

Genetic Diversity Study of Steudneri Clover (*Trifolium steudneri*) Accessions of Ethiopia Using Inter Simple Sequence Repeat (ISSR) Markers

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Abstract

Inter simple sequence repeat (ISSR) markers were employed to study the genetic diversity of 48 accessions of *T. steudneri*, representing two populations from Ethiopia. DNA was extracted from a bulk of fresh young leaves from three plants per accession using a modified CTAB method. Four selected di-nucleotide ISSR primer combinations generated a total of 65 bands (200-1000bp), of which 63 (96.92%) were polymorphic. The Nei's gene diversity (h) and Shannon's index (I) were high at species level ($h = 0.26$, $I = 0.39$). But, the genetic diversity was relatively low at population level, and average within-population diversity was $h = 0.22$, $I = 0.33$. Overall, Gojam population showed higher genetic diversity than Shewa. Clustering using UPGMA and PCo based on jaccards coefficient of similarity grouped the accessions according to the geographical origin where they were sampled. The study clearly indicates the presence of variable genotypes with their unique identity that deserve conservation attention.

Keywords: DNA extraction, Molecular markers, Polymerase chain reaction (PCR)

INTRODUCTION

Trifolium steudneri Schweinfur belongs to the genus *Trifolium* and section Vesicastrum (Ellison *et al.* 2006). *Trifolium steudneri* is a diploid with $2n = 16$ (Badr, 1995) and self pollinated (ILCA, 1990) forage legume. It is indigenous to the highlands of Ethiopia, Eritrea, Kenya and Uganda at 1100 to 2800 m altitude (Thulin, 1983). Due to this broad altitudinal distribution, it is exposed to a wide range of temperatures, radiation and rainfall (Griffiths, 1972). It performs well under varying edaphic conditions, widely adapted to acid and low fertility soils in the tropical highlands (Gillett *et al.*, 1971) and able to tolerate seasonal water logging.

T. steudneri is a fast growing annual clover that can provide good biomass within 2-3 months. It is a good forage legume for grazing in native pastures with potential for pasture improvement. It is suitable for hay and silage making, which can be used to increase the quality of straw-based diets and to overcome seasonal feed shortage. Under favourable environmental conditions, *T. steudneri* showed a high yield (9.1 t DM ha⁻¹) in a relatively short period (104 days) (Weise, 1989). It has some potential for hay production on bottom land sites with clay soil subject to seasonal water logging and for good production moderate inputs of P-fertilizer are necessary (Kahurananga and Asres Tsehay, 1984). Thus, it can be managed to increase forage production without competing for land with food crops. *T. steudneri* is an excellent fixer of atmospheric nitrogen in symbiosis with the indigenous Rhizobium population that helps to improve fertility of the soil. Under favorable environmental conditions, *T. steudneri* fixed a large quantity of nitrogen (165 kg ha⁻¹) in a relatively short period (104 days) (Weise, 1989). This species can grow and fix nitrogen even under severe water logging conditions, although the nitrogen yield may be low due to poorer growth (Weise, 1989). It can be used for intercropping with wheat or barley in tropical highlands to improve soil nitrogen and quality of residues for livestock feed. It 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 (Zewdu, 2004).

Information about genetic variation of germplasms is great interest of many population geneticists and plant breeders (Badr *et al.*, 2002). In recent years, different types PCR based DNA markers have been developed and widely used to study genetic diversity at intra and interspecific levels (Wolfe and Liston, 1998). It is important, however, to understand that each type of markers have different properties and will reflect different aspects of genetic diversity (Karp and Edwards, 1995). In *Trifolium* species, primarily Random Amplified Polymorphic DNA (RAPDs) have been used for genetic diversity study in *T. pratense* (Ulloa *et al.*, 2003), *T. resupinatum* (Arzani and Samei, 2004). However, the Inter simple Sequence Repeat (ISSR) markers (Wolfe and Liston, 1998) based on any di, tri, tetra, penta nucleotide repeats of a microsatellite primers with no need of genome sequence information seems particularly suitable for germplasm comparison. ISSRs are semi arbitrary markers found in between two microsatellite regions oriented in opposite direction and amplified by PCR in the presence of one primer complementary to a target microsatellite region. The ISSR markers are used for genetic diversity study in *Trifolium* species (Rizza *et al.*, 2007; Dabkevičienė *et al.*, 2011) and these findings suggested

that ISSR markers are suitable for genetic diversity study of *Trifolium* species.

Based on the above ground, ISSR marker was chosen for genetic diversity study of *T. steudneri* accessions collected from Ethiopia. Despite the importance of this species as livestock feed and contribution to soil fertility, so far, there were no published reports concerning ISSR markers for analyzing its genetic diversity. Hence, the present study aimed to evaluate the genetic diversity of *T. steudneri* accessions using ISSR markers. The results of the study can give 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, 48 accessions of *T. steudneri* collected from two different administrative regions (AR) of Ethiopia were kindly provided by International Livestock Research Institute (ILRI) Forage Germplasm Bank, Addis Ababa, Ethiopia (Table 1) 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 (Gojam and Shewa) having an equal sample size of 24 accessions each. 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 (nearly one month).

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

Acc. No	District	Zone/Adm. region	Latitude	Longitude	Altitude (m)	Lab. Code
8471	Chebo and Gurage	Shewa	08°21' N	037°49' E	1800	Ts Sh1
8489	Menagesha	Shewa	08°51' N	038°30' E	2080	Ts Sh2
9720	Selale	Shewa	09°45' N	038°47' E	2880	Ts Sh3
9966	Chebo and Gurage	Shewa	08°30' N	037°57' E	2000	Ts Sh4
10134	Jibat and Mecha	Shewa	09°03' N	038°11' E	2220	Ts Sh5
10139	Menagesha	Shewa	09°02' N	038°18' E	2240	Ts Sh6
10130	Jibat and Mecha	Shewa	08°59' N	037°48' E	2220	Ts Sh7
8370	Jibat and Mecha	Shewa	09°02' N	037°21' E	2210	Ts Sh8
8482	Menagesha	Shewa	08°39' N	038°07' E	2300	Ts Sh9
7614	Selale	Shewa	09°53' N	038°23' E	2560	Ts Sh10
6221	Addis ababa	Shewa	08°45' N	038°57' E	1800	Ts Sh11
9970	Chebo and Gurage	Shewa	08°24' N	037°52' E	1870	Ts Sh12
8465	Chebo and Gurage	Shewa	08°17' N	037°47' E	1880	Ts Sh13
7620	Selale	Shewa	09°58' N	038° 18' E	2550	Ts Sh14
8341	Jibat and Mecha	Shewa	08°59' N	037°48' E	2000	Ts Sh15
6253	Menagesha	Shewa	09°02' N	038°45' E	2380	Ts Sh16
8477	Chebo and Gurage	Shewa	08°35' N	038°00' E	2080	Ts Sh17
8357	Jibat and mecha	Shewa	08°01' N	037°03' E	2370	Ts Sh18
9961	Chebo and Gurage	Shewa	08°39' N	038°08' E	2370	Ts Sh19
8319	Menagesha	Shewa	08°48' N	038°54' E	1870	Ts Sh20
8087	Yerer and Kereyu	Shewa	08°35' N	039°08' E	1780	Ts Sh21
8084	Yere and Kereyu	Shewa	08°39' N	039°04' E	1850	Ts Sh22
6222	Addis Ababa	Shewa	08°45' N	038°57' E	1800	Ts Sh23
8488	Chebo and Gurage	Shewa	08°48' N	038°20' E	2080	Ts Sh24
9452	Agew Midir	Gojam	10°57' N	036°32' E	1850	Ts Goj25
9424	Debre Markos	Gojam	10°00' N	038°39' E	2500	Ts Goj26
9456	Debre Tabor	Gojam	11°50' N	037°35' E	1950	Ts Goj27
7637	Debre Markos	Gojam	10°18' N	038°09' E	2420	Ts Goj28
2179	Debre Markos	Gojam	10°28' N	038°09' E	2550	Ts Goj29
8520	Debre Markos	Gojam	10°30' N	038°10' E	2420	Ts Goj30
14602	Mota	Gojam	11°05' N	037°05' E	2450	Ts Goj31
2177	Mota	Gojam	10°54' N	038°02' E	2610	Ts Goj32
9700	Debre Markos	Gojam	10°15' N	038°06' E	2400	Ts Goj33
7697	Debre Markos	Gojam	10°16' N	037°49' E	2350	Ts Goj34
7647	Bichena	Gojam	10°41' N	038 °11' E	2500	Ts Goj35
9704	Debre Markos	Gojam	10°13' N	038°08' E	2500	Ts Goj36
7658	Mota	Gojam	11°05' N	037°53' E	2450	Ts Goj37
2050	Debre Markos	Gojam	10°12' N	037°52' E	2400	Ts Goj38
7645	Bichena	Gojam	10°37' N	038°10' E	2520	Ts Goj39
7659	Bichena	Gojam	10°15' N	038°08' E	2510	Ts Goj40
7652	Mota	Gojam	10°58' N	037°55' E	2520	Ts Goj41
7667	Bichena	Gojam	10°37' N	038°01' E	2500	Ts Goj42
7671	Bichena	Gojam	10°27' N	037°33' E	2610	Ts Goj43
7677	Debre Markos	Gojam	10°14' N	038°06' E	2400	Ts Goj44
2163	Debre Markos	Gojam	10°13' N	038°08' E	2410	Ts Goj45
7633	Debre Markos	Gojam	10°14' N	038°08' E	2500	Ts Goj46
2185	Debre Markos	Gojam	10°14' N	038° 04' E	2420	Ts Goj47
9380	Debre Markos	Gojam	10°13' N	038°02' E	2450	Ts Goj48

(Source: International Livestock Research Institute (ILRI))

Acc. = Accessions, E= East, N= North, m= meter, Adm. = Administrative

Ts Sh = *Trifolium steudneri* from Shewa, Ts Goj = *Trifolium steudneri* from Gojam

DNA extraction

Fresh young leaves from three individuals per 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 using the modified Cetyl Trimethyl Ammonium Bromide (CTAB) method (Borsch *et al.*, 2003) with some modifications. Finally pellets were dissolved in 100 µl 1x TE solutions and kept in freezer at -

20 °C until used.

PCR amplification and gel electrophoresis

A total of 13 ISSR primers obtained from Genetics Laboratory, Addis Ababa university (originally bought from University of British Columbia) 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 (Table 2) and used for the analysis of 48 accessions based on the number of amplification products, the quality of the profile

The level of polymorphism and the reproducibility of bands. 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 ddH₂O, 0.8 µl dNTPs (25 mM each), 2.5 µl of reaction buffer (10x taq polymerase buffer with 15 mM MgCl₂), 3µl MgCl₂ (25mM), 0.4µl primer (20 pmol/µl) and 0.3µl Taq Polymerase (1.5 unit) and 1µl 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°C, then 40 x 15 seconds denaturation at 94°C, 1 minute primer annealing at 48°C, 1.30 minutes extension at 72°C and the final extension for 7 minutes at 72°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, destained 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 (°C)	Motives
818	CACACACACACACAG	48	Dinucleotide
841	GAGAGAGAGAGAGAYC	48	Dinucleotide
844	CTCTCTCTCTCTCTRC	48	Dinucleotide
848	CACACACACACACARG	48	Dinucleotide

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

Data analysis

Only those clearly resolved amplified bands of ISSR markers derived from four primers were scored. Scoring was performed manually for each primer based on presence (1) and absence (0) or as a missing observation (?) of bands across accessions. POPGENE version1.32 software (Yeh *et al.*, 1999) was used to calculate percentage of polymorphic loci (PPL), gene diversity (h), and Shannon's information index (I). NTSYS- pc version 2.02 (Rohlf, 2000) software was 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 line *i* and *j*,

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

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

The unweighted pair group method with arithmetic average (UPGMA) was used in order to determine the genetic relationship among accessions of the two populations and generates phenogram using NTSYS- pc version 2.02(Rohlf, 2000).

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

The four ISSR primers amplified 65 well defined and reproducible bands among 48 accessions of *T. steudneri*

(Table 3). The number of bands generated by individual primer varied from 15 to 18 with the average number of bands per primer being 16.25. Primer 848 and 844 exhibited the highest and the lowest number of bands, respectively. The size of the amplified bands ranged from 200 to 1000bp. Figure 1 shows an example of ISSR amplification pattern generated by primer 848

Table 3. List of ISSR primers, amplification pattern and Number of scorable bands (NSB) used in *T. steudneri* accessions

ISSR Primer	Amplification pattern	NSB
818	polymorphic, reproducible	16
844	polymorphic, reproducible	15
841	polymorphic, reproducible	16
848	polymorphic, reproducible	18
Total		65
Average		16.25

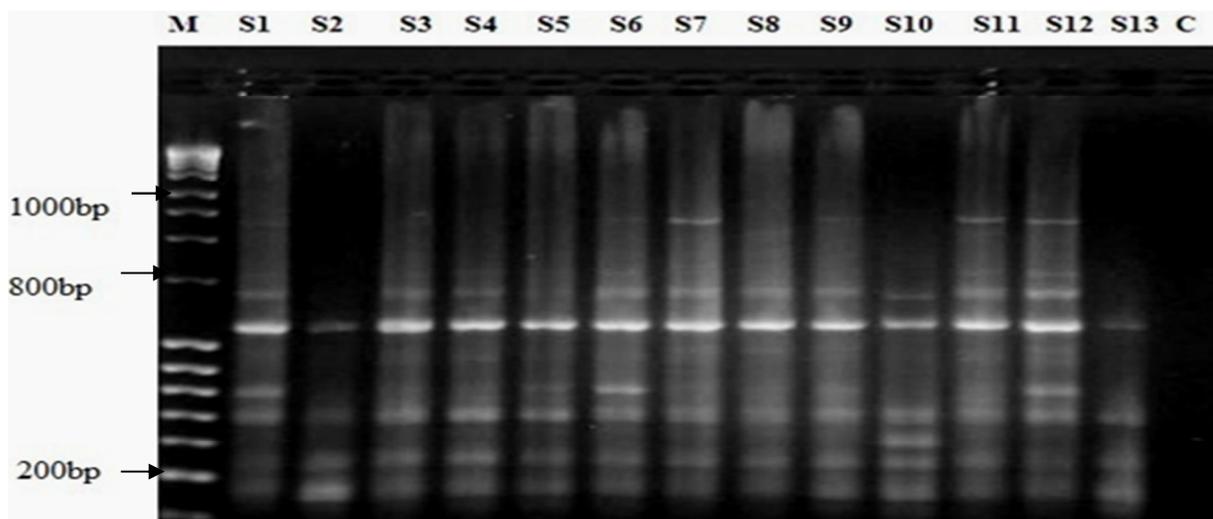


Figure 1. Banding pattern of primer 848 in 13 *T. steudneri* accessions. M represents a 100 bp marker; S stands for *T. steudneri*, while the numbers associated with these letters represent accessions, C represents control.

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).

A total of 65 scorable bands were obtained, of which 63 (about 96.92%) were found to be polymorphic among the 48 accessions of *T. steudneri* (Table 4). Two primers, namely 841 and 848 showed 100% polymorphism, while primer 844 showed the least polymorphism, accounting for 93.33%. The overall gene diversity (*h*) and Shannon's information index (*I*) by four ISSR primers were 0.26 and 0.39, respectively. Primer 818 showed the highest gene diversity (0.30) and Shannon's information index (0.44), while primer 848 showed the least for the two indices, 0.21 and 0.34, respectively (Table 4).

Table 4. Number of polymorphic Allele (NPA), Percent of polymorphic Allele (PPA), Gene diversity (h) and Shannon's information index (I) revealed by the four ISSR primers in *T. steudneri* accessions

ISSR Primer	NPA	PPA (%)	h (\pm SD)	I (\pm SD)
818	15	93.75	0.30 (0.20)	0.44(0.28)
844	14	93.33	0.25(0.20)	0.38(0.28)
841	16	100	0.27(0.18)	0.41(0.24)
848	18	100	0.21(0.17)	0.34(0.24)
Over all	63	96.69	0.26(0.19)	0.39(0.25)

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 loci, varying from 15-18 fragments per primer displaying 96.92% polymorphism with high mean gene diversity ($h=0.26$) and Shannon's information index ($I=0.25$). This indicates the existence of high level of genetic diversity among the two populations of *T. steudneri* in Ethiopia. High genetic diversity found among the population could be due to lack of selection and strict domestication under low overgrazing.

When each population was taken into account, Gojam population was more polymorphic and diverse (PPA = 78.46%, $h=0.25$, $I=0.37$) than Shewa (PPA= 70.77%, $h=0.20$, $I=0.30$) (Table 5). This may be due low overgrazing because of protection of the grazing areas during the rainy season for hay and silage production.

Table 5. Number of polymorphic Allele (NPA), Percent of polymorphic Allele (PPA), Gene diversity (h) and Shannon's information index (I) within and among populations of *T. steudneri*

Populations	NPA	PPA (%)	h (\pm SD)	I (\pm SD)
Shewa	46	70.77	0.20(0.20)	0.30(0.28)
Gojam	51	78.46	0.25(0.20)	0.37(0.28)
Average	48.5	74.61	0.22(0.20)	0.33(0.28)
Species level	63	96.92	0.26(0.19)	0.39(0.25)

Up to now, there are no published reports concerning ISSR method for analyzing the genetic diversity of *T. steudneri*. However, Rizza *et al.*, (2007) studied the genetic diversity of 34 genotypes from six *Trifolium* species using ISSR markers and found 89.5% polymorphism in *T. medium*, 0.0% in *T. pratense*, 52.4% in *T. repens*, 92.9% in *T. argentinense*, 46.4% in *T. polymorphum* and 78.0% in *T. riograndense*. The level of polymorphism in *T. argentinense* and *T. medium* were comparable with the present result, while the other four *Trifolium* species showed a lower level of polymorphism than the present result.

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. pratense* 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).

Genetic relationships

The UPGMA and PCo dendrograms were constructed on the basis of Jaccard's coefficient of similarity to investigate the relationship of *T. steudneri* accessions. From the UPGMA dendrogram three major clusters were identified at about 62% similarity level (Figure 2). Moreover, the range of genetic similarity among the total accessions was in the range of 27-97%, resulting in a high inter-population differentiation. The first major cluster (I) comprised 22 accessions belonging to Shewa population, the second major cluster (II) was formed by 10 accessions belonging to Gojam population and the third major cluster (III) was formed by 10 accessions belonging to Gojam population. From the UPGMA dendrogram few accessions were observed to be distinct and ungrouped at 62% similarity level that also showed clearly different genetic amplification pattern from other individuals of the population. In this case, a study with different primers and the inclusion of more individuals would be necessary to verify the clustering.

The higher levels of genetic variation found in this study may be due to altitudinal and ecological difference of the origin of the accessions. In addition, the dendrogram also highlights the intra-population

diversity. Higher genetic similarity was observed among accessions of Shewa population than Gojam. Two accessions from Shewa population (TsSh21 and TsSh22) in cluster 1 collected from central highland showed the highest level of genetic similarity (97%). The UPGMA tree topology was also further confirmed by the two and three dimensional PCO plot, in which most of the accessions from the same population formed a separate cluster. In general, this indicates that ISSR markers have the power to distinguish accessions of the same species collected from different geographical region.

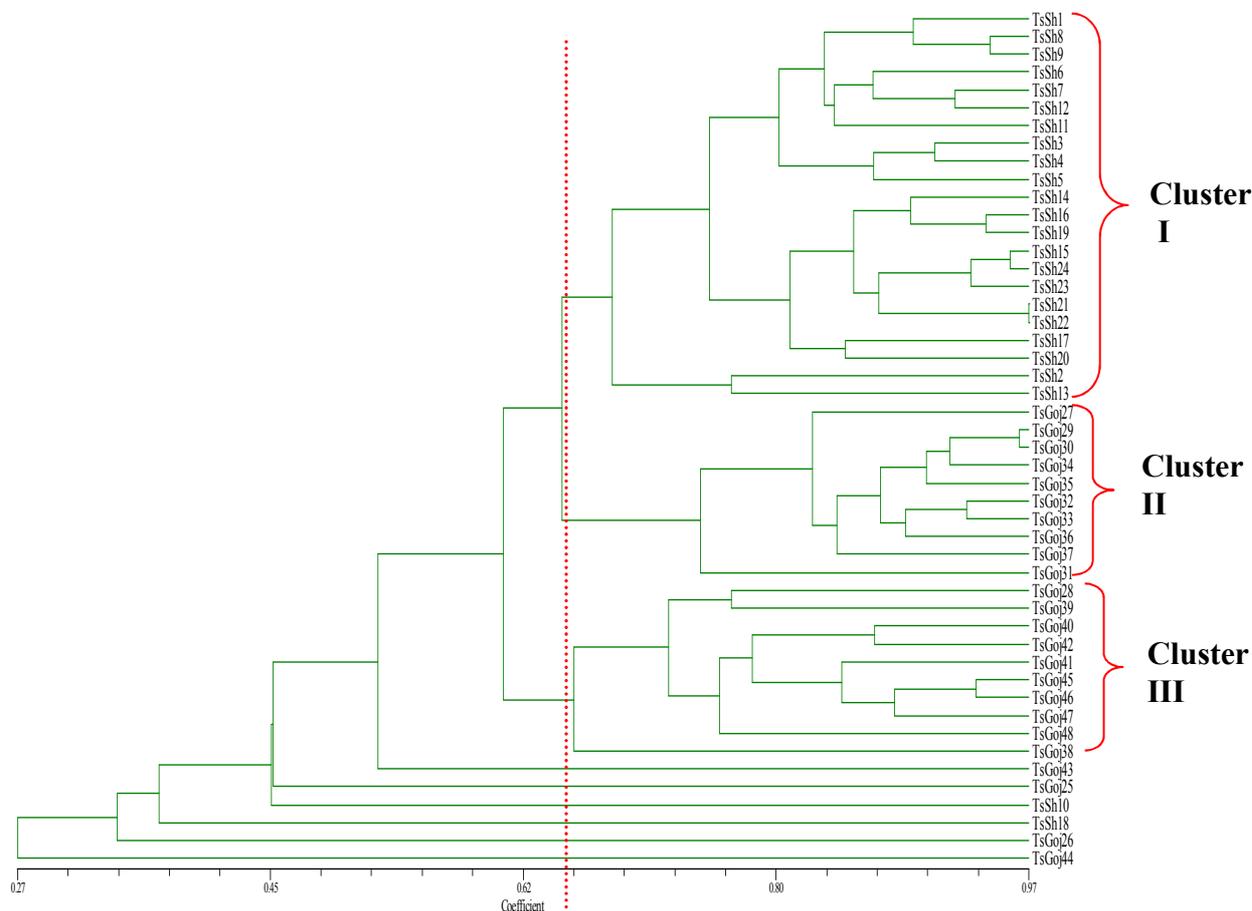


Figure 2. UPGMA dendrogram based on Jaccard's similarity coefficient among 48 *T. steudneri* accessions with data generated by four ISSR primers.

Principal Coordinate (PCO) analysis

Principal coordinate analysis (PCoA) was carried out on all the ISSR data obtained from 48 accessions of *T. steudneri* to evaluate the relationship among accessions (Figure 3). The first three coordinates of the PCo had Eigen values of 3.90, 3.38 and 2.39 with percentage of variation 13.51, 11.72 and 8.30, respectively, were used to show the grouping all of the accessions using two and three coordinates. Most of the accessions from the same geographical origin clustered together in the two dimensional PCoA plot. This was further confirmed by the three dimensional plot (Figure 4).

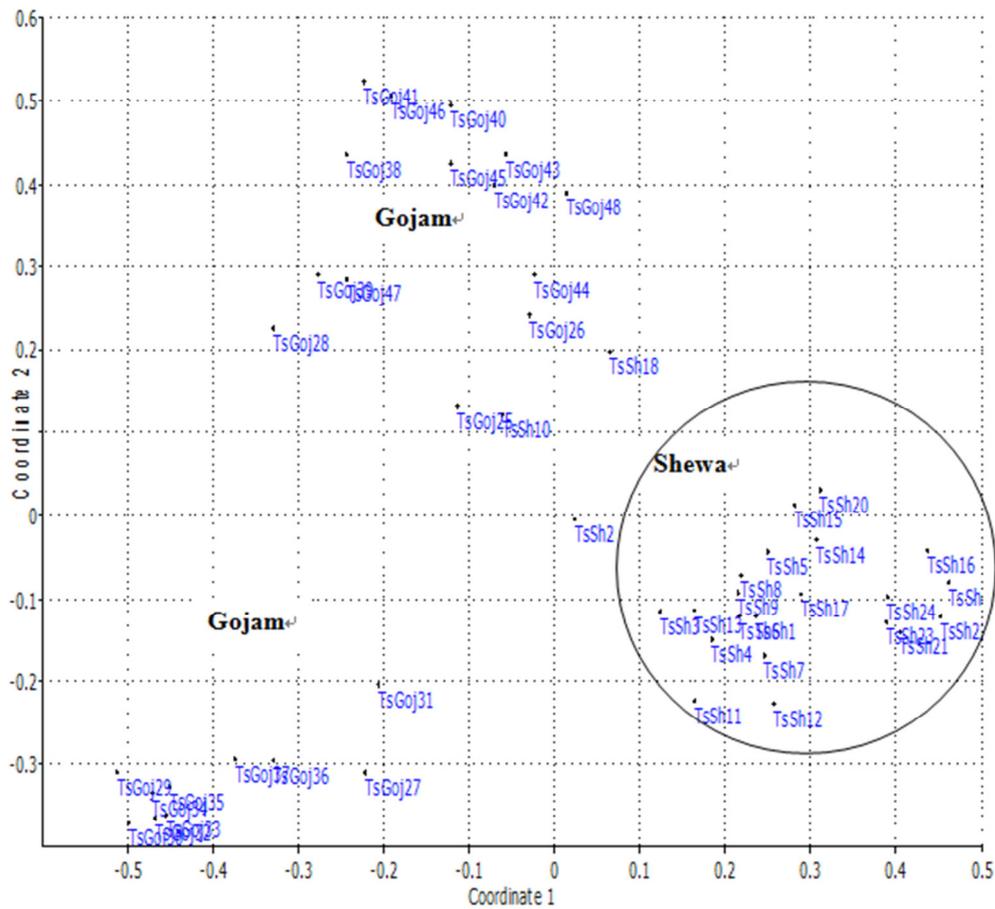


Figure 3. Two-dimensional plot obtained from principal coordinate analysis of 48 *T. steudneri* accessions using 65 ISSR markers with Jaccard's coefficient similarity.

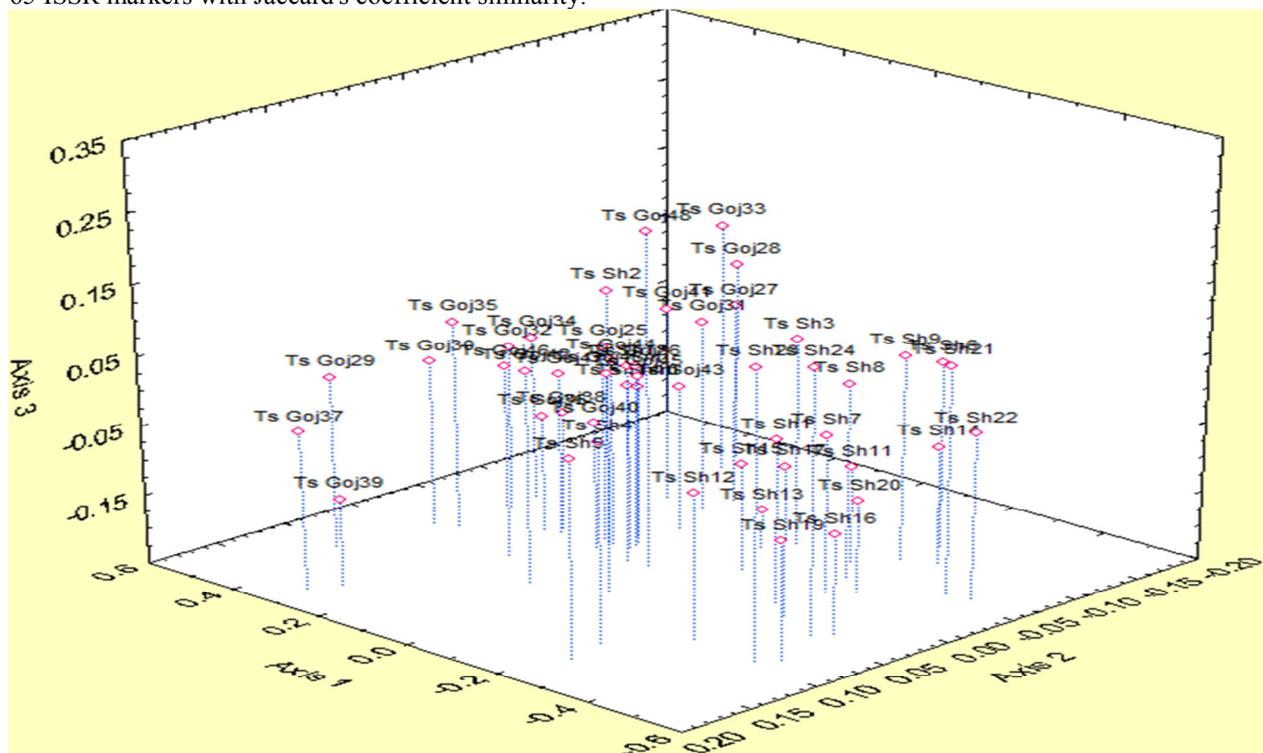


Figure 4. Three-dimensional plot obtained from principal coordinate analysis of 48 *T. steudneri* accessions using 65 ISSR markers with Jaccard's coefficient of similarity.

CONCLUSION

The present study is the first report on inter and intra population genetic diversity and relationships of *T. steudneri* 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. UPGMA cluster analysis and PCO supported the grouping of accessions to the respective population. 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. steudneri* accessions.

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