

Full Length Research Paper

Analysis of the impact of domestication of *Warburgia ugandensis* (Sprague) on its genetic diversity based on amplified fragment length polymorphism

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Warburgia ugandensis Sprague (Canellaceae) occurs in East and Central Africa and is an important multipurpose tree species. Over-exploitation of natural forests for medicinal purposes and clearance for farming threaten the species survival. Cultivation of the tree species would ensure sustainable medicinal source and its conservation. However, on-farm genetic diversity of the species is currently unknown. The genetic diversity of the on-farm *W. ugandensis* populations and their proximate natural populations were analyzed using the amplified fragment length polymorphism (AFLP). Four primer combinations produced a total of 223 polymorphic bands. Both the natural and on-farm populations had high genetic diversity ranging from $H = 0.2892$ to $H = 0.1278$. Principal co-ordinates analysis and dendrogram separated the ten populations into two major groups corresponding to Kenyan and Tanzanian populations, respectively. Ugandan populations were shared between the two major groups; this is probably because Uganda is believed to be the centre of diversity for *W. ugandensis*. Close genetic relationships between the on-farm and their proximate natural population were revealed. Analysis of molecular variance (AMOVA) revealed that a total of 54% AFLP variation resided within populations with 46% reside among populations. The high genetic diversity of *W. ugandensis* on-farm populations could be useful in germplasm collection and conservation strategies.

Key words: *Warburgia ugandensis*, amplified fragment length polymorphism (AFLP), domestication, genetic diversity, on-farm, natural.

INTRODUCTION

Warburgia ugandensis Sprague (Canellaceae) is a multi-purpose tree found in the lowland rainforest and upland

dry evergreen forest of Eastern and Southern Africa (Orwa et al., 2009) and is highly valued for its medicinal

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properties. It has a mixed mating system, predominantly outcrossing and pollinated by small bees (e.g. *Trigona*) and other insects (Muchugi et al., 2008). Natural forests inhabited by this species are under threat of destruction by clearing for farming and unsustainable exploitation for medicinal purposes (Maundu and Tengnas, 2005). Cultivation of the species in plantations and on-farm has been advocated to ensure sustainable supply of the herbal products and its conservation (Okigbo et al., 2008). The species is propagated through seeds, stem cuttings (Akwatuilina, 2011) and tissue culture (Kuria, 2012). A prerequisite for the efficient use of genetic resources in any planting program is a detailed understanding of the genetic diversity and the extent of distribution of genetic variation available within a species (Muchugi et al., 2012). A wide genetic base provides the ability to withstand potential inbreeding depression through future generations of farmer propagation (Frankham, 2010). However, little is known about the influence of cultivation on genetic diversity within the species and whether on-farm stands are suitable sources of material for further planting.

Molecular markers have proven to be invaluable tools for assessing plants' genetic resources by improving our understanding with regards to the distribution and the extent of genetic variation within and among species (Ilga and Yousry, 2014). Previously, genetic diversity studies on *W. ugandensis* have been carried out using random amplified polymorphism (RAPDS) (Wamalwa et al., preliminary report) and amplified fragment length polymorphism (AFLP) (Muchugi et al., 2012, 2008).

AFLP technique is a powerful method for marker analysis genetic diversity investigations. It targets large loci due to several primer pair combination that can be used. In addition, AFLP markers are highly polymorphic reproducible and do not require prior sequence information of the species under investigation.

In the present study, the genetic diversity in on-farm and proximate natural populations of *W. ugandensis* was investigated. Genetic partitioning among 10 populations in East Africa was evaluated using AFLP markers in an attempt to determine possible effects of domestication on the genetic structure of this species and to provide backing for management and conservation of these populations. The data will be important for future exhaustive germplasm core collections for breeding and conservation purposes.

MATERIALS AND METHODS

Sample collection

Samples were collected from natural forest populations and their proximate on-farm stands in East Africa (Table 1). Sampling for the natural trees was done at random, separated by a minimum distance of 100 m while farm trees were collected from single individuals from a series of separate small farms and did not have a minimum distance requirement. Three young leaves were

collected from each tree, cleaned using 70% ethanol, dried with silica gel and stored at -20°C, prior to DNA isolation.

DNA extraction and AFLP analysis

Total genomic DNA was extracted from approximately 30 mg of silica-dried leaves following a modified cetyltrimethyl ammonium bromide (CTAB) (Doyle and Doyle, 1987). The integrity and quality of the DNA was checked using 0.8% (w/v) agarose gel and NanoDrop[®] nd-1000 spectrophotometer (Thermo Fischer Scientific, Wilmington, DE, USA) and diluted in TE buffer (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA) to a final concentration of 200 ng/μl. The AFLP method was carried out following the standard procedure described by Vos et al. (1995) and adapted in the AFLP[™] Plant mapping protocol of the Applied Biosystems (ABI), USA. First, 200 ng of genomic DNA was digested with *EcoRI* and *MseI* restriction enzymes, followed by ligation of adapters to the restriction fragments. A pre-amplification of the primary templates was done with *EcoRI* and *MseI* primers having three selective nucleotides at the 3' end. A selective amplification was performed on the pre-amplified fragments with fluorescent-labeled *EcoRI* primers having three selective nucleotides at the 3' end and *MseI* primers having three selective nucleotides at the 3' end. The fluorescent-labeled primers were synthesized by Applied Biosystems (ABI), USA. The selective amplification was performed in a MJ Research PTC-200 DNA Engine thermocycler using the following amplification profile. The selective PCR Amplification was programmed for an initial 2 minutes at 94°C followed by one cycle of 94°C for 20 s, 66°C for 30 s and 72°C for 2 min. This cycle was repeated 12 times with a lowering of the temperature of 1°C per cycle. This was followed by 20 cycles of 94°C for 20 s, 56 °C for 30 s and 72°C for 2 min and a further hold time of 30 min at 60°C. The AFLP product was prepared for analysis in ABI prism 3730 DNA analyzer by making a 1:3 dilution. A loading buffer mix was prepared by adding 10 μl of gene scan 500 LIZ internal size standard ABI to 990 μl deionised formamide. Nine microliters (9 μl) of the size standard mix was added to 1 μl of the diluted selective amplification products in a micro-Amp PCR plate. The amplification products were first denatured by heating at 95°C then cooling in ice for 5 min before loading onto the genetic analyzer. From the ABI PRISM 3730, the sample data was directed to the GeneMapper[™] software to analyze and display the sizing results as electrograms and tabular data. The four *EcoRI:MseI* primer combinations were selected based on the previous AFLP analyses in *W. ugandensis* (Muchugi et al., 2008).

Statistical analysis

The resulting fragments from the ABI Prism 3730 automated sequencer (Applied Biosystems) were described and screened for quality using Gene Mapper 3.7 software. GeneMapper generated allele frequency data for all individuals as product presence (1) or product absence (0) which were then converted into Microsoft Excel spread sheet. Population allele frequency diversity values and genetic distance (D) between population were computed in TFPGA 1.3 (Miller, 1997). Principal coordinates analysis (PCoA) was performed using GenAIEx 6.3 (Peakall and Smouse, 2009) software to provide visual representation of genetic structure. Cluster analysis using an unweighted pair-group method with arithmetic averaging (UPGMA) (Sneath and Sokal, 1973) was used to generate a dendrogram showing relationship among populations based on genetic distance (Nei, 1978). The analysis of molecular variance (AMOVA) (Excoffier et al., 1992) based on pHP-T-statistic was generated by GenAIEx 6.3 using 999 permutations.

Table 1. Details of ten populations sampled for *W. ugandensis* for AFLP analysis

Country	Population	GPS reading	Altitude (M)	Population type	Number of samples	Population analysis
Kenya	Cherangani	N 01° 00' E 035° 01'	2189	On-farm	15	1
	Rumuruti	N 00° 07' E 036° 25'	2100	On-farm	15	2
	Kitale	N 01° 00' E 035° 01'	2189	Natural	15	3
	Rumuruti	N 00° 07' E 036° 25'	2100	Natural	15	4
Uganda	Mabira	N00° 22.798' E033° 04.804'	1215	Natural	22	5
	Mabira	N00° 22.798' E033° 04.804'	1215	On-farm	12	6
	Kibale	N00°13' E30° 19'	1300	Natural	20	7
Tanzania	Lushoto	S 04° 35' 05"E 38° 11' 33"	1780	Natural	16	8
	Kagera	S 01° 05' 07"E 31° 31' 06"	1167	Natural	16	9
	Lushoto	S 04° 38' 54" E 38° 14' 42"	1950	On-farm	17	10

Table 2. Mean diversity estimates (H) for 10 populations of *W. ugandensis* generated from 223 AFLP markers (the percentage polymorphic loci and sample size (N) are shown).

Country	Provenance	N	H	Polymorphic loci (%)
Kenya	Rumuruti on-farm	15	0.1278	37.22
	Rumuruti natural	15	0.1787	59.64
	Cherangani on-farm	15	0.2119	69.96
	Kitale natural	15	0.1464	50.22
Uganda	Mabira natural	22	0.1946	63.23
	Mabira on-farm	12	0.1963	57.85
	Kibale natural	20	0.2892	88.79
Tanzania	Lushoto natural	17	0.2210	63.68
	Lushoto on-farm	16	0.2431	74.89
	Kagera natural	15	0.2195	61.88

RESULTS

Estimation of genetic diversity

The mean Nei's genetic diversity for all the 223 loci in the 10 populations ranged from $H = 0.1278$ (Rumuruti on-farm) to $H = 0.2892$ (Kibale natural) as shown in Table 2. The percentage of polymorphic loci was proportional to the genetic diversity estimate; populations with higher diversity estimates showed higher percentages of polymorphic loci.

Generally, higher genetic diversity was observed for on-farm stands than in their proximate natural counterparts. For instance, Cherangani on-farm had higher genetic diversity estimate ($H = 0.2119$) than its proximate Kitale natural population ($H = 0.1464$), same results were shown for Lushoto on-farm ($H = 0.2467$) and Lushoto natural ($H = 0.2210$). Mabira on-farm ($H = 0.1963$) was slightly higher than proximate Mabira natural ($H = 0.1946$) while Rumuruti natural ($H = 0.1787$) was

higher than proximate Rumuruti on-farm ($H = 0.1278$). Among all the natural populations, Kitale had unusually low genetic diversity estimate ($H = 0.1464$) with Kibale natural having the highest ($H = 0.2892$). The level of genetic diversity as shown by Shannon's diversity index was average for most of the populations ranging from 0.1920 (Rumuruti on-farm) to 0.4000 (Kibale natural) (Table 2). Based on the mean polymorphism of the loci within population, the diversity ranged from 37.22 (Rumuruti on-farm) to 88.79% (Kibale natural) (Table 2).

Genetic relationships and population structure

Principal coordinates analysis (PCoA) was performed to visualize regional genetic structure of *W. ugandensis* based on the 10 populations. PCoA resolved the genetic groups on two axes, accounting for 73.93% of the overall variation (Figure 1). The first and second axes explained 60.70 and 13.23%, respectively, of the total variation

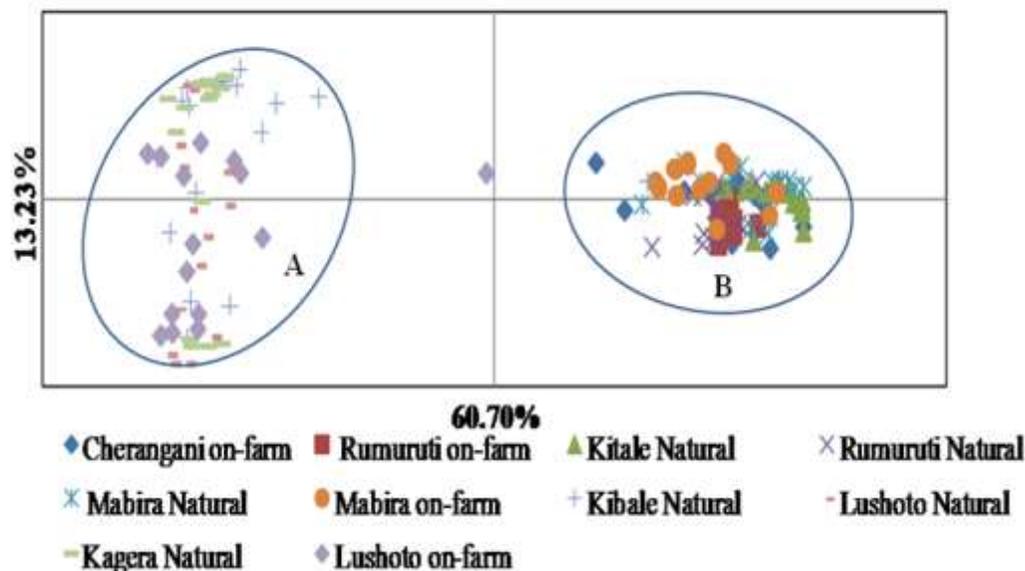


Figure 1. Principal co-ordinate analysis of 163 *W. ugandensis* from Kenya, Uganda and Tanzania.

Table 3. Matrix of unbiased genetic distance according to Nei (1978) among 10 populations of *W. ugandensis* based on 223 AFLP markers.

Population	Cherangani-on-farm	Rumuruti-on-farm	Kitale-Natural	Rumuruti-Natural	Mabira-Natural	Mabira-on-farm	Kibale-Natural	Lushoto-Natural	Kagera-Natural
Cherangani-on-farm									
Rumuruti-on-farm	0.1622								
Kitale-Natural	0.0485	0.1472							
Rumuruti-Natural	0.1498	0.0531	0.1399						
Mabira-Natural	0.0612	0.1428	0.0425	0.1014					
Mabira-on-farm	0.0929	0.1813	0.1182	0.1215	0.0463				
Kibale-Natural	0.4367	0.5231	0.5342	0.4979	0.4959	0.4823			
Lushoto-Natural	0.6269	0.6721	0.7587	0.6424	0.7059	0.6651	0.0642		
Kagera-Natural	0.6042	0.6621	0.7412	0.6282	0.6866	0.6442	0.0445	0.0496	
Lushoto-on-farm	0.5796	0.6565	0.7185	0.6221	0.6718	0.6333	0.0566	0.0246	0.0365

between cluster A and B. One cluster consisted of Lushoto (natural), Lushoto (on-farm) and Kagera (natural) while the other consisted of Mabira (on-farm), Mabira (natural), Rumuruti (on-farm), Rumuruti (natural), Cherangani (on-farm), Kitale (natural), with Kibale population splitting in the two groups (Figure 1).

Genetic variation within and among the 10 populations of *W. ugandensis* populations was quantified by analysis of molecular variance (AMOVA). Partitioning of genetic variability by AMOVA revealed that most of the AFLP variation was found within populations (54% $P < 0.01$) while the remaining resided among populations (46%

$P < 0.01$) (Table 3).

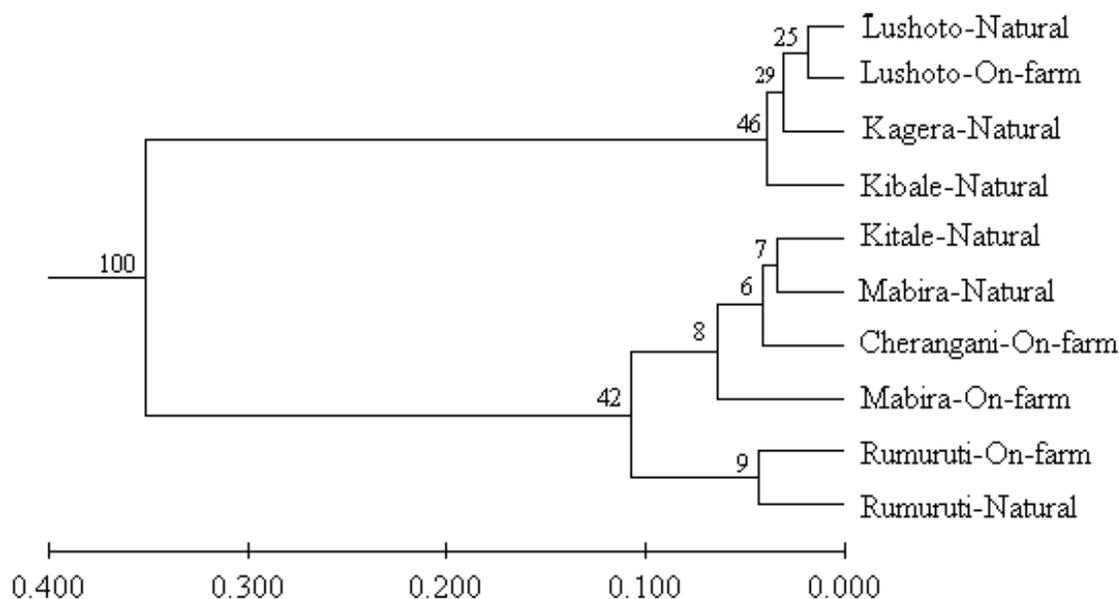
Genetic differentiation

A dendrogram generated using Nei's genetic distance (Table 4) showed two major clusters (Figure 2). The first cluster consisted of Kibale natural, Kagera natural, Lushoto natural and Lushoto on-farm while the other comprised Cherangani on-farm, Kitale natural, Mabira natural, Mabira on-farm, Rumuruti on-farm and Rumuruti natural. Each of the two major clusters had two distinct

Table 4. Analysis of molecular variance (AMOVA) for AFLP among *W. ugandensis* sampled from Lake Victoria region in Kenya, Uganda and Tanzania.

Source of variation	Df	SS	MSD	Total variance (%)	P-value
Among populations	9	2882.06	320.22	46	0.01
Within populations	131	3277.38	25.01	54	0.01
Total	140	6159.39	345.24		0.01

Analysis based on all stands. Df=degrees of freedom, SS=sums of squares, MSD=mean square deviations and P=significance of variance. Significant values were based on the random permutations 99 times.

**Figure 2.** Phenogram based on Nei (1978) genetic distance for 10 populations of *W. ugandensis* from Kenya, Uganda and Tanzania generated using 223 AFLP markers.

subgroups with the first cluster having Lushoto natural and Lushoto on-farm separated from Kagera and Kibale natural and the second having Rumuruti on-farm separated from Kitale natural, Cherangani on-farm, Mabira natural and Mabira on-farm. Most on-farm populations clustered with the proximate natural populations. The clustering pattern based on unweighted pair-group method with arithmetic averaging (UPGMA) was consistent with the PCoA results (Figure 1). The shortest genetic distance was observed between Lushoto natural and Lushoto on-farm (0.0246), while the most distant populations were Lushoto natural and Kitale Natural (0.7587) (Table 4).

DISCUSSION

Evaluation of genetic diversity

Genetic diversity estimation is useful for optimization of

sampling strategies, conserving and management of genetic diversity of trees (Hamrick and Godt, 1996). AFLP markers are able to detect high levels of polymorphism, thus making it a powerful tool for assessing genetic diversity in many species (Bensch and Åkesson, 2005).

In the present study, the genetic diversity estimate of the sampled populations was relatively high, consistent with Muchugi et al. (2008) who found high genetic diversity among the sampled *W. ugandensis* populations. Levels of genetic diversity observed within a population may be influenced by population size, isolation and gene flow (Godt and Hamrick, 1989). Cherangani on-farm population showed higher genetic diversity than its proximate population Kitale natural. A significant difference was observed in genetic diversity between Rumuruti on-farm and Rumuruti natural populations. This may be an indication that the planting materials were sourced from a few mother plants from the natural population. However, during the collection, most farmers

clearly stated that they did not plant the trees and that the on-farm trees had regenerated from remnants of natural forest trees. In this case, the loss in genetic diversity in on-farm trees can be linked to selection pressure (e.g. and firewood) in the now cultivated land, resulting to significant loss of alleles (Ratnam and Boyle, 2000). In addition, restricted gene flow could have contributed to the difference as the barrier between the two populations is expected to increase with increased farming and settlement, resulting in differences in genetic diversity. On-farm populations analyzed in this study remain relatively diverse; therefore genetic erosion concerns that could result to loss of adaptability and potential inbreeding did not appear to have occurred.

Natural populations are expected to have high genetic diversity as shown by Kagera and Kibale populations; however, Kitale showed a relatively low genetic diversity. The same population showed the lowest genetic diversity among the populations during an earlier study (Muchugi et al., 2008). The low genetic diversity in Kitale population could be as a result of stochastic loss of alleles probably as a result of strong selective pressures due to deforestation for settlement causing changes in landscape, cutting down of big trees for timber, habitat destruction altering population density, diversity and abundance of pollinator communities thereby impinging on the mating systems (Lowe et al., 2005). These factors could further lead to increased genetic differentiation and potentially increase inbreeding (Lowe et al., 2004). However, some forest tree species are able to ameliorate these population genetic pressures through a variety of mechanisms such as extensive gene flow in many cases over tens of kilometers (Bacles et al., 2005), which serves to maintain connectivity even in highly fragmented and degraded landscapes where trees are at very low densities (Lander et al., 2011). The long lived nature of trees and existence of overlapping generations in a single site serves to retard the loss of genetic diversity (Davies et al., 2010). Flexible mating systems in some species circumvent self incompatibility to allow selfed progeny to form (Ward et al., 2005) particularly when faced with lack of compatible mates within a landscape. The impact of fragmentation varies by species and context, but not merely simple loss of genetic diversity and increased genetic differentiation among populations (Lowe et al., 2015). The key consequence of fragmentation has been identified as increased inbreeding depression in progeny sired in fragmented landscape (Breed et al., 2012b) and fitness loss due to low numbers of partners and low pollen diversity (Breed et al., 2014). Therefore, in the case of *W. ugandensis*, a study focusing on the progeny stands have experienced any inbreeding depression. Studies that compared genetic diversity of both natural populations of tropical trees and cultivated trees Meru oak (*Vitex fischeri*, synonym *Vitex keniensis*) using RAPDS (Lengkeek et al., 2005b) and sheanut (*Vitellera*

paradoxa) using microsatellite markers (Kelly et al., 2004) found little difference in diversity levels between agroforestry trees and the natural populations stand categories, with no evidence of genetic bottleneck events in agroforestry. In both cases, majority of trees tested from agroforests stands were expected to represent remnants or dispersed natural regenerants, thus providing an appropriate comparison with the present study. However, studies on *Inga edulis* comparing natural and planted stands at five locations in Peruvian Amazon, found limited but significant bottlenecks in the planted trees (Hollingsworth et al., 2005).

Population genetic structure

Analysis of molecular variance analysis (AMOVA) indicated low genetic differentiation between natural and cultivated populations. Partitioning genetic variation of populations of *W. ugandensis* showed a slightly higher within-population component as compared to the among-population component, which is characteristic of many widely distributed, abundant out-breeding tree species (Hamrick et al., 1992). *W. ugandensis* has a mixed mating system which is predominantly out-crossing. Thus, the transfer of pollen by insects between the different individuals and dispersal of seeds by wild animals (Muchugi et al., 2007) may increase the possibility of sexual recombination and subsequently increase within-population genetic variance. The high levels of variation within populations suggest that sampling from a few localities for either breeding or conservation could capture a large proportion of the variation within the species.

Genetic differentiation

The dendrogram and principal coordinates' analysis clustered the on-farm populations together with their proximate natural populations, implying that they are genetically related. When considered separately, the natural populations did not necessarily follow the geographical distance trend. This also does not agree with the predicted vegetation classification. Mabira and Kagera fall in the Lake Victoria Mosaic while the Kibale and Kitale fall in the Guineo-Congolian phytochoria (White, 1983). The Kibale and Kitale samples displayed this affinity previously (Muchugi et al., 2008). The clustering of geographically distant populations of Lushoto, Kibale and Kagera suggests a different migration theory from the previous study (Muchugi et al., 2008). A probable explanation is the effect of Lake Victoria as a gene flow barrier, especially in the case of Mabira and Kagera populations. Interestingly, the genetic disjunction revealed among Kenyan populations in Muchugi et al. (2008) is also revealed here with the

Rumuruti populations clustering separately from the Kitale population despite their geographical closeness, pointing to the Rift valley as the most probable barrier between the two populations hence separate evolution. In addition, the Kitale population belongs to the Guineo-Congolian vegetation block and Rumuruti falls outside this vegetation. It has been found that isolated populations evolve separately as they adapt to new ecological habitat leading to changes in allele frequencies hence the genetic differentiation (Epperson, 1992). This study further confirmed the implied theories of African floral evolution (White, 1983), which considers western Kenya as the most eastern remnant of the Guineo-Congolian phytochoria while eastern Kenya populations fall within the Somalia-Maasai centre of endemism. Such genetic differentiation reflects the different evolutionary histories of the species in the different ecological niches combined with different gene dispersal mechanisms (White, 1983).

This revelation calls for more genetic analysis in understanding the species migration and colonization from its centre of diversity which is suggested to be Uganda (Muriira, 2012). Human activities such as logging, slash-and burn agriculture, roads construction and cities together with human mediated dispersal of seeds could have also played an important role in the distribution of genetic diversity in this species as much as natural barriers which could have resulted in the current differentiation observed.

Conclusion

Despite the concerns that *W. ugandensis* trees currently on-farm might be of low genetic base, these results showed that they have high genetic diversity comparable to their proximate natural populations. Therefore, materials currently on-farm can be used as seed sources and conservation of the species. It is however, crucial to conserve the genetic variation of *W. ugandensis* by protecting the existing natural forests. High genetic structuring was evident among the populations with most genetic variation residing within populations, thus sampling intensively from a few populations would capture most genetic diversity.

Conflict of interest

The authors declare that there is no conflict of interest.

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