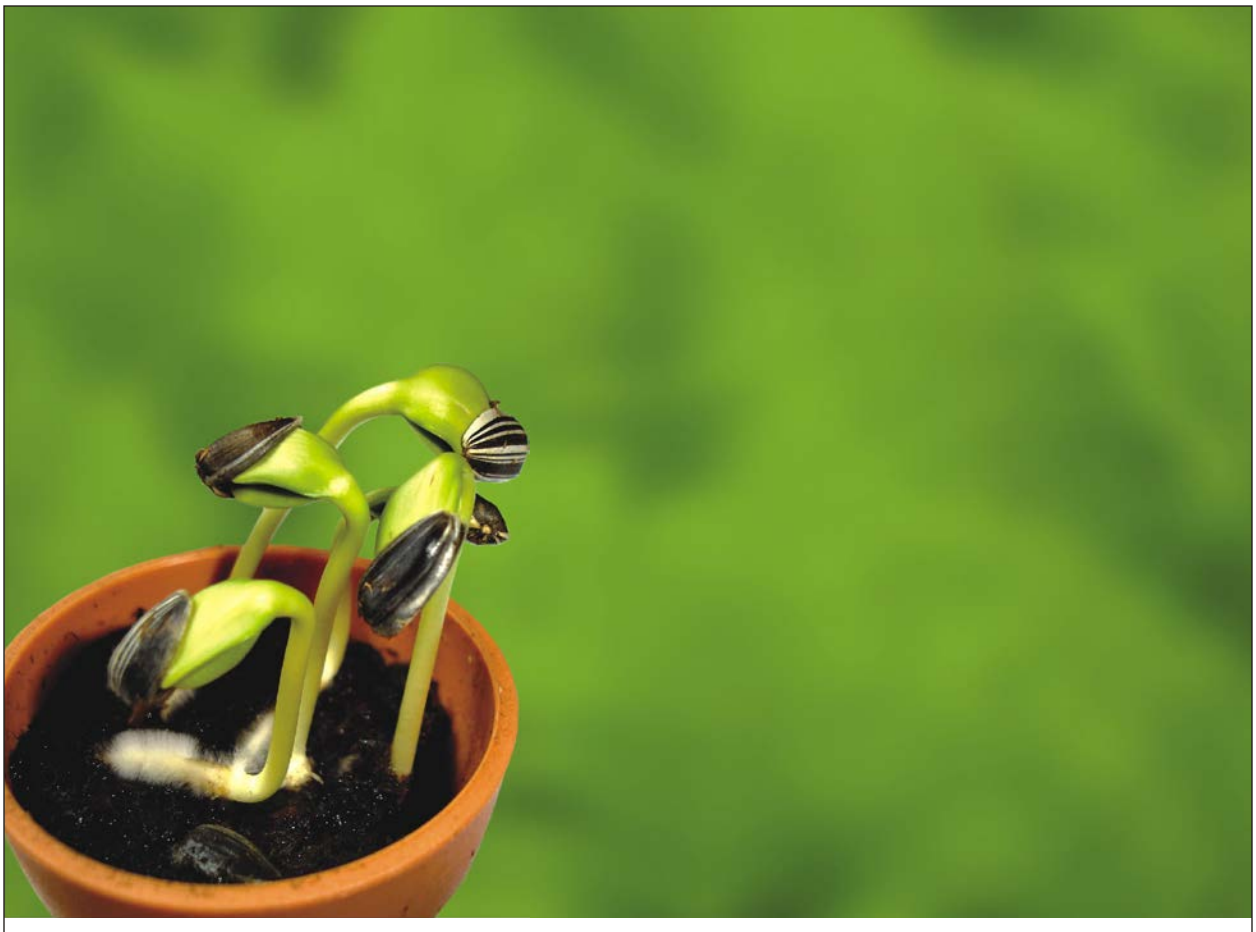

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Genetic Diversity in *Pterocarpus angolensis* Populations Detected by Random Amplified Polymorphic DNA Markers

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ABSTRACT

Pterocarpus angolensis is one of the most valuable timber trees in Tanzania currently extensively exploited. This study was conducted to obtain baseline information on its genetic diversity and population structure in order to formulate effective conservation plans. Random amplified polymorphic DNA (RAPD) markers were used to assess the genetic diversity in *Pterocarpus angolensis* DC. collected from six natural populations in eastern part of Tanzania. A total of 42 individual plants were analysed using 11 most informative RAPD primers which amplified 73 scored DNA bands showing 75.3% polymorphism. Cluster analysis is used unweighted pair group method with arithmetic average (UPGMA) formed three major clusters which illustrated that most individuals from a given population tended to cluster together and were therefore more genetically similar than individuals from different populations. Most of the genetic variations were found to be high within population 77.13% and low among populations 22.86%. The genetic diversity information of *P. angolensis* populations reported in this study is very important component for efficient conservation and effective management of its genetic resources.

Key words: *Pterocarpus angolensis*, conservation, genetic diversity, RAPD markers, over exploitation, baseline information

INTRODUCTION

Pterocarpus angolensis DC. is a leguminous tree species which belongs to the family Fabaceae. The species grows naturally in the miombo woodlands of sub-Saharan Africa and is one of the most valuable timber species within its distribution. *P. angolensis* is used for a range of products such as furniture, veneer, carving, general-purpose timber and traditional medicine (Monela *et al*., 1993; Van der Reit *et al*., 1998). The natural stock of large *P. angolensis* trees is dwindling in many areas due to commercial logging throughout their range also as a result of the valuable products derived from the species (Schwartz *et al*., 2002; Shackleton, 2002; Caro *et al*., 2005). The species has poor seed germination and collection of seed for the purpose of raising plants in a nursery is difficult because many pods are empty (Msanga, 1988; Chisha-Kasumu *et al*., 2007). Little also has been achieved through vegetative propagation (Magingo and Dick, 2001). High exploitation pressure to the species is threatening its genetic diversity and future existence in its natural habitats (WCMC, 1998).

Continued deforestation of tropical tree species has not only reduced size of natural populations or eliminate local populations but also may contribute to the declining genetic diversity of these species (Schwartz *et al*., 2002; Newton *et al*., 2002). The situation is particularly grave with regards to some species of economic importance, such as the *P. angolensis*. Efficient conservation strategies for a particular plant species can drafted if baseline information of its genetic diversity and population genetic structure is known (Newton *et al*., 2002). Preservation of genetic variation and evolutionary processes in viable populations has an ecological importance of preventing potential extinction for the species (Segarra-Moragues *et al*., 2005).

Different methodologies of assessing variability at the DNA level have been widely used, there are Restriction Fragment Length Polymorphisms (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphisms (AFLP), Simple Sequence Repeats (SSR) and others. Among the various used markers, Random Amplified Polymorphic DNA (RAPD) is the most popular DNA based markers (Bekessy *et al*., 2002; Jena *et al*., 2004). These markers have several advantages over other DNA marker as they are technically least demanding, relatively low cost, use small amounts of target DNA and offer a fast method for providing information from a large number of loci, particularly in species where no studies have previously been undertaken (Fontaine *et al*., 2004). Moreover, in terms of results, the diversity assessed with RAPDs is comparable with that obtained with Restriction Fragment Length Polymorphism (RFLP) or allozymes (Esselman *et al*., 2000). The use of RAPD markers in population genetic studies has been well established for tree species such as *Melia volkensii* (Runo *et al*., 2004), Vitellaria paradoxa (Bouvet *et al*., 2004), *Rachylaena huillensis* (Machua *et al*., 2007), *Oroxylum indicum* (Jayaram and Prasad, 2008) and *Dalbergia melanoxylon* (Amri *et al*., 2009).

Previous studies in assessing diversity of *P. angolensis* have used morphological methods (Chisha-Kasumu *et al*., 2009a). However, compared to molecular markers the uses of morphological markers for estimating genetic diversity in plants have limitations due to effect of stage of development or environment (Moyib *et al*., 2008). Morphological traits are also controlled by a relatively small number of loci (Bruschi *et al*., 2003). Although, Chisha-Kasumu *et al*. (2009b) has previously reported genetic diversity of *P. angolensis* populations from Zambia and Zimbabwe, there is limited knowledge available on the basic biology and genetic diversity *P. angolensis* populations in other areas including those from Tanzania. The information on genetic variability of *P. angolensis* is important for effectively formulating conservation, tree improvement and domestication plans for long term survivor of the species. This work was carried out using RAPD markers to evaluate the genetic diversity within and among *P. angolensis* populations in order to suggest appropriate conservation and management strategies.

MATERIALS AND METHODS

Study populations and sampling methodology: This study was conducted from August 2008 to September 2009. Leaf samples were collected from six natural populations of *P. angolensis* located in Morogoro region, in the eastern part of Tanzania; namely Iwonde and Nyanganje in Kilombero district, Kihiliri in Kilosa District. Other populations were Dindili, Mkindo and Kitulanghalo in Morogoro District. The information pertaining to the locations of the populations such as co-ordinates and altitudes were recorded using Global Positioning System (GPS) for each population (Table 1). Within each population, seven individual plants of *P. angolensis* were randomly selected at a distance of at least 100 m, in order to avoid sampling of closely genetically related individuals. Five young leaves were collected from each tree and dried rapidly in a zip

Table 1: Description of sampling sites of *P. angolensis* populations

Population	Code	Latitude	Longitude	Altitude (m a.s.l)
Iwonde	IWD	S 07°55' 12"	E 036°32' 22"	620
Nyanganje	NGJ	S 07°57' 16"	E 036°38'18"	580
Kihiliri	KHR	S 06°48' 07"	E 036°57' 23"	935
Mkindo	MKD	S 06°13' 04"	E 037°28' 12"	710
Dindili	DNL	S 06°42' 03"	E 037°54' 09"	610
Kitulanghalo	KTL	S 06°43' 15"	E 037°57' 10"	570

locked plastic bag in the field using silica gel. The collected leaf samples were stored at room temperature until DNA extraction.

DNA extraction: DNA was extracted from dried leaves, following the modified protocol as described by Dellaporta *et al.* (1983).

RAPD analysis and primers screening: A Polymerase Chain Reaction (PCR) for RAPD profile was carried out in a final volume of 25 μL containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mm mixture of dNTPs (dATP, dCTP, dGTP and dTTP), 2 μL of primer, 1 unit of Taq DNA polymerase (Sigma) and 1 μL of genomic DNA. Amplification of RAPDs was performed in Eppendorf Mastercycler (Applied Biosytems, USA) thermocycler. Optimal amplification PCR conditions used for RAPD were 1 cycle of 3 min at 94.5°C (initial denaturation), followed by 35 cycles of 1 min at 94.5°C (denaturation), 1.30 min at 40°C (annealing) and 2 min at 72°C (extension). A final step of 5 min at 72°C ensured full extension of all amplified products. Amplified PCR products were separated alongside a molecular weight marker (100-bp DNA ladder, Biolabs, USA) by electrophoresis in 1.4% agarose gel run in 0.5X TBE (Tris Borate EDTA) buffer, with subsequent staining with ethidium bromide (0.5 μg mL^{-1}) at 100 volts for one hour. The gels were photographed under UV light and the images transferred to microcomputer for further analysis of amplified bands.

Thirty decamer primers of arbitrary sequence ('Kit KFP' provided by Integrated DNA Technologies Inc., USA) were initially screened to determine the suitability of each primer for the study. Eleven primers were selected for further analysis of all individuals based on their ability to amplify DNA, band intensity, number of loci amplified and reproducibility of the RAPD products (bands) over two independent runs. Only data from clear, intensely staining unambiguous bands were used for analysis.

Data analysis: The RAPD products (bands) were aligned and data were scored in a binary manner as either presence (1) or absence (0) of a DNA band at a particular locus to compile a binary matrix for cluster analysis. Only intensely stained bands were scored. The resulting data matrices were analysed using the NTSYS-pc software Version 2.10 t (Rohlf, 2000). The genetic similarity matrix of individuals was calculated using Dice coefficient (Dice, 1945) and cluster analysis run on Sequential, Agglomerative, Hierarchical and Nested (SAHN) using the Unweighted Pair Group Method with Arithmetic average (UPGMA). The binary data matrix was treated with POPGENE software Version 1.32 (Yeh *et al.*, 1999) to calculate the genetic diversity parameters which include the gene diversity (He), Shannon' s information indices (I), observed number of alleles (Na), effective number of alleles (Ne) and percentage of polymorphic loci. The mean Shannon's information indices (I) was used as (H_{pop}) for the average diversity of the six populations

and the total diversity of the whole sample population was estimated as (H_{sp}). Then the proportion of diversity within populations was calculated as H_{pop}/H_{sp} and the proportion of diversity among populations as $(H_{sp}-H_{pop})/H_{sp}$.

RESULTS

Eleven most informative RAPD primers out of thirty primers screened were selected for DNA amplification reactions because they yielded many highly repeatable polymorphic bands. These primers generated a total of 73 reproducible RAPD bands with fragments ranging from 200 to 1200 bp, of which 55 (75.3%) were polymorphic (Table 2). The bands per primer produced ranged from 4 to 9 and the average band loci per primer were 6.6. Primers KFP04 gave the highest degree of polymorphic bands (87.5%) followed by 03 and KFP24 with 83.3% each, respectively. Primers 18 and KFP10 gave low polymorphism indices 62.5 and 66.6%, respectively (Table 2).

Cluster analysis: The UPGMA dendrogram based on Dice's similarity coefficient obtained using the 73 RAPD markers exhibited three main clusters, cluster I-III, when truncated at (0.82 coefficient) 82% similarity level (Fig. 1) cluster I was the largest cluster which consisted of two sub-clusters mainly with individuals from MKD and DNL and some individuals from KTL. Genetic similarities for cluster I ranged from 85 to 94%. Cluster II was formed by individuals from population NGJ and IWD in which similarity level ranged from 83 to 97%. In cluster III, 90% all individuals were from population KHR at similarity level ranged from 82 to 92%. Based on the UPGMA dendrogram, similarity coefficients ranged from 75 to 97% among the 42 samples of *P. angolensis*. The dendrogram generally showed clustering reflecting both the population origin and levels of diversity, as determined by the Nei's genetic diversity indices.

Genetic diversity estimates of the populations: Estimates of Nei's genetic diversity, based on RAPD data, varied from 0.1142 to 0.1916 with an average of 0.1468 and the total gene diversity parameter was 0.2775. The Shannon's indices (I) average diversity was estimated to be 0.2156 ranging from 0.1685 to 0.2769 at the population level and 0.4084 at the species level. The lowest diversity value was for Iwonde and highest value was for Kihiliri (Table 3).

At the population level, the observed number of alleles (Na) in the six populations of the *P. angolensis* varied from 1.3014 to 1.4521, with an average of 1.3813 (Table 3). The effective

Table 2: Number of scored bands per primer and polymorphism in *P. angolensis*

Primer code	Sequence of primer 5' to 3'	Total No. scored bands	No. of polymorphic bands	Polymorphism (%)
KFP01	GGC TCG TAC C	9	6	66.6
KFP02	CGT CCG TCA G	4	3	75.0
KFP03	GTT AGC GGC G	6	5	83.3
KFP04	CGG AGA GTA C	8	7	87.5
KFP05	CCT GGC GAG C	9	7	77.7
KFP07	CCA GGC GCA A	5	4	80.0
KFP08	ACG CGC TGG T	5	4	80.0
KFP10	ACG GTG CGC C	9	6	66.6
KFP16	GCA TGG AGC T	4	3	75.0
KFP18	ACC CAT TGC G	8	5	62.5
KFP24	ACT CGT AGC C	6	5	83.3
Total		73	55	75.3

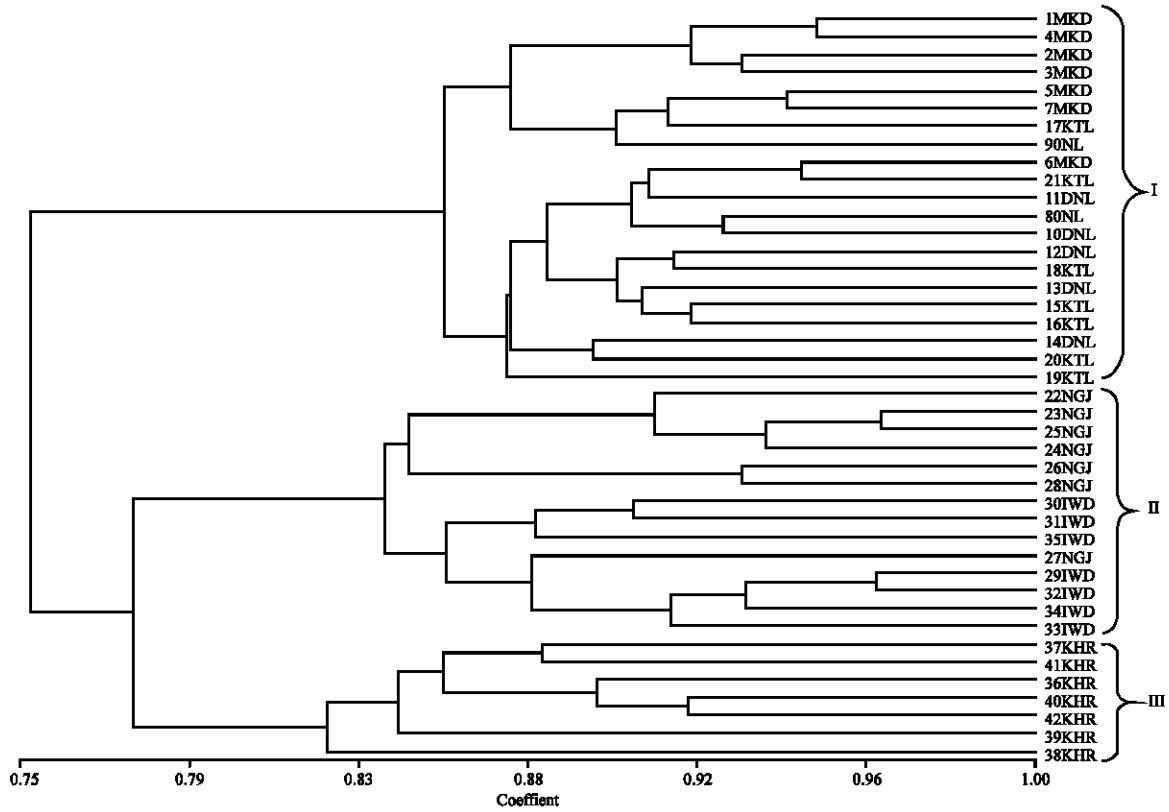

Fig. 1: UPGMA dendrogram computed from genetic similarity matrix using Dice's similarity coefficient for six populations of *P. angolensis*. Populations denoted as MKD: Mkindo, DNL: Dindili, KTL: Kitulanghalo, NGJ: Nyanganje, IWD: Iwonde and KHR: Kihiliri. Numbers I-III are clusters

Table 3: Genetic parameters variation in populations of *P. angolensis* based on RAPD analysis

Populations	Nei's gene diversity (He)	Shannon's Information index (I)	Observed number of alleles (Na)	Effective number of alleles (Ne)	Polymorphic loci (%)
Iwonde	0.1142	0.1685	1.3014	1.2007	30.14
Nyanganje	0.1489	0.2197	1.3973	1.2629	39.73
Kihiliri	0.1916	0.2769	1.4521	1.3441	45.21
Mkindo	0.1240	0.1851	1.3562	1.2164	35.62
Dindili	0.1298	0.1961	1.3836	1.2187	38.36
Kitulanghalo	0.1724	0.2473	1.3973	1.3173	39.73
Mean	0.1468	0.2156	1.3813	1.2601	38.13
Species	0.2775	0.4084	1.7397	1.4891	73.97

IWD	NGJ	KHR	MKD	DNL	KTL	H_{pop}	H_{sp}	H_{pop}/H_{sp}	$G_{ST} = H_{sp}-H_{pop}/H_{sp}$
Genetic diversity (H_j) of each population									
0.1685	0.2197	0.2769	0.1851	0.1961	0.2473	0.2156	0.2795	0.7713	0.2286

number of alleles (Ne) ranged from 1.2007 to 1.3441, with an average of 1.2601 (Table 3). At species level, the observed number of alleles (Na) was 1.7397 and the effective number of alleles was 1.4891 (Table 3). At the population level, the percentage of polymorphic loci ranged from 30.14 to 45.21%, with an average of 38.13%. The Kihiliri population had the highest value of polymorphism while the Iwonde population had the lowest (Table 3). A total of 73.97% polymorphic

Table 4: Genetic distance matrix for *P. angolensis* populations based on RAPD analysis

Population	IWD	NGJ	KHR	MKD	DNL
IWD	****				
NGJ	0.0989	****			
KHR	0.1788	0.1952	****		
MKD	0.3294	0.2332	0.2611	****	
DNL	0.3077	0.2478	0.2784	0.0799	****
KTL	0.2816	0.2388	0.2637	0.0706	0.0412

Populations denoted as: IWD: Iwonde, NGJ: Nyanganje, KHR: Kihiliri, MKD: Mkindo, DNL: Dindili, KTL: Kitulanghalo

loci were found at the species level in the six populations indicating that *P. angolensis* had high genetic diversity. The mean diversity within the six populations (H_{pop}) of *P. angolensis* was 0.2156 and the total diversity of the whole sample population (H_{sp}) was 0.2795. The proportion of average diversity within populations (H_{pop}/H_{sp}) was 0.7713 (77.13%) while the proportion of diversity among populations (H_{sp}-H_{pop})/H_{sp} was 0.2286 (22.86%) (Table 3).

The genetic distance of populations: To further evaluate the relationships among the populations, individual RAPD haplotypes were used to estimate population differentiation. The result for the genetic distances between populations of *P. angolensis* ranged from 0.0412 to 0.3294. The maximum value appeared between the samples from Mkindo and Iwonde, while the minimum value appeared between the samples from Kitulanghalo and Dindili (Table 4).

DISCUSSION

Analysis of the RAPD markers using cluster analysis and genetic parameters measure techniques demonstrated similar interpretations of the genetic diversity and structure of *P. angolensis* populations. The study revealed a high level of polymorphic loci (73.9%) at the species level in the six populations of *P. angolensis*. The UPGMA dendrogram illustrated that most individuals from a given population tend to cluster together and are therefore more genetically similar than individuals from different populations. Clustering of individuals from one population illustrate that there is an effective gene flow between populations. The clustering pattern found in this study is in agreement with Chisha-Kasumu *et al.* (2009b) and ascribed such pattern as a result of effective gene flow contributed by flower density, timely synchronisation of flowering periods and plant-pollinator interactions. The genetic distance among the populations is also important for effective gene flow. The movement of genes among populations has a significant influence on the distribution of genetic variation (Hamrick and Godt, 1990). The pattern of isolation by distance is often observed in insect-pollinated tree species when long distances within the natural range are considered (Bekessy *et al.*, 2002; Juchum *et al.*, 2007).

The results of the partition of variation within populations of *P. angolensis* by Shannon's information measure showed high genetic diversity within populations (77.13%) although slightly lower to (80.82%) reported by Chisha-Kasumu *et al.* (2009b) for *P. angolensis* populations from Zambia and Zimbabwe. The genetic diversity within populations of *P. angolensis* is within the range that has been estimated for other tree species reported by Newton *et al.* (2002) and Bouvet *et al.* (2004) in which Shannon's diversity index within populations ranged from 42 to 89.7%. It has been reported that a close association exists between breeding system and the distribution of genetic diversity among population (Hamrick and Godt, 1996; Lacerda *et al.*, 2001; Nybom, 2004). Inbreeding species are generally characterized by high levels of genetic

differentiation among populations, whilst outcrossing species tend to retain considerably low variability among populations (Hamrick and Godt, 1990). According to Frankham *et al.* (2004), reproductive system is the most important factor in determining the genetic structure of plant populations along with the effects selection, genetic drift, mutation and migration. Studies of the biology of flowering and pollination in *P. angolensis* indicate that it is an outcrossing (Mbuya *et al.*, 1994). Hamrick *et al.* (1991) and Nybom and Bartish (2000) reported that long-lived woody plants, outcrossing in natural ranges are likely to have high genetic variability within and low among populations. The genetic variability within populations is a very important measure of species adaptation to environmental changes and of species survival (Sofia *et al.*, 2006).

Genetic differentiation estimates among populations for outcrossing species have been found to range between 15-38% when RAPD markers were analysed using Shannon's index (Bussell, 1999). The genetic diversity among populations of *P. angolensis* 22.86% is within the range and is in agreement with other outcrossing plant species that have been reported with similar range of values of diversity among populations, e.g., *Cedrela odorata* (37%; Gillies *et al.*, 1997), *Alkanna orientalis* (32%; Wolff *et al.*, 1997), *Silene tatarica* (36.9%; Tero *et al.*, 2003). An explanation for the rather low, among population diversity for these species is the outcrossing nature of species which is the most important determinant of population genetic structure (Nybom and Bartish, 2000).

Plant species, especially the perennials like trees, rely on the available genetic diversity for stability and survival under the ever-changing environments. Populations with high level of genetic variation are valuable since they offer a diverse gene pool from which gene conservation and improvement programs can be made (Machua *et al.*, 2007). The decrease in the genetic diversity limits species ability to keep pace with the changing selection pressure and their roles in the ecological and evolutionary development of the biosphere (Runo *et al.*, 2004; Machua *et al.*, 2007). Consequently, maintenance of genetic diversity is important as the diversity carries forward both ecological adaptation and microevolution. The over exploitation of *P. angolensis* thus threatens their genetic diversity and hence might limit their ecological and evolutionary development of the remaining populations. Therefore, knowledge of the levels and distribution of genetic diversity is important for designing conservation strategies for threatened and endangered species (Francisco-Ortega *et al.*, 2000). Based on the information available for *P. angolensis* the following conservation strategies are proposed.

In situ conservation plan that would guarantee that gene flow is occurring to maintain most of the species' genetic variation. *In situ* conservation is very useful for preserving inherent diversity to capture the existing local adaptation (Almeida *et al.*, 2003). For the less diverse populations, efforts should be made for *ex situ* conservation based on seeds and stem cuttings collections from the population and subsequent reintroduce them into their original parental localities. Domestication of *P. angolensis* in field plots also proposed as means of conservation and utilization that will ensure local people needs for cash incomes from sale of tree products. Domestication will also decrease pressure on the few natural populations and protect its genetic diversity.

CONCLUSION

Genetic diversity of *P. angolensis* populations revealed by the use of RAPD data has indicated that there is a still high level of genetic variation in its natural populations studied. Estimates of genetic variation and structure reported herein provide a basis for both *in situ* and *ex situ* conservation strategies and effective management of its genetic resource.

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