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Transferability and application of microsatellites (SSRs) from *Juniperus communis* L. to *Juniperus procera* Hochst. Ex endl.

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ABSTRACT

Transferability of five nuclear microsatellite markers (*Jc*-16, *Jc*-31, *Jc*-32, *Jc*-35 and *Jc*-37) that were originally developed for *J. communis* was tested to *J. procera*. *Jc*-31 & *Jc*-37 showed successful amplifications and polymorphism in *J. procera*. *Jc*-35 which had been reported as polymorphic in *J. communis* was monomorphic in *J. procera* while the primer pair for *Jc*-32 failed to record any amplification. The remaining one primer pair (*Jc*-16) showed double loci amplification in both *J. procera* and the control *J. communis* suggesting further examination of the primer pair and its binding sites. Genetic variation of six Ethiopian *J. procera* populations: Chilimo, Goba, Menagesha-Suba, Wef-Washa, Yabelo and Ziquala was assessed based on the two polymorphic loci (*Jc*-31 & *Jc*-37) in 20 - 24 individuals of each population. From these two loci, a total of 41 alleles could be retrieved. Two populations that are located south east of the Great Rift Valley together harboured 75% of private alleles signifying their deviant geo-ecological zones and suggesting special consideration for conservation. Chilimo, which is at the western margin of *Juniper* habitat in Ethiopian central highlands scored the highest fixation (F_{IS} = 0.584) entailing lower immigrant genes and hence higher inbreeding. The *AMOVA* revealed that 97% of the variation resided within the populations while still among population variation was significant ($p < 0.05$).

Keywords: Ethiopia; *Juniperus procera*; Microsatellites; Transferability

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1. INTRODUCTION

Due to their high polymorphism and co-dominant mode of inheritance, microsatellite (SSR) markers provide high resolution and precise information in genetic analysis such as gene flow, mating system and paternity [1,2]. Hence, they have become one of the important genetic markers that are being widely used for genetic studies of many important plants and animals. However, microsatellites developed for a species usually are transferable to only closely related other species [3]. As a result, a specific species may require development of specific microsatellite markers. A number of microsatellites have been developed and employed in genetic studies of tree species [4-8].

Juniperus procera is one of the biggest trees in its genus reaching to a height of over 40 m and a diameter of above 3 m [9,10]. The tree naturally grows between the Arabian Peninsula in Asia to Zimbabwe in Africa [11]. It is believed to have been evolved from *J. excelsa* [12] or a pre-existing common ancestral species for both *J. excelsa* and *J. procera* [13]. *J. procera* is the only species that succeeded south of equator in the genus *Juniperus* which comprises 67 taxa [11]. The separate success of *J. procera* to the south may entail its divergent evolution to its unique geographic regions which has likely led it having deviant genomic structure. The possible distinct pattern of variation with different genome structure adapted and specialized to the unique geographical region may limit the transferability rate of microsatellite markers that have been developed for other species in the genus. However, some microsatellites primer developed for species in other genus: *Chamaecyparis*; *Chamaecyparis nootkatensis* [14] and *Chamaecyparis obtusa* [15] were reported to score strong amplification in *J. procera*

[16]. This on the other hand, may suggest a more successful transferability of microsatellites within the genus.

Recently, microsatellite markers were developed for three species in the genus *Juniperus*, namely, *J. communis* [17], *J. przewalskii* [18] and *J. tibetica* [19]. This has promised detail genetic studies of species in the genus for high resolution data generation which will have significant role in setting up of their conservation strategies. *J. procera* is a high use value tree chosen for a variety of constructions, furniture and outdoor uses [10,20]. Consequently, the species has been subjected to extensive logging which has led its current status to be threatened in many of its habitats [21-23]. This justifies the need for a systematic study of the genetic structure of the populations of *J. procera* in order to design appropriate conservation strategies. The objectives of the present study were therefore, to assess the transferability rate of microsatellites developed for *J. communis* to *J. procera* and to use transferable markers in genetic variation analysis of *J. procera* populations in Ethiopia and accordingly, to recommend possible conservation measures.

2. MATERIALS AND METHODS

2.1. Sampling Technique

Leaves (needles) of 20 - 24 trees from each six representative *J. procera* populations: Chilimo, Goba, Menagesha-Suba, Wef-Washa, Yabelo and Ziquala (**Figure 1**) in Ethiopian highlands were collected in separate plastic bags containing silica gel. In order to avoid the collection of relatives, a minimum 50-meter distance between any sampled trees in a population was maintained using GPS. Further leaves of *J. communis* from Forestry Botanical Garden of the Georg-August University of Goettingen were collected as experimental control.

2.2. DNA Extraction

Total genomic DNA was extracted from silica gel dried leaf samples of both *J. procera* and *J. communis* using DNeasy 96 plant kit (Qiagen, Hilden, Germany). In order to check the quality and quantity of the DNA, 5 µl from each probe mixed with 2 µl of 6× orange loading dye solution (Fermentas) was electrophoresized for 25 minute

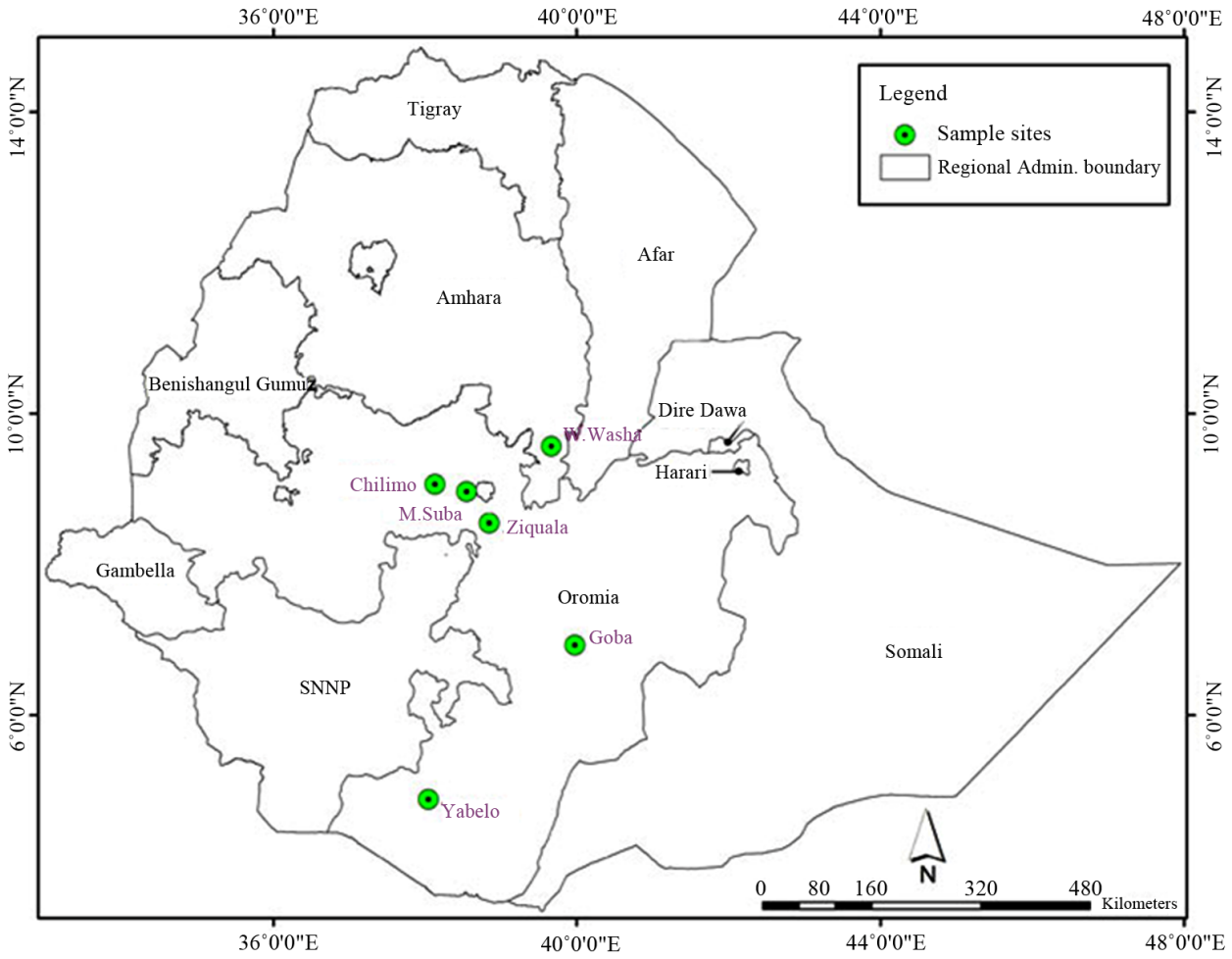

Figure 1. Map of the populations studied.

on 1% (w/v) agarose, in 1× Tris-Acetate-Ethylenediaminetetraacetic acid (TAE) buffer and about 0.003% (v/v) ethidium bromide as dye. After confirming the quality and quantity, the remaining DNA was stored at 20˚C for further investigation.

2.3. Primer Testing

Five primer pairs (*Jc*-16, *Jc*-31, *Jc*-32, *Jc*-35 and *Jc*-37) that were designed for microsatellite loci in *Juniperus communis* [17] were tested for amplification in a sample from each *J. procera* population and *J. communis* as positive control. PCR was performed in a final volume 15 µl containing 2 µl (10 ng) genomic DNA template, 1.5 µl 10× buffer (promega), 0.07 mM of each dNTP, 2 µl each primer (5 piko mol/µl), 2.5 mM $MgCl_2$ for two primer pairs (*Jc*-16 and *Jc*-31) and 1.5 mM $MgCl_2$ for the rest three primer pairs and 1 U Hot star *Taq* polymerase (Qiagen). PCR was performed following the profile reported in [17] using a Peltier Thermal Gradient Cycler (PTC-200 version 4.0, MJ Research).

In order to observe amplifications in the expected regions of the respective primer pairs, PCR products were subjected to gel electrophoresis. Fragments of PRC products that showed amplification in the expected size ranges were separated on ABI Genetic analyzer 3100 with internal size standard fluorescent dye ROX (Gene Scan 500 ROX) from Applied Biosystems. The above PCR and fragment separation procedures were performed for all samples for the primer pairs that showed clear amplification and polymorphism thereby variation analysis of the *J. procera* populations. The allele sizes of the polymorphic loci were scored using Gene scan 3.7® and Genotyper 3.7® software (Applied Biosystems) and the scores were appended to excel for further analysis.

2.4. Data Analysis for Polymorphic Loci

Population variation analysis was made based on polymorphic microsatellite loci. Genetic variation parameters such as, allele frequencies, private alleles, fixation indices (F_{IS}) and genetic differentiation based on F_{ST} via AMOVA were computed using GenAlEx version 6.4. In order to compute population pair wise Nei's unbiased genetic distance [24], the program POPGEN version 1.32 [25] was applied. Un- weighted pair group method using arithmetic averages (UPGMA) dendrogram was generated following the method of SAHN clustering [26] using NTSYSpc 2.0 program based on the unbiased genetic distance.

3. RESULTS

3.1. Test of Primer Pairs

Two microsatellites (*Jc*-31, *Jc*-37) produced clear and polymorphic amplifications in *J. procera*. Another loci, *Jc*-35, which was polymorphic in *J. communis* [17] appeared to be monomorphic in *J. procera* (**Figure 2**). *Jc*-16 primer pair amplified double loci in both *J. procera* and the control *J. communis* which the additional amplification was stronger than the amplification in the expected size ranges (**Figure 3**). On the other hand, the rest prime pair, *Jc*-32 did not record any amplification in *J. procera*.

3.2. Allelic Frequencies

A total of 41 alleles were revealed from the two polymorphic loci *Jc*-31 and *Jc*-37 (See Appendix). *Jc*-37 was found to be a highly variable locus with a total of 35 alleles revealed within the expected size range of 164 to 232 bps. The population, Menagesha-Suba, with its origin from the central part of Ethiopia, stood the first in terms of the total number of alleles revealed, which was 24 in this case (**Table 1**). The other locus, *Jc*-31, which was found to be less polymorphic in this study, contributed six alleles in a confined sizes ranged between 155 to 161 bps. At microsatellite locus *Jc*-37, allele sizes 188, 196 and 200 bps were observed in all the populations, with the most frequent observation of alleles being at the size of 196 bps. Two alleles with sizes of 155 and 156 bps accounted over 90% of at locus *Jc*-31 (see Appendix).

3.3. Private Alleles

A total of 12 private alleles were revealed for the overall six populations at the two loci. All private alleles were observed at locus *Jc*-37 except one at locus *Jc*-31 in Ya- belo, a population which is from the extreme southern block of Ethiopia (**Table 2**). A private allele with a size of 164 bps at *Jc*-37 contributed for more than 10% of allelic frequency in this same population (**Table 2**). Two populations which are south east of The Great Rift valley, namely Goba and Yabelo, har bored about 75% of the private alleles observed in this study.

3.4. Heterozygosity and Fixation Index

Among the populations tested in this study, Ziquala

Table 1. Number of alleles in each population.

Locus	Number of alleles per population					
	Chilimo	Goba	M.Suba	W.Washa	Yabelo	Ziquala
***Jc*-31**	3	4	4	3	5	5
***Jc*-37**	17	17	20	17	16	16
Total	**20**	**21**	**24**	**20**	**21**	**21**

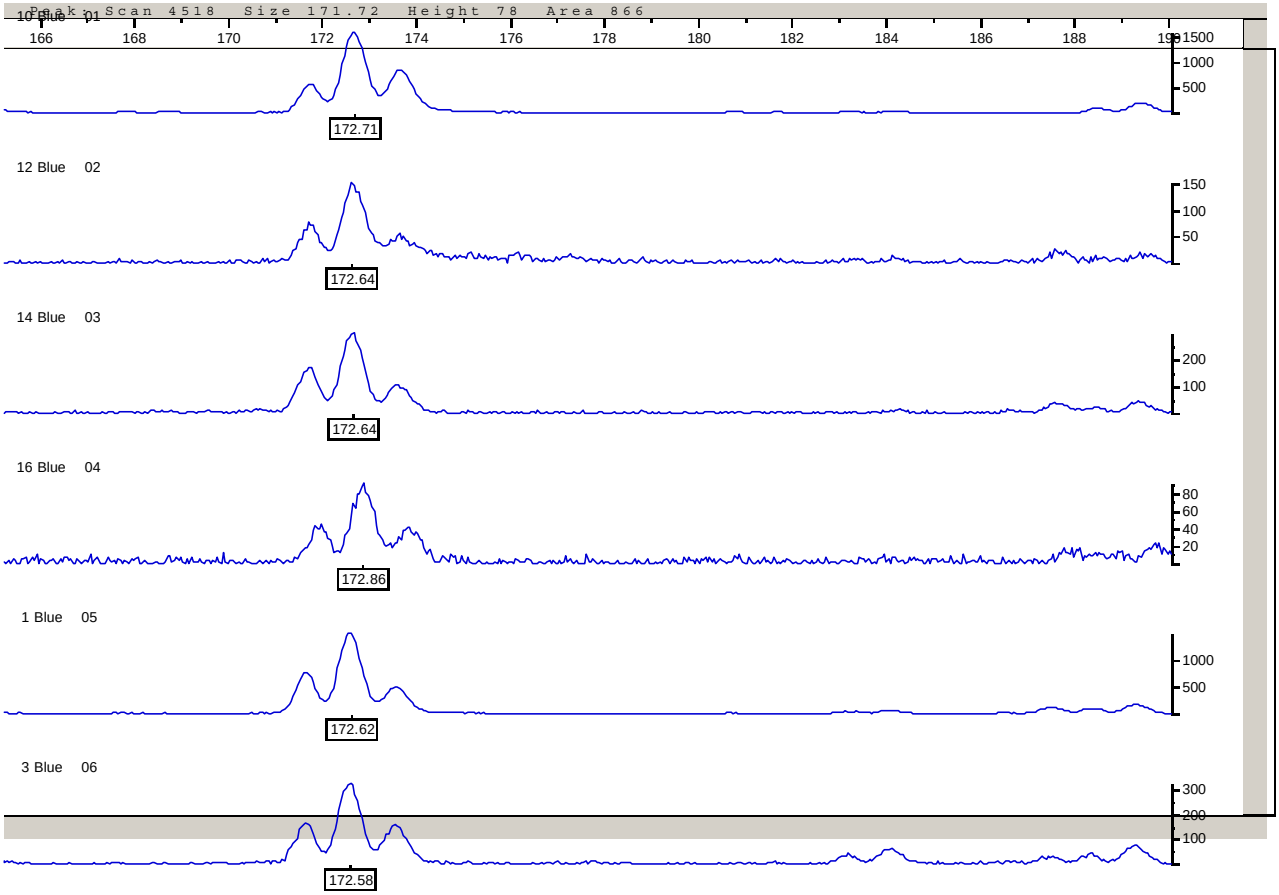

Figure 2. Monomorphic and homozygous locus *Jc*-35.

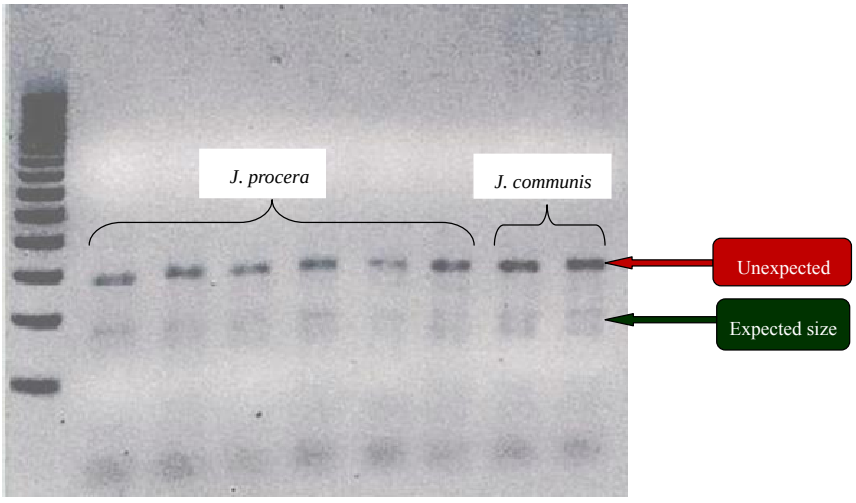

Figure 3. Loci amplified by single primer pair *Jc*-16.

scored the highest heterozygosity (Ho = 0.604) followed by Goba (Ho = 0.595) and congruently, these two populations showed lowest inbreeding, *i.e.* F_{IS} = 0.283 & 0.296 in that order. The lowest heterozygosity (Ho = 0.354) and hence the highest fixation index (F_{IS} = 0.584) was computed for Chilimo population (**Table 3**).

3.5. Among Populations Variation

3.5.1. AMOVA

AMOVA based on the two polymorphic loci combine puts 97% of the variation within the populations (**Table 4**). Nevertheless, the result indicated that there is highly

Table 2. Private alleles and their respective genotypes.

Population	Private allele	Genotypes with private allele at both loci				Locus with Private Alleles
		Jc-31		*Jc*-37		
Chilimo	210	155	155	178	210	*Jc*-37
Chilimo	208	157	157	208	211	*Jc*-37
Goba	204	155	155	188	204	*Jc*-37
Goba	204	155	155	202	204	*Jc*-37
Goba	204	0	0	196	204	*Jc*-37
Goba	204	156	158	202	204	*Jc*-37
Goba	204	156	158	182	204	*Jc*-37
Goba	204	155	157	188	204	*Jc*-37
Goba	218	158	158	186	218	*Jc*-37
Goba	223	156	158	188	223	*Jc*-37
Goba	225	155	155	166	225	*Jc*-37
Goba	232	155	155	170	232	*Jc*-37
M.Suba	191	157	155	184	191	*Jc*-37
Yabelo	161	155	161	184	190	*Jc*-31
Yabelo	164	156	156	164	190	*Jc*-37
Yabelo	164	155	155	164	194	*Jc*-37
Yabelo	164	155	155	164	194	*Jc*-37
Yabelo	164	0	0	164	198	*Jc*-37
Yabelo	164	156	156	164	192	*Jc*-37
Yabelo	168	156	156	168	190	*Jc*-37

Table 3. Within population genetic variation based on the two microsatellite loci.

Locus	Parameters	Populations						Mean	Total
		Chilimo	***Goba***	***M.Suba***	***W.Washa***	***Yabelo***	***Ziquala***		
Jc-37	n	24	23	24	24	24	24	23.833	143
	A	17	17	20	17	16	16	17.167	35
	AE	11.636	10.907	11.294	9.931	11.294	9.846	10.82	-
	Ho	0.625	0.826	0.833	0.75	0.875	0.958	0.81	-
	He	0.914	0.908	0.911	0.899	0.911	0.898	0.91	-
	F_{IS}	0.316	0.091	0.086	0.166	0.04	-0.067	0.105	-
Jc-31	n	24	22	24	23	22	20	22.5	135
	A	3	4	4	3	5	5	4	6
	AE	0.907	1.344	1.076	0.972	0.988	1.307	1.1	-
	Ho	0.083	0.364	0.250	0.174	0.091	0.250	0.202	
	He	0.559	0.729	0.598	0.0.590	0.546	0.679	0.622	
	F_{IS}	0.851	0.501	0.582	0.705	0.834	0.632	0.684	
Mean Values	n	24	22.5	24	23.5	23	22	23.167	139
	A	10	10.5	12	10	10.5	10.5	10.583	41
	AE	6.952	7.301	6.891	6.184	6.750	6.479	6.760	-
	Ho	0.354	0.595	0.542	0.462	0.483	0.604	0.507	-
	He	0.737	0.819	0.755	0.745	0.729	0.789	0.762	-
	F_{IS}	0.584	0.296	0.334	0.436	0.437	0.283	0.395	-

n: No. of samples; A: Observed Alleles; AE: Effective Alleles; Ho: Observed Heterozygosity; He: Expected Heterozygosity; F: Fixation Index.

Table 4. AMOVA results at microsatellite loci.

Locus	Source of variation	df	SS	MS	Est. Var.	%	F_{ST} Value	Prob.
	Among Pops	5	5.05	1.01	0.01	2	0.024	0.000
***Jc*-37**	Within Pops	282	130.65	0.46	0.46	98		
	Total	287	135.70	1.47	0.48			
	Among Pops	5	4.526	0.91	0.01	4	0.037	0.002
***Jc*-31**	Within Pops	282	88.98	0.32	0.32	96		
	Total	287	93.50	1.22	0.33			
	Among Population	5	9.58	1.92	0.02	3	0.030	0.000
Combined (*Jc*-37 + *Jc*-31)	Within Population	282	219.63	0.78	0.78	97		
	Total	287	229.21	2.69	0.80			

significant ($P < 0.01$) variation among the populations.

3.5.2. Pair Wise Analysis

The pairwise F_{ST} analysis based on the polymorphic loci showed significant ($P < 0.05$) differentiations between all pairs except Chilimo with Yabelo, Goba with Ziquala and Menagesha-Suba with Wef-Washa (**Table 5**). Menagesha-Suba and Wef-Washa were the least differentiated ($F_{ST} = 0.005$).

3.5.3. Population Clustering

The populations from the different eco-geographical sources were grouped into two distinct clusters based on Nei's unbiased genetic distance UPGMA (**Figure 4**). Two of the populations from the central part of Ethiopia, namely Menagesha-Suba and Wef-Washa, were grouped into one cluster, whereas the remaining four populations, *i.e.*, Chilimo, Goba, Yabelo and Ziquala, were grouped into the second cluster.

4. DISCUSSION

4.1. Transferability of Microsatellites from *J. communis* to *J. procera*

The low transferability of the microsatellite markers from *J. communis* to *J. procera* may indicate the deviance in the genomic structure of the two species, which enabled the species to have success in their current respective eco-geographic zones. The success of *J. procera* in the south unlike the rest of the species in the genus [11,27] was already postulated to be due to evolutionary changes in the species to adapt to its unique ecogeographical region [11-13]. The polymorphism in *J. communis* [17] and the complete monomerphism in *J. procera* at locus *Jc*-35 may also indicate the degree of genetic divergence exist between the two species, justifying the taxonomic classification of the two species into different groups [11]. Reference [18] who checked polymorphism of the five microsatellite loci [17] in *J. przewalskii* reported polymorphism for locus *Jc*-32 which failed to show amplification in *J. procera*. Conversely, [18] failed to observe amplification record in *J. przewalskii* at locus *Jc*-37 which was found highly polymorphic in *J. procera* and they got monomorphism in *J. przewalskii* at locus *Jc*-31 which was polymorphic in *J. procera*. This might be an indication that *J. procera* is

Table 5. Pairwise population differentiation (F_{ST}) based on the two microsatellite loci.

population	Chilimo	Goba	M.Suba	W.Washa	Yabelo	Ziquala
Chilimo		**	**	*	Ns (P = 0.156)	*
Goba	0.036		**	**	**	Ns (P = 0.061)
M.Suba	0.038	0.032		Ns (P = 0.22)	**	**
W.Washa	0.029	0.040	0.005		**	**
Yabelo	0.008	0.039	0.038	0.041		*
Ziquala	0.018	0.013	0.047	0.032	0.024	

*P < 0.05; **P < 0.01; Ns: P > 0.05.

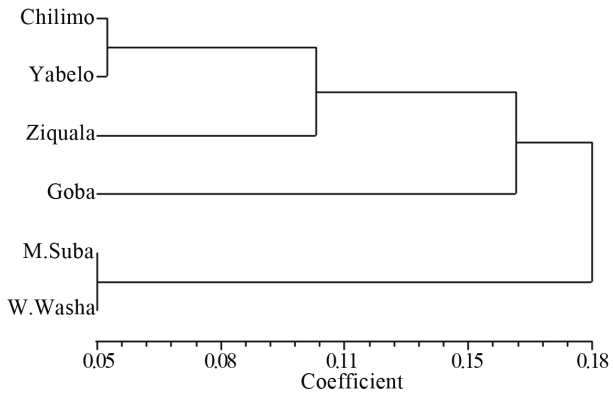

Figure 4. UPGMA dendrogram based on pair wise Nei's unbiased genetic distances.

genetically divergent not only from *J. communis* but also from other species within the genus Juniperus. Reference [18] also failed to produce any successful amplification with *J. przewalskii* at the locus *Jc*-16.

The double loci amplification by primer pair *Jc*-16 in both *J. procera* and *J. communis* (control) is in accordance with the report of [28] who observed multiple binding sites of the primer in her further investigation though [17] reported it as successful microsatellite primer. The frequency of microsatellite regions in the genome increases with the genome size of an organism [29-31]. Hence, primer sets designed for a single target microsatellite region may encounter more than one region in large genomes, thereby possibility of amplifying additional non target regions [31]. Conifers in general are reported to have large genome with a highly repetitive DNA that has made difficulty for recovery of microsatellite markers from their genome [14,32-34]. Accordingly, the experiences of single primer pair amplifying multiple loci in conifer genomes have also been commonplace [14,31,35].

4.2. Population Genetic Variation

J. procera is diocious and hence it is obligatorily outcrossing wind pollinated species [10,11,27]. The high within population variation in this study is likely attributable to these mating features of the species which was also reported for many species having similar reproductive biology [5,36-40). In addition, the strong gene migration of the species via seed which is mediated by long traveling birds [11,13] likely pronounced the within population genetic variation by narrowing down the variation among populations [39,41].

The lowest pairwise genetic distance which was realized between Menagesha-Suba and Wef-Washa may confirm the historical record that claims Menagesha-Suba as plantation of wildlings from Wef-Washa back in 15th century [42,43]. These two populations were also the only undifferentiated pairs in AFLP analysis of the six populations [44]. The low differentiation between Chilimo and Yabelo, however; is contrary to the one reported by [44] between which the authors computed the highest differentiation. The result in this study is likely due to the allele size 155 bps at *Jc*-31 which was shared between the two populations at higher frequency. The allele sizes 155 and 156bps at *Jc*-31 seem to have been evolved through insertion and deletions process that has gone in the flanking region of the locus [45]. Therefore, by chance, the two populations (Chilimo and Yabelo) probably have made relatively same rate of evolution in favor of the allele size 155 bps which can be considered as Homoplasy [46,47].

4.3. Heterozygosity and Fixations

The relatively and consistently higher heterozygosity at both of the loci for the Goba population might indicate the strong reproductive contact of this population with other populations. The Goba population is a sub population of an extensive mega Juniper population over the Bale Mountains [48,49]. Nevertheless, this population was found with inferior gene diversity of all the populations investigated through AFLP analysis, which was assumed to suffer from bottleneck associated with sever disturbance [44]. Bottlenecks have little effect on heterozygosity [50-52]. This likely has enabled the Goba population in maintaining its superiority in heterozygosity in this study while the population was reported with lower gene diversity at AFLPs [44].

On the other hand, the higher fixation (F_{IS} = 0.584) computed for Chilimo might be an indication of the higher inbreeding [53,54] in this population which might be resulted from limited geneflow [55-57]. Chilimo was also reported with lower gene diversity among the central highland populations based on AFLP analysis [44]. Chilimo is located in the western margin of Juniper habitats in Ethiopia [58].

4.4. Geographic Isolations and Private (Endemic) Allele Concentrations

The reason for higher concentration of endemic alleles in the south eastern populations (Goba and Yabelo) may somehow be related to the exclusive isolation of these populations from the rest by the Great Rift Valley as a physical barrier to gene flow. Reference [44] also reported that these two populations were genetically divergent from the other populations. Yet, the two south easterners exist at substantially long geographic distance apart each other which likely enabled them to harbor large number of endemic alleles independently. The Bale Mountains, where the Goba population belongs are known to have high concentration of endemic faunas and floras resulting from its unique ecology [49,59] experiencing temperature extremes between −15˚C to +26˚C [60]. The unique ecological features of the area may also contribute for change of certain genetic factors in the non-endemic organisms as well which perhaps have resulted in high level of private allele in Goba population. The Yabelo population harbored a total of three endemic alleles of which two were at locus *Jc*-37 and the other at *Jc*-31. The high allelic frequency (>10%) of the endemic allele with the size of 164 bps (locus *Jc*-37) in Yabelo may indicate the uniqueness of this population. Yabelo is recognized as lowland Juniper population in Ethiopia and it was reported for having unique seed morphology with a higher germination rate [61,62]. The two endemic alleles observed in Chilimo population may also be attrib-

uted to the geographic position of the population, which is at the western margin of the ecological zones for the Juniper forests in Ethiopia [58].

4.5. Conservation Implication of the Genetic Structure

Although most of the variations resided within the populations, due mainly to the mating system and gene flow mechanism of the species, the significant differentiation among the populations entails the level of variation of genetic information harbored in each population. Furthermore, the high private allele concentration for the geographically isolated populations clearly suggested that these populations deserve a special attention for genetic conservation. Earlier [61,62] reported the Juniper at Yabelo having unique morphological and physiological traits signifying the need of special conservation attention of this population. Contrary to [44] who reported low diversity in Goba population, the present study scored both the highest heterozigocity and private alleles in this population. This, as stated above, may emanate from bottleneck problems that have little effect on heterozygosity but likely be associated with the excessive disturbances of the population. Therefore, appropriate steps must be taken to put in place a proper genetic resources conservation strategy before such populations, particularly those with high concentration of private alleles are genetically eroded as their private alleles might not be regained from anywhere else once they are lost. The higher fixation score in Chilimo population seem to be due to selective logging of the elite genotypes with higher diameter [63,64] and due to its geographic isolation with the other populations [58]. In order to avoid genetic erosion and inbreeding depression, this population may require further genetic enrichment involving the introgression of new genetic backgrounds into the existing population by inter-planting of seedlings from other populations.

5. CONCLUSION

The less transferability of the microsatellites in *J. communis* to *J. procera* elucidates the genome structure differences of the two species. It is likely that the genome structure of *J. procera* has evolved in response to its current unique habitats, which may make it divergence not only from *J. communis* but also from other species of the genus. Though only two polymorphic loci were involved in this study for the assessment of *J. procera* populations in Ethiopia, results were able to generate precisely valid information relevant for conservation strategy set up and history reconstruction. However, we suggest more microsatellite markers either specifically developed for the species or those that could be transferred from related taxa to be involved for detail investigation and generate reliable data in further studies.

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APPENDIX

Allelic frequencies at microsatellite loci *Jc*-31 and *Jc*-37.

Locus	No.	Allele size (bps)	Allele frequencies in each population					
			Chilimo	Goba	M.Suba	W.Washa	Yabelo	Ziquala
***Jc*-31**	1	155	0.542	0.341	0.313	0.348	0.591	0.475
	2	156	0.375	0.295	0.542	0.522	0.318	0.250
	3	157	0.083	0.159	0.083	0.000	0.045	0.100
	4	158	0.000	0.205	0.063	0.130	0.000	0.150
	5	159	0.000	0.000	0.000	0.000	0.023	0.025
	6	161	0.000	0.000	0.000	0.000	0.023	0.000
***Jc*-37**	1	164	0.000	0.000	0.000	0.000	0.104	0.000
	2	166	0.000	0.022	0.000	0.000	0.000	0.000
	3	168	0.000	0.000	0.000	0.000	0.021	0.000
	4	170	0.000	0.022	0.042	0.000	0.000	0.000
	5	172	0.000	0.000	0.063	0.021	0.000	0.000
	6	176	0.000	0.000	0.021	0.021	0.000	0.021
	7	178	0.063	0.043	0.021	0.000	0.000	0.000
	8	180	0.083	0.000	0.021	0.000	0.042	0.021
	9	182	0.042	0.109	0.125	0.021	0.125	0.000
	10	184	0.000	0.065	0.188	0.063	0.146	0.021
	11	186	0.000	0.087	0.063	0.000	0.021	0.083
	12	188	0.167	0.174	0.021	0.042	0.083	0.104
	13	190	0.063	0.000	0.021	0.125	0.125	0.063
	14	191	0.000	0.000	0.021	0.000	0.000	0.000
	15	192	0.083	0.000	0.063	0.042	0.021	0.104
	16	194	0.125	0.000	0.083	0.125	0.063	0.000
	17	196	0.083	0.043	0.104	0.208	0.042	0.146
	18	198	0.000	0.043	0.042	0.063	0.042	0.000
	19	200	0.021	0.065	0.021	0.042	0.042	0.188
	20	202	0.000	0.087	0.021	0.083	0.063	0.063
	21	203	0.021	0.000	0.000	0.021	0.000	0.000
	22	204	0.000	0.130	0.000	0.000	0.000	0.000
	23	205	0.042	0.022	0.000	0.021	0.000	0.000
	24	207	0.000	0.000	0.021	0.000	0.000	0.021
	25	208	0.021	0.000	0.000	0.000	0.000	0.000
	26	209	0.021	0.000	0.021	0.021	0.000	0.021
	27	210	0.021	0.000	0.000	0.000	0.000	0.000
	28	211	0.063	0.000	0.000	0.042	0.021	0.021

Continued

29	213	0.000	0.000	0.021	0.042	0.000	0.042
30	215	0.063	0.000	0.000	0.000	0.042	0.063
31	217	0.021	0.000	0.000	0.000	0.000	0.000
32	218	0.000	0.022	0.000	0.000	0.000	0.021
33	223	0.000	0.022	0.000	0.000	0.000	0.000
34	225	0.000	0.022	0.000	0.000	0.000	0.000
35	232	0.000	0.022	0.000	0.000	0.000	0.000

