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Genetic Diversity Study of Quarin Clover (T. quartinianum) Accessions of Ethiopia Using ISSR Markers

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Abstract
Genetic diversity of *Trifolium quartinianum* accessions from Ethiopia were studied using Inter simple sequence repeat (ISSR) markers. A total of 24 accessions, divided into three populations were used for the present study. DNA was extracted from a bulk of samples using a modified CTAB method. A total of 84 bands were amplified by the four di-nucleotid ISSR primers in the overall experimental materials. Genetic diversity was high at the species level (PPL = 100%, h = 0.29, I = 0.44). Comparison of population-based genetic diversity showed that Gojam population was the most diverse. Analysis of molecular variance (MOVA) revealed high level of within-population variation with 83.13%. This could be caused by high pollen and seed flow among-populations. Unweighted pair group method with arithmetic average (UPGMA) and Principal Coordinate (PCo) analysis showed that only accessions of *T. quartinianum* from Gondar formed separate cluster. The study clearly indicates the presence of variable genotypes with their unique identity that deserve conservation attention.
Keywords: Genetic diversity; Ethiopia; ISSR markers; *T. quartinianum*

INTRODUCTION

Trifolium quartinianum A. Rich belongs to the genus *Trifolium* and section Vesicastrum (Ellison *et al.* 2006). It is a diploid with 2n =16 (Badr, 1995) and self pollinated (ILCA, 1990) species indigenous to east African highlands. It is suitable for hay and silage making to increase the quality of straw-based diets and to overcome seasonal feed shortage. Compared to other native Ethiopia clovers, it is the most productive with vigorous growth that can produce 7800kg dry matter per ha within three months when growing conditions are favorable (Kahurananga and Asres Tsehay, 1991). It has higher seed production capacity compared to other *Trifolium* species and may adapt to a wide range of soils from heavy to clay vertisols and nitosols to loams and sandy loams (Akundabweni and Njuguna, 1996). It tolerates seasonal water logging. It has higher biomass production under different moisture condition (Friedericks *et al*., 1991). It nodulates well with most of the *Rhizobium* strains (Myton *et al.,* 1988). This species has potential to improve natural or sown pastures in the tropical highlands of Africa (Lulseged Gebrehiwot *et al.,* 1996). It combines well with other annual clovers and short-growing grasses. When grown in mixture with *T. quartinaianum,* grasses are known to accumulate more dry matter (DM), crude protein (cp) and *in vitro* digestable dry matter (IVDDM) than grass monoculture (Lulseged Gebrehiwot *et al.,* 1997). This species can be intercropped with wheat without significant reduction of grain yield (Kahurananga, 1987). According to Zewdu Tesema (2004), *T. quartinianum* can be undersown with barley simultaneously or at first weeding without affecting the grain and straw yield of barley but significantly increasing the total fodder yield.

In recent years, a number of PCR-based DNA markers have been developed to evaluate genetic variation at the intraspecific and interspecific levels (Wolfe and Liston, 1998). In *Trifolium*, primarily Random Amplified Polymorphic DNA (RAPDs) was used to evaluate genetic diversity in *T. pratense* (Ulloa *et al*., 2003) and *T. resupinatum* (Arzani and Samei, 2004). However, the ISSR-PCR method (Wolfe and Liston, 1998) using primers based on di, tri, tetra, penta nucleotide repeats without the requirement for prior knowledge of the genome sequence seems particularly suitable for germplasm comparison. For the *Trifolium* species studied, the ISSR markers were used for the first time to study genetic diversity of three South American and three Eurasiatic species (Rizza *et al.,* 2007). ISSR markers were used to asses genetic diversity of four clover species from Europe (Dabkevičienė *et al.,* 2011).These research findings suggest that ISSR markers system are suitable for trifolium genetic diversity study.

Based on the above ground, ISSR marker was chosen for genetic diversity study of *T. quartinianum* collected from different geographical locations of Ethiopia. Despite the importance of this clover as livestock feed and contribution to soil fertility, so far, there were no published reports for its genetic diversity study using ISSR markers. Few genetic diversity studies based on morphological and agronomic traits were conducted. However, morphological variability is often has limitations; since characters may not be observed at all stages of the plant development and traits may be affected by environment. Hence, the present study aimed to determine the genetic relationship and pattern of variation among three populations of *T. quartinianum* as well as intra population genetic diversity using inter simple sequence repeat (ISSR) markers. The study can give a base line information for efficient preservation, exploitation of the existing genetic resources and assist for germplasm

management.

MATERIALS AND METHODS

Plant material

For this study, 24 accessions of *T. quartinianum* collected from three different administrative regions (AR) of Ethiopia were kindly provided by International Livestock Research Institute (ILRI) Forage Germplasm Bank, Addis Ababa, Ethiopia. Accessions collected from each AR were considered as a single population and, therefore, samples (accessions) collected from randomly selected districts of an AR were used to represent each population (Table 1). Seeds of each accession were sown in plastic pots and grown in the greenhouse of the College of Natural Sciences, Addis Ababa University, until the seedlings produced young leaves sufficient for DNA extraction.

Table 1. List of ***T.*** quartinianum accessions with their associated information used for the present study

Acc. No	District	Zone/Adm. region	Latitude	Longitude	Altitude (m)	Lab. Code
9428	Debre Markos	Shewa	10˚08´ N	037 ˚58´ E	2430	Tq Sh49
9975	Chebo & Gurage	Shewa	08 ˚15´ N	037 ˚40´ E	1840	Tq Sh50
8321	Menagesha	Shewa	08˚48 ´N	038˚54´ E	1870	Tq Sh51
9968	Chebo & Gurage	Shewa	08 ˚24´ N	037 ˚52´ E	1870	Tq Sh52
6297	Menagesha	Shewa	09˚02´ N	038˚45 ´E	2380	Tq Sh53
14408		Shewa	-	-	-	Tq Sh54
8473	Chebo & Gurage	Shewa	08˚21´ N	037˚49´ E	1880	Tq Sh55
8464	Chebo & Gurage	Shewa	08˚17´ N	037 ˚47´ E	1880	Tq Sh56
13716		Shewa	10˚ 02´ N	038 ˚14 ´E	2010	Tq Sh57
7675	Debre Markos	Gojam	10˚14´ N	038˚06´ E	2400	Tq Goj58
2049	Debre Markos	Gojam	10˚12´ N	037˚52´E	2400	Tq Goj59
2047	Debre Markos	Gojam	10˚15´ N	037˚57´ E	2500	Tq Goj60
8521	Debre Markos	Gojam	11˚26´ N	037 ˚36´ E	2200	Tq Goj61
6277	Bahir Dar	Gojam	11˚40´ N	037˚28´ E	1900	Tq Goj62
9378	Debre Markos	Gojam	10 ˚07´ N	038˚ 09´ E	1980	Tq Goj63
8540	Debre Markos	Gojam	10˚13´ N	037˚52´ E	2450	Tq Goj64
7693	Debre Markos	Gojam	10˚14´ N	037˚52´ E	2360	Tq Goj65
8535	Debre Markos	Gojam	10 ˚42 ´N	037˚04´ E	2100	Tq Goj66
7771	Libo	Gondar	12˚03´ N	037 ˚44´ E	1840	Tq Gon67
14586		Gondar	-	-	-	Tq Gon68
13808		Gondar	-	-	1950	TqGon69
7759	Gondar	Gondar	12˚22´N	037˚17´ E	1860	TqGon70
7746	Debre Tabor	Gondar	11˚53´ N	037˚41´ E	1860	Tq Gon71
7768	Gonder	Gondar	12˚27´N	037˚31´ E	1940	Tq Gon72

(**Source:** ILRI)

Acc. = Accession, E = East, N = North, m = meter, Adm. = Administrative, Lab. = Laboratory, TqSh = *T. quartinianum* from Shewa, TqGoj = *T. quartinianum* from Gojam, TqGon = *T. quartinianum* from Gondar

DNA extraction

Fresh young leaves of three individuals of an accession were bulked and ground to a fine powder by pestle and mortar in liquid nitrogen and thereafter transferred to 1.5 ml eppendorf tubes. Total genomic DNA was extracted from the fine powder following the Cetyl Trimethyl Ammonium Bromide (CTAB) protocol with slight modification established by (Borsch *et al.,* 2003). Finally pellets were dissolved in 100 µl 1x TE solutions and kept in freezer at -20 °C for subsequent use.

PCR amplification and gel electrophoresis

A total of 13 ISSR primers were screened based on previously published results in *Trifolium* and related species for their ability to generate consistently amplified band patterns (Rizza *et al*., 2007, Dabkevičienė *et al.*, 2011, Arslan and Tamkoc, 2009) and to access polymorphism in two random DNA samples from each population.

Only four primers were selected and used for the analysis of 24 accessions based on the number of amplification products, the quality of the profiles, the level of polymorphism and the reproducibility of bands (Table 2). Furthermore, the PCR-reaction components were optimized in such a way that the optimum amount of reagents would produce clear and unambiguous banding patterns. Each PCR reaction of ISSR markers had a final reaction volume of 25 µl , containing 17 µl ddH_20, 0.8 µl dNTPs (25 mM each), 2.5 µl of reaction buffer (10x taq polymerase buffer with 15 mM $MgCl_2$), 3µl $MgCl_2$ (25mM), 0.4µl primer (20 pmol/µl) and 0.3µl Taq Polymerase (1.5 unit) and1µl diluted template DNA. Amplifications were performed in Biometra 2003 T3

Thermo cycler programmed to run the following temperature profile: a preheating and initial denaturation for 4 minutes at 94 $^{\circ}C$, then 40 x 15 seconds denaturation at 94 $^{\circ}C$, 1 minute primer annealing at 48 $^{\circ}C$, 1.30 minutes extension at 72 $^{\circ}C$ and the final extension for 7 minutes at 72 $^{\circ}C$. For each primer a negative control reaction was included where the template DNA was replaced by ddH2O to check for absence/presence of contamination. Agarose gel of 1.67 % concentration was prepared in 1X TBE buffer (0.86g agarose in 50 ml 1X TBE and 2 µl Ethidium bromide). PCR product (11 µl) and 1 µl 6X loading dye were loaded into the wells. Fifteen wells comb was used for each gel slab. The first lane was loaded with 100 bp ladder by loading 2 µL (peq gold range mix) with loading dye in that well as a size standard and the last lane was control (without template DNA).The electrophoreses were done for about 3 hours at constant voltage of 80V. After electrophoresis, the gel was stained in ethidium bromide, distained with ddH2O, visualized under UV light, photographed and documented.

Table 2. List of primers, sequence and repeat motives used for the study

Code of primers	Primer sequence	Annealing temperature ($^{\circ}C$)	Motives
818	CACACACACACACACAG	48	Dinucleotide
841	GAGAGAGAGAGAGAGAYC	48	Dinucleotide
844	CTCTCTCTCTCTCTCTRC	48	Dinucleotide
848	CACACACACACACACARG	48	Dinucleotide

Source: Primer kit 900 (UBC 900); Y = Pyrimidines (C or T), R = purines (A or G).

Band scoring and data analysis

The ISSR band profiles were treated as dominant markers and each locus was considered as a bi-allelic locus with one amplifiable and one null allele. Scoring was performed manually for each primer based on presence (1) and absence (0) or as a missing observation (?), and each band was regarded as a locus. Only amplified bands that were clearly resolved were recorded, and a "0" and "1" data matrix was established. The resulting presence/absence data matrix of the ISSR phenotype was analysed using POPGENE version1.32 software (Yeh *et al.*, 1999) to calculate the following genetic diversity parameters: percentage of polymorphic loci (PPL), gene diversity (h), and Shannon's information index (*I*). The genetic structure was investigated using Analysis of Molecular Variance (AMOVA). The AMOVA analysis was carried out using the software ARLEQUIN version 3.01 (Excoffier *et al.,* 2006) to estimate genetic variability within and among populations without grouping. NTSYS- pc version 2.02 (Rohlf, 2000) and Free Tree 0.9.1.50 (Pavlicek *et al.,* 1999) software's were used to calculate Jaccard's similarity coefficient which is calculated with the formula:-

$$S_{ij} = \frac{a}{a+b+c}$$

Where,

'a' is the total number of bands shared between individuals i and j,

'b' is the total number of bands present in individual i but not in individual j and

'c' is the total number of bands present in individual j but not in individual i.

The unweighted pair group method with arithmetic average (UPGMA) (Sneath and Sokal, 1973) was used in order to determine the genetic relationship among accessions and generates phenogram using NTSYS- pc version 2.02 (Rohlf, 2000). The neighbor joining (NJ) method (Saitou and Nei, 1987; Studier and Keppler, 1988) was used to compare individual accessions and evaluate patterns of accession clustering using Free Tree 0.9.1.50 Software (Pavlicek *et al.,* 1999).

To further examine the patterns of variation among individual samples, a principal coordinated analysis (PCO) was performed based on Jaccard's coefficient (Jaccard, 1908). The calculation of Jaccard's coefficient was made with PAST software version 1.18 (Hammer *et al.,* 2001). The first three axes were later used to plot with STATISTICA version 6.0 software (Hammer *et al.,* 2001; Statistica soft, Inc.2001).

RESULTS AND DISCUSSION

ISSR primers and banding patterns

An initial screening of 13 ISSR primers enabled selection of four primers, which produced satisfactory amplification profiles in *T. quartinianum* accessions. All the selected four primers have dinucleotide repeat motives. In total, 84 very clear identifiable ISSR fragments and informative patterns were amplified using the four ISSR primers. The number of bands amplified by each primer ranged from 18 to 24 with an average of 21 bands per primer. Primer 848 produced the highest number of bands, while the lowest number of bands was amplified by primer 844 (Table 3). The size of the fragments amplified with these primers was in the range of 200 to 1000bp. Representative gel illustrating the amplification profiles produced by the ISSR marker assay by employing primers 844 is shown in figure 1.

Table 3. List of ISSR primers used and overview of their banding pattern

Primers	Repeat motif	Annealing temperature (°C)	No of scorable bands
818	$(CA)_8G$	48	22
844	$(CT)_8RC$	48	18
841	$(GA)_8YC$	48	20
848	$(CA)_8RG$	48	24
Total			84
Average			21

Y = (C, T); R = (A, G).

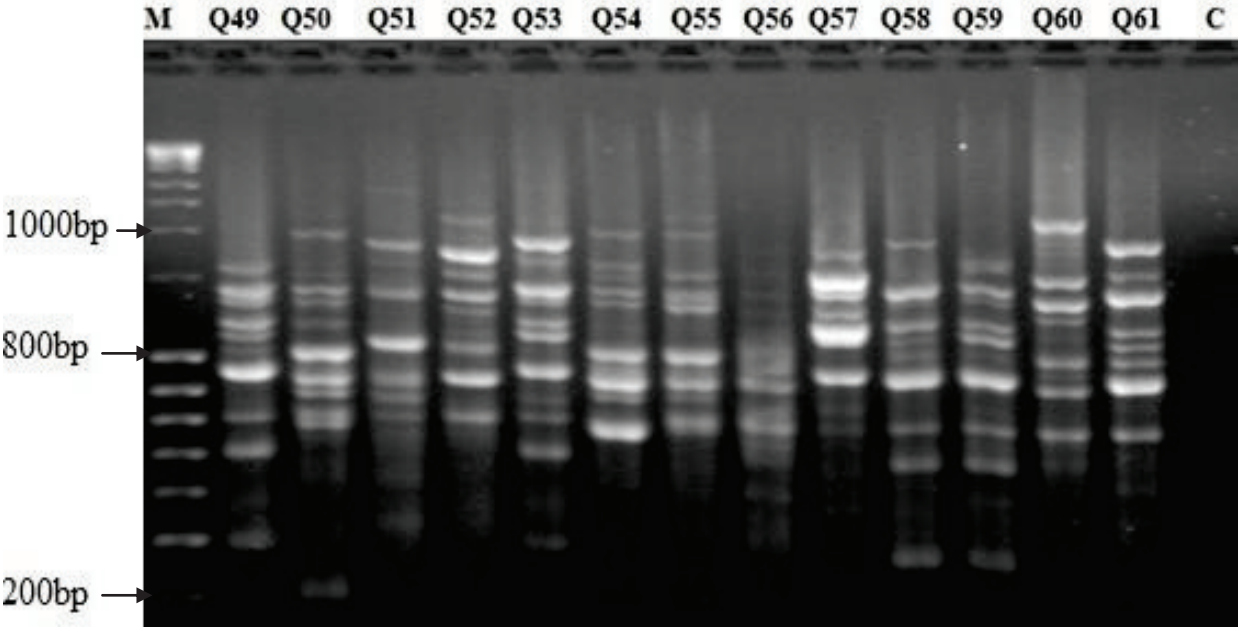

Figure 1. Banding pattern of primer 844 in T. quartinianum accessions. M represents a 1000 bp DNA ladder; Q stand for *T. quartinianum*, while the numbers associated with these letters represent accessions, C represents control.

Genetic diversity

Inter-simple sequence repeat (ISSR) markers have become widely used in population studies because they have been found to be highly variable, to require less investment in time, money and labor than other methods (Wolfe and Liston, 1998), and to have the ability to be inherited (Gupta *et al.,* 1994; Tsumura *et al.,* 1996). Several researchers used ISSR markers to study genetic diversity within and among populations of forage crops (Bolourchian *et al.,* 2013; Jonaviclene *et al.,* 2009; Zarrabian *et al.,* 2013; Shirvani1 *et al.,* 2013). Better reproducibility of products of ISSR bands compared to other markers such as RAPD could be due to its longer SSR-based primers with higher annealing temperatures (Huangfu *et al.,* 2009). Moreover, as microsatellites are frequent and widely distributed throughout the genome, the ISSR targets are abundant. Compared with SSR markers, where the flanking regions of the SSR motifs have to be known in advance, ISSR amplification takes advantage of the fact that no prior sequence information is required, and the results are therefore obtained more rapidly and cost effectively (Wang *et al.,* 2008; Yang *et al.,* 1996; Bornet and Branchard, 2001).

The present study employed ISSR markers to assess genetic diversity within and among populations of *T. quartinianum* accessions from Ethiopia. A total of 84 bands were amplified, all of which showed 100% polymorphism. All of the four primers used also showed 100% polymorphism. Over all gene diversity (h) and Shannon's information index (I) by the four ISSR primers were 0.29 and 0.44, respectively. The higher values for gene diversity (h) and Shannon's information index (I) were in primer 844, while 848 showed the lowest indexes (Table 3).

Table 3. Level of genetic diversity revealed by the four ISSR primers

Primer	NPL	PPL	h	I
818	22	100	0.29	0.44
844	18	100	0.31	0.48
841	20	100	0.29	0.45
848	24	100	0.27	0.41
Over all	84	100	0.29	0.44

NPL = Number of polymorphic Loci; PPL = Percent of polymorphic Loci; h = gene diversity; I = Shannon's information index

In this study the di-nucleotide ISSR primers 818 and 848 with CA repeat, and 844 and 841 with CT and GA motives, respectively, detected genetic diversity within and among populations. Generally primers with (AG), (GA), (CT), (TC), (AC), (CA) repeats show higher polymorphism in plants than primers with other di-, tri- or tetra nucleotides (Reddy *et al.,* 2002). Moreover, the choice of appropriate primer motives in ISSR fingerprint is critical to detect high polymorphism and reveal relationship within and among populations. The four ISSR primers chosen for this study amplified large number of bands, varying from 18-24 per primer displaying 100% polymorphism with high mean gene diversity (h= 0.29) and Shannon's information index (I= 0.44). This indicates the existence of high level of genetic diversity among the three populations of *T. quartinianum* in Ethiopia. High genetic diversity found among the population could be due to lack of selection and strict domestication under low overgrazing.

The ISSR survey of three populations of *T. quartinianum* revealed a high level of genetic variation at the species level (PPL = 100%; h = 0.29; I = 0.44). The least polymorphic and genetically unique population was Gonder (PPL = 57.14%; h = 0.18; I = 0.27), while Gojam was the most polymorphic and diverse (PPL = 82.14%; h = 0.28; I = 0.41) (Table 4). Previous assessments of genetic diversity in 34 *T. quartinianum* accessions of Ethiopia based on eight morphologic and agronomic traits have reported that most of the accessions showed similarity in morphological characteristics (Basweti and Hanson, 2012). This finding contradicted with the present study, mainly because morphological features have a number of limitations including low polymorphism, low heritability, late expression, and vulnerability to environmental influences (Smith, and Smith, 1992; Konarev, 2000; Muthusamy *et al.,* 2008), which, in turn limits their utility for assessing real genetic diversity.

Up to now, there are no published reports concerning ISSR method for analyzing the genetic diversity of *T.quartinianum*. However, ISSR marker was used to assess the level and pattern of genetic diversity in four *Trifolium* species represented by two varieties, one breeding sample and two wild population of *T. pratense,* four wild populations of *T. medium,* two varieties and one population of *T. resupinatum*, and two varieties and three wild populations of *T. repense*. The study showed 69.5% polymorphism in *T. medium,* 68.9% in *T. resupinatum,* 76.2% in *T. pretense* and 73.6% in *T. repense* (Dabkevičienė *et al.,* 2011). Recently, ISSR marker also used for the genetic diversity study of 14 accessions of three species of *Trifolium* and 60% polymorphism in *T. fragiferum*, 58.67% in *T. hybridum* and 77.32% in *T. pratense* was found (Aryanegad *et al.,* 2013*)*. The genetic diversity investigated in the present study was higher than the ones reported by Dabkevičienė *et al.,* (2011) and Aryanegad *et al.,* (2013*)*. Generally, the higher level of genetic variation found in this study may be due to the fact that geographically isolated populations in certain geographic locations could accumulate genetic differences and evolve unique genotypes as they adapt to different environment (Souframanien and Gopalakrishn, 2004).

In general, high level of genetic diversity is not expected with strictly limited distribution and a small population size. Nevertheless, ISSR markers used in this study generated higher level of polymorphism in 24 accessions of *T. quartinianum* from limited geographical location in North West Ethiopia. This shows that small populations or individuals are not always associated with a lack or low level of genetic variation (Yingjuan and Ting, 2009).

Table 4. Genetic diversity within populations of *T. quartinianum*

Population	**NPL**	**PPL**	**h**	**I**
Shewa	**64.0**	**76.19**	**0.25**	**0.38**
Gojam	**69.0**	**82.14**	**0.28**	**0.41**
Gonder	**48.0**	**57.14**	**0.18**	**0.27**
Average	**60.3**	**71.80**	**0.24**	**0.35**
Over all	**84.0**	**100**	**0.29**	**0.44**

NPL = Number of polymorphic loci; PPL = Percent of polymorphic Loci; h = Gene diversity; I = Shannon's information index

Analysis of Molecular Variance (AMOVA)

The AMOVA without grouping indicated that most of the total genetic variation in *T. quartinianum* populations exists within populations (83.13%), while among population variation (16.87%) was observed to be low (Table 5). High genetic variation within populations indicated that high genetic dissimilarities among the individual

plants sampled from a single population. The vast majority of diversity studies across members of the Trifolieae have shown a generally high level of diversity within populations, even among other inbreeding species such as *T. subterraneum* (Pecetti and Piano, 2002; Piluzza *et al*., 2005). The AMOVA results obtained in the present study do not contradict with the above findings. It is a prevalent view that self-pollinated species maintain higher genetic variation among populations than within populations. Though *T. quartinianum* is self-pollinated plant, higher within populations genetic variation than among populations was obtained for this species in the present study, contrary to this prevalent view. High genetic exchange or gene flows, which actually have a more homogenizing effect on the genetic variation among populations by the dispersal of the seeds, can likely explain these higher within population genetic variation. Some seeds may be harvested as weeds together with those of crops and distributed with the crop seeds via market channels. Moreover, the pods and mature calyx of *T. quartinianum* tend to have a coarse surface and points which may attach to passing animals and be transported to other places.

Table 5. Analysis of molecular variance (AMOVA)

Source of variation	**d.f**	**Sum of** squares	**Variance** components	**Percentage** of variation	**P-Value***
Among populations	**2**	**69.66**	**2.59**	**16.87**	**P< 0.001**
Within populations	**23**	**281.49**	**12.80**	**83.13**	**P< 0.001**
Total	**25**	**351.16**	**15.40**		

d.f = degree of freedom, * significance tests after 1023 permutations

Genetic relationship within and among population

The UPGMA dendrogram of *T. quartinianum* accessions showed that most accessions of the same population formed a unique cluster, while others were distributed over the tree. Moreover, all accessions from Gonder population grouped in one major cluster and the genetic similarity values among accessions were relatively high. All of the accessions from each population of Gojam and Shewa were not grouped in a single major cluster but formed different clusters involving most of the accessions from the same populations and the similarity value among the accessions was relatively low. The two dimensional plot (PCO) showed that accessions collected from Gonder grouped together and appear separate from other populations, while accessions of Gojam and Shewa were not clustered based on populations of origin, rather intermixed in one group. Accessions of Gojam and Shewa in one group present an important genetic similarity despite of their different geographical origins. This was further confirmed by the three dimensional plot and the pattern could be explained by long distance seed flow facilitated by human for forage cultivation for livestock feed production.

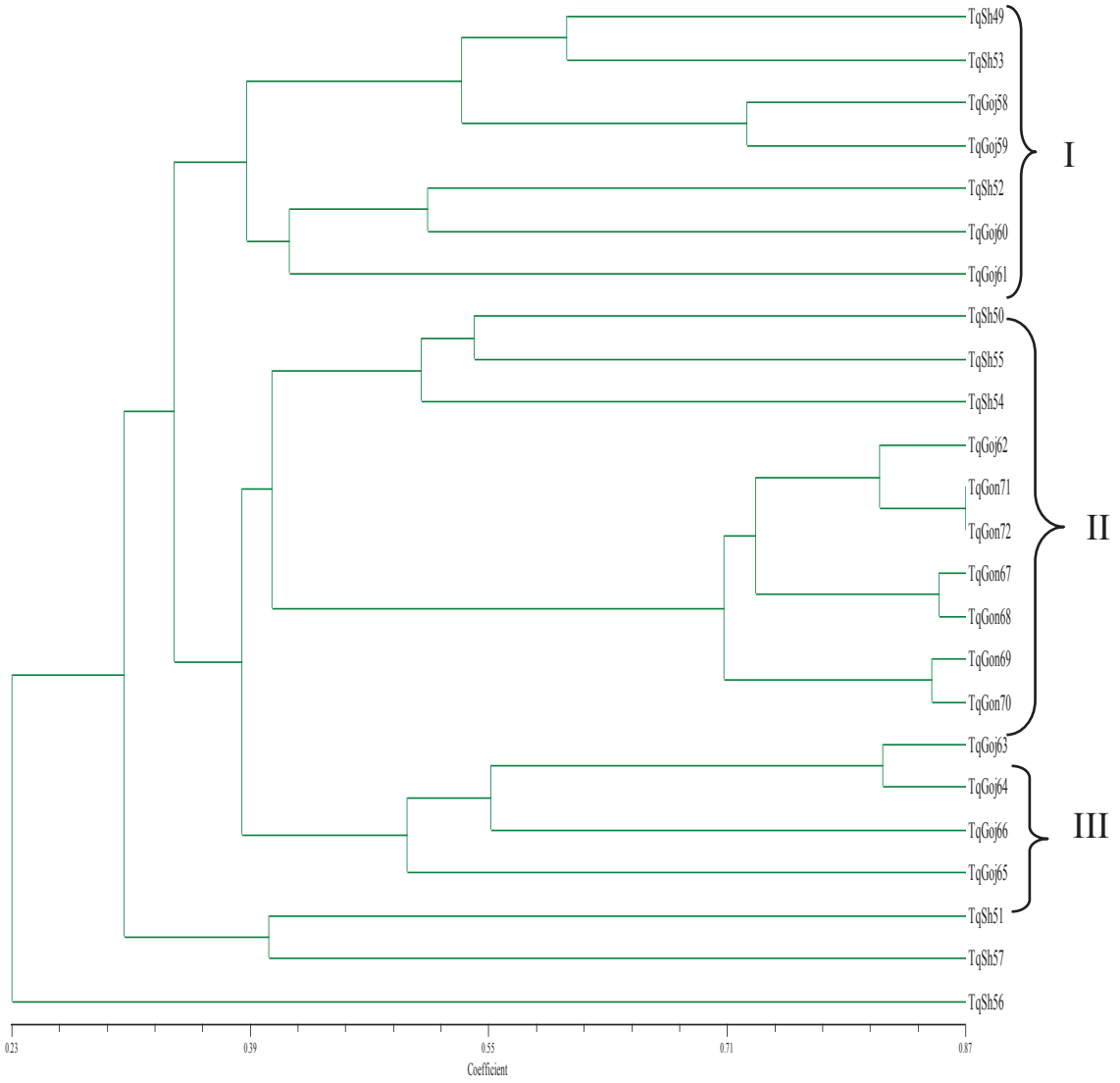

Figure2.UPGMA dendrogram based on Jaccard's similarity coefficient among 24 *T. quartinianum* accessions using data generated with four ISSR primers.

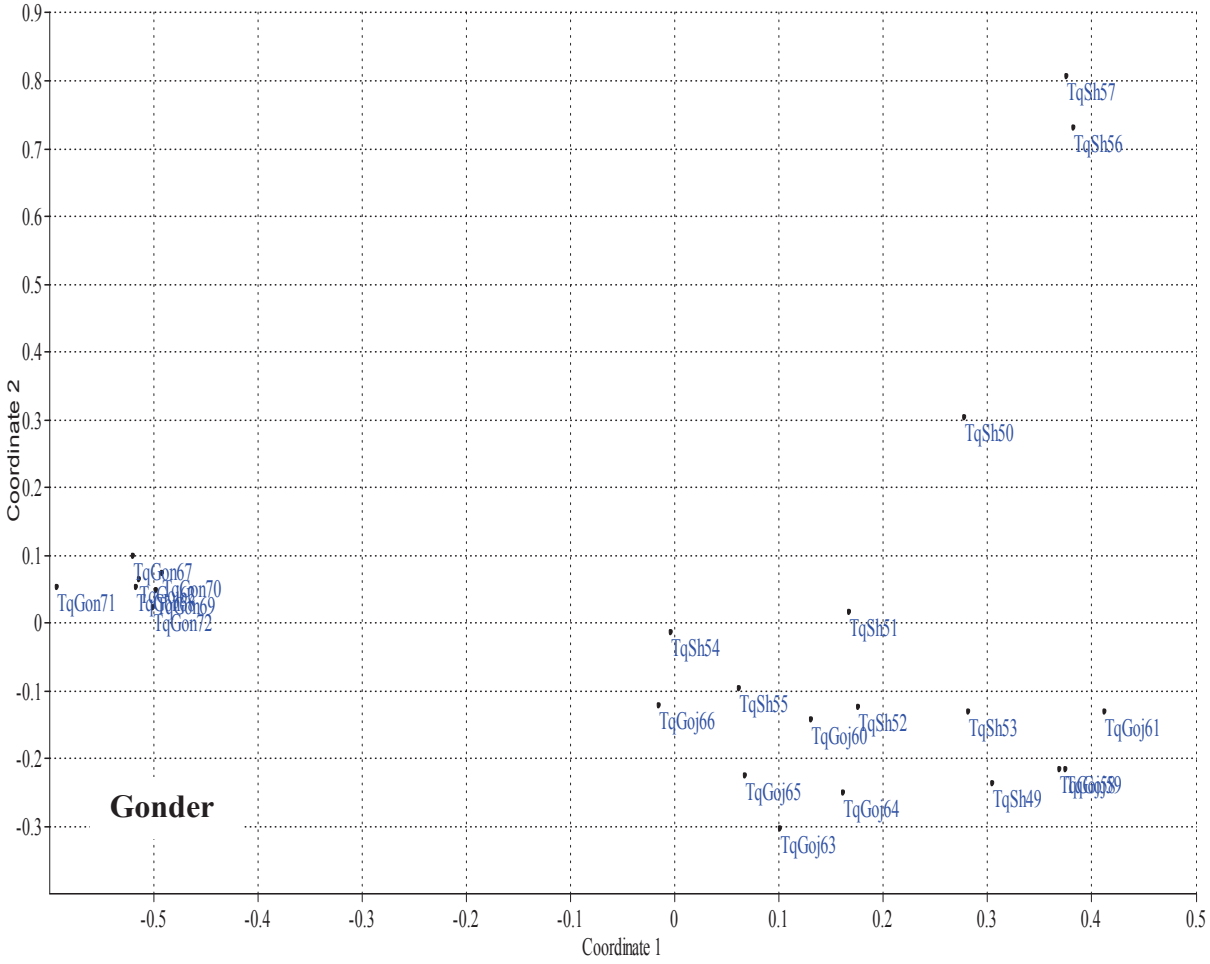

Figure 3. Two-dimensional plot obtained from principal coordinate analysis of 24 T. quartinianum accessions using 84 ISSR markers with Jaccard's coefficient similarity.

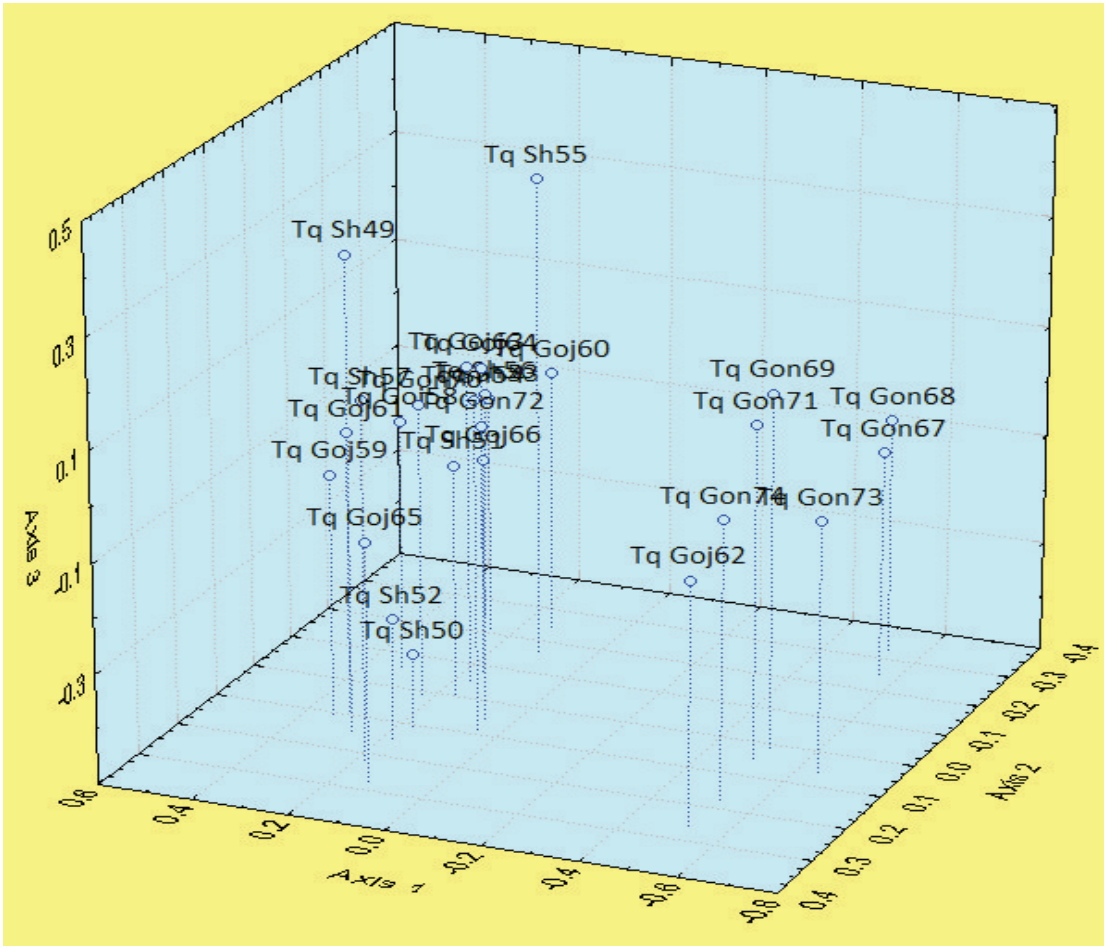

Figure 4. Three-dimensional plot obtained from principal coordinate analysis of 24 T. quartinianum accessions using 84 ISSR markers with Jaccard's coefficient similarity.

Conclusion

The present study is the first report on inter and intra population genetic diversity and relationships of *T. quartinianum* accessions of Ethiopia using ISSR markers. The genetic diversity data generated by four ISSR primers revealed that high genetic diversity exists at the species level. The assessed genetic diversity level varied among populations, which could be due to different environmental conditions in which they are growing and naturally distributed. Gojam population showed relatively higher genetic diversity than Shewa and Gonder. UPGMA and PCo showed that only accessions of *T. quartinianum* from Gondar formed separate cluster. The findings of this study indicates that ISSR markers could be good tools to assess the genetic diversity and relationship at inter and intra population level of *T. quartinianum*accessions.

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