Genetic Variability Analysis of the Polyploid Complex of *Acacia nilotica* (L.) Willd. Using RAPD Markers

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Summary

The genetic variability of nine Acacia nilotica *subspecies of various origins was analyzed by thirty-six RAPD primers. Sixteen among them produced polymorphic bands and generated 166 polymorphic markers. The amplified bands were separated by electrophoresis on 1.8% agarose gel. The analysis of 166 RAPD markers allowed to distinguish essentially three main groups in* Acacia nilotica *complex: (i) The first group comprised subspecies,* indica*,* cupressiformis*,* nilotica*,* tomentosa*. Subspecies* subalata *and* jacquemontii *of which the systematic position is unclear seems to belong to this group. (ii) The second group comprised subspecies* adstringens *and* leiocarpa*. (iii)* Kraussiana *subspecies distinguishes itself from first two groups. The genetic variability within populations (H) was calculated through Shannon index. Subspecies* adstringens *presents the lowest within population variability (H= 0.015) while* kraussiana *and* leiocarpa *subspecies showed high variation index (H= 0.095) and (H= 0.096) respectively. The genetic variability analysis of* Acacia nilotica *revealed large differences between subspecies but no correlation between geographic distances and genetic distances could be established. Subspecies native of east Africa presented higher allelic richness than west African and Indian ones.*

Résumé

Analyse de la variabilité génétique du complexe polyploïde *Acacia nilotica* (L.) Willd. à l'aide de marqueurs RAPD

*La diversité génétique de neuf sous-espèces d'*Acacia nilotica *de différentes origines a été analysée à l'aide de trente-six amorces RAPD. Seize parmi elles ont produit des bandes polymorphes. Les amorces choisies ont ainsi généré un total de 166 marqueurs polymorphes. Les bandes amplifiées ont été séparées par électrophorèse sur gel d'agarose à 1,8%. L'analyse des 166 marqueurs RAPD a permis de distinguer essentiellement trois groupes dans le complexe* Acacia nilotica *quel que soit l'indice de similarité utilisé: (i) le premier groupe est constitué par les sous-espèces,* indica*,* cupressiformis*,* nilotica*,* tomentosa*. Les sous-espèces* subalata *et* jacquemontii *dont la position systématique est confuse semblent appartenir à ce groupe; (ii) le second groupe est constitué par les sous-espèces* adstringens *et* leiocarpa *et (iii) la sous-espèce* kraussiana *se distingue des deux premiers groupes. La valeur de la diversité génétique intra population (H) a été calculée grâce à l'index de Shannon. La sous-espèce* adstringens *présente le plus faible indice de diversité intra population (H= 0,015) tandis que les sous-espèces* kraussiana *et* leiocarpa *ont montré respectivement des indices élevés (H= 0,095) et (H= 0,096). Ainsi, le pourcentage de loci polymorphes varie de 3,72% (ssp* adstringens*) à 25,53% (ssp* leiocarpa *et* kraussiana*). L'analyse de la variabilité génétique des sous-espèces d'*Acacia nilotica *a révélé de grandes différences entre sous-espèces mais aucune corrélation entre les distances géographiques et les distances génétiques n'a été établie. Les sous-espèces originaires d'Afrique de l'est ont présenté une plus grande richesse allélique que les sous-espèces ouest africaines et indiennes.*

Introduction

A major goal of conservation biology is the preservation of genetic diversity in order to maintain the evolutionary potential of the species (4). The use of genetic data to determine evolutionary relationships between populations and species can make significant contribution to conservation (15).

Acacia nilotica is a multipurpose tree mainly distributed in the warm arid and semiarid regions of the world (11, 26). It is also a polyploid complex, which plays an essential role in the rural economy through the supply of wood, by-products, stabilization and the fertilization of soils (13, 29). Taxonomy was studied in detail by Brenan (5) who divided the species in nine subspecies among which 3 are native from India and 6 from Africa. The subspecies can be differentiated mainly on the shape, the size and degree of pubescence of pods and shape of the crown. Subspecies *adstringens*, *kraussiana*, *subalata*, *hemispherica* and *leiocarpa* are characterized by pods not necklace-like, the margins of the pods are straight, crenate or sometimes irregularly constricted. Subspecies *nilotica*, *tomentosa*, *cupressiformis* and *indica* are characterized by pods necklace-like, narrowly and regularly constricted between the seeds (5, 10).

Presently, there are very few studies concerning the genetics of polyploid populations especially at the level of forest trees (3, 8, 13). Genetic variability of natural populations of *Acacia* is still little known (7, 9, 17, 16, 21, 26). The knowledge of this parameter is however an indispensable preliminary to conservation strategy definition, management and durable use of these forest resources which are threatened with disappearance in tropical dry lands (14). Genetic diversity is required for populations to evolve to cope with environmental change, and loss of genetic diversity is often associated with reduced reproductive fitness (15).

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Table 1
Location of the origin of *Acacia nilotica* subspecies seed samples analysed

Subspecies	Country	Locality	Latitude	Longitude
Tomentosa	Senegal	Guanguel	15°23'00 N	12°57'14 W
Tomentosa	Senegal	Donaye	15°36'37 N	14°52'92 W
Adstringens	Senegal	Dahra	15°21'00 N	15°27'00 W
Nilotica	India	Pune	18°32 N	73°51 E
Indica	India	Pune	18°32 N	73°51 E
Cupressiformis	India	Pali	18°34 N	73°18 E
Subalata	India	Pune	18°32 N	73°51 E
Leiocarpa	Kenya	Sabaki	03° 09 S	40° 08 E
Kraussiana	Zimbabwe	Chabalala	20° 00 S	30° 00 E
Jacquemontii	India	Pune	18°32 N	73°51 E
Jacquemontii	India	Ajamgahr	26°03 N	83°30 E

In the current paper, we focus on the analysis and interpretation of genetic diversity of nine *Acacia nilotica* subspecies and examine the population's structure of this polyploid complex. This is the first genetic analysis on almost all *A. nilotica* subspecies.
The study is realized on the populations of Senegal and other origins from east Africa and India to support the comparison and have a global vision of the genetic diversity organization of this polyploid complex on all its geographical distribution area (Table 1).

Materials and methods

- Plant material
A total of 180 seeds from the 9 subspecies were analyzed. Each subspecies was represented by 20 seeds originating from one or two populations. When the descents were separated, one seed was selected by tree and for twenty trees. In the opposite situation, seeds came from bulk.

- Seed germination and DNA extraction
Seeds were treated with concentrated sulphuric acid (95% v/v) for one or two hours (according to subspecies) and then soaked in water overnight to break dormancy. Seeds were allowed to germinate in an incubator (temperature maintained at 27 °C) during two days. Seeds were then transferred in boxes containing a sand and compost mixture and kept at room temperature (around 25 °C). After two or three weeks, the first leaves were available for genomic DNA extraction. A total of 100 mg of fresh leaves was taken from young plantlets for extraction using Bousquet *et al*. (6) protocol. Total DNA was estimated on 1% agarose gels and the quantity was determined using a fluorimeter. DNA was then diluted in sterile water to a concentration of 3 ng/µl for use in amplification reactions.

- DNA amplification
Thirty-six arbitrary primers were screened for their suitability for RAPD analysis using a small number of samples from different *Acacia nilotica* subspecies. Sixteen primers able to detect clearly resolved within or between subspecies

Table 2
Number of RAPD *loci*, primers sequences, amplified polymorphic fragments and phenotype generated by 16 arbitrary primers

Primers code	Primers sequences 5' to 3'	No. of RAPD bands generated	No. of polymorphic bands
SC 10/14	TCCCGACCTC	9	9
SC 10/16	CCTGGCGAGC	11	10
SC 10/17	GTTAGCGGCG	10	10
SC 10/19	CGTCCGTCAG	8	8
SC 10/22	CTAGGCGTCG	10	9
SC 10/23	GGCTCGTACC	9	9
SC 10/24	ACCCATGCGG	11	11
SC 10/25	CGGAGAGTAC	12	11
SC 10/30	CCGAAGCCCT	15	13
OPA-M05	GGGAACGTGT	9	9
OPA-N12	CACAGACACC	14	13
OPA-Y04	GGCTGCAATG	12	12
OPA-Y05	GGCTGCGACA	17	17
OPA-Y11	AGACGATGGG	13	13
OPA-Y15	AGTCGCCCTT	13	13
OPA-Y17	GACGTGGTGA	10	10
Total		183	177

polymorphism were selected for further analysis (Table 2).
PCR was carried out in a final volume of 25 µl containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2 mM $Mgcl_2$, 0.1 mM of each dNTP, 0.56 µM primer, 25 ng genomic DNA and one unit of Taq DNA polymerase. Amplifications were performed in 96-well plates using a Techne-Cyclogene p96 thermal cycler programmed as follows: an initial denaturation step at 94 °C for 4 min, 35 cycles each at 92 °C for 45 sec, 40 °C for 45 sec and 72 °C for 1.45 mn and a final extension step at 72°C for 10 min.
Amplifications were performed twice for some samples to ensure reproducibility of banding patterns. (The usual precaution was taken to prevent contamination of PCR experiments with previously amplified fragments. In particular, pre- and post- amplification procedures were carried out separately and fresh aliquots of reagents were used for each experiment wherever possible). To test the reliability of PCR products, two controls were included in each set of reactions, one control containing all components except genomic DNA and the second control containing genomic DNA and all components except Taq polymerase. No amplification occurred in any of these controls. Amplification products were separated on 1.8% agarose gels stained using ethidium bromide and photographed under UV light with Polaroid film.

- Data analysis

The presence and absence of RAPD bands were coded [1] or [0] respectively regardless of band intensities and used to construct data matrix. The Sokal and Michener index of similarity S (25) was calculated between all individuals using the NTSYS-PC software (24). This coefficient considers both positive and negative matches. We calculated distances as 1-S and we constructed a neighbour-joining (NJ) dendrogram with the unweighted pair group method (UPGMA) using the DARwin software (23).

The Shannon's index ($Hs= -\sum p_i \ln p_i$) and Nei's index ($H= n(1-\sum p_i^2)/n-1$) where pi in both indices is the frequency of the *i*th RAPD band, as well as the percentage of polymorphic loci (P) were calculated for each subspecies using Popgen 1.32 (30).

The AMOVA analysis was conducted to estimate variance components at different hierarchical levels, partitioning the variation among groups of subspecies (group "pods necklace-like" *vs.* group "pods not necklace-like"), among subspecies and among individuals within subspecies using WINAMOVA 1.55 (12). Input files of dominant RAPD markers were prepared using AMOVA-PREP (20).

Factorial correspondence analysis: In order to obtain a synthetic picture of the organization of the genetic diversity a multiple correspondence analysis was carried out on a matrix of presence [1] and absence [0] of all the RAPD bands using the Corresp procedure of the SAS software Version 8.

Results

- Genetic diversity

The data for RAPD analysis were scored from photographs of the ethidium bromide stained agarose gels. Bands were considered to be the same if they occurred at exactly the same position on the electrophoresis gel (Figure 1). The sixteen primers chosen for analysis were assumed to be a random sample of the genome and generated a total of 188 bands ranging from 8 with SC10/19 to 17 with OPA-Y05 and an average of 11.8 bands per primer. The patterns of RAPD fragments produced by the primer SC10/30 is shown in figure 1 as exemple. Of the 188 bands, 177 (94.2%) were polymorphic in at least one of the subspecies studied.

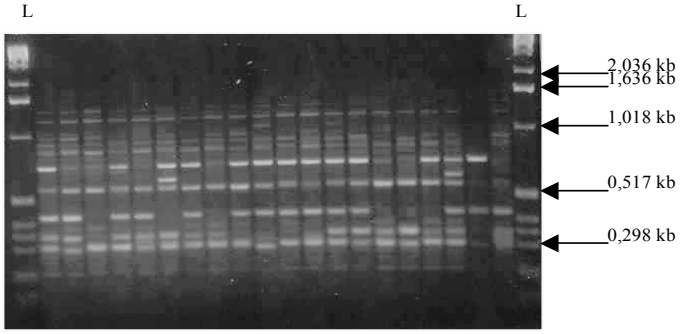

Figure 1: Example of RAPD profile obtained with the primer SC 10/30 on *Acacia nilotica* ssp *kraussiana*. L = ladder.

Table 3
Estimates of phenotypic diversity for *Acacia nilotica* ssp.

Subspecies	N	No. polymorphic *loci*	PI (%)	Na	Ne	H	I
adstringens	20	7	3.7	1.037	1.027	0.015	0.022
				0.190	0.143	0.079	0.114
kraussiana	20	48	25.5	1.255	1.166	0.095	0.140
				0.437	0.322	0.175	0.252
leiocarpa	20	48	25.5	1.255	1.169	0.096	0.141
				0.437	0.323	0.177	0.254
subalata	20	48	25.5	1.255	1.125	0.078	0.121
				0.437	0.259	0.147	0.220
group 1	80	167	88.8	1.888	1.614	0.347	0.509
				0.316	0.336	0.163	0.220
cupressiformis	20	10	5.3	1.053	1.027	0.016	0.024
				0.225	0.143	0.078	0.113
Indica	20	15	8.0	1.080	1.052	0.029	0.043
				0.272	0.196	0.108	0.156
jacquemontii	20	13	6.9	1.069	1.041	0.024	0.036
				0.254	0.164	0.095	0.140
nilotica	20	13	6.9	1.069	1.050	0.027	0.040
				0.254	0.204	0.107	0.152
tomentosa	20	27	14.4	1.144	1.095	0.053	0.079
				0.352	0.262	0.141	0.202
group 2	100	114	60.6	1.606	1.332	0.199	0.301
				0.490	0.354	0.193	0.278
all ssp	180	176	93.6	1.936	1.561	0.328	0.491

N: Number of analysed seeds
No: Polymorphic *loci* number of polymorphic loci
PI: Polymorphic *loci*: Percentage of all *loci* that are polymorphic regardless of allele frequencies.
Na: Allele Number, counts the number of alleles with non zero frequency.
Ne: Effective Allele Number, estimates the reciprocal of homozygosity.
H: Gene Diversity: Estimates Nei's gene diversity.
I: Shannon Index, Estimates Shannon's information index as a measure of gene diversity.

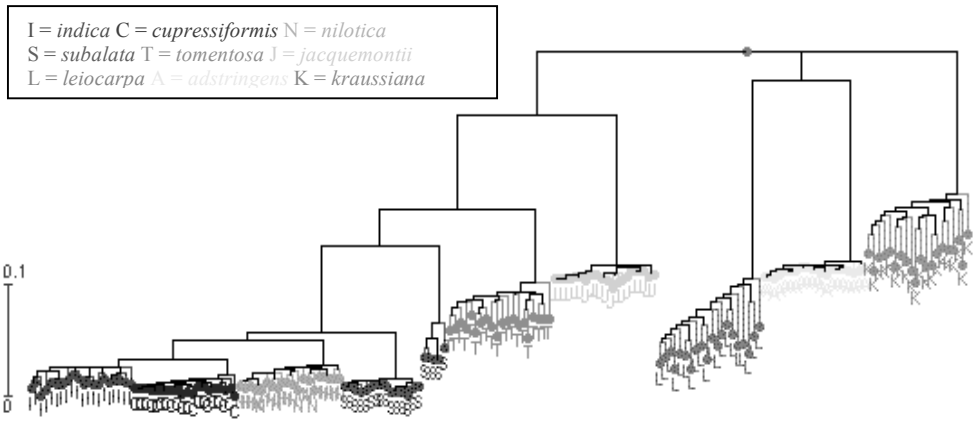

Figure 2: Dendrogram showing dissimilarities (Sokal and Michener) among *Acacia nilotica* subspecies based on RAPD data from 16 primers. The phenogram was build according to the unweighted pair-group method with neighbour joining mean.

Pourcentage of polymorphic RAPD loci varied from 3.7% (*adstringens*) to 25.5% (*subalata*, *leiocarpa* and *kraussiana*). The average percentage of polymorphic *loci* was lower in the group of subspecies with necklace like pods (41%) compared to the group of subspecies with not necklace like pods (90%).

- Genetic diversity within subspecies

The subspecies *adstringens* showed the lowest within population diversity (H= 0.015), whereas, ssp. *kraussiana* and ssp. *leiocarpa* were the most diverse with respectively (H= 0.095) and (H= 0.096) while the percentage of polymorphic RAPD *loci* varied from 3.72% (ssp. *adstringens*) to 25.53% (ssp. *subalata*, *leiocarpa* and *kraussiana*) (Table 3).

- Genetic diversity among subspecies

The cluster phenogram obtained by the UPGMA algorithm divided the subspecies of *Acacia nilotica* into three main groups (Figure 2). One comprises the necklace-like pods (*tomentosa*, *cupressiformis*, *indica*, *nilotica*), and the other the non necklace-like ones. Ssp. (*adstringens* and *leiocarpa*). Ssp. *kraussiana* differs from other subspecies and forms a separate group. The ssp. *jacquemontii* and *subalata* which systematic position is not clear seems to belong to the first group.

The multiple correspondence analysis confirms the great differentiation between subspecies on the 1-2 plan (Figure 3). Subspecies with whole pods (*leiocarpa*, *adstringens* and *kraussiana*) and subspecies with pearl pods (*tomentosa*, *nilotica*, *cupressiformis, indica* and *jacquemontii*) are clearly differentiated and distributed into two different groups.

The partitioning of genetic variation examined by AMOVA (12) showed that, although most of the variation (62%) was found among subspecies within groups. A significant proportion was attributable to differences among groups (26%) and only (12%) represent the variation within subspecies. The differentiation assessed among subspecies was marked, 87% of the variation was present among subspecies and only 13% within subspecies. Nearly 60% of the variation was among groups (Table 4).

Discussion

RAPDs constitute a successful tool for the analysis of both among and within subspecies diversity.

- Impact on *Acacia nilotica* taxonomy

Patterns of genetic diversity using RAPD markers have shown that: (i) the majority of RAPD variations detected was partitioned among subspecies; (ii) the necklace-like pods group present lower variability than the none necklace-like ones; (iii) subspecies from east Africa present a higher variability than those native of western Africa.

In spite of subspecies *subalata* and *jacquemontii* which have a systematic vague position, the genetic diversity analysis of *Acacia nilotica* complex made from 166 RAPD markers allowed classifying the nine subspecies in three groups. A first group would gather subspecies *nilotica*, *cupressiformis*, *indica*, *tomentosa*, *subalata* and *jacquemontii* whereas the second group would be established by subspecies *adstringens* and *leiocarpa*. Ssp. *kraussiana* distinguishes itself from other subspecies and forms a separate group.

The existence of individuals belonging to both genetically different under groups within the *subalata* subspecies could confirm the hypothesis of Ali and Feruqi (1, 2), according to which *Acacia nilotica subalata* is an hybrid between ssp. *hemispherica* and ssp. *indica*. To verify this hypothesis, it would be interesting to analyse later samples of the ssp. *hemispherica*

Table 4
AMOVA (12) for all 180 individuals of *Acacia nilotica* from 9 subspecies, using 166 RAPD markers

Sources of variation	d.f.	Variance component	% variation	P-value
Nested analysis				
Among groups	1	10.02	26	0.0280
Among subspecies within group	6	23.58	62	< 0.0002
Within subspecies	152	4.69	12	< 0.0002
Analysis among subspecies				
Among subspecies	8	30.04	87	< 0.0002
Within subspecies	171	4.36	13	
Analysis among groups				
Among groups	1	15.69	41	
Within groups	158	22.60	59	

Degrees of freedom (d.f.) and the significance (P-value) of the variance components are shown.

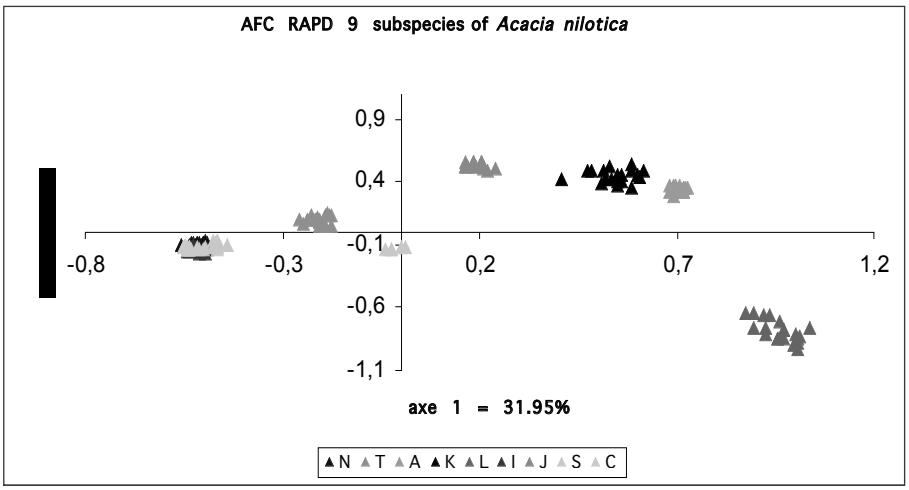

Figure 3: Factorial Correspondence Analysis of nine *Acacia nilotica* subspecies using the CORRESP of SAS software version 8.

Moreover, there does not seem be correlations between the genetic distances and the geographic distances. In fact, *tomentosa* subspecies native from Senegal is genetically closer to subspecies from India. It notably presents many similarities with the *nilotica* subspecies from Pali (India) and the subspecies *indica* and *cupressiformis* from India. The subspecies *adstringens* native from Senegal is also genetically close to *leiocarpa* subspecies native from east Africa. Subspecies *adstringens* and *tomentosa* native from Senegal are genetically very remote from each other in the dendrogramm, they belong to two different groups. The same observation was made during previous studies on the genetic variability of seven *Acacia nilotica* subspecies by isoenzymatic electrophoresis (22).

These studies confirm that the subspecies *jacquemontii* belongs to the *Acacia nilotica* complex in opposition to what was advanced by Joustra and Ben Salem in Brenan (5) who considered it as a separate species. Indeed, according to the revealed RAPD markers, there are several common fragments between this subspecies and subspecies with necklace-like pods. The axis 1/2 seems to confirm this tendency while individualizing the *subalata* subspecies although the axis 1/3 put in evidence the genetic similarities of subspecies *nilotica*, *indica*, *tomentosa* and *cupressiformis*.

The subspecies *leiocarpa* and *kraussiana* native respectively from Kenya and Zimbabwe (East Africa) presents the highest percentages of polymorphic *loci* (PI= 25%) in *A. nilotica* complex. If we consider this strong rate of polymorphism as resulting from very advanced level of polyploidy of these subspecies, we can emit the hypothesis that according to the samples analysed during this study; these two subspecies are the most evolved in the *A. nilotica* complex. Certainly, this strong rate of polymorphism is a high factor of differentiation, which plays an essential role in the evolutionary process of the species. These results are in concordance with those from Mandal *et al*. (18) and Mandal and Ennos (19).

In parallel to this high allelic richness, we notice a low polymorphism index particularly at the level of the subspecies *adstringens* with only 3.72%. This low percentage of polymorphic *loci* was already evidenced by isoenzymatic electrophoresis technique (22); which let us suppose that this subspecies, in spite of its polyploidy, would have a preferentially autogame reproduction system. Molecular data cannot prove that the low genetic variation present in some populations of *A. nilotica* subspecies is a cause of concern for its viability in the wild.

- Implication of RAPD data for conservation management of the species

Effective and rigorous measurements are necessary to reverse the current tendencies leading to an impoverishment of genetic resources. Those must be based on a better understanding of the species as well as the ecosystems, extent of biological diversity and genetic variation. It is crucial to support the dynamic interaction between subspecies and their environment because it constitutes a genetic source of diversity.

The organization of genetic variability of *Acacia nilotica* complex revealed in our study is a parameter, which will have to be integrated in the future into any strategy of durable management of these natural resources. Our researches lead to a better understanding of the mechanisms of evolutionary polyploid complex with interesting possible applications in the management of the genetic resources.

Conclusion

The management of *Acacia nilotica* genetic resources should pass by the creation of protected areas even from natural reserves in the zones of strong anthropological pressure such as the valley of Senegal River. So, the creation of seed bank and the elaboration of replacements plantations will allow meeting the needs of timber local populations and by-products. These conservation strategies as well *in situ* and *ex situ* will have to support the gene flow between subspecies and populations. They will maintain their adaptation and evolutionary potentials in their environment. *Acacia nilotica* is a potentially interesting species for the reforestations programs. However, it is important to select subspecies to be targeted. Indeed, the interpretation of data relative to *Acacia nilotica* raises problems if we do not clarify the subspecies about which we speak. Nowadays, the contribution of rural communities in forest resources management begins to appear as an inevitable element in the forest resources management strategies. Conservation and management of natural *Acacia nilotica* populations is essential in environment stabilisation and fighting against drought.

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