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Research Article

Analysis of Morphological and Molecular Genetic Diversity in Stinging Nettle (*Urtica simensis*) from Northern Ethiopia

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Abstract

Urtica simensis (Samma) is among the species of nettle which belongs to the family *Urticaceae* that is endemic to Ethiopia and consumed as vegetable in some places and traditionally people used in treating different types of diseases including infectious diseases. This study was conducted to evaluate the genetic diversity of *Urtica simensis* to address and fill the gap on its genetic variability using morphological characters and ISSR DNA markers. A total 133 plant samples were collected from Northern growing areas of Ethiopia (Gondar and Mekelle), based on variability in morphological characters. Morphological characterization was conducted on six morphological traits which demonstrated variation based on PCA and correlation matrix analysis. A total of 5 good amplified ISSR markers were selected out of 16 ISSR primers for molecular characterization. Highest genetic divers6ity (H) was found to be 0.4286 and Shannon Information index (I) was 0.6197 with respect to 100% polymorphism. Dendrogram based on Jaccard's similarity coefficients generated by UPGMA cluster analysis using morphological and ISSR data shows major and minor clusters with broad distribution of *Urtica simensis* individuals over the entire tree which indicates the low divergence in morphological appearance among populations of both study areas. Based on the results of this study, morphological and ISSR markers were effective in studying genetic diversity of *Urtica simensis* since they shows variations in morphological appearances and genetic variability and this have valuable effect on characterization of *Urtica simensis* genetic resources in different parts of Ethiopia for conservation purposes.

Keywords: Genetic diversity, *Urtica simensis*, morphological characters and or markers, cluster analysis.

Introduction

Stinging nettle belongs to the family *Urticaceae* is one of the widely available wild perennial plants in temperate regions of Asia, America and Europe. It is representative for 30-45 species of common wild flowers that belong to the genus *Urtica* and it commonly grows in rich soils in forest clearings, old fields and wasted places (Mamta and Preeti, 2014). *Urtica simensis* is among the widely available species of nettle that is endemic to Ethiopia locally known as Samma (Amharic). It grows around the highlands of Ethiopia, specifically in the North and South Gondar, North and South Wello, North Shewa, Wag Hamra, Tigray region, highland of Sidama zone in Southern region and Arsi zone of Oromia region at 1500-3500 meter above sea level (Dereje *et al.*, 2016; Erenso and Maryo, 2014). It is mostly found in grassland areas common in disturbed localities, plentiful near houses and can be harvested whenever there is a need (Gebrezgabiher *et al.*, 2013; Assefa *et al.*, 2013; Alemayehu *et al.*, 2015).

Urtica simensis (Samma) is dark green perennial wild species of plant predominantly leaves and young shoot parts collected by women and children and traditionally cooked and consumed as vegetable in some places of the country since it have high nutritive contents and grows throughout the year and available on demand in nearby areas and also usually used as emergency famine food during food shortage. It has a great potential and contribution to food security to meet nutritional demand of human. It is one of nontoxic and well known locally accessible protein feed resources which contains all of the essential amino acids so, its crude protein content is bounded from 25.1 to 26.3% in addition it contains iron, calcium, phosphorus, potassium, sulphur, magnesium and it is also rich in vitamins A, C, K D, and B and up to 20% mineral salts, mainly salts of calcium, potassium, silicon, and nitrates (Friis, 1989; Assefa *et al.*, 2013; Dereje *et al.*, 2016; Keflie *et al.*, 2017).

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It has medicinal properties and used in the treatment of diseases including infectious diseases. Traditionally people use different part of the plant (leaves and root) in different forms for the treatment of ailments such as allergic rhinitis, blood pressure, prostate hyperplasia, rheumatoid arthritis, diarrhea, cough and other problems (Lahigi *et al*., 2001; Dar *et al*., 2012) and it also use for treatment of diabetes, malaria and peptic ulcer disease (Kefalew *et al.,* 2015; Wubetu *et al.,* 2017). Despite of its potential in solving food security, nutrition and health there is limited information on genetic diversity of *Urtica simensis*. Therefore, the aim of this study was to evaluate and characterize the genetic diversity of stinging nettle (*Urtica simensis*) using morphological and ISSR markers.

Materials and methods

Description of study area: This study was carried out and sample collected in two major growing areas of northern Ethiopia (Mekelle (Tigray region) and Gondar (Amhara region)), which are known for their high potential in *Urtica simensis* production.

Field survey and sample collection: Field surveys were conducted in the aforementioned Northern, areas of Tigray (Mekelle) and Amhara (Gondar) region, Ethiopia. Followed by purposive sampling methods and selection of study areas, depend on availability of different targeted morphological appearances which are helpful in the evaluation of morphological diversity analysis and genetic variability according to (Lizawati *et al*., 2018). A total of 133 (65 from Gondar and 68 from Mekelle) young leave samples were collected using polybags.

Plant materials and morphological characterization: *Urtica Simensis* plant species used in the study were collected based on society/community prior knowledge on its importance and availability in addition to its population. A total of six different which are two quantitative (Plant Height (PH), Stem Length (SL)) and four qualitative (Flower Colour (FC), Leave Shape, Leave Arrangement and Plant Growth Habit) Morphological characters were evaluated and recorded with two replications from fresh plant material during field survey and ten plants were randomly selected for scoring (Figueredo-Urbina *et al*., 2017; Lizawati *et al*., 2018).The two quantitative characters (plant height and stem length) were measured in cm from ground level to the top of spike for plant height and from the soil level to end of flower part for stem length with meter stick respectively. The four qualitative characters (flower color, leaf arrangement, leaf shape and plant growth habit) were recorded and scored following to the standard descriptors reported on the International Union for the Protection of New Varieties of plants (UPOV, 2006) for scoring of flower color, (Chaki *et al*., 2018) for scoring of leaf shape and leaf arrangement and (Bioversity International, 2007) for scoring of plant growth habit.

DNA extraction and qualification: Young leaves of each *urtica simensis* sample (two to three grams) were harvested and lyophilized then ground using mortar and pestle and stored in Eppendorf tube/PCR tube (Inqaba, Nairobi, Kenya), sample preparation, DNA extraction were performed according to DNA isolation kits (Terzopoulos and Bebeli, 2008) and instructions using (Inqaba, Nairobi, Kenya). Quality of the extracted DNA was measured by running on 0.8 % agarose gel and the gel was stain using ethidium bromide solution. Appropriate dilutions of DNA were made for further amplification and ISSR analysis.

ISSR amplification and gel electrophoresis: Among sixteen random primers (Haghpanah *et al.,* 2016) utilized, only five well amplified primers showing polymorphic results were selected for the purpose of diversity and similarity analysis. ISSR assays were performed using PCR kit or beads (Bioneer, Korea) which is PCR premix in a final volume of 20 µL containing 2 µL template DNA, 2 µL random primer, and 16 µL PCR water. The mixture was amplified in a thermal cycler that was programmed for one cycle of initial denaturation at 94°C for 5 min, 35 cycles of 94°C for one min, followed by specific annealing at 50°C (based on the primers annealing temperatures) for 1 min, and a final extension at 72°C for 1 min; and a final extension cycle at 72°C for 5 min. the PCR machine was adjusted to hold the product at 4°C. The PCR products and 1kb DNA ladder were electrophoresed on 1.8% agarose gel (stained with Ethidium bromide). The separated fragments were visualized using gel doc and photographed.

Data analysis: The banding patterns obtained from ISSR were scored as present (1) or absent (0). Principal component analysis (PCA) was carried out using the original numerical data of quantitative characters and the assigned qualitative trait data and excluding the constant variables with similar morphological appearances among the populations using R studio software. Jaccard's coefficient similarity was measured and a dendrogram based on similarity coefficients were generated by the Unweighted Pair Group Method of Arithmetic means (UPGMA) cluster analysis using NTSYS software (Rohlf, 2000). POPGENE software were used to calculate Nei's (1978) unbiased genetic distance- among different cultivars with all markers, genetic parameters including genetic diversity for each population as number of polymorphic loci, percent polymorphism, Genetic diversity (H) and Shannon diversity index (I). An analysis of molecular variance (AMOVA) pair-wise procedure was used to calculate the genetic variance among and within population or varieties.

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Results

Analysis on Morphological characterization and genetic diversity of Urtica simensis: Results of the analysis of morphological traits showed that significant (p<0.001) differences were found among samples for all the measured traits, indicating levels of variation in the studied quantitative morphological traits. The mean, range, standard deviation and percentage of coefficient of variation (CV) were calculated for the two quantitative morphological traits of populations (Mekelle and Gondar). For Samples recorded in Mekelle, the Plant Height mean value was 102.8 cm and ranged between 25 cm and 160 cm, with SD of 47 cm, and 45.71% of Coefficient of Variance and the Stem Length mean value was 37 cm and ranged between 20 cm and 500 cm, SD was 8.8 cm, and Coefficient Variance was 23.8%. For the samples recorded in Gondar, Plant Height mean value was 146.8 cm and ranged between 80 cm and 200 cm, Std Dev. was 43.6 cm, and CV was 29.7%. The Stem Length mean value was 63.9 cm and ranged between 50 cm and 800 cm, SD was 9.8 cm, and CV was 15.3% as presented in Table 1. Qualitative morphological traits of *Urtica simensis* among the populations of Mekelle and Gondar indicated according to standard discriptors. The morphological appearances Leaf Shape, Leaf Arrangement and Plant Growth Habit in 133 of total sample size were lenceolate, opposite and erect respectively with 100% similarity between the populations whereas the trait Flower Color (FC) showed variation in both study areas, out of 68 recorded samples in mekelle there were 30 samples grey green (6) (44.12% of the total), and the rest 38 samples were grey brown (46) (55.88%). For morphological data recorded in Gondar regarding the FC trait in 65 total samples were 34 samples brown green (9) (52.31% of the total) and the remaining 31 samples were grey (48) (47.69%) (Table 2). Due to the variation and ability of creating diversity of this trait (FC) compared to the other recorded traits was included during correlation coefficient analysis for the formation of correlation matrix and PCA analysis for the calculating variations among the targeted morphological traits. The Pearson correlation coefficient output data indicated statistical significant correlations between the three morphological recorded variables (Table 3). PH and SL were positively correlated, and PH and FC were negatively correlated, as well as SL with FC was also negatively correlated. The correlation matrix was the base for performing in the PCA analysis. The PH, SL, and FC were shown negative vector value and this indicated that all the traits negatively correlated with PC1, with an eigenvalue of 2929.80, 75.669% of variance and 0.512 proportion of variance of the total morphological variation; In PC2, only SL loaded with 0.125 eigenvector compared to other traits with eigenvalue of 851.66, 21.996% of variance and 0.334 of proportion of variance.

Fig. 1. Data Representation using Scree plot based on eigenvalue among the major morphological traits.

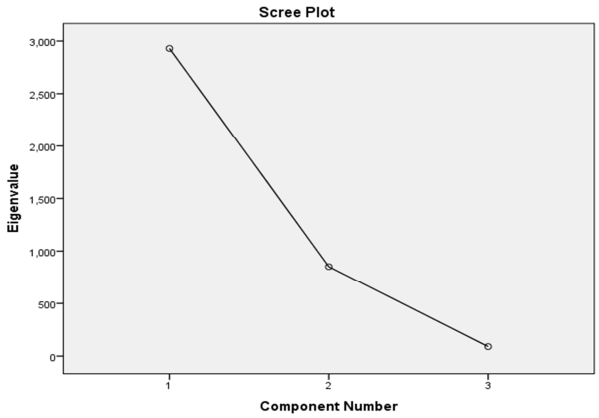
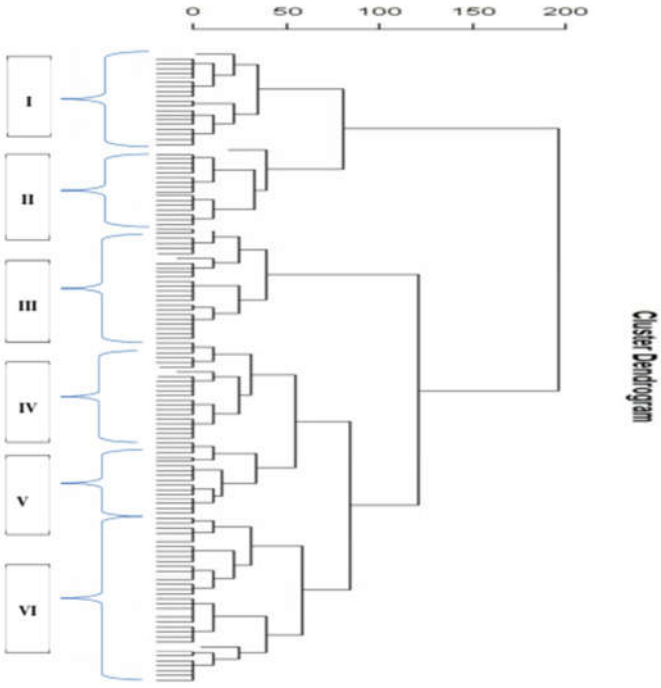


Fig. 2. UPGMA dendrogram Cluster analysis of *Urtica simensis* generated using morphological characters.



In PC3 the eigenvalue was 90.38, 2.33% of variance and 0.15 proportion of variance of the total variation; SL was highly loaded following FC with eigenvector of 0.701 and 0.102 respectively (Table 4). This indicates the PC3 was reflected as major component of plant type's indicator. Depend on the total variance which were summarized the variations among this major morphological traits expressed in Scree plot (Fig. 1) which represented the data output of eigenvalue versus the number of Components (Principal components) and indicated the retained amount of principal components.

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Table 1. Descriptive analysis of two quantitative morphological appearances in *Urtica simensis*.

Components	Sample location	Plant height (in cm)	Stem length (in cm)	Over all (133 No. of samples)	
				Plant height (in cm)	Stem length (in cm)
Minimum value	Mekelle	25.0	20.0	25.0	20.0
	Gondar	80.0	50.0		
Maximum value	Mekelle	160.0	50.0	200.0	80.0
	Gondar	200.0	80.0		
Mean	Mekelle	102.8	37.0	124.3	50.2
	Gondar	146.8	64.0		
SD	Mekelle	47.0	8.8	50.3	16.4
	Gondar	43.6	9.8		
Variance	Mekelle	2207.7	77.1	2527.3	269.4
	Gondar	1897.2	97.0		
CV (%)	Mekelle	45.7	23.8	40.5	32.7
	Gondar	29.7	15.3		

% CV = SD/mean * 100 Where, CV= Coefficient of variance, SD= Standard deviation.

Table 2. Analysis of qualitative morphological trait of *Urtica simensis* (Flower Color) in Percentage (%).

Sample location	Amount of samples	Flower Color representative No.	Percentage (%)	Cumulative percentage	Total samples
Mekelle	30	6	44.12	44.12	68
	38	46	55.88	100	
Gondar	34	9	52.31	52.31	65
	31	48	47.69	100	

Table 3. Correlation matrix among the three morphological characters.

	PH	SL	FC
PH	1.00		
SL	.533	1.00	
FC	-.426	- .754	1.00
Significant at p – value =0.00, PH = Plant Height, SL = Stem Length and FC = Flower Color.			

Table 4. Principal component analysis based on covariance matrix and total variance.

	PC1	PC2	PC3
Plant Height	-0.708	-0.019	-0.706
Stem Length	-0.702	0.125	0.701
Flower Color	-0.075	-0.992	0.102
Eigen values	2 929.80	851.66	90.38
% of Variance	75.669	21.996	2.334
Cumulative %	75.669	97.666	100.00
Std dev.	1.239	1.001	0.679
Prop. of Variance	0.512	0.334	0.154
Cumulative Prop.	0.512	0.846	1.00

*Corresponding author

Table 5. Overall Cluster analysis among the morphological traits.

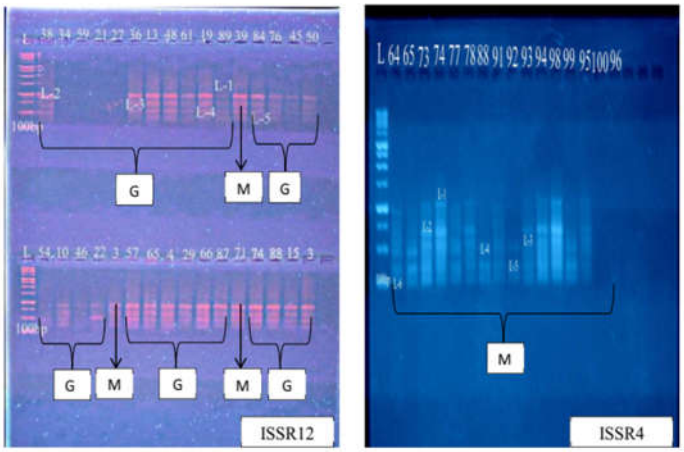
Component	Sample traits	Clusters (Groups) Number					
		I	II	III	IV	V	VI
Mean	PH	95 5	141.2	147.9	106.0	143.1	119.1
	SL	37 4	63.1	63.6	36.8	55.4	47.0
SD	PH	49.7	45.9	43.8	41.6	40.6	54.8
	SL	9.1	12.3	8.3	8.9	13.4	17.9
CV %	PH	52.0	32.6	29.6	39.2	28.4	46.0
	SL	24.3	19.5	13.1	24.2	24.2	38.1

PH = Plant Height, SL = Stem Length.

Based on morphological character recorded data results dendrogram cluster analysis (Fig. 2) and the mean value of each group (Table 5), with respect to the values of coefficient of variances (CV) evaluated *Urtica simensis* traits were grouped into six major clusters with significant differences in the morphological characteristics and intermixing of individuals of both populations. The sub- clusters at the same line within the major and minor clusters associated with same morphological appearance like plant growth habit, leave shape and leave arrangements. Cluster I contained 20 sample traits collected only from Mekelle were classified in this cluster. The main features were PH between 25 and 160 cm, and SL between 20 and 50 cm with mean value of PH and SL 95.5 and 37.4 respectively with respect CV value of PH 52% and SL 24.3%. Cluster II contained 17 sample traits collected from Mekelle (1) and Gondar (16), with PH between 50 and 200 cm, and SL between 45.2 and 80 cm. with mean value of 141.2 and 63.1 respectively and PH 32.6 % and SL 19.5% of CV value. Cluster III consisted of 24 sample traits collected only from Gondar. The main features were PH between 80 and 200 cm, and SL between 50 and 80 cm with mean value of PH and SL 147.9 and 63.6 respectively 29.6% PH and SL 13.1% value of CV. Cluster IV consisted of 21 individual traits only from mekelle. The main features were PH between 25 and 160 cm, and SL between 20 and 50 cm with mean value of PH and SL 106 and 36.8 respectively, 39.2% of CV of PH and SL CV value of 24.2%. Cluster V had 16 traits collected from Mekelle (5) and Gondar (11) province with PH between 80 and 200 cm, and SL between 33.9 and 80 cm. with mean value of 143.1 and 55.4 respectively with CV values of 28.4% PH and 24.2% of SL. Cluster VI had 35 traits, and this group was collected from Mekelle (22), and Gondar (13). with PH between 25 and 200 cm, and SL between 21.9 and 80 cm as well as mean value of 119.1 and 47 respectively with respect of 46% PH and 38.1% SL of CV values.

Analysis of molecular genetic diversity of Urtica simensis: Molecular genetic diversity analysis of 133 *Urtica simensis* samples were determined using ISSR markers (Fig. 3).

Fig. 3. Representatives for gel electrophoresis PCR product of ISSR markers which shows polymorphism.



(G= samples from Gondar, M= samples from Mekelle).

The results expressed in terms of Number of Amplified Bands (NAB), Number of Polymorphic Locus (NPL), Nei (genetic diversity) values (H), percent of polymorphism (PP) and Shannon information index (I) based on five ISSR primers and among the two study areas of northern Ethiopia (Table 6 and 7). The highest and good number of amplified bands was recorded primers ISSR12, ISSR4, ISSR14 and ISSR 8 respectively, while the lowest number of amplified bands was recoded primer ISSR13. Primer ISSR4 and ISSR13 recorded the same Number of Polymorphic Locus (6), the higher number of Polymorphic Locus was registered at primer ISSR8 (8) and ISSR12 and ISSR14 were scored 5 Polymorphic Locus (Table 6). The present study revealed that, out of 16 ISSR markers only 5 primers (markers) were showing good amplification. Primer ISSR14 shows highest H and I (0.43 and 0.62 respectively) was following to primer ISSR13 which showed (0.42 and 0.59 respectively) and least H and I was produced by ISSR8 (0.29 and 0.45 respectively) (Table 6). The 100% polymorphism was detected in both populations within 5 primers (Table 6 and 7).

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Table 6. General Diversity analysis of *Urtica Simensis* based on 5 ISSR markers.

Primer	Sequence (5'→3')	NAB	NPL	PP	H	I
ISSR4	$(GA)_8C$	106	6	100	0.38	0.55
ISSR8	$(GA)_8A$	75	8	100	0.29	0.45
ISSR12	$(TG)_8A$	111	5	100	0.36	0.54
ISSR13	$(AC)_8C$	54	6	100	0.42	0.59
ISSR14	$(ATC)_6T$	99	5	100	0.43	0.62
Total		445	30	100	1.88	2.75

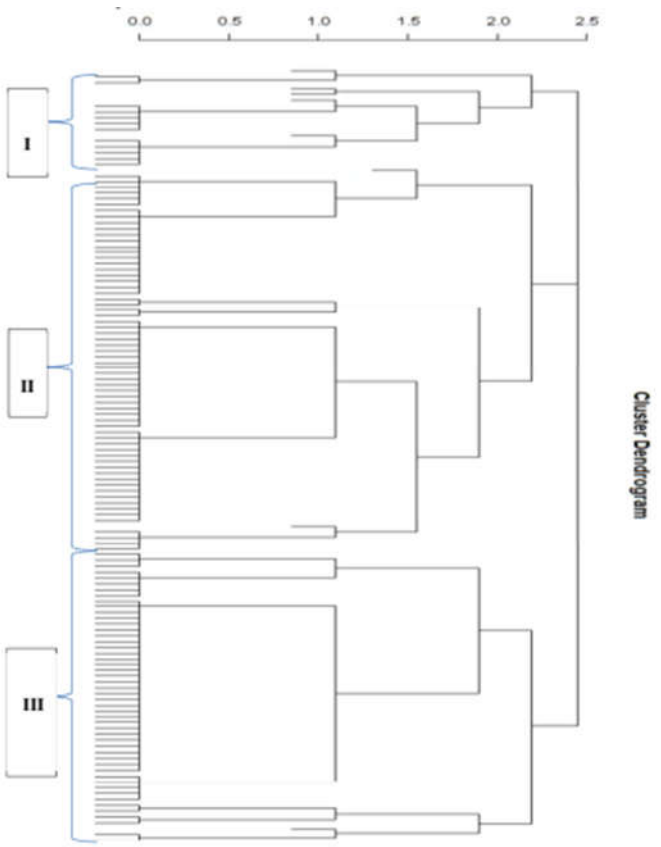
NAB=Number of Amplified Bands, NPL=Number of Polymorphic Locus, H=Nei (genetic diversity) values, PP(%)= Percent of polymorphism and I=Shannon information index.

Table 7. Genetic diversity of *Urtica simensis* based on ISSR results among the two populations.

Populations	NPL	PP	H	I
Gondar	25	100	0.3152	0.484
Mekelle	29	100	0.3462	0.502

Number of Polymorphic Loci (NPL), Percent Polymorphism (PP), Genetic diversity (H) and Shannon information index (I).

Fig. 4. UPGMA dendrogram Cluster analysis of *Urtica Simensis* generated using ISSR markers.



The highest H was indicated in samples from Mekelle compared to samples from Gondar (0.3462 (34.62%) and 0.3152 (31.52%) respectively) with respect to 3.10 % of difference diversity value (H) as shown in (Table 7).

The Dendrogram based on Jaccard's similarity coefficients generated by UPGMA (Fig. 4). Cluster analysis of ISSR recorded data of all 133 samples without grouping the data to locations generated three major clusters (Fig. 4). Those sub clusters at the same line within the main clusters associated with the genetic similarity among and within the individuals of each population. Most of the individuals collected from both areas (Gondar and Mekelle) enclose to arise over the entire tree without making group. The broad distribution of *Urtica simensis* individuals over the entire tree were indicated the low divergence among populations of both study areas.

Discussion

Approachable genetic diversity in genetic resource collections can highly make possible well founded classification and determination main traits with possible importance in maintaining biodiversity (Majidi *et al*., 2009). The results of this present study showed that both populations (Mekelle and Gondar) have variations in the quantitative traits (Plant Height (PH) and Stem Length (SL)). The qualitative traits (Leaf Shape, Leaf Arrangement and Plant Growth Habit) were similar in both study populations while the trait Flower Color (FC) shown some variations in both areas and this indicated variability and reflected major plant type with combination of the quantitative traits. Whereas the study reported by (Shen *et al*., 2018) on morphological diversity of Job's-tears (*Coix lacryma-jobi* L.) revealed that morphological variation like PH(Plant Height), SNN(stem node number, and PBN (primer branch nodes) reflected as the main factor of plant type with respect to PCA. Therefore, the variation of those both quantitative and qualitative traits could be leaded for excellent or good gene resources conservation strategies due to its capacity in building diversity.

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Most of the morphological appearances recorded in this study supported to the *urtica simensis* morphological characters reported in Flora of Ethiopia book (Volume 3) (Friis, 1989). The analysis of simple correlations among traits is significant. The results revealed that traits with high PH and SL, positive correlation among traits shows these traits could be used as excellent selection criteria to evaluate traits and this indicated as PH increased SL also increased. The significant correlation among the traits PH with FC and SL with FC but its negative correlation. Regarding PCA it generated based on the correlation coefficients matrix with eigenvalues which summarized variation among the measured traits of morphological appearances through Principal Components. Many studies reported that PCA was an effective method for understanding of plant evaluation (Wang *et al.,* 2013). In the present study revealed that the three morphological traits (quantitative traits PH, SL and qualitative traits FC) of the 133 *Urtica simensis* formed three Components and the morphological traits PH, SL and FC were indicated negatively correlated in PC1 with negative eigenvectors and 75.669% and the PC 2 loaded with SL trait. The PC3 loaded both with SL and FC morphological traits with eigenvalue value of 90.38 and 2.33% and reflected the main factor of plant type. Population genetic diversity using morphological traits in a species is constrained through a number of natural factors including gene flow, geographic range among the traits (Hamrick and Godt, 1996). The variations in morphological traits can be used to classify materials in different groups. According the cluster analysis of this study morphological trait of 133 sampled traits showed closer resemblance among populations of *Urtica simensis* consisted of six clusters compared to ISSR polymorphism contained of three clusters as indicated by the distributed mean values with their percentage of variance and distance on the trees distance scales.

Based on study conducted in Genetic diversity of *U. dioca* using ISSR molecular markers (Haghpanah *et al.,* 2016) revealed that the highest Number of Amplified Bands and Number of Polymorphic Loci was produced in primer ISSR8 with respect to 72% polymorphism and compared to results of this current study and results by (Haghpanah *et al.,* 2016) revealed that, the percentage polymorphism ranged from 17-92% among 16 primers and 68% average polymorphism was reported in genetic diversity of nettle plant *U. dioca*. Whereas this study revealed that ISSR12 produced highest Number of Amplified Bands and ISSR8 shown highest NPL with respect to total 100% average polymorphism, but all the primers with samples collected from both Northern parts revealed that high percent of polymorphism (100%) and genetic diversity as well compared to *U. dioca*. UPGMA analysis based on both locations (Gondar (Amhara) and Mekelle (Tigray) regions) with individuals appears intermixing of individuals to different groups.

Analysis of Molecular Variance (AMOVA) of this study conducted on overall ISSR data recorded on *Urtica simensis* showed high variation was indicated within individuals in each populations rather than among populations (in both study areas). Study reported by Hassen (2018) in genetic diversity of *L. sativum* using ISSR markers and morphological characters revealed that the dendrogram formed four clusters and two sub-clusters of *L. sativum* with samples collected from five different regions including Amhara and Tigray regions and UPGMA shows intermixing of individuals to different groups.

Conclusion

This study revealed some variations in *Urtica simensis* collected from the study areas indicates variability and it leads for existence of diversity among populations (in both study areas) but also within individuals in each populations due to the morphological appearances and genetic variability. ISSR markers like (ISSR12 and ISSR8) utilized in this study in evaluating genetic diversity of *Urtica simensis* the results indicated that they are effective markers in evaluating genetic diversity of *Urtica simensis* with respect to 100% average polymorphism, so useful for marker based studies of *Urtica simensis* for the future and this have valuable effect on characterization of *Urtica simensis* genetic resources in different parts of Ethiopia, and used in providing knowledge and generating information for *Urtica simensis* improvement and conservation policies.

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