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Full Length Research Paper

Analysis of genetic diversity in *Eucalyptus grandis* (*Hill* ex Maiden) seed sources using inter simple sequence repeats (ISSR) molecular markers

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Eucalyptus grandis **is an economically important tree species that is native to the Australian continent and its northern neighbours, where it is grown primarily for its hard wood timber and pulp for paper industries. It is widely grown in tropical countries such as South Africa, Kenya, Angola, Ghana, and Zimbabwe. Five ISSR primers generated 41 scorable polymorphic bands which were used to analyse genetic diversity between and within the seed sources and for construction of neighbour-joining phenogram. Mean Genetic Diversity per each primer loci based on Nei (1987) statistics indicated significant genetic variation between seed sources with 26.4%, (Gst = 0.264) of the total variation attributed to differences between seed sources. The variation between populations could be due to ecological, geographical association and gene flow rates and hence they should be conserved to retain the full breadth of genetic variation of the species. Thus, ISSR-PCR technology is a reliable, rapid (high throughput) and cost effective marker system that can be used to study genetic variation and genetic relationships among *E. grandis* seed sources.**

Key words: *Eucalyptus grandis*, inter simple sequence repeat (ISSR), genetic variation, seed sources.

INTRODUCTION

Eucalyptus grandis belongs to the Myrtaceae family, a large diverse, very distinct and isolated family of mostly woody plants (Jacobs, 1981). The species is native to the Australian continent and its northern neighbours where it is grown primarily for hard wood timber and pulp for paper industries. *E. grandis* is an evergreen tree that measures 40-60 m high with a tall straight trunk and 1 - 2 m in diameter (Little, 1983). The species is widely cultivated in many countries such as Angola, Argentina, Australia, Brazil, Cuba, Ghana, Indonesia, South Africa, Peru, Sri Lanka, Zimbabwe and Kenya (Mariani et al., 1981). The rotation period of the species in Kenya ranges from 6 years for domestic fuel woods, 7 - 8 years for telephone poles and 10 – 12 years for industrial fuel woods. The species is generated by seed or coppice from stumps (Webb, et al., 1980).

Currently, forests are among the most threatened ecosystems in the world (National Research Council, 1991). The majority of the highly exploited tropical forests are not under any conservation programmes. This trend of over exploitation of the forest trees posses a threat to the highly demanded tropical forest trees like *E. grandis*. Population genetic theory predicts that the decrease in the genetic diversity limits species ability to keep pace with the changing selection pressure (Young and Merrian, 1992).

The release of genetically diverse material is a prerequisite for preventing inbreeding depression in future generations. Hence, unless diversity is ensured, inbreeding depression is likely to happen as farmers distribute seeds from initial introduction to their neighbours. Tropical forest trees typically occur in assemblages of very high diversity, with the result that the majority of species occur at low density (Black et al., 1950; Lieberman and Lieberman, 1993).

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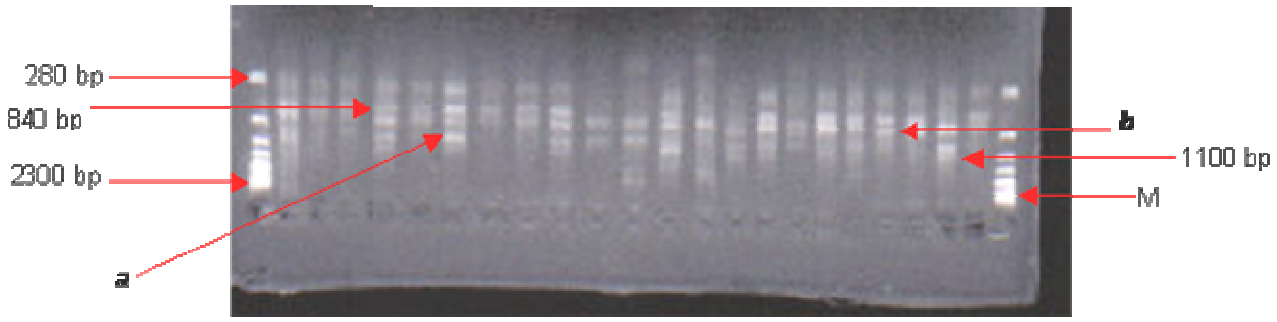

Figure 1. A section of a horizontal gel electrophoresis of amplification products generated from arbitrary primer IS15. Some of the scored polymorphisms are shown by letters *a*and *b*. M is *Eco*RI/*Hind*III-restricted - λ DNA (1kb) size marker.

Table 1. The sequence of primers used for the analysis of genetic diversity of *E. grandis*.

Primer code	Sequence (5' to 3')
ISO1	CACACACACACAGG
ISO2	CTCTCTCTCTCTCTCTAC
ISO9	GTGTGTGTGTGTGG
ISO11	GTGTGTGTGTGTCC
ISO15	GTGGTGGTGGC

Improved varieties of *E. grandis* can enhance production and help meet the local demands of its wood products. Similarly, planting in agroforestry systems can provide revenue for small-scale farmers. Although knowledge of genetic variation is vital for the sustainable management of the species under cultivation, little information on genetic variation is currently available. The present work was therefore carried out using Inter-Simple Sequence Repeats (ISSR) marker analysis to assess the genetic variation within and between the *E. grandis* seed sources in order to suggest appropriate conservation and management strategies.

MATERIALS AND METHODS

Plant material and DNA isolation

Leaf samples used for analysis were randomly collected from five seed sources of *E. grandis* in Kenya namely Kericho, Muguga, Turbo, Ex-Zimbabwe, and Kaimosi (Figure 1). Improved *E. grandis* seeds from South Africa, which are currently being introduced into the country, have also been included in the study. DNA was isolated following the CTAB method (Doyle and Doyle, 1990).

ISSR-PCR and Gel electrophoresis

The ISSR amplification reaction was carried out in a total volume of 20 µl consisting of 10 mM Tris-HCL pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$ (Fermentas), 1.3% BSA 0.2 mM dNTPs (Roche*, Applied Biosystems), 2% formamide, 1 µl of primer (MWG-Biotech AG), 1U of *Taq* polymerase (Thermopol, Biolabs) and 10 ng of genomic DNA. Hybaid OMN-E thermal cycler was programmed as follows: initial denaturation step for 7 min at 94°C, followed by 45 cycles of 30 s at 94°C, 45 s at 52°C, 2 min at 72°C and a final 7 min extension at 72°C. PCR products were resolved on 1.4% agarose gels. PCR products were visualised under UV light after staining with ethidium bromide and then photographed using a Polaroid camera. The products were sized using 1 kb DNA ladder. The primer sequences used is indicated in Table 1 below.

Data analysis

Each polymorphic band was scored for the presence or absence of the band. The ISSR products were scored by visual inspection of gel images as a matrix of product presence and product absence. Diversity values were calculated according to Nei's unbiased statistic (Nei, 1987), using POPGENE V1.31 (Yeh et al., 1999). Genetic distance (*D*) between population frequency data was generated according to Nei (1972). Cluster analysis based on genetic distances was undertaken according to equations of Nei (1978) using unweighed pair-group method with arithmetic averaging (UPGMA; Sneath and Sokal, 1973) to generate a dendrogram showing relationships among populations. The degree of polymerphism was also quantified using Shannon's index of phenotypic diversity (King and Schaal, 1989). Nei's analysis of gene diversity of subdivided populations was used to partition genetic variation within and between the populations (Nei, 1987). Significance values were assigned to variance components based on the random permutation (5000 times) of individuals.

RESULTS

Analysis of the six seed sources of *E. grandis* generated 41 ISSR amplification products out of which 27 (65.9) were polymorphic. The molecular profile generated by one of the primers (IS15) is shown in Figure 1. The levels of genetic diversity within seed sources polymorphism detected with ISSR primers in the six seed sources ranged from 0.2494 (Kaimosi) to 0.3010 (Zimbabwe) with a mean value of 0.2659 (Table 2). The number of polymorphic amplification products ranged from 3 (IS09) to 8 (IS02 and IS15) while the size of the amplified products scored ranged from 220 base pairs (primer IS11) to 2100 base pairs (primer IS15). The highest percentage of polymorphism was detected with primer IS02 (77.8%)

Table 2. Mean diversity estimates (*H*) for *E. grandis* seed sources analyzed using ISSR primers, based on Nei (1987) unbiased diversity estimates.

Population name	GPS Reading	Altitude (m)	Soil type	Population type	*H*
South Africa	0.2503	-	-	-	Orchard
Kericho	0.2517	00º08', 35°36'	2430	Loam	Plantation
Muguga	0.2694	01º13', 36°36'	2000	Nitisoil	Orchard
Turbo	0.2736	01º40', 30°02'	1800	Loam	Orchard
Ex-Zimbabwe	0.3010	01º40', 30°02'	1800	Loam	Orchard
Kaimosi	0.2494	00º06', 34°56'	2100	Loam	Plantation
Mean	0.2659				

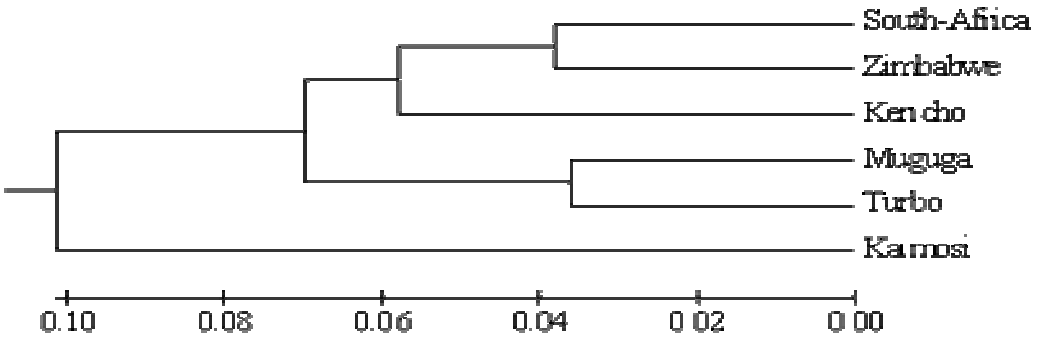

Figure 2. A dendogram (Neghbour – joining phenetic tree) based on Nei (1978) genetic distance for 6 seed sources of *E. grandis.*

Table 3. Mean genetic diversity per each primer based on Nei (1987) statistics.

Primer loci	Sample size	Ht	Hs	Gst	Nm*
ISSR1	161	0.3016	0.2082	0.2723	5.5876
ISSR2	167	0.3341	0.2453	0.2481	14.8511
ISSR11	168	0.4015	0.2885	0.2324	21.080
ISSR15	159	0.3280	0.2511	0.2043	16.24
ISSR9	162	0.3251	0.2501	0.1752	19.2165
Mean	163	0.3385	0.2491	0.2643	1.3921
St. D		0.0180	0.0106		

Nm = Estimate of gene flow from Gst or Gcs. E.g., Nm = 0.5(1 - Gst)/Gst.

while the lowest was detected with primer IS09 (42.85%).

A dendrogram based on Nei's genetic distance showing the clustering between the six seed sources is shown in Figure 2. The neighbour joining phylogenetic tree based on Nei's genetic distance (Nei, 1978) grouped the seed sources into two main clusters. One cluster contained five seed sources (South African, Zimbabwe, Muguga, Turbo and Kericho) while the other cluster contained the Kaimosi seed source. In the dendrogram, seed sources of the first crossability group (South African, Zimbabwe and Kericho), having considerably small genetic distances clustered together. Within this group Zimbabwean seed source was closer to the South African seed sources and distant from the seed source from Kericho which in turn was relatively distant to the South African seed source. The Muguga and Turbo seed sources were clustered together. The Kaimosi seed source was grouped distantly from all the other seed sources.

The results of the Nei's Analysis of gene diversity in seed sources (Nei, 1987) are shown in Table 3. The unstructured analysis (no consideration given to the seed sources) based on the six seed sources indicated that most of the variation was found within the seed sources (73.6%) while the rest (26.4%) was attributed to variation between seed sources.

DISCUSSION

The majority of ISSR variation (73.6%, Gst = 0.264) in *E. grandis* was found within seed sources. The fact that most genetic diversity existed within seed sources is consistent with the general trend in other out-crossing species based on RAPD variation such as *E. globulus* (Potts and Jordan 1994), *E. grandis* (Huff et al., 1993; Nesbit et al., 1995), *Prunus africana* (Mwangi, 2001) and *Melia volkensii* (Runo et al., 2004).

Plant species differ markedly in the way genetic diversity is partitioned between populations. The pattern of partitioning is correlated with the mating systems and life history parameters (Hamrick and Godt, 1989). Species that are primarily out-crossing and long-lived have most of their genetic diversity partitioned within populations, and the present results are consistent with this pattern. *E. grandis* is an out crossing species (see House, 1997) and therefore most of the variations seem to occur within populations as found in the present study.

However, contrary to these findings, higher values for between populations variation using RAPDs have been found in some species such as *M. stenopatela* (Muluvi, 1998), *Hordeum spontaneum (*Dawson et al 1993) and *Gliciridia sepium* (Chalmers et al., 1995).

The association between breeding system and levels of genetic diversity have been documented. Generally, most self-pollinated species are characterised by high variation among populations where as predominantly out-crossing wind pollinated species exhibit less variation among populations (Loveless and Hamrick, 1984; Falk and Holsinger, 1991). The present findings have revealed that *E. grandis* is therefore an out-crossing tree species and that gene flow occurs over long distances.

The levels of genetic variation detected by ISSR markers within and between populations of *E. grandis* was relatively higher than that found using RAPD markers (Grattapaglia and Sedroff, 1994). Previous work on genetic diversity in *E. grandis* using RAPD molecular markers revealed that the percentage polymorphism was 53% (Grattapaglia and Sedroff, 1994). However the present study revealed high levels of diversity with a mean of 68.9%. This can be attributed to the hypervariable nature of the ISSR markers, which are expected to reveal high levels of variation. These findings corroborate with those of Van der Nest et al. (2000) who employed ISSR markers to access micro-sattelite-rich regions of *E. grandis.* ISSRs have been considered to be highly informative. In rice, a high percentage of polymerphic bands were produced with the ISSR technique (73.3%) than with AFLP (55.5%) as was observed by Blair et al. (1999). Galvan et al. (2003) also found that ISSR markers were more informative than RAPDs in evaluation of genetic diversity in common beans.

Results from the current work indicate that the seeds obtained from the current seed sources are of wide genetic base. However, it is still recommended that strategies that can capture high genetic variability be adopted. These strategies include sampling across the seed sources as well as mixing of the planting stock. The results of the study indicate that the Kenyan populations were genetically diverse, with the diversity levels ranging from Kaimosi (*H*= 0.2494), Kericho (*H*= 0.2517), Muguga (*H*= 0.2694), Turbo (*H*= 0.2736) to the naturalised Zimbabwean population (*H* = 0.3010). A comparison was made between the introduced South African (*H*= 0.2503) forms. The above results clearly demonstrate that the South African seed source was not of narrow genetic base and that the genetic diversity of that seed source compares well with the Kenyan populations.

The neighbour joining phylogenetic tree showed differentiation in two groups in which the Kaimosi seed source was distant from all the other seed sources. This could be attributed to the origin of the planting material and the sampling methods were used to obtain the planting materials by the collectors. This could also have resulted due to the continuous propagation of a unique allele within the population. The ISSR analysis of the accessions also revealed genetic similarity within the crossability group consisting of South African and Zimbabwean seed sources. This could be attributed to the close proximity of their geographical origin. However, given its geographical position, it is somewhat surprising that the Muguga seed sources from central part of Kenya clustered together with the Western Kenyan seed sources (Turbo, Kericho and Zimbabwe). This could be probably due to introduction or transfer of materials from or to the two regions thereby resulting to the mix up of planting material. Ecological and geographical differentiations are important factors that influence the breeding and sampling strategies for tree crops. Namkoog (1988) stressed the importance of high levels of genetic variation as a safeguard against co-evolving biotic factors such as pests and diseases.

The present work is the first report where ISSR markers have been used in the analysis of genetic variation and determination of genetic relationships between and within seed sources of *E. grandis* that are currently being used for planting in Kenya. The ISSR technique was found very reproducible and polymorphic in detecting genetic variability in *E. grandis* seed sources. These findings are in agreement with earlier work by Ratnaparkhe et al. (1998) and Wambui (2005) who used the ISSR technique in detecting genetic diversity in chickpea and sweet potato, respectively.

The present study revealed significant variations among all the seed sources studied, thus indicating that both national and international based conservation strategies of the species are crucial. In order to sample germplasm adequately for evaluation or conservation purposes and to maintain a wide genetic base of the materials distributed to farmers, sampling strategies that collect extensively within seed sources must be employed, since significant differences exist among seed

sources both within and among countries. Further work will need to be carried out on seed sources which are highly divergent to determine if there are important quantitative traits associated with these differences or not.

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