microsatellites are short tandem repeat lengths of a few base pairs[2] for example $(AT)_{10}$, $(GA)_8$, $(TG)_{12}$. These repeats can be dinucleotides, trinucleotides, and tetranucleotides and so on with variable number of repeats. Microsatellites can be amplified by a PCR machine and the variation in the number of repeats can be detected with agarose gel electrophoresis. Microsatellite primers are developed from conserved DNA regions of sequences flanking the microsatellite repeat. Microsatellites have become the molecular markers of choice for a wide range of applications in genetic mapping and genome analysis[2], genotype identification and variety protection[3] and diversity studies[4]. In each application the variation in number of repeats is exploited as an indicator of uniqueness of the individual understudy.

In this study, the aim was to determine the genetic diversity of Namibian cowpeas colleted and stored in the Namibian National Botanical Research Institute (NBRI) genebank and also to infer the extent of geneflow using microsatellites as a first dedicated step towards a focused cowpea improvement strategy in Namibia.

Table - 1: Accession numbers, region of origin, source of the cowpeas and date of collection from source for the cowpeas used in this genetic study

Sample number	NBRI number	Provincial Accession	District of Region	Source collection	Date of collection
1	1543	Kunene	Opuwo	wild	17/5/1991
2	1110	Caprivi	Katima Mulilo	farmland	24/10/1992
3	1637	Caprivi	Katima Mulilo	village market	5/5/1995
4	1638	Caprivi	Katima Mulilo	village market	6/5/1995
5	1639	Caprivi	Katima Mulilo	farmland	5/5/1995
6	1640	Caprivi	Katima Mulilo	village market	5/5/1995
7	1692	Caprivi	Katima Mulilo	farm store	5/5/1995
8	1694	Caprivi	Katima Mulilo	farm store	5/5/1995
9	1872	Oshana	Ondangwa	village market	8/8/1996
10	2022	Omusati	Outapi	farmland	1/1/1997
11	2023	Omusati	Outapi	farmland	1/1/1997
12	2024	Omusati	Outapi	farmland	1/1/1997
13	2024	Omusati	Outapi	farmland	1/1/1997
14	2025	Omusati	Outapi	farmland	1/1/1997
15	2026	Omusati	Outapi	farmland	1/1/1997
16	2027	Omusati	Outapi	farmland	1/1/1997
17	2028	Omusati	Outapi	farmland	1/1/1997
18	2029	Omusati	Outapi	farmland	1/101997
19	2030	Omusati	Outapi	farmland	1/1/1997
20	2031	Okavango	Rundu	seed fair	1/10/1997
21	2032	Okavango	Rundu	seed fair	1/10/1997
22	2033	Okavango	Rundu	seed fair	1/10/1997
23	2034	Okavango	Rundu	seed fair	1/10/1997
24	2035	Okavango	Rundu	seed fair	1/10/1997
25	2036	Okavango	Rundu	seed fair	1/10/1997
26	2037	Okavango	Rundu	seed fair	1/10/1997
27	2038	Okavango	Rundu	seed fair	1/10/1997
28	2039	Okavango	Rundu	Seed fair	1/10/1997
29	2137	Ohangwena	Eenhana	seed fair	24/10/2000

MATERIALS AND METHODS

Plant material

Twenty-eight domesticated cowpea accessions and one wild-type were used in this study (Table 1). The seeds were obtained from the Namibian National Botanical Research Institute. The seeds were representatives of the seeds collected from northern regions of Namibia where the cowpeas are grown. The seeds were grown in the University of Namibia greenhouse.

DNA extraction and microsatellite PCR amplification

DNA was extracted from the cowpea leaves using the Fermentas Genomic DNA Purification Kit. Microsatellite PCR was carried out for each sample using 17 microsatellite primers. The following PCR mixture was used in a volume of 25 µl: Sterile distilled water (14.75µl), 10x PCR buffer (2.5µl), 25mM $MgCl_2$ (1.5µl), 2.5mM dNTP mixture (2.0µl), 10µM forward and reverse primer (1.0µl each) and *Taq* DNA polymerase (one unit). The microsatellite PCR profile used was as follows: initiation denaturation at 94 ºC for 2 minutes followed by 40 cycles of denaturation at 94ºC for 10 seconds, appropriate annealing temperature for each primer pair (see Table 2) for 30 seconds and extension at 72ºC for 1.5 minutes. After the 40 cycles there was a final elongation step of 10 minutes at 72ºC and holding until at 4ºC. The PCR amplifications were repeated four times for each primer for reproducibility.

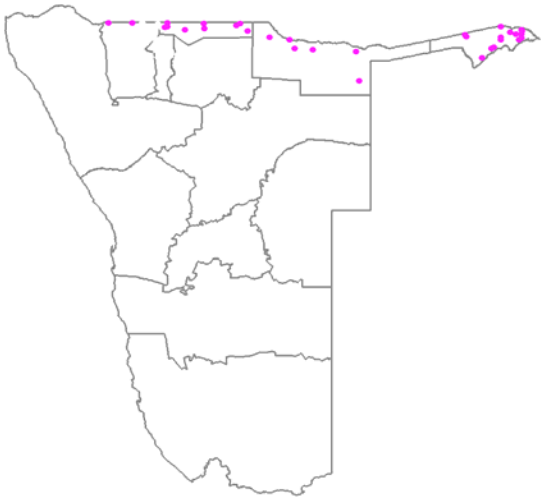


Fig. - 1: Political map of Namibia showing the locations where the cowpea accessions included in the study where collected in dots.

The PCR reaction products were evaluated for polymorphism on a 3% superfine Fermentas agarose gel. The gels were stained with 0.5% of ethidium bromide for 20 minutes.

Table - 2: Summary of microsatellite primer pairs used in the study

Primerpair	Repeat	Product size(bp)	Annealing Temperature(°C)	References
VM5	$(AG)_{32}$	188	55	[1]Li *et al.*, 2001
VM9	$(CT)_{21}$	271	61	[1]Li *et al.*, 2001
VM10	$(AC)_3(CT)_{10}(AC)_3$	278	65	[1]Li *et al.*, 2001
VM20	$(GT)_{17}$	246	61	[1]Li *et al.*, 2001
VM31	$(CT)_{16}$	200	60	[1]Li *et al.*, 2001
VM35	$(AG)_{11}.(T)_9$	127	58	[1]Li *et al.*, 2001
VM36	$(CT)_{13}$	160	59	[1]Li *et al.*, 2001
VM39	$(AC)_{13}.(AT)_5.(TACA)_4$	212	57	[1]Li *et al.*, 2001
VM69	$(AG)_{19}$	217	60	[1]Li *et al.*, 2001
VM72	$(AG)_{20}$	310	58	[1]Li *et al.*, 2001
H15	$(GAAA)_8$	300	60	[5]Chimwamurombe *et al.*, 2007
H16	$(GT)_3..(GT)_{4..}(GT)_{2...}(GT)_{2...}(GT)_3$	200	58	[5]Chimwamurombe *et al.*, 2007
VM21	$(AT)_{17}$	217	58	[1]Li *et al.*, 2001
VM32	$(AG)_{10}$	177	60	[1]Li *et al.*, 2001
VM15	$(AG)_4...(GT)_{10}$	162	58	[1]Li *et al.*, 2001
VM16	$(CT)_7..(CT)_7$	203	61	[1]Li *et al.*, 2001
VM73	$(AG)_{15}$	201	65	[1]Li *et al.*, 2001

Band scoring and cluster analysis

The gels were observed under ultraviolet light and their images were captured using a UVIdoc gel documentation system. The bands were coded by 1 or 0 for their presence or absence for each allele. A Plymouth Routines in Multivariate Ecological Research (PRIMER version 5) software program was used to draw dendograms. The dendrograms drawn were based on similarity matrices computed through the Bray-Curtis similarity method. A two-dimensional cluster principle coordinate analysis of the cowpea samples was also constructed with the Hierarchical Cluster analysis. All the data analysis was performed using the PRIMER5 program.

RESULTS

DNA extraction and microsatellite PCR

Good quality DNA was extracted from all the 29 samples included in the study. Seventeen primer pairs were used to amplify the DNA of cowpea (Table 3). Fourteen primers showed polymorphism among the individuals, the other three primers produced monomorphic bands. All primers successfully amplified DNA from cowpea. All the primers yielded 349 clearly identifiable bands. The average number of bands produced by each primer pair was 20.5.

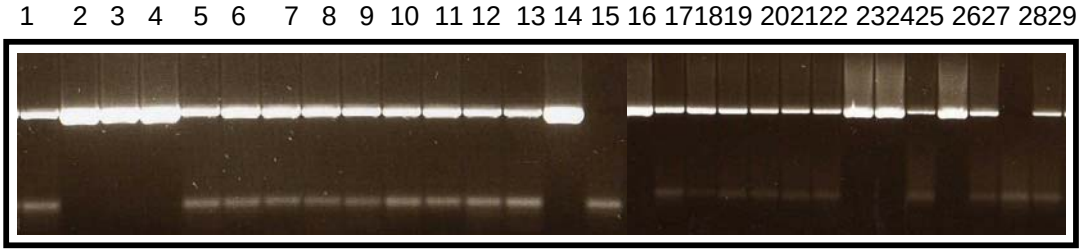


Fig. - 2: A representative 3% agarose gel electrophoresis profile showing microsatellites amplification of 29 samples included in the study amplified using primer VM 72. Numbers of the lines are listed in Table 1, the wild cowpea relative is number 1.

Cluster analysis

A dendrogram representing all seventeen primers was constructed on a basis of genetic similarity of accessions (Figure 3). The dendrogram showed that the accession 1543, of the wild sample from Opuwo and accession 1694, of the domesticated cowpea from Caprivi were the most diverse among the cowpea samples. A two-dimensional principle coordinate analysis of the cowpea samples was constructed and it shows five clusters existed in the accessions used in the study. These clusters were a mixture of samples from all the regions where the cowpea were collected. There was no way of distinguishing the different clusters except for the single entry of the wild cowpea. Hierarchical cluster analysis (Figure 3) showed an average similarity of 78% of the samples used for total 17 primer sets used in the study, indicating a low genetic diversity of the samples included in the study.

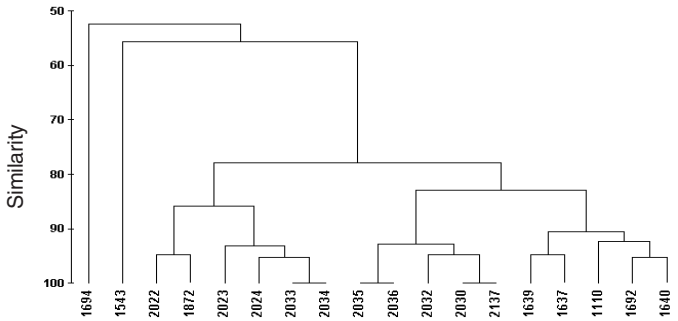
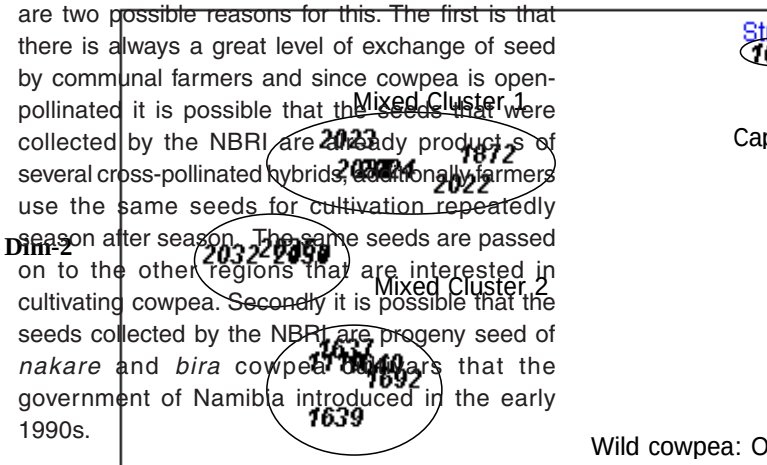


Fig. - 3: A single most parsimonious tree generated from PRIMER 5 showing the clusters of 18 of the samples included in the study.

Fig. - 4: A 2-dimension principle coordinate analysis for the samples used in study. The numbers indicate the accession numbers.

DISCUSSION

The main objective of this project was to determine the genetic diversity of cowpea accessions in Namibia, which included a wild sample from Opuwo in Kunene region and domesticated samples from Omusati, Okavango, Ohangwena, Oshana and Caprivi regions of Namibia. The results show high genetic similarity in the accessions that were used. All primers successfully amplified DNA from cowpea and the 349 bands obtained were scorable. Primers VM5, VM10, VM20, VM31 and VM36 were monomorphic and they did not show any variation among the cowpea samples. A single dendrogram was constructed combining all the primer results (Figure 3) it produced 5 clusters. The results produced by the dendrograms were consistent with the two-dimensional principle coordinate analysis (Figure 4.), that detected that samples 1543 and 1694 were clustered separately from the rest of the accessions. Five mixed sub-groupings were produced on the two-dimensional principal coordinate analysis; these groupings consist of the samples that are closely related genetically even though they were collected from different regions of Namibia (see Figure 1). There are two possible reasons for this. The first is that there is always a great level of exchange of seed by communal farmers and since cowpea is open-pollinated it is possible that the seeds that were collected by the NBRI are already products of several cross-pollinated hybrids, additionally farmers use the same seeds for cultivation repeatedly season after season. The same seeds are passed on to the other regions that are interested in cultivating cowpea. Secondly it is possible that the seeds collected by the NBRI are progeny seed of *nakare* and *bira* cowpea cultivars that the government of Namibia introduced in the early 1990s.



In other studies, microsatellite markers have shown high levels of polymorphism in crops such as rice[4], wheat[6], barley[7], oat[8], maize[2], sorghum[2], soybean[9], and beans[10]. Out of the 17 microsatellite primers used in this study, 14 produced polymorphic alleles even though genetically similarity was still high (78%).

The wild cowpea share similar DNA banding patterns in microsatellite profiles of more than five primers pair used in the study (see Figure

2 for example). This is an indication that there is a level of exchange of alleles between the wild and the domesticated cowpeas. This may have implications in geneflow if genetically modified cowpeas were to be introduced into the Kunene region of Namibia. It means the introduced genes can easily escape into wild relatives by pollination.

Microsatellite markers have been used to investigate genetic diversity of a large number of cultivars in rice[1], soybean[11], wheat[6], and sorghum[2]. The number of alleles amplified for each primer pair was 3 to 25 for rice, 11 to 26 for soybean, 3 to 16 for wheat, and 2 to 23 for sorghum. In this study one to three alleles per primer were amplified from the 29 cowpea samples. This shows that the level of microsatellite polymorphism in cowpea is much lower than other crops. Similarly, in a study carried out in Nigeria with 91 cowpea breeding lines and one wild cowpea relative[2], 46 microsatellite primer pairs were used to amplify the DNA of the cowpea. The allele number per primer pair varied from two to seven that is also relatively low compared to that of other crops.

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