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Full Length Research Paper

Genetic diversity in Kenyan populations of *Acacia senegal* (L.) willd revealed by combined RAPD and ISSR markers

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Acacia senegal **belongs to the subgenus,** ***Aculeiferum.*** **It is an African arid and semi arid zone multipurpose tree species, highly valued for gum arabic production, agroforestry and desertification control besides other multiple uses. Genetic variation and resulting variable groupings were assessed using combined RAPD+ISSR markers within and among four Kenyan populations of** ***A. senegal.*** **Using 10 RAPD and 5 ISSR primers, a total of 55 polymorphic bands with an average of 3.6 polymorphic loci per RAPD+ISSR primer were detected. The mean Nei's gene diversity index (*H*) for the populations was 0.283 and mean observed number of alleles per locus (*A*) was 1.982. Much of the genetic variation resided within the populations based on the Analysis of Molecular Variance (AMOVA) (86%). The dendrogram derived using the Unweighted Pair-Group Method with Arithmetic averaging (UPGMA) clustered the Garissa and Wajir populations in one group and the Samburu and Baringo populations in the other, reflecting geographical sub-structuring of the genetic diversity. It was therefore recommended that selection of desired important economic traits for improvement and conservation should target individual trees within populations and cover the entire ecological amplitude of the populations.**

Key words: *Acacia senegal*, genetic diversity, combined RAPD+ISSR markers, multipurpose, Kenya, selection, conservation.

INTRODUCTION

Acacia senegal (L.) Willd is a leguminous multipurpose African tree species belonging to the subgenus, *Aculeiferum* (Arce and Blanks, 2001). There is currently an undergoing debate on retypifying *Acacia* with a new type which would place most species ascribed to the present subgenus *Aculeiferum* into the genus *Senegalia*, if the international botanical community accepts the split (Orchard and Maslin, 2003; Seigler et al., 2006). The species grows to 2-15 m tall with a flat or rounded crown (Maundu et al., 1999). The tree is highly valued for centuries for gum arabic production, which is used in food, pharmaceuticals and other industries in the USA and Europe (Anderson and Weiping, 1992; ICRAF, 1992). Gum arabic is approved for use as food additives by the US Food and Drug Administration and is on the list of substances that are generally recognized as safe (Dondain and Phillips, 1999). Other uses include soil fertility restoration by way of atmospheric nitrogen fixation, provision of wood for fuel, local construction and poles for fence posts (Fagg and Allison, 2004). Fiber from the root bark is used to make ropes and fish nets (NAS, 1984). In addition, the species seeds are used as vegetables by humans while pods and leaves provide fodder for livestock (FAO and UNEP, 1983). The species is also used in agroforestry systems and in desertification control through sand dune stabilization and as wind breaks (Cossalter, 1991).

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A. senegal is widely distributed because it tolerates aridity and eroded soils. It is wide spread in tropical and sub tropical Africa, from South Africa northwards to Sudan (Raddad et al., 2005). In Kenya, the *A. senegal* tree grows on Homa hill in the rift valley, Lokitaung and Mutha hill in dry *Acacia* commiphora bush land in dry areas. High densities and sometimes pure stands of this species have been found in parts of Turkana and Baringo districts. The tree is also spread in Samburu district and North Eastern Province; in Garissa and Wajir districts (Maundu et al., 1999).

Not much effect has been made in Kenya to develop management strategies for utilization and genetic conservation of *A. senegal*, despite the fact that it is a source of livelihood for many people in areas where it grows naturally. Efforts have been seen in gum quantity and quality determination. For instance, gum chemical composition based on sources of origin has been reported within some Kenyan populations of the species (Chikamai and Odera, 2002). Fagg and Allison (2004) reported variation in chemical composition, molecular as well as morphological characteristics between Ugandan and Sudanese populations of *A. senegal*. In addition, Al-Assaf et al. (2005) and Motlagh et al. (2006) observed high variability in gum arabic quality and molecular weight from different sources which was suspected to be due to the diverse geographic origins. However, no clear study has been done so far to ascertain sources of these variations. Lack of knowledge of the causes of these variations and their relationship with genetic factors has made it difficult to improve gum arabic yield and quality in Kenya. A coordinated effort to conserve *A. senegal* germplasm resources would be useful to complement gum quantity and quality researches, which would lead to effective utilization of the resources to improve productivity and quality. This would make a contribution to sustainable development in the lowly developed countries, especially in East and central Africa, where *A. senegal* grows naturally.

Our knowledge on the structure of genetic diversity of *A. senegal* in Kenya is still limited and adequate conservation methodologies require further development. There is thus a need to generate and share knowledge towards the development of a technological base for the safe conservation and active use of *A. senegal* genetic resources.

Based on morphological attributes, *A. senegal* has been grouped into several varieties; var. *senegal*, var. *kerensis* Schweinf, var. *leiorhachis* Brenan and var. *rostrata* (Sim) Brenan (Brenan, 1983). However, these varietal group identification show limited levels of inter and intra-varietal polymorphism and may not account for all the diversity in the species. Indeed, retypifying the genus *Acacia* as a whole might help resolve some of the taxonomic complexities within the genus.

The diversity of environmental conditions, especially moisture, under which *A. senegal* occurs naturally suggest that there is a great genetic variability among the populations of the species (Brenan, 1983; Oleghe and Akinoufesi, 1992). Therefore the understanding of the genetic variation of this important species is imperative for selection activities, provenance trials, management, conservation and utilization of the species (Oleghe and Akinoufesi, 1992).

Molecular and biochemical studies have been conducted on African and Australian *Acacia* species to provide markers useful for plant breeding and conservation programs (Playford et al., 1993; McGranaham et al., 1997; Butcher et al., 1998). In most African species of *Acacia*, isozymes have been used (Boer, 2002). However, data from RAPD analysis have indicated that their diversity is usually similar to or greater than diversity from allozymes in plant species (Esselman et al., 1999, 2000). RAPD and ISSR are high-through put marker technologies which allow the analysis of a large number of individuals with a large number of markers in relatively short time, as only a few primers allow the generation of sufficient data to obtain a robust estimate of diversity index. RAPD markers are based on amplified arbitrary sequences and sample a wider part of the genome than the ISSR primers, while the ISSR primer sequence is usually larger, allowing for a higher primer annealing temperature which results in greater band reproducibility than RAPD markers. The ISSR primers may also be anchored at the 5' or 3' end with a few nucleotides to increase specificity of priming. Mainly, RAPD has allowed the resolution of complex taxonomic relationships (Cottrell et al., 1997; Casiva et al., 2002). Accordingly, ISSR markers have been used successfully in a number of recent studies of genetic diversity in plants (Blair et al., 1999; Huang and Sun, 2000).

This study has utilized the advantages of the two molecular marker techniques by combining the two marker's data in estimating the genetic diversity in four Kenyan populations of *A. senegal*, in order to provide a guide for effective management, conservation and selection for genetic improvement.

MATERIALS AND METHODS

Tree sampling design

Sample collection was carried out in four districts of Kenya: Kutulo division in Wajir District, Bulla Sambul Region of Garissa District, Wamba Division in Samburu District and Meisori Division-Lokortabim Area in Baringo District (Table 1 and Figure 1) representing the major regions where the species is found and much of the gum trade is carried out.

Trees within a locality were sampled at intervals of at least 100 m to minimize the risk of sampling closely related individuals. In total, the samples represented four localities, 19 samples from each of the Garissa, Wajir and Samburu populations, and 38 samples from the Baringo population. Young healthy leaf samples were picked from every sampled tree for eventual DNA analysis in the laboratory. In total, 95 samples were analyzed for molecular work and all were accommodated at the same time by the thermocycler during the amplification process. This was purposely done to ensure reproducibility of the results within the laboratory for the four

Table 1. Collection site data of Kenyan populations of *A. senegal* used in this study.

Population	Latitude	Longitude	Altitude (m)	Mean annual rainfall (mm)	Mean annual temperature (ºC)
Garissa	0º27'25'S	39º39'30'E	235	350	29.5
Wajir	1º44'05'N	40º04'08'E	205	320	28
Samburu	0º40'31'N	36º02'10'E	975	475	26.5
Baringo	0º28'00'N	36º00'00'E	1000	650	24

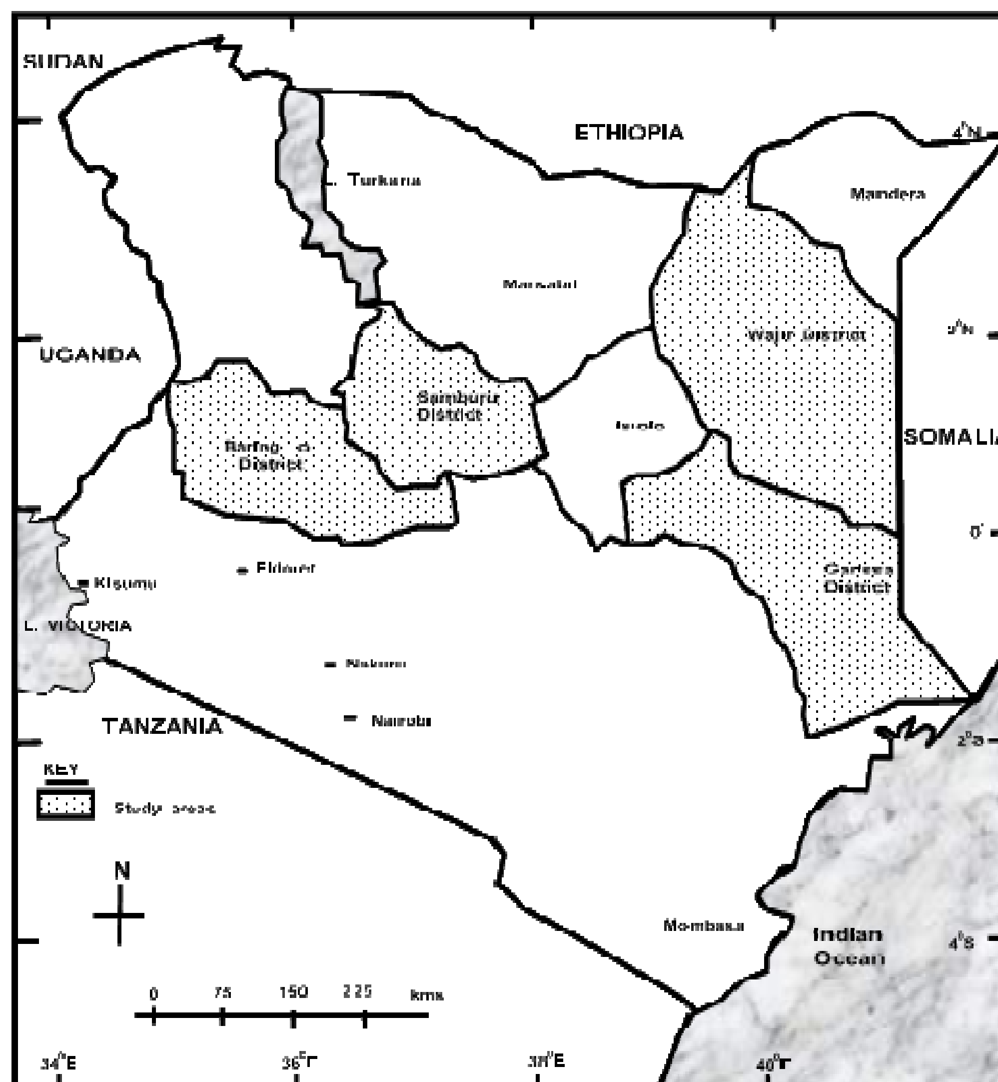

Figure 1. Locations of study areas for genetic diversity study of *A. senegal* in Kenya situated in four districts of Kenya

populations simultaneously.

RAPD and ISSR methods and data analysis

DNA was isolated from 0.5 g of the fresh leaf material using the modification of the CTAB method of FAO/IAEA (2002). The DNA quantity and quality (intactness) was visually quantified using the agarose gel electrophoresis method. Uncut, unmethylated Lambda (λ) phage DNA was used as the standard. A working stock of λ DNA (20 ng/µl) was prepared and used for quantification of *A. senegal* DNA extracts. Different concentrations of λ DNA (40-200 ng) were electrophoressed with the test DNA samples (5 µl), on 1.5% agarose in 1 X TBE running buffer (89 mM Tris-HCl, pH 8.0; 89 mM boric Acid; 2.5 mM EDTA) at a constant voltage of 50 V for 40 min. After electrophoresis, the gel was stained in ethidium bro-

Table 2. Primer code, primer sequence, GC content, number of amplified bands, number of polymorphic bands and percent polymorphic bands for each RAPD and ISSR primer used.

Primer code	Primer sequence	GC content (%)	No. of amplified bands	No. of polymorphic bands	Polymorphic bands (%)
AB4-16	AAGTCCGCTC	60	7	3	42.9
OPD-18	GAGAGCCAAC	60	6	1	16.7
OPU-03	CTATGCCGAC	60	7	3	42.9
OPU-10	ACCTCGGCAC	70	6	2	33.3
OPU-16	CTGCGCTGGA	70	12	8	66.7
OPU-18	GAGGTCCACA	60	10	4	40.0
OPU-20	ACAGCCCCCA	70	5	1	20.0
OPV-15	CAGTGCCGGT	70	11	6	54.6
OPW-11	CTGATGCGTG	60	12	6	50.0
OPW-18	TTCAGGGCAC	60	8	4	50.0
808	AGAGAGAGAGAGAGAGC	47	10	3	30.0
810	GTGTGTGTGTGTGTGTCA	47	9	5	55.6
813	CTCTCTCTCTCTCTCTT	47	7	2	28.6
817	CACACACACACACACAA	47	8	4	50.0
849	GAGAGAGAGAGAGAGAT	47	7	3	42.9

mide (0.1% w/v) before viewing under ultraviolet light (312 nm) on a transilluminator. The concentrations of the samples were determined by comparing band sizes and staining intensities of the test DNA samples with those of the standard λ DNA. The DNA samples were then standardized to concentrations of between 10-30 ng/µl by appropriate dilutions using sterile distilled water.

A total of 27 RAPD and 14 ISSR primers (ISSR primers anchored at the 3' end) were screened for polymorphism, reproducibility and their capacity to differentiate among the populations of *A. senegal.* Eventually, only the best 10 RAPD and 5 ISSR primers were chosen for use in this study.

DNA amplification reactions were performed in a total volume of 10 µl, each containing 40 ng template genomic DNA; dNTPs (dATP, dCTP, dGTP and dTTP mixture) at 100 nM final concentration; 200 nM oligonucliotide primers (Operon Technologies, USA); 1X Taq DNA polymerase buffer, 2.5 mM $MgCl_2$; and 0.5 units of Taq DNA polymerase (Amplitaq Gold, Perkin Elmer, USA). Reactions were set in 0.2 ml PCR tubes with a mineral oil overlay. Each PCR run included a negative control of sterile distilled water. The amplification was then performed in a thermocycler (Techne PHC-3, UK). The thermocycler was programmed as follows: one cycle of 93ºC for 5 min (Hot start step); 40 cycles of 93ºC for 1 min (denaturation step), 42ºC for 1.5 min (primer/template annealing step), 72ºC for 1 min (polymerization step); and a final extension phase of 10 min at 72ºC for RAPD amplification reaction. Similarly, one cycle of 94ºC for 5 min (Hot start step); 40 cycles of 94ºC for 30 s (denaturation step), 52ºC for 45 s (primer/template annealing step), 72ºC for 2 min (polymerization step); and a final extension phase of 10 min at 72ºC for ISSR amplification reaction (Williams et al., 1990). The PCR samples were mixed with 3 µl of gel loading dye (50% Glycerol, 250mM EDTA (pH 8.0), 0.01% bromophenol blue) and separated by electrophoresis on 1.5% agarose gels in an electrophoretic tank containing 1 X TBE (89 mM Tris-HCl, pH 8.0; 89 mM Boric Acid; 2.5 mM EDTA) both as electrode and gel buffer. The running voltage was 150 volts for three hours. The agarose gels were then stained in ethidium bromide (50 µl of 10 mg/ml solution in 1litre 1 X TBE buffer) for 45 min, and visualized and photographed on a UV transilluminator at 312 nm.

Only intensely stained unambiguous polymorphic bands were scored for presence (1) and absence (0). Missing data was recorded as ".". Scoring was carried out twice to ensure correct typing. The binary matrix data file created was then configured as an input file for data analysis. Nei's (1973) gene diversity (*H*), mean number of alleles per locus (*A*), percent of polymorphic loci (*P*), and genetic distance (*D*) for each population was derived using POPGENE 3.2 software (Yeh et al., 1999) assuming Hardy-Weinberg equilibrium. The genetic distance matrix was also used to generate the diversity trees using UPGMA method. The data matrix was also subjected to AMOVA to partition the genetic variation into within and among the population components using GenAlEx software (Peakall and Smouse, 2006).

RESULTS

Molecular analysis in *A. senegal*

Out of the 27 RAPD and 10 ISSR primers screened for their polymorphism, reproducibility and their capacity to differentiate among the four populations of *A. senegal*, the best 10 RAPD and 5 ISSR primers were chosen and allowed us to distinguish the populations (Table 2). Figure 2 shows some of the polymorphic bands generated by RAPD primer OPU-18 and ISSR primer 810. The generated band sizes had diverse molecular mass, with the RAPD primers ranging from 564 bp for primers OPU-20, OPU-18, OPU-10, OPD-18, OPW-18, OPW-11 and OPV-15 to 1690 bp for primer OPU-16. The largest number of polymorphic RAPD bands was detected by primer OPU-16 (eight bands), while the least was for primers OPD-18 and OPU-20 (one band each) (Table 2). The amplification products for ISSR primers ranged from 564 bp for primers 808, to 983 bp for primer 813. The largest number of polymorphic ISSR bands was amplified by primer 810 (five bands), while the least was amplified by primer 813 (two bands) (Table 2).

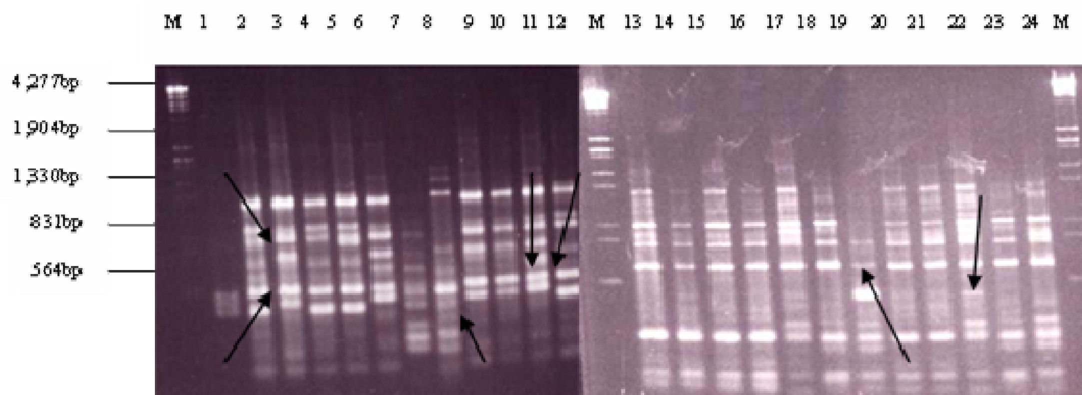

Figure 2. Profiles of *A. senegal* on agarose gel, amplified with RAPD primer OPU-18 (lane 1-12) and ISSR primer 810 (lane 13-24). M=Molecular marker, λ EcoRI/Hind III DNA. The arrows indicate some of the polymorphic bands

Table 3. The number of polymorphic loci (*N*), percentage of polymorphic loci (*P*), mean observed number of alleles (*A*) and Nei's gene diversity index *(H)* of the four populations of *A. senegal* based on the combined RAPD+ISSR data.

Population	*N*	*P* (%)	*A*	*H*
Garissa	46	82.1	1.821	0.260
Wajir	47	83.9	1.839	0.237
Samburu	42	75.0	1.750	0.210
Baringo	54	96.4	1.964	0.285
Mean	47	84.4	1.982	0.283

The species exhibited a high level of polymorphism, which was reflected in the number of polymorphic loci (*N*), percent polymorphic loci (*P*), mean observed number of alleles per locus (*A*) and Nei's (1973) gene diversity index *(H)* (Table 3). The number of polymorphic loci (*N*), ranged from 42 to 54 for the Samburu and Baringo populations, respectively. The combined markers also estimated high diversity indices in *A. senegal* based on Nei's (1973) gene diversity index (*H*). The gene diversity index ranged from 0.210 in the Samburu population to 0.285 in the Baringo population with a total population mean diversity of 0.283. Similarly, the percent polymorphic loci (*P*) was highest in the Baringo population (96.4%) and least in the Samburu population (75.0%).

Genetic differentiation

Genetic differentiation was assessed according to AMOVA and the genetic distance (*D*). The AMOVA performed on the 95 samples for the populations revealed that 14% of the variation was apportioned among the populations while 86% resided within the populations (Table 4). Though both the diversity partitioned components (i.e. between and within populations) were statistically significant, the data suggested that most of the genetic diversity in *A. senegal* occurs within populations.

The matrix of Nei's (1978) unbiased measures of genetic distance (*D*) is presented in Table 5. The smallest genetic distance was observed between the Samburu and Baringo populations (0.020), while the largest was between the Samburu and Garissa populations (0.063). The mean genetic distance for all the populations was 0.049. From the matrix, it is evident that the populations share most of the common alleles, since the genetic distances between them are so short. The relationships between the populations of *A. senegal* depicted by UPGMA dendrogram (Figure 3) constructed from Nei's genetic distance (Nei, 1978) was suggestive of a geographic grouping of the populations. Two groups were evident from the clustering, i.e., the Samburu and Baringo populations in one group while the Garissa and Wajir populations in the other (differentiated North Eastern province populations from those of Rift valley province populations).

DISCUSSION

Molecular variation within *A. senegal*

Much of the genetic diversity studies on African acacias have involved the use of isozymes, in which the gene diversity index (*H*) scores are mostly lower than that obtained in this study (mean *H*=0.283). For instance, in *Faidherbia albida* (syn. *Acacia albida)*, Dangasuk and Gudu (2000) found low levels of diversity (*H*=0.141) using isozymes. Isozyme studies have also indicated that the West African provenances of *A. senegal* var. *Senegal* show little variation (Boer, 2002). Lower *H* values were also obtained in four Argentinean species of *Acacia* by Casiva et al. (2002) using isozymes and RAPD markers. Similar to the range of our *H* value, Playford et al. (1993) found high levels of genetic diversity (0.208) in *Acacia*

Table 4. AMOVA for the four populations of *A. senegal* based on combined RAPD+ISSR data.

Source of variation	DF	SS	MS	Variance component	% Total variation	*p-value**
Among populations	3	127.119	42.373	1.457	14	<0.001
Within populations	91	833.747	9.162	9.162	86	<0.001
Total	94	960.866	51.535	10.619		

*After 999 random permutations.

Table 5. Combined RAPD+ISSR data Matrix of Nei's (1978) unbiased measures of genetic distance (*D*) for the four populations of *A. senegal*.

	Garissa	Wajir	Samburu
Wajir	0.049		
Samburu	0.063	0.047	
Baringo	0.062	0.052	0.020

melanoxylon population in association with a great genetic differentiation among geographic areas.

The percent polymorphic loci (*P)* values obtained in this study were by far higher than those observed in *Acacia caven* (29.4%) (Casiva et al., 2002), *Acacia anomala* (43%) (Coates, 1988) and *Faidherbia albida* (42.7%) (Dangasuk and Gudu, 2000). However, similar results were obtained in *Haloxylon ammodendron* (74.9%) by Sheng et al. (2005) using ISSR markers, in *Changium smyrnioides* (69%) by Fu et al. (2003) using RAPD markers and in *F. albida* (90%) reported by Joly et al. (1992) using isozymes.

The average number of alleles per locus (*A*) was 1.982 and less than that reported for *F. albida* (2.5) (Dangasuk and Gudu, 2000), and 3.02 reported by Joly et al. (1992) for *F. albida.* It was however, higher than that reported by Casiva et al. (2002) in *A. aroma* (1.2), *A. macracantha* (1.2) and *A. caven* (1.3).

The high level of genetic variation in *A. senegal* observed in this study is consistent with its wide geographic range as is also the case with other acacias. Genetic studies of other domesticated *Acacia* species throughout the world have identified many species with moderate to high levels of population differentiation that was geographically structured (Butcher et al., 1998). Genetic structuring reflecting the disjunct geographical distribution was ob-served in the tropical *A. mangium* (Butcher et al., 1998). Another tropical species, *A. auriculiformis*, can be differentiated into three geographical regions (Wickeneswari and Norwati, 1993) and some genetic differentiation was also observed in the tropical *A. tumida* (McDonald et al., 2003). The species complex *A. aulacocarpa*, which also occurs in tropical regions showed high levels of differen-tiation into five geographical regions (McGranahan et al., 1997) and a taxonomic revision of this complex has identified the presence of five taxa in the complex (McDonald and Maslin, 2000).

The Baringo population receives much rainfall (650 mm annually) as compared to the other three populations which receive 320, 350 and 475 mm for the Wajir, Garissa and Samburu populations, respectively (Table 1). The Baringo population is also widespread and extensive and occurs in pure stand (Maundu et al., 1999). The adequacy of rainfall especially for this arid and semi arid species within the Baringo population seems to have favored seed germination (dispersed by livestock ingestion and humans from other local populations) and subsequent growth of different genotype combinations of the tree species, resulting in a pure stand with a greater variability. This observation also supports the notion that tropical trees, especially those that are wide spread and occur at high densities maintain a high level of genetic diversity (Houston and Houston, 1993). So, the conclusion that spatial organization of local populations and the concomitant patterns of gene flow are important determinants of the level of genetic diversity within each population and of a species becoming genetically differentiated over its range (Yeh, 1989), might also apply to *A. senegal.*

The *A. senegal* populations in Kenya naturally occur in Arid and Semi Arid Lands (ASALs) comprising the savannah grasslands. These environments normally experience intensive natural selection pressure to ensure survival and adaptation to the harsh environmental conditions of the Sahel region (Dangasuk and Gudu, 2000; Pohlman et al., 2005), and this might have led to the high genetic diversity. In addition, the animal form of seed dispersal, common in the Sahelian region (Corbasson et al., 1985; Dangasuk and Gudu, 2000), might have contributed to greater genetic diversity as a result of increased level of gene flow between the local populations.

Genetic differentiation among populations is principally a function of gene flow among populations via pollen and seed dispersal. The significant ($p< 0.001$) proportion of among populations diversity (accounting for 14% of the total detected diversity) in this study could be a reflection of disjunction and lack of connectivity between populations due to geographical isolation. Accordingly, *A. senegal* maintains a high level of genetic diversity within populations, as expected of an out crossed widespread species. These results concur with those reported by Olng'otie (1992) in *Acacia tortilis* subspecies *heteracantha* of which 24% of the variation existed among the populations while 76% resided within the populations, and in *Acacia raddiana* where 11% of the variation was

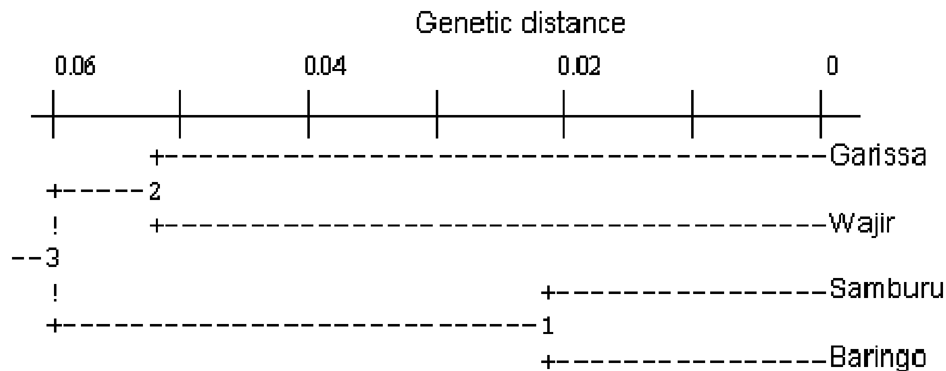

Figure 3. RAPD+ISSR Neighbour-joining dendrogram based on Nei's (1978) genetic distances.

found among the populations while 89% within the population. Plant species with outcrossed mating system and a wide geographic range have higher levels of genetic diversity than do selfers and endemic species. Likewise, species whose seeds are dispersed by animal ingestion or by wind maintain high levels of within-population genetic variability (Wachira et al., 2001; Tian et al., 2004). Except for wind pollination, *A. senegal* exhibits all these traits and is thus expected to maintain a high level of genetic diversity both at the species and population levels.

Based on the UPGMA dendrograms generated from Nei's (1978) genetic distance (*D*) matrices, two groups were evident; Garissa and Wajir populations clustered in one group, with Baringo and Samburu populations clustering in the other. This agreed with the geographic distribution of the species, with the geographically close populations having short genetic distance internodes in the dendrogram.

Differentiation of the populations at the loci level is a result of historical population processes, such as natural selection, mutation, isolation and genetic drift (Reed and Frankham, 2001). The molecular data found much of the species' variation residing within the populations. Interestingly, Karamalla et al. (1998) observed significant variation in gum arabic chemical properties among single trees within populations of *A. senegal*. This therefore, calls for correlation and provenance studies to be carried out to confidently give an insight into any congruence between the genetic relationships of this species and gum arabic composition. With the recent elucidation of the gum composition and property studies, selection should focus on high yields and quality as the case may be. Molecular markers have roles to play in constructing a useful population classification of the species, as well as providing important baseline information for breeding strategies and gene pool management plans as has been depicted in this study. Although rapid genetic gain could be attained through selection of individual trees within populations, sampling strategies for improvement and conservation should incorporate a high number of individual trees within populations and also ensure a comprehensive coverage of the entire population ecological amplitude.

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