ASSESSING GENETIC DIVERSITY AND POPULATION STRUCTURE OF THYME (*THYMUS SCHIMPERI* RONNIGER) IN EASTERN, CENTRAL AND NORTHERN HIGHLANDS OF ETHIOPIA

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ABSTRACT

Thymus schimperiRonniger (Ethiopian thyme) is wild-growing endemic perennial herb which is rich in medicinally important metabolites. However, little is known about its genetic diversity and population genetic structure. Nine T. schimperi populations were collected from Bale, North Shewa and East Gojam zones of Ethiopia and analyzed using five ISSR markers. Seventy-seven amplicons showed an overall 100% polymorphism, corresponding to an average of 15.4 bands per primer. At the individual population level, the percentage of polymorphic loci (PPL) within population ranged from 63.64 % for Dargegne to 87.01 % for Rira population, with an average of 74%. Nei's genetic diversity (H) was 0.25 on average at the population level and 0.36 at the species level, while Shannon indices (I) were 0.39 and 0.54, respectively. Percentage of polymorphic bands (PPB) varied from 15.79% to 100% in different primers with an average of 75.2%. The Gst value for the overall loci was 0.31 indicating moderate differentiation among populations and lower gene flow (Nm = 1.133). AMOVA showed that total genetic variance, partitioned as 4%, 27% and 69% (P < 0.00) between populations from different regions, among populations within regions and within individual populations, respectively. Mantel's test results with significance detected using ISSRs among all of the tested populations was; r = 0.304 (P < 0.001,999 permutations). UPGMA cluster analysis indicated grouping of the populations regardless of their geographical locations. This is the first report to demonstrate the use of molecular markers for diversity analysis in Ethiopian thyme and the result obtained suggests an urgent need for conservation of the existing natural population and implement alternative measures to meet the market demand.

Keywords: *Ethiopian thyme,* Endemic species, Genetic Diversity, Genetic Differentiation, ISSR marker, medicinal plant

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INTRODUCTION

Ethiopian thyme, *Thymus schimperi*,is a wild-growing perennial herb which is rich in medicinally important metabolites. It belongs to the family Lamiaceae to the tribe Mentheae within the subfamily Nepetoideae. Lamiacae includes about 236 genera, 7173 species native to Europe, and grown in the Mediterranean basin and northern Europe, as well as other parts of the world such as Asia, South America, and Australia (Morales, Stahl Biskup, & Sáez, 2002). The Mediterranean region, in particular the West Mediterranean region, can be described as the center of origin of the genus (Hardman, Stahl-biskup, & Sáez, 2002). Thymus is considered a well-defined genus, based on the morphological and chemical features of its species, and is widely distributed throughout the Old World. Two species, *T. Schimperi*Ronniger and *T. serrulatus*Hochst.exBenth, both locally known as *Tosign*, are endemic species represented in Ethiopia.Both species are perennial herbs, woody at the base and 5-40 cm high (Ray Harley, Paton, Harvey, & Gardens, 1998). *T. schimperi* has ovate to elliptic leaves with entire margins. It occurs in open grasslands, between bare rocks, on slopes and tops of mountains between 2250-4000 m.a.s.l(Nigist & Sebsebie, 2009).It is also well known in Central, Eastern and Northern highlands of Ethiopia and is harvested and dried by people living close to the towns of Dinsho (Bale Zone) and Menz (North Showa Zone)and sold at local markets (Sebsibe, 1993).

There is remarkable variation in chromosome number in the genus as well as within the same species where the chromosome numbers in the genus *Thymus* ranges from 2n=24 to 26,28,30,32,42,48,50,52,54,56,58,60,84 and 90 corresponding to the diploid, tetraploid and hexaploid levels probably from a basic numbers x=14 and x=15 origin. The most frequent numbers are

2n=28, 30, 56 and 60. Polyploidy is extremely common throughout the family (ranging from 51.4% to 68.8%) and allopolyploidy is most frequent than autopolyploidy(RM Harley & Brighton, 1977).

Thyme is propagated by seeds or by stem-cuttings and pollination is mainly effected by bees (Brabant, LEFORT, VALDEYRON, & VERNET, 1980). Male sterile and male fertile plants have been identified in several populations leading to high levels of polymorphism (Assouad, Dommée, Lumaret, and Valdeyron (1978). According to Huck (1992) cross-pollination in Labiatae is effected by animals, of which by far the most diverse groups are the insects.

Like other aromatic plants, thyme has volatile essential oils due to the presence of countless glandular hairs of different forms. Damage to these glandular hairs leads to the production of a fragrance which attracts humans and leads to the exploitation of these aromatic plants (Hardman et al., 2002).

Essential oils and extracts from Thymus plants exhibit antioxidant, antibacterial, antifungal, antiviral, cytotoxicity, anti-parasitic and other properties (Ait M'Barek et al., 2007; Jukic, Politeo, Maksimovic, Milos, & Milos, 2007). Oral treatment of coughs, upper respiratory infections, acute and chronic bronchitis, whooping cough, and catarrh are among the internal health problems that herbal medicines belonging to the genus thymus are used as treatment in traditional medicine (Basch, Ulbricht, Hammerness, Bevins & Sollars, 2004). For external use, thyme is used as mouthwash to treat laryngitis, as anti-acne and anti-stomatitis agents, and in the topical treatment of minor injuries.

Recent data suggest that thyme’s essential oil, and its main component carvacrol, show anti-inflammatory effects that have been attributed to the

inhibition of inflammatory edema and leukocyte migration (Fachini-Queiroz, et al., 2012). In Ethiopia, the dried leaves of *T. schimperi* are also used in traditional medicine for the treatment of headache, inflammation, spasm, thrombosis, urinary retention, mental illness, eye disease, toothache, stomachache, earache, liver disease, gonorrhea, leprosy, lung TB, acne and ascaris(Nigist & Sebsebie, 2009). Tea made by the herb in water is also recommended as a local medicinal remedy for respiratory problems, gastrointestinal disorders, and liver disease (Dawit & Ahadu, 1993).The dried leaves are also used to flavor tea, coffee and different kinds of stew (Jansen, 1981).

Factors, such as urbanization, and the replacement of traditional agricultural systems by modern mechanized agriculture have reduced biological diversity (Khlestkina, Varshney, Röder, Graner, & Börner, 2006). Unfortunately, the increased demands on *T. schimperi* have been met by random and destructive harvesting, which has resulted in severe loss of habitat and genetic variation. Therefore, sustainable management and conservation of these endangered species have become a necessity. Jackson, Bawa, Pascual, and Perrings (2012)have stated that an effective conservation strategy for a medicinal plant requires studying the plant's genetic variation and the relationship among its different populations.

So far, most studies on *T. schimperi*have focused mainly on its essential oil content. Though understanding of genetic relationships and variability is crucial for protection and conservation of Thymus plants, the genetic relationships of Ethiopian Thyme have never been studied. Hence, assessment of the genetic variability of this species has important consequences for making informed decision in breeding and conservation practices. Therefore, the purpose of this study was to detect the genetic

variation, population structure and the relationship among populations of Ethiopian Thyme.

MATERIALS AND METHODS

Study sites and plant sample collection

A total of 108 samples of Thyme plants were collected from Bale, North Shewa and EastGojamzones (36 samples per Zone, 12 from each kebele). The sampling sites represented a wide range of geographic distribution of Ethiopian thyme. Bale mountain, in the Southern part of Ethiopia, receives an annual rainfall between 1500 and 2000 mm and belongs to the moist evergreen forest (Friis, 1992). Guassa Community Park in the central highlands of Ethiopia (North ShewaZone) is a small patch of land which has persisted in its original condition for the past four hundred years through traditional conservation system. Guassa area ranges from 3200 to 3700 m.a.s.l. and has a total area of 100 km^2(Tefera, 1975). Mount Choqa (also known as Ch'ok'eTerara and Mount Birhan at 4,100 meters, found in the northern part of Ethiopia, aretwo of the highest mountains of Gojam, a region of Ethiopia located south of Lake Tana. The geographic location of the study area lies in the range of 06046’ 32.7"-10^0 43' 59.7" N Latitude, to 037^0 46' 03.3" - $039^0$55'35.8"E longitude. Samples were collected from the highest elevation of 3606 m.a.s.l for Bale and the lowest average elevation of 3421 m.a.s.l for North Shewa. Within each zone,the altitude ranges from 3207-3881, 3476-3701 and 3304 -3493m.a.s.l. for Bale, East Gojam and North Shewa zones, respectively (Table 1).

The nine populations of samples were grouped into three, based on their geographic origin, Eastern group (Bale Zone) consisting Fassil (F), Rira (R) and Geremba (G) populations from Goba and Dinshoworedas. The Central

group (North Shewa Zone) consists Dargegne (D), Alfameder (M) and Yedi (Y) populations from Mama woreda, and Northern group (East Gojam Zone) consists Dangule (N), Shewakidanemeheret (S) and Waber (W) populations from Sinan and Bibugneworedas, respectively (Table2).

Genomic DNA Extraction

Total genomic DNA was isolated from 108 silica-gel-dried fresh leaf samples of *T. schimperi* following a modified version of CTAB (Borsch, et al., 2003). Double CTAB extractions (2% cetyltrimethyl ammonium bromide, 0.4mM polyvinylpyrrolidone, 0.1M Tris-HCl (pH 8), 20 mM EDTA, 2 M NaCl) at high salt and 28 mM β-mercaptoethanol were followed to eliminate the excess content of carbohydrates, phenolic compounds, and proteins present in the leaves, which may cause inhibition on Taq polymerase action during PCR and to yield optimal quantities of high-quality DNA. DNA quality was checked using 2% agarose gel. Thermo Scientific Nano Drop 2000/2000c spectrophotometer was employed to further quantify the concentration and confirm the purity of the DNA.

ISSR Primer Selection and Amplification

Nineteen ISSR primers previously selected for *T. daenensis*(Markers, et al., 2009) and *T. vulgaris, T. vilosus*, *T. praecox*, *T. serpyllum*, *T. serpyllum* 'Aureum' and *T. citriodorus* 'Silver'(Smolik, Jadczak, & Korzeniewska, 2009) were tested against three randomly selected *T. schimperi* samples collected from the three distant geographic locations to screen the primers. Of the 19 primers screened, five of them that produced a higher number of polymorphic bands, which are reproducible and intense, were selected for the ISSR analysis.

The reaction mixture for ISSR-PCR amplification assay had a total volume of 25 µl, which contained ~100 ng μl^{-1} of template DNA, dH_2O, 10x reaction buffer S, 3.2 mM of each dNTPs, 25 mM $MgCl_2$, 1.5 units of Taq DNA polymerase, and 0.2 µM primer. A sample without genomic DNA was used as a negative control to rule out the possibility for self-amplification of the primers or the contamination of genomic DNA.

DNA amplification was carried out on a thermocycler (Biometra 2003T3) with pre-heating at 99°C, followed by initial denaturation at 94^oC for 4 min, subsequent denaturation at 94°C for 15 secs, annealing for 1 min at optimum temperature for each primer, extension for 1.5 min t 72°C, and 7 min at 72°C for a final extension for a total of 40 cycles. The amplified ISSR-PCR products were separated on a 1.67% w/v agarose gel at 80 V, stained with ethidium bromide and visualized with Gel Doc™ EZ Imaging system (BIORAD).

SCORING AND DATA ANALYSIS

Only clearly distinctive bands in the range of 100–2000 bp and with strong intensities were used for scoring using Image Lab™ software version 5.0 from BIORAD. The gels were scored for the presence or absence of polymorphic and reproducible ISSR bands. Each band was regarded as a locus with two alternative alleles.

The data from the ISSR analysis were converted into a binary data matrix as discrete variables (1' for band presence, '0' for band absence and '?' for ambiguous bands). Thus, clear and unambiguous bands were estimated using 100bp DNA ladder with standard size marker.

GenAlEx version 6.5 software (Peakall & Smouse, 2012) was used to convert the binary ISSR data into a data that can be used by POPGENE. The genetic variability at the individual and population levels was analyzed under the assumption of Hardy-Weinberg equilibrium. The percentage of polymorphic bands (PPB), the numbers of effective alleles (Ne), Shannon's information index (I), and Nei's gene diversity index (H)(Nei, 1973) were calculated using the POPGENE (v 1.32) (Yeh, Yang, Boyle, Ye, & Mao, 1999). The parameters like Nei's coefficient of population differentiation (G_{ST}) with the formula: G_{ST} = (Ht −Hs)/ Ht(Nei, 1978) and Gene flow among populations (Nm) which was calculated using the formula of Nm = 0.5(1–GST)/GST were included to evaluate gene differentiation between populations.

According to the method developed by Nei, the gene diversity statistics, including the total allelic diversity (Ht),the mean allelic diversity within populations (Hs), the proportion of the total allelic diversity found among populations (Gst), and the gene flow among populations (Nm), were estimated using the POPGENE software (Ellstrand, 2014).

The genetic relationships among populations were analyzed by NTSYs pc version 2.02h (Rohif, 2000) using Jaccard's coefficient of similarity (Jaccard, 1908) matrix to construct unweighted pair group method with arithmetic average (UPGMA) dendrogram(Sneath & Sokal, 1973) and neighbor joining tree (Saitou & Nei, 1987) by further employing free tree software 0.9.1.50 (Pavlicek, Hrda, & Flegr, 1999).

GenAlEx 6.5 software was used to perform the two dimensional Principle Coordinate Analysis (PCoA). The total genetic variation among the samples was calculated by GenAlEx 6.5 software using the Phi-statistic. To assess percent distribution of genetic variation among and within populations, a hierarchical AMOVA was performed and the

total genetic variation was partitioned at three levels; within populations (PhiPT=Fct), among populations within regions (PhiPR=Fst), and among regional populations (Phi-RT=Fsc). Population polymorphism, and Nei's genetic distance and gene flow (Nm) was estimated using POPGENE and Genetic distance was tested against geographic distance by Mantel test with 999 random permutations using GenAlEx 6.5 software.

RESULTS

ISSR Marker Polymorphism and Genetic Diversity

The ISSR analysis of 108 samples from nine populations of Ethiopian thyme resulted in a total of 77 unambiguous score-able markers using five primers. Band sizes ranged from 300 to 1300 bps corresponding to an average of 15.4 bands per primer. All the five primers [$(GA)_{8C}$, $(GA)_{8}A$, $(TC)_{8}G$, $(AG)_{8}YA$, $(AG)_{8}YC$] were informative as 100% of the amplicons at species level were polymorphic (Table 1). Population genetic analysis for the single population showed that the number of polymorphic loci per primer under each population ranged from 3 to 19 and the percentage of polymorphic loci (PPL) varied from 15.79% in primer 824 to 100% in four different primers (811,812,835,836) with an average of 11.36 and 75.2%, respectively (Table 1).

When diversity and polymorphism among primers over the entire populations is compared, both the highest gene diversity index (H) and Shannon Information index (I) values were obtained for primer 811(H=0.397, and I=0.581) and the lowest (H= 0.315, I=0.477) were obtained for primer 812 and for primer 824, respectively (Table 1).

At the individual population level, the percentage polymorphic loci (PPL) within population ranged from 63.64% at Dargegne population of the North Shewa Zone to 87.01% at Rira population from Bale Zone, with an average of 74% (Table 2). Genetic diversity estimates for geographic populations showed the highest values in the Eastern (Fassil, Rira and Geremba) populations of the Bale Zone (PPL=96.1%, H=0.34 and I=0.51) followed by Northern (Dargegne, Alfameder and Yedi) populations (PPL=93.5%, H=0.34, I=0.50) and the lowest value (PPL=88.3%, H=0.32, I=0.47) was attained from the central geographic groups having Dangule, Shewakidanemeheret and Waber populations (Table 2).

Table 1 list of ISSR primers used, their nucleotide sequence annealing temperature(Ta), Number of Polymorphic Loci (NPL) as well as Percent PolymorphicLoci (PPL).

S. No.	Primer Code	Primer sequence	Repeat motif	Ta (°C)	No of bands per primer	NPL	PPL	H ±SD	I ±SD
1	811	GAGAGAGAGAGAGAGAC	$(GA)_8C$	48	15	11.8	78.5	0.397 (0.1026)	0.581 (0.1205)
2	812	GAGAGAGAGAGAGAGAA	$(GA)_8C$	45	14	12.1	86.5	0.315 (0.753)	0.491 (0.0884)
3	824	TCTCTCTCTCTCTCTCG	$(TC)_8G$	48	19	10.3	54.4	0.316 (0.1670)	0.477 (0.2099)
4	835	AGAGAGAGAGAGAGAGYA	$(AG)_8YA$	48	16	12.3	77.1	0.384 (0.0864)	0.569 (0.0967)
5	836	AGAGAGAGAGAGAGAGYC	$(AG)_8YC$	45	13	10.3	79.5	0.396 (0.0914)	0.581 (0.1028)
Total					77	56.8			
Mean					15.4	11.36	75.08		

Y= (A, C).

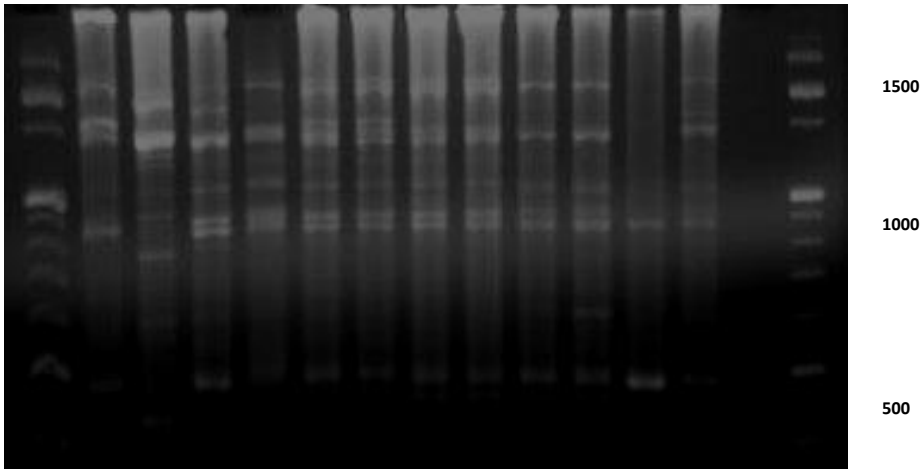


Figure 1. ISSR fingerprint generated from 12 individuals of *T. schimperi*by primer 824. L=100bp DNA ladder, C=negative control and 1-12 are samples of Rira population

Table 2. Genetic variation statistics for all loci; NPL, PPL, Effective number of alleles (Ne),Gene diversity (H) and Shannon's information index (I) for each population

Population	NPL	PPL	N_e ±SD	H±SD	I ±SD				
Fassil	55	71.43	1.49 (0.455)	0.28 (0.198)	0.41 (0.280)				
Rira	67	87.01	1.48 (0.343)	0.29 (0.170)	0.43 (0.229)				
Geremba	64	83.12	1.50 (0.345)	0.30 (0.176)	0.44 (0.242)				
Mean	62	80.52	1.49	0.29	0.43				
Dargegne	49	63.64	1.38 (0.371)	0.22 (0.201)	0.33 (0.285)				
Alfameder	53	68.83	1.37 (0.346)	0.22 (0.185)	0.34 (0.264)				
Yedi	54	70.13	1.41 (0.378)	0.24 (0.197)	0.36 (0.276)				
Mean	52	67.53	1.39	0.22	0.35				
Dangule	60	77.92	1.43 (0.356)	0.26 (0.183)	0.39 (0.254)				
Shewa	53	68.83	1.34 (0.338)	0.21 (0.182)	0.33 (0.259)				
kidanemeheret									
Waber	57	74.03	1.40 (0.350)	0.24 (0.184)	0.37 (0.259)				
Mean	57	72.08	1.39	0.23	0.36				
Population level	57	74	1.4(0.36)	0.25 (0.18)	0.39 (0.25)	Ht	Hs	Gst	Nm*
Species level	77	100	1.61	0.36	0.54	0.36	0.25	0.31	1.13
			(0.2645)	(0.1174)	(0.1421)	(0.014)	(0.010)		

Ne=Effective number of alleles, H= gene diversity, I=Shannon's informationindex, Ht=Average total gene diversity, Hs Genetic Diversity

within ppn, Gstrelative degree of Gene differentiation among ppn, * Nm=estimate of gene flowfromGst, E.g., Nm = 0.5(1 - Gst)/Gst

Diversity Index and Population Level Diversity Estimate

Assuming Hardy Weinberg equilibrium, different levels of gene diversity were observed. The mean Nei's genetic diversity (H) at the population level was estimated to be 0.25 and at the species level 0.36 while Shannon indices (I) were 0.39 and 0.54, respectively. Among the nine populations, Geremba exhibited the highest level of diversity (H = 0.30; I = 0.44), while Shewakidanemeheret population exhibited the lowest level (H = 0.21; I = 0.33) (Table 2).

Table 3. Polymorphism, Gene Diversity, Shannon's Information Index, Gene Differentiation and Estimates of Gene Flow obtained by grouping populations based on their geographic origin.

Region	Zone	Geographic	Population code	Sample	NPL	PPL	H±SD	I±SD			
Oromia	Bale	Eastern Ethiopia	F R G	36	74	96.1	0.338 ±0.125	0.509 ±0.161	0.34 ±0.02	0.29 ±0.01	0.16
Amhara	North Shewa	Central Ethiopia	D M Y	36	68	88.31	0.320 ±0.175	0.474 ±0.236	0.32 ±0.03	0.23 ±0.02	0.29
Amhara	East Gojam	Northern	N S W	36	72	93.51	0.335 ±0.156	0.498 ±0.204	0.34 ±0.02	0.24 ±0.02	0.29
Average					71.33		0.33	0.49	0.33	0.25	0.25

F=Fassil, R=Rira, G=Geremba, D=Dargegne, M=Alfameder, Y=Yedi, N=Dangule, S=Shewakidanemeheret and W=Waber

Genetic differentiation and gene flow

The relative degree of gene differentiation among the nine populations of T. *schimperi* was high (Gst=0.31) showing that31% of the genetic diversity was between populations (among regions and populations within regions) while 69% of the genetic diversity was within

populations. The result of AMOVA also revealed that 69% of the total genetic variance (PhiPT) was attributed to differences within individual populations (Table 4). The total genetic variance (PhiPR) among populations within regions contributed 27% and 4% of the total genetic variance (PhiRT) was distributed among populations from different geographic regions. However, all of the three levels of variance significantly contributed to the overall genetic variation, as determined through the permutation analyses at p≤0.001value. Deduced from the Gst value, the level of gene flow (Nm) was computed to be 1.133, representing a relatively low level of migration rate between populations resulting in high gene differentiation within, as well as, among populations. Our result also indicated that there is a strong differentiation within geographic groups. Table 4. Summary of the AMOVA results for 108 individuals of *T. schimperi* from three zones (Nine populations)

Source	Df	SS	MS	Estimated Variance	Percentage variation	Phi statistic	Value	P-value
Among	2	199.519	99.759	0.706	4%	PhiRT	0.038	0.001
Among Populations Within region	6	446.000	74.333	5.125	27%	PhiPR	0.285	0.001
Within	99	1270.583	12.834	12.834	69%	PhiPT	0.312	0.001
Total	107	1916.102		18.665	100%			

d.f., degree of freedom; SS, sum of squared observations; MS, mean of squared observations; PhiRT, proportion of the total genetic variance that is due to the variance between regions; PhiPR, proportion of the total geneticvariance that is due to the variance among populations within a region; PhiPT, proportion of the total genetic variance which is due to the variance among individuals within populations. Probability, (P-value), for PhiRT, PhiPR and PhiPTare based on standard permutation across the full data set.

Genetic Similarity and Cluster Analysis

The genetic similarity was calculated for all 108 combinations of nine *T. schimperi* populations based on 77 polymorphic ISSR markers. The similarities varied from 0.213 (Rira vs. Dargegne) to 0.364 (Shewakidanemeheret vs. Yedi populations) with an average of 0.415 (Table 6).

In order to further illustrate relationships among populations, a dendrogram was generated by UPGMA algorithm based on Jaccard's similarity, which clustered the nine populations into four major groups (Fig. 3). The first group contains population from eastern (Fassil), central (Yedi) and northern (Shewakidanemeheret and Dangule) geographic groups. Rira populations as separate group from Bale Zone came under Cluster II, Alfameder and Waber populations from central and northern geographic group were included under Cluster III and Populations of Geremba and Dargegne from eastern and northern geographic groups of Ethiopia were placed together in cluster IV.

Furthermore, dendrogram based on NJ analysis (Fig 4) grouped the 108 individual of *T. schimperi* into three major clusters (A, B and C); cluster A-I sub-cluster consisted Dargegne, Dangule and solitary R12 from Rira, A-II sub-cluster consisted Yedi and Shewakidanemeheret which were grouped together in almost all cases, A-III sub-cluster consisted Alfameder and Waber populations and A-IV sub-cluster have exclusively Gerembapopulation. Fassil population and four other individuals were assembled in the B major group while C major group consisting only Rira population. In order to evaluate how well the nodes of NJ phylogenetic tree is supported, a 1000 times replicated bootstrapping procedure was followed

and the nodes showed up 100% value in all bootstrap replicates indicating that the nodes were well supported.

Table 5: Jaccard's coefficient based pair wise similarity matrix calculated for the nine populations of T. schimperi

	F	R	G	D	M	Y	N	S	W
F	1.000								
R	0.291	1.000							
G	0.226	0.261	1.000						
D	0.246	0.213	0.299	1.000					
M	0.250	0.260	0.235	0.258	1.000				
Y	0.270	0.271	0.247	0.307	0.295	1.000			
N	0.306	0.233	0.222	0.259	0.239	0.316	1.000		
S	0.333	0.300	0.253	0.215	0.255	0.364	0.301	1.000	
W	0.276	0.261	0.221	0.267	0.309	0.283	0.252	0.282	1.000

F=Fassil, R=Rira, G=Geremba, D=Dargegne, M=Alfameder, Y=Yedi, N=Dangule, S=Shewakidanemeheret and W=Waber

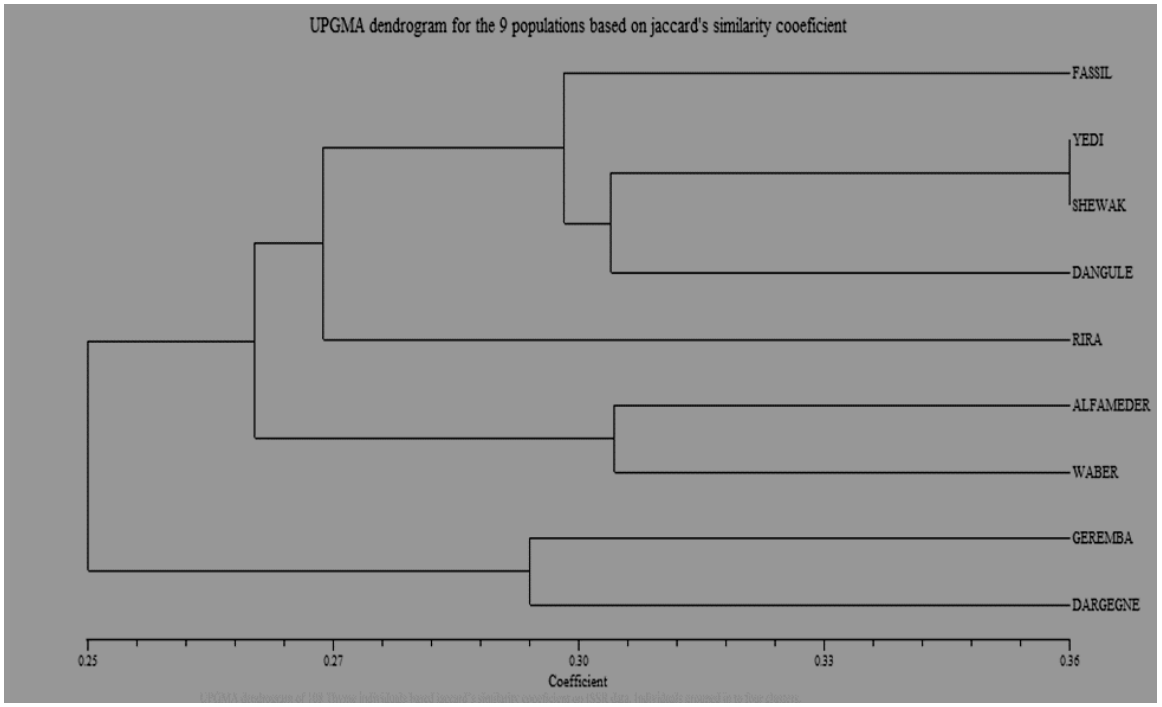
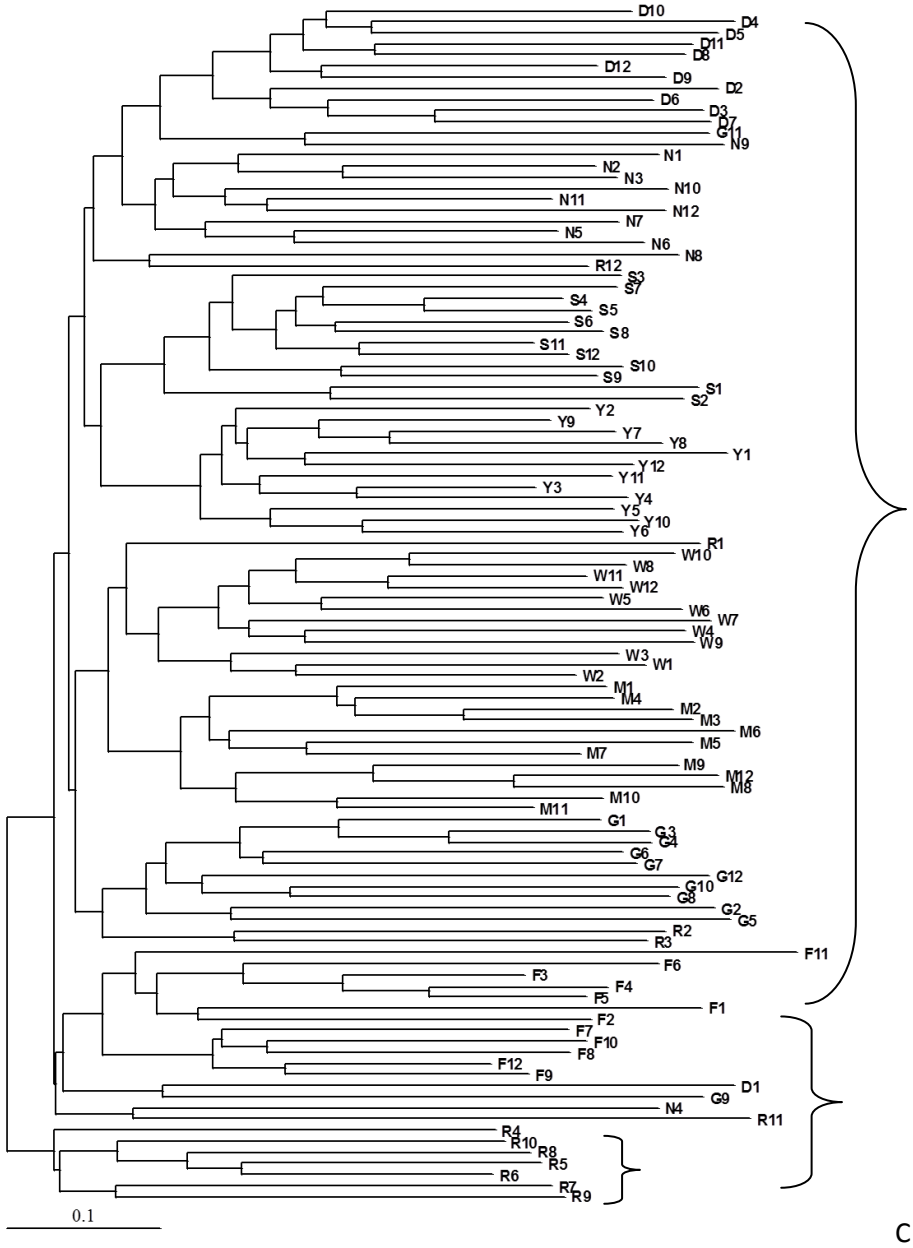


Figure 2. UPGMA dendrogram based on Jaccard's similarity coefficient on ISSR data of the nine *T. schimperi* populations from Bale, North Shewa and East Gojam Zones in Ethiopia.



C

Figure 3. Neighbor joining (NJ) dendrogram of the nine *T. schimperi* populations (108 individuals) based on jaccard's similarity coefficient on ISSR data.

Principal Coordinate Analysis (PCoA)

Principal coordinate analysis (PCoA) as a complementary technique for cluster analysis showed that the PCoA fully supported the UPGMA cluster analysis and also provided grouping of the populations regardless of their geographical locations. Populations from the same had a tendency to remain together.

Similar to the UPGMA clustering pattern, the 108 individuals of *T. schimperi* were grouped into four major groups (clusters) on the principal coordinate (PCoA) (Figure 5). Quadrate I containing most of Waber and Alfameder, quadrate II comprised populations of Shewakidanemeheret and Yedi, quadrate III Fassil and Rira, while populations of Dargegne and Dangule were grouped together in quadrate IV together with the isolated Geremba population.

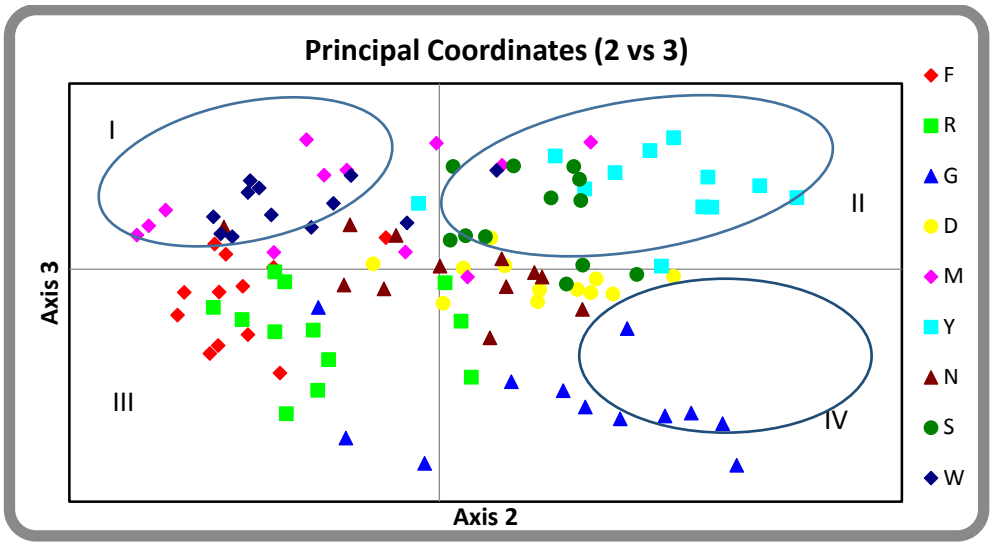


Figure 4. Two dimensional Principal Coordinates Analysis (PCoA) for the 108 individuals via Genetic Distance matrix with data standardization using axis one and three

DISCUSSION

ISSR Markers Polymorphism and Genetic Diversity

Several different molecular markers have been used in previous studies that have examined diversity among cultivated and wild Thymus populations (Bibi, Alamdary, Safarnejad, & Rezaee, 2011; Khalil, Khalil, & Li, 2012;Trindade, 2010). However, no genetic diversity using molecular markers were available for *T. schimperi.* Five ISSR markers used in this study produced informative bands. Primers containing TC and GA repeat motifs generated sharper bands in *T. schimperi*accessions than those with other motifs. Primer 812 (PPL=86.5%) and 811 (PPL=78.5%) with the repeat units of GA showed stable amplification and rich polymorphism than those with other motifs tested. Similar result has been reported by Liu, Liu, and Huang (2011) on *T. triquetrum.* In addition, with AG motifs viz, primers 836 and 835 generated sharper bands with PPL value of 79.5% and 77.1%, respectively, followed by TC motifs with PPL value of 54.4%. Hence, in terms of generating genetic variability in Ethiopian thyme, primers with di-nucleotide repeat, especially the "AG" repeat motifs, could be considered highly informative. The percentage of polymorphic bands (PPB) varied from 15.79% to 100% with an average of 75.2%. Such level of polymorphism is comparable to the results of some similar studies on *Thymus* species and other medicinal plants of Lamiaceae family including *Lamiophlomis rotate*, *Cunilaspp* and other *Thymus spp*(Agostini, Echeverrigaray, & Souza-Chies, 2008; J. Liu, et al., 2006; V. Yousefi, et al., 2015).

Genetic Diversity and Population Differentiation

***T. schimperi*showed high variability as estimated by the average (at population level) percentage of polymorphic loci (PPL=73.88%), Nie's gene diversity (H=0.25) and Shannon's information index (I=0.39). This could be attributed to the out-crossing mating system and floral biology of the species. Indeed, *T. schimperi*is a gynodioecious species in which hermaphrodite (male fertile) and female (male sterile) can occur in the same or in different plant, this establishes out breeding gradient beyond its genetic consequences (Ali, Guetat, & Boussaid, 2012). The different ecological conditions in the three different zones (Bale, East Gojam and North Shewa) of Ethiopia also affect the genetic variations of this species.**

The results for the overall population also showed that *T. schimperi* from the Geremba population of Bale Zone (Ne = 1.50, H = 0.30, I = 0.44) was the richest in the three genetic diversity estimates and the lowest diversity (Ne = 1.34, H = 0.21 and I = 0.33) was exhibited in the Shewakidanemeheret population of East Gojam Zone.

The level of gene flow (Nm) in this study was computed to be 1.13 reflecting lower pollen or seed dispersal among populations and exchange of gene between populations, ultimately promoting the large genetic differentiation among populations as argued by Aiqing Ji1, Guoliang Wu1*, Wenjiang Wu3, Hongyan Yang4, & Wang1, N (2014). As a result, a significant amount of genetic differentiation (*Gst*= 0.31) was observed among the nine populations of *T. schimperi*indicating a substantial differentiation among populations and possibly due to the fixation of a single unique allele in each population (Wright, 1978). Our

result also demonstrated that the populations were subjected to genetic isolation as the higher genetic differentiation of population within a given species is driven by various factors: first, it may be because of low gene flow in the *T. schimperi*habitat (Nm =1.13); second, the mountains found between and within regions contribute to the geographical isolation, which hindered dispersal of pollen or seeds and exchange of genes between populations, ultimately promoting the large genetic differentiation within and among populations; third, in order to adapt to the diverse habitats of the region in the evolutionary process, variants to some extent occurred, which may have been preserved and fixed gradually due to limited gene flow, thereby, genetic differentiation among populations occurred (Aiqing Ji1, et al., N 2014 ; Cao, et al., 2013). According to Handel (1983) as the relative size of populations exchanging genes divergence, gene flow relationships are expected to be increasingly asymmetric. Likewise, selfers tend to have lower gene flow rates than out-crossers and wind-pollinated species have higher gene flow rates than those plants like *T. schimperi*pollinated with an animal pollen vector.

There are extremely few examples of studies of genetic differentiation and genetic diversity in natural populations of gynodioecious species like *Thymus schimperi*. Treuren, Bijlsma, Van Delden, and Ouborg (1991)quantified genetic diversity and population structure in the protandrous perennial Labiatae*Salvia pratensis* (Hs = 0.1 15. H_T = 0.136. G_{ST} = 0.156). This G_{ST} value is similar to that obtained for two populations of *Origmumvulgare* (G_{ST}= 0.178) as reviewed by (Govindaraju, 1988). The populations of these two Labiatae species thus

have noticeably smaller among-population differentiation compared to the current study conducted on *T. schimperi*populations (G_{ST} = 0.31).

The presence of strong differentiation with in geographic groups is further confirmed by AMOVA analysis of the Nine *T. schimperi*populations considered in this study. Accordingly, a total of 69% of the total genetic variation was attributed to intra-populations. This high intra-population genetic diversity suggests the predominance of open pollination system within *T. schimperi*populations.The mating system of a species has implications for the patterns of intraspecies genetic diversity (Hamrick & Nason, 1996). Even though most of the genetic variationswere present among individuals within populations, the result also revealed that there is a significant amount of variation among populations within the three sampled mountain systems (27%) at P < 0.001. However, all of the three levels contributed significantly to the overall genetic variation, as determined through the permutation analyses.

Genetic Relationships among Populations

Geographic proximity contributed to genetic similarity between populations in the case of populations from Oromia region since populations collected from Bale Zone of this region were clustered close to each other. However, this was not always the case as reflected in the sub-clusters that contains populations from geographically distant populations.

The cluster result not necessarily shows the relationship with geographic separation. For example, the Waber population from EastGojamhad a much greater geographic separation (510km) from

north Shewa Zone's populations but clustered together with Alfameder population and not with the East Gojam populations in the dendrogram. Therefore, geographical distance alone cannot account for the genetic distance between populations. These PCoA results showthat the first three principal coordinates explain 28.67%, 21.99and 22.18% cumulative variation, and they correspond largely to the results obtained through cluster analysis. In the, PCoA as well as the UPGMA analysis, the geographic groups were separated from each other and showed a clustering pattern regardless of their geographic origin unlike the result found by Yousefi, Najaphy, Zebarjadi, & Safari (2015), where both the methods classified and presented analogous grouping of fourteen thymus accessions into five groups with minor variation. The Rira population, as it was isolated in the UPGMA and NJ dendrogram, also appeared separated in the PCoA plot, too, and this population might have a larger possibility of heterosis in a breeding program to improve favorite medicinal properties or agronomical traits in Thymus species.

CONCLUSION

The analyzed ISSR markers created sufficient polymorphism and reproducible fingerprinting profiles. They proved to be informative and provided reliable molecular tool for detecting genetic variation and relationships of thyme populations. In addition, this research provided information about geographic distribution and genetic distance of the *T. schimperi*. The results of the PCoA corresponded largely to the results obtained by cluster analysis. Both PCoA and UPGMA cluster analysis approved the clustering of all nine populations into four groups, regardless of the geographic distribution patterns of the

populations. The genetic variation data would be very useful for the improvement of Ethiopian thyme through conventional breeding programs as well as molecular breeding approaches such as marker assisted selection and also design sustainable use strategy. This study also supports the hypothesis that variations in ecological conditions affect genetic variations of *T. schimperi.*

The observed patterns of molecular variation can be used to draw important taxonomic and phytogeographic conclusions which are of great importance to the conservation of genetic resources and for breeding programs. Our results highlight the need to study the genetic variation of unexplored medicinal and aromatic plants in Ethiopia using molecular techniques.

Information generated thorough such studies are useful for developing appropriate conservation strategies which would ensure that there is less anthropogenic destruction of existing habitats, increase in the natural population size, optimization and improvement of cultivation practices resulting in constant supply of plant material without overexploiting the natural populations.

According toCrawford, et al. (2001) and Zaouali and Boussaid (2008) the analysis of the genetic diversity of species is an important tool for conservation programs. The ability of populations to respond to selection forces is related to the level of genetic variation available (Reed, 2007). Corresponding to the estimates of genetic diversity parameters found in this study, the majority of populations showed a high variation suggesting that populations constitute a valuable germplasm for conservation and sustainable use.

REFERENCES

Agostini, G., Echeverrigaray, S., & Souza-Chies, T. (2008). y. *Agostini, G, 274*(3-4), 135-141.

Aiqing Ji1, Yina Wang1, , Guoliang Wu1*, Wenjiang Wu3, Hongyan Yang4, & Wang1, Q. (N 2014). <Genetic Diversity and Population Structure.pdf>. *Am J Plant Sci*,(5), 3194-3202. doi:10.4236/ajps.2014.521335

Ait M'Barek, L., Ait Mouse, H., Jaâfari, A., Aboufatima, R., Benharref, A., Kamal, M., . . . Gamouh, A. (2007). Cytotoxic effect of essential oil of thyme (Thymus broussonettii) on the IGR-OV1 tumor cells resistant to chemotherapy. *Brazilian Journal of Medical and Biological Research, 40*(11), 1537-1544.

Ali, I. B. E. H., Guetat, A., & Boussaid, M. (2012). Genetic diversity, population structure and relationships of Tunisian Thymus algeriensis Boiss. et Reut. and Thymus capitatus Hoffm. et Link. assessed by isozymes. *Ali, Imen Ben El Hadj, 36*(1), 149-163.

Assouad, M., Dommée, B., Lumaret, R., & Valdeyron, G. (1978). Reproductive capacities in the sexual forms of the gynodioecious species Thymus vulgaris L. *Bot J Linn Soc, 77*(1), 29-39.

Basch, E., Ulbricht, C., Hammerness, P., Bevins, A., & Sollars, D. (2004). Thyme (Thymus vulgaris L.), thymol. *Journal of Herbal Pharmacotherapy, 4*(1), 49-67.

Bibi, S., Alamdary, L., Safarnejad, A., & Rezaee, M. (2011). Evaluation of Genetic Variation between Thymus Accessions Using Molecular Markers. *1*(12), 2552-2556.

Borsch, T., Hilu, K., Quandt, D., Wilde, V., Neinhuis, C., & Barthlott, W. (2003). Noncoding plastid trnT-trnF sequences reveal a well resolved phylogeny of basal angiosperms. *Journal of Evolutionary Biology, 16*(4), 558-576.

Brabant, P., LEFORT, P. G. G., VALDEYRON, G., & VERNET, P. (1980). Pollination studies in Thymus aulgarir L.(Labiatae).

Cao, Y., Zhang, Y., Yu, Z., Mi, F., Liu, C., Tang, X., . . . Xu, J. (2013). Structure, gene flow, and recombination among geographic

populations of a Russula virescens Ally from southwestern China. *PloS One, 8*(9), e73174.

Crawford, D. J., Ruiz, E., Stuessy, T. F., Tepe, E., Aqeveque, P., Gonzalez, F., . . . Baeza, C. M. (2001). Allozyme diversity in endemic flowering plant species of the Juan Fernandez Archipelago, Chile: ecological and historical factors with implications for conservation. *American Journal of Botany, 88*(12), 2195-2203.

Dawit Abebe, & Ahadu Ayehu. (1993). Medicinal plants and enigmatic health practices of Northern Ethiopia.

Ellstrand, N. C. (2014). I S Gene Flow the Most Important Evolutionary Force. *101*(5), 737-753. doi:10.3732/ajb.1400024

Fachini-Queiroz, F. C., Kummer, R., Estevao-Silva, C. F., Carvalho, M. D. d. B., Cunha, J. M., Grespan, R., . . . Cuman, R. K. N. (2012). Effects of thymol and carvacrol, constituents of Thymus vulgaris L. essential oil, on the inflammatory response. *Evidence-Based Complementary and Alternative Medicine, 2012*(A.M.A. Archives of Industrial Hygiene and Occupational Medicine).

Friis, I. (1992). *Forests and forest trees of northeast tropical Africa: their natural habitats and distribution patterns in Ethiopia, Djibouti and Somalia*: London (UK), Her Majestry's Stationery Office, 1992.

Govindaraju, D. R. (1988). Relationship between dispersal ability and levels of gene flow in plants. *Govindaraju, Diddahally R*, 31-35.

Hamrick, J. L., & Nason, J. D. (1996). Consequences of dispersal in plants. *Population dynamics in ecological space and time, 1*, 203.

Handel, S. N. (1983). Pollination ecology, plant population structure, and gene flow. *Pollination biology, 163*, 211.

Hardman, R., Stahl-biskup, E., & Sáez, F. (2002). Thyme The genus Thymus. *15*.

Harley, R., & Brighton, C. (1977). Chromosome numbers in the genus Mentha L. *Botanical Journal of the Linnean Society, 74*(1), 71-96.

Harley, R., Paton, A., Harvey, T., & Gardens, R. B. (1998). Lamiales newsletter. *Harley, Ray*(6).

Huck, R. (1992). Overview of pollination biology in the Lamiaceae. *Huck, R*, 167-181.

Jaccard, P. (1908). *Nouvelles recherches sur la distribution florale*.

Jackson, L., Bawa, K., Pascual, U., & Perrings, C. (2012). Agrobiodiversity: a new science agenda for biodiversity in support of sustainable agroecosystems. DIVERSITAS report N 4 (pp. 4-40).

Jansen, P. C. M. (1981). *Spices, condiments and medicinal plants in Ethiopia, their taxonomy and agricultural significance*: PudocInternational Journal of Agriculture and Biology.

Jukic, M., Politeo, O., Maksimovic, M., Milos, M., & Milos, M. (2007). In vitro acetylcholinesterase inhibitory properties of thymol, carvacrol and their derivatives thymoquinone and thymohydroquinone. *Phytotherapy Research, 21*(3), 259-261.

Khalil, R., Khalil, R., & Li, Z. (2012). Determination of genetic variation and relationship in Thymus vulgaris populations in Syria by random RAPD markers. *Journal Plant Biosystems, 146*, 217-225.

Khlestkina, E. K., Varshney, R. K., Röder, M. S., Graner, A., & Börner, A. (2006). A comparative assessment of genetic diversity in cultivated barley collected in different decades of the last century in Austria, Albania and India by using genomic and genic simple sequence repeat (SSR) markers. *Khlestkina, Elena K, 4*(02), 125-133.

Liu, D., He, X., Liu, G., & Huang, B. (2011). Genetic diversity and phylogenetic relationship of Tadehagi in southwest China evaluated by inter-simple sequence repeat (ISSR). *Tileye,Feyissa, 58*(5), 679-688.

Liu, J., Wang, L., Geng, Y., Wang, Q., Luo, L., & Zhong, Y. (2006). Genetic diversity and population structure of Lamiophlomis rotata (Lamiaceae), an endemic species of Qinghai–Tibet Plateau. *Genetica, 128*(1-3), 385-394.

Markers, R. I., Rahimmalek, M., Bahreininejad, Æ. B., Khorrami, M., Ebrahim, Æ. B., & Tabatabaei, S. (2009). Genetic Variability and Geographic Differentiation in Thymus daenensis subsp . daenensis , an Endangered Medicinal Plant , as Revealed by Inter Simple Sequence. 831-842. doi:10.1007/s10528-009-9281-z

Morales, R., Stahl Biskup, E., & Sáez, F. (2002). The history, botany and taxonomy of the genus Thymus. *Morales, Ramón*, 1-43.

Nei, M. (1973). Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences, 70*(12), 3321-3323.

Nei, M. (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics, 89*(3), 583-590.

Nigist Asfaw, & Sebsebe Demissew. (2009). Aromatic plants of Ethiopia: Shama books, Addis ababa, Ethiopia.

Pavlicek, A., Hrda, S., & Flegr, J. (1999). Short Communication FreeTree-Freeware Program for Construction of Phylogenetic Trees on the Basis of Distance Data and Bootstrap/Jackknife Analysis of the Tree Robustness. Application in the RAPD Analysis of Genus Frenkelia. *Folia Biologica (Praha), 45*, 97-99.

Peakall, & Smouse. (2012). GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. *Bioinformatics, 28*, 2537-2539.

Reed, D. (2007). Selection and the rate of loss of genetic variation: Natural selection and genetic diversity. *Heredity, 99*(1), 1-2.

Rohif, F. (2000). NTSYS-pc: Numerical taxonomy and multivariate analysis system, version 2.1. *Rohif, FJ*.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4*(4), 406-425.

Sebsebe Demissew. (1993). The Genus Thymus (Labiatae) in Ethiopia. *Sebsebe Demissew*,(121), 57-60.

Smolik, M., Jadczak, D., & Korzeniewska, S. (2009). Assessment of morphological and genetic variability in some Thymus accessions using molecular markers. *Smolik, Milosz, 37*(2), 234.

Sneath, P. H., & Sokal, R. R. (1973). *Numerical taxonomy. The principles and practice of numerical classification*.

Tefera, Z. (1975). Community management of Afroalpine highlands in Ethiopia. 10-11.

Trindade, H. (2010). Molecular biology of aromatic plants and spices. A review. *Bagci, Ey*[illegible]*p, 25*(5), 272-281. doi:10.1002/ffj.1974

Van Treuren, R., Bijlsma, R., Van Delden, W., & Ouborg, N. (1991). The significance of genetic erosion in the process of extinction. I. Genetic differentiation in Salvia pratensis and Scabiosa columbaria in relation to population size. *Heredity, 66*, 181-189.

Wright, S. (1978). Vol. 4: Variability within and among natural populations. *Wright, S.*

Yeh, F. C., Yang, R., Boyle, T., Ye, Z., & Mao, J. X. (1999). POPGENE, version 1.32: the user friendly software for population genetic analysis. *Yeh, Francis C.*

Yousefi, V., Najaphy, A., Zebarjadi, A., & Safari, H. (2015). Molecular Characterization Of Thymus Species Using Issr Markers. *Yousefi, V, 25*(4), 1087-1094.

Yousefi, V., Najaphy, A., Zebarjadi, A., Safari, H., Resources, N., & Resources, N. (2015). Molecular Characterization Of Thymus Species Using Issr Markers. *25*(4), 1087-1094.

Zaouali, Y., & Boussaid, M. (2008). Isozyme markers and volatiles in Tunisian Rosmarinus officinalis L. (Lamiaceae): A comparative analysis of population structure. *Biochemical Systematics and Ecology, 36*(1), 11-21. doi: http://dx.doi.org/10.1016/j.bse.2007.08.005