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Genetic diversity of seed storage protein in the Ethiopian garden cress (*Lepidium sativum* L.)

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The Ethiopian garden cress (*Lepidium sativum* L.) is an important crop extensively used as food and medicine. In this study, total seed storage proteins of 112 garden cress genotypes collected from diverse growing regions in Ethiopia were investigated to assess patterns of genetic diversity and relationships. Using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), a total of 1774 stable protein bands were identified through discontinuous electrophoresis. Of these, 1597 bands were polymorphic. A maximum of 20 protein sub-units in the range of 15 to 75 kDa were observed per genotype. The similarity coefficient among these genotypes ranged from 0.25 to 1.00 with an average genetic dissimilarity of 0.2754. On the basis of Ward Euclidian distance, the genotypes were grouped into five major clusters, the largest one comprised of 62 genotypes (Cluster III) followed by 26 genotypes in Cluster I. Cluster IV and V contained a total of 14 genotypes that were the most distantly related to other groups, and thus can be potentially used as parents for exploitation of heterotic effects in hybrid breeding programs. Our findings using SDS-PAGE profiles revealed no obvious association between geographic region of origin and germplasm clustering. However, the polymorphism and cluster analysis indicated that garden cress genotypes differed greatly in the composition of seed proteins. This shows that protein profiling could be used as a rapid and reliable method for genetic diversity studies. In order to fully explore the protein based genetic diversity in garden cress germplasm, techniques such as 2-D gel electrophoresis are recommended in future studies.

Key words: Cluster analysis, dissimilarity index, garden cress, *Lepidium sativum*, protein polymorphism, protein profiling, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), seed storage protein.

INTRODUCTION

Garden cress (*Lepidium sativum* L.) belongs to the family Brassicaceae and the genus *Lepidium* which contains about 150 species that are distributed throughout almost all temperate and subtropical regions of the world (Bermejo and Leon, 1994; Wadhwa et al., 2012; Rava, 2016). Garden cress is widely cultivated in Africa, Europe, Russia and North America. It is a fast growing, edible herb and an important medicinal plant having a wide range of desirable effects on human health since the Vedic era about 3000 years ago (Manohar et al.,

2012; Doke and Guha, 2014). Various beneficial effects have been observed with the consumption of garden cress. Different parts of the plant including roots, seeds and leaves have been used as a source of functional food and/or medicine (Rava, 2016).

The seeds and leaves of garden cress contain volatile oils and have been consumed as salad and as spice (Amare, 1976; Wadhwa et al., 2012; Rava, 2016). Its seeds are rich source of proteins, carbohydrate, fat (for example omega-3 fatty acids), dietary fiber, vitamins (tocopherol, β--carotene and ascorbic acids), minerals (K, Mg, P, Ca, Fe), and other essential nutrients and phytochemicals (Gokavi et al., 2004; Doke and Guha, 2014). This indicates that the seeds of garden cress play vital role as a promising multipurpose medicinal and nutritional plant. Nowadays, garden cress is becoming popular not only because of its superior medicinal and nutritional values but also due to its contribution in the biofortification of nutritionally inferior crops (Manohar et al., 2012; Singh et al., 2015) in order to ensure the nutritional security of the global population.

Diversity existing in germplasms of crops collected from diverse growing regions need to be properly characterized and evaluated to improve strategies for conservation and utilization towards cultivar development (Parashar et al., 2015; Sharma and Krishna, 2017). Several strategies have been adopted for germplasm characterization including the use of morphological markers (Kancherla and Bhalla, 2003), seed storage protein markers and DNA molecular markers (Rahman and Hirata, 2004). Using quantitative, biochemical and molecular markers suitable germplasm for future plant breeding programs can be identified. Studies in 49 Ethiopian garden cress landraces using morphological and yield-related traits revealed huge variability at diverse agro-ecological zones (Temesgen et al., 2013a, b). Similarly, large diversity was recorded among 85 Ethiopian garden cress genotypes using inter simple sequence repeat (ISSR) (Said and Kassahun, 2015).

Storage or structural seed proteins, encoded by families of polymorphic genes (Mandal and Mandal, 2000) have been extensively used as a genetic marker as they are largely independent of environmental fluctuations (Hameed et al., 2009). Unlike morphological markers (Siddiqui and Naz, 2009), the banding patterns of protein markers are stable (Iqbal et al., 2005; Nasar et al., 2006; Iqbal et al., 2014). Due to these benefits, protein markers have been widely applied in the analysis of genetic diversity within and between accessions, in studying plant domestication in relation to genetic resource conservation and breeding, and in establishing genome relationships (Kakaei and Kahrizi, 2011; Hameed et al., 2012; Sharma and Krishna, 2017). Seed storage protein profiling has been used in investigating diversity among selected varieties of *Brassica napus* (Nasar et al., 2006; Choudhary et al., 2015). Similarly, seed protein profiles were instrumental in the identification of intra-specific genetic divergence in rape seed (Khan et al., 2014).

The use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in studying plant proteins was found to be simple and inexpensive; hence, it has applications in the improvement of the crop of interest through breeding (Zada et al., 2013; Sharma and Krishna, 2017). However, so far, only few studies were made regarding the diversity of storage proteins in the garden cress germplasm. Gianazza et al. (2007) examined the influence of different concentrations of cadmium on the garden cress plants using seed protein storage marker. However, genetic diversity in the seed protein of garden cress was so far not made using the SDS-PAGE, at least in the Ethiopian germplasm collections. Hence, the current study was conducted to investigate using SDS-PAGE diversity in the seed storage protein of the Ethiopian garden cress accessions collected from diverse agro-ecological regions in the country.

MATERIALS AND METHODS

Plant materials and protein samples

Lists of plant materials used in the study and their origin are presented in Table 1. For extraction of proteins, mortar and pestle were used to crush and grind seeds of each genotype. Defatting of about 0.1 g of flour was carried out with chloroform, methanol and acetone in the ratio of 2:1:1 as described by Geetha and Balamurugan (2011) for mustard genotypes. Next day, about 0.05 g of flour was suspended in 1 ml extraction buffer (0.125 M Tris-Cl, 4% SDS, 20% v/v glycerol, 1% 0.2 M DTT, 25 mM EDTA, pH 6.8). Homogenized mixture was incubated at room temperature for 3 h. Protein extraction buffer was properly mixed by vortexing for 5 to 10 min intermittently. The solubilized samples were centrifuged at 14000 rpm for 10 min at 4°C, and the clear supernatant saved at 4°C until they were run gel electrophoresis following the methods of Roy and Kumar (2014) and Buckseth and Singh (2016) with some modification. The gel electrophoresis was run by mixing of 30 µl of the protein extract with 30 µl of sample loading buffer (0.1% of bromophenol blue, 2% SDS, 6% v/v glycerol, 2.5% 0.5 M Tris-HCL PH 6.8 and 1% 0.5 M DTT) followed by vortexing and heating at 65°C for 10 min to ensure complete denaturation, and then with brief vortexing and spinning at room temperature just before loading on gel (Bollag et al., 2002; Nisar et al., 2016).

The SDS-PAGE was carried out in various combinations and optimized to 12.5% acrylamide gel concentration and by loading 30 µl of samples to obtain the best resolution. The procedure

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Table 1. Names and origin of plant materials used in the study.

Accession ID	Origin		
	Regional state	Zone	District/location
229799	Amhara	East Gojam	Enbise SarMidir
229798	Amhara	East Gojam	Hulet Ej Enese
CG14	Amhara	North Gojam	Goncha Siso Enese
235892	Amhara	North Gondar	Addi Arkay
214243	Amhara	North Gondar	Debark
205163	Amhara	North Gondar	Debark
205162	Amhara	North Gondar	Debark
208030	Amhara	North Gondar	Gondar Zuria
CG12	Amhara	North Shewa	Efratana Gidim
229203	Amhara	North Shewa	Lay BetnaTach Bet
229202	Amhara	North Shewa	Lay BetnaTach Bet
229200	Amhara	North Shewa	Lay BetnaTach Bet
229201	Amhara	North Shewa	Lay BetnaTach Bet
229204	Amhara	North Shewa	Lay BetnaTach Bet
229199	Amhara	North Shewa	Siyadebrina Wayu Ens
229205	Amhara	North Shewa	Weremo Wajetuna Mid
241777	Amhara	North Wello	Guba Lafto
207542	Amhara	South Gondar	Kemekem
90004	Amhara	South Gondar	Tach Gayint
90018	Amhara	South Wello	Debresina
CG7	Amhara	South Wello	Debresina
90020	Amhara	South Wello	Dessie Zuria
212628	Amhara	South Wello	Kutaber
215714	Amhara	South Wello	Werebabu
215713	Amhara	South Wello	Werebabu
CG11	Amhara	South Wello	Werebabu
CG22	Amhara	South Wello	Werebabu
90012	Unknown		
233679	Unknown		
233370	Unknown		
240579	Unknown		
90010	Unknown		
90009	Unknown		
90014	Unknown		
90017	Unknown		
90007	Unknown		
90008	Unknown		
205141	SNNP	Gurage	Goro
242916	SNNP	Keficho Shekicho	Chena
240396	SNNP	Keficho Shekicho	Decha
240397	SNNP	Keficho Shekicho	Decha
202116	SNNP	Keficho Shekicho	Ginbo
CG17	SNNP	North Omo	Basketo
225725	SNNP	North Omo	Bonke
CG13	SNNP	North Omo	Damot Wayde
8604	SNNP	North Omo	Damot Weyde
240808	SNNP	North Omo	Damot Weyde
90016	SNNP	North Omo	Gofa Zuria
CG16	SNNP	North Omo	Goffa Zuria

Table 1. Contd.

225799	SNNP	North Omo	Kemba
CG18	SNNP	North Omo	Mareka Gena
240578	SNNP	North Omo	Melokoza
CG21	Tigray	West Tigray	Shire
233985	Tigray	West Tigray	Laelay Adiyabo
219959	Tigray	West Tigray	Medebay Zana
216885	Oromia	Arssi	Merti
216886	Oromia	Arssi	Merti
CG15	Oromia	Arssi	Tiyo
CG20	Oromia	Bale	Meliyu
237991	Oromia	Bale	Adaba
CG4	Oromia	Bale	Agarfa
CG5	Oromia	Bale	Dinsho
CG9	Oromia	Bale	Gaserana Gololcha
19001	Oromia	Bale	Ginir
212852	Oromia	Bale	Goro
19002	Oromia	Bale	Goro
212853	Oromia	Bale	Goro
90002	Oromia	Bale	Sinanana Dinsho
CG6	Oromia	Bale	Sinanana Dinsho
230524	Oromia	East Hararghe	Girawa
CG10	Oromia	East Shewa	Akaki
90006	Oromia	East Hararghe	Deder
90005	Oromia	East Hararghe	Deder
230831	Oromia	East Hararghe	Girawa
208693	Oromia	East Hararghe	Gursum
216816	Oromia	East Hararghe	Gursum
230830	Oromia	East Hararghe	Jarso
208669	Oromia	East Hararghe	Kersa
234828	Oromia	East Wellega	Diga Leka
CG8	Oromia	Jimma	Limu Seka
18843	Oromia	North Shewa	Debre Libanos
208666	Oromia	West Hararghe	Mieso
18841	Oromia	West Shewa	Bako
90021	Oromia	West Shewa	Cheliya
19000	Oromia	West Hararghe	Chiro
CG2	Oromia	West Hararghe	Chiro/Wachu
CG3	Oromia	West Hararghe	Gemechis
208667	Oromia	West Hararghe	Habro
CG19	Oromia	West Hararghe	Mesela
CG1	Oromia	West Shewa	Wolmera
90022	Oromia	West Wellega	Dale Lalo
208769	Oromia	West Wellega	Sayo
215808	Oromia	West Wellega	Sayo
215807	Oromia	West Wellega	Sayo
230829	Somalia	Jigjiga	
230523	Somalia	Jigjiga	
216815	Somalia	Jigjiga	
231210	Somalia	Jigjiga	
233982	Tigray	Central Tigray	Adwa
237512	Tigray	Central Tigray	Adwa

Table 1. Contd.

219961	Tigray	Central Tigray	Adwa
207910	Tigray	Central Tigray	Adwa
219958	Tigray	Central Tigray	Laelay Maychew
233984	Tigray	Central Tigray	Werielehe
233983	Tigray	Central Tigray	Werielehe
238273	Tigray	Central Tigray	Adwa
219962	Tigray	Central Tigray	Naeder Adet
233981	Tigray	East Tigray	Ganta Afeshum
242609	Tigray	East Tigray	Ganta Afeshum
234355	Tigray	East Tigray	Ganta Afeshum

SNNP: Southern Nations and Nationality of People Regional State.

developed by Laemmli (1970) was followed for gel preparation and running. 30 µl of samples digested was loaded into the wells of 4% acrylamide stacking gel (1.5 mm thick) for protein separation. Electrophoresis was carried out at a constant 250 V for the medium slab vertical gel apparatus until bromophenol blue marker crossed bottom of the gel. Pre-stained protein marker, ranging from 10 to 250 kDa (precision plus protein dual color standard supplied by BIO-RAD) was run for reference to molecular weight of respective protein bands in KDa. After complete run, the gels were fixed and stained with 0.5% coomassie brilliant blue (CBB) R-250 in acetic acid: methanol: water (10:40:50 volume ratio) for 3 h and destained in the same acetic acid-methanol-water solution except CBB for overnight (Bollag et al., 2002; Sadia et al., 2009) with constant and gentle shaking.

Data analysis

Gel evaluation for data scoring was done on a light box and rechecked by using photograph that was taken by high resolution camera supported by white light illuminator. The experiment was repeated twice to check the reproducibility of the protein bands. A band presence was coded (1), while the absence of bands scored as (0). Only reproducible bands occurring in high frequency were scored by identifying each protein band carried out according to standard proteins. The intensity of bands was not taken into consideration but only the presence of the bands was taken as indicative. Presence and absence of the bands were entered in a binary data matrix. Based on results of electrophoretic band spectra, similarity index was calculated for all possible pairs of protein type's electrophore-grams. Similarities among genotypes were estimated using Jaccard coefficient of similarity (Jaccard, 1908). Depending on the electrophoretic band spectra, similarity index (S) was designed for all pairs of protein band pattern by the subsequent formula: $S = a/(a + b)$ where S = similarity index, a = Number of bands common to a and b protein types, b = Number of bands in protein type 'b'. The similarity matrix was generated and converted to a dissimilarity matrix. The different bands in the range of 15-75 kDa were used for calculation of similarity indices. Polymorphism % was calculated using the formula:

Polymorphism (%) = [Number of polymorphic bands/Total number of bands] ×100.

The generated data matrix was then used for descriptive statistics and for constructing dendrogram by the ward method using Darwin version 6 Software.

RESULTS AND DISCUSSION

Genetic diversity in seed storage protein

A typical electrophoretic banding patterns and their distribution is presented in Figure 1. A maximum of 20 protein sub-units were observed per genotype within the range of protein molecular weight of 15 to 75 kDa. A total of 1774 polypeptide bands with an average of 15.8 bands per genotype were obtained (Table 2). Out of the total of 1774 bands, 1597 were polymorphic while 177 were monomorphic (data not shown). The banding patterns revealed large variations among genotypes in the low molecular weight protein profiles. This revealed considerable variations in five regions (A to E) (Figure 1). Region A contains relatively high molecular weight proteins ranging from 50 to 75 kDa while in Region B, three protein sub-units ranging in size from 37 to 50 kDa are observed. Five protein bands were found for Region C which ranged from 25 to 37 kDa while Region D comprised of four protein sub units ranging from 20 to 25 kDa. The last part, Region E, contains small size proteins with molecular weight ranging from 15 to 20 kDa. All these five regions showed both light and dark stained bands and were polymorphic except for the last two bands of Region E which were monomorphic (Table 1). This indicates that the proportion of polymorphic bands over the total bands detected were 90%. Ten genotypes (namely, 241777, 229203, 235892, 229205, 214243, 208030, 229199, 237991, 216816 and CG2) showed the highest number of protein bands (each 20 bands) followed by 12 genotypes (207542, 229202, 229799, 212628, 229204, CG14, 238273, 90021, 208667, 219961, 242609, and CG6) each with 19 bands. Three genotypes (90002, 229799 and 90022) had minimum number of protein bands ranging from 7 to 9. Protein sub units located on band number 19 and 20 were the most frequent (Table 2). Similar banding patterns were reported for Brassica species (Rabbani et al., 2001; Nasar et al., 2006; Turi et al., 2010).

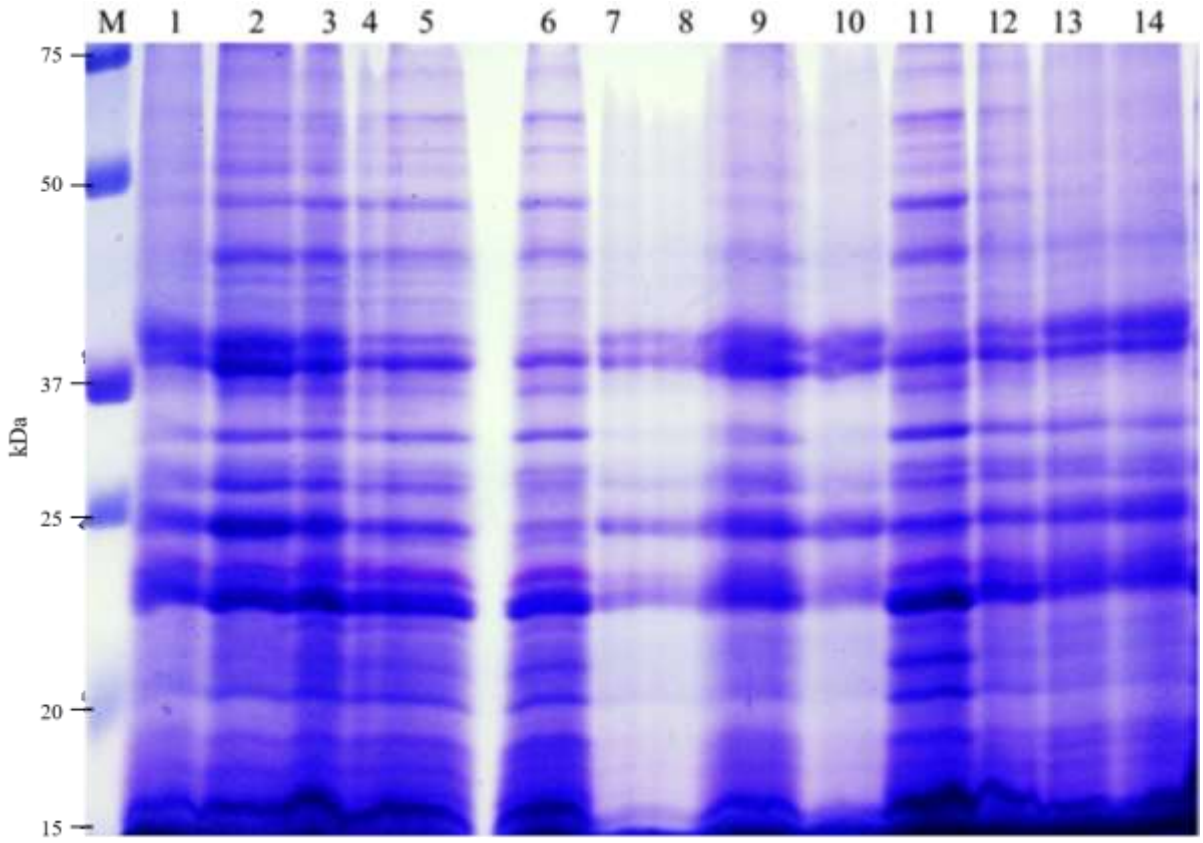

Figure 1. Atypical electrophoretic banding pattern of genotypes generated through SDS-PAGE M represents molecular size marker, while numbers from 1-14 represent selected land cress genotypes.

SDS-PAGE has of paramount importance in separating and characterizing the proteins and estimating the extent of genetic diversity in the present set of garden cress germplasm. The banding pattern in the total seed protein showed close relationships among these studied genotypes. Similarity coefficient among these genotypes ranged from 25 to 100%. This is in agreement with earlier study on *Brassica carinata* where a similarity coefficient of 50 to 100% was reported (Zada et al., 2013).

Proteins have been used as markers for the assessment of genetic diversity in many crops (Iqbal et al., 2005; Nisar et al., 2016; Singh et al., 2017). The seed protein fragments exhibited appreciable polymorphism among the Ethiopian genotypes of garden cress, being used for the study of variability (Table 2). Consequently, electrophoretic analysis of the seed proteins had direct relationship to the genetic background of the proteins, and hence it is a potential marker for the study of genetic diversity and varietal identification. Similar studies have been carried out using protein marker for the study of genetic diversity and/or varietal identification in many crops, as mentioned above (Iqbal et al., 2005; Netra and Prasad, 2007; Nisar et al., 2016; Singh et al., 2017). In general, DNA markers are more robust to detect variability among different genotypes. The only diversity study on garden cress using DNA markers was the one using less efficient inter simple sequence repeat (ISSR) (Said and Kassahun, 2015).

Cluster analysis of seed storage proteins

The cluster analysis showed that the genotypes were divided into three main groups (SG1-SG3) consisting of five major clusters and several sub-clusters (Figure 2). According to the magnitude of this genetic distance, genotypes from Cluster IV and V (SG3) were most divergent from the other two groups (SG1 and SG2). Such divergent genotypes should be used for designing effective breeding programs for evolving genetically vigor and variable breeding lines. Similarly, Cluster I and II represent relatively diverse group as compared to Cluster III. Thus, crossing between genotypes from Cluster I, II, III and genotypes from Cluster IV and V gene pools could create more genetic variability than crosses within genotypes of each main group (Figure 2). They are used

Table 2. The distribution and presence of bands in SDS-PAGE for 112 garden cress genotypes

Region	Code of protein band	Number of genotypes	
		Present	Absent
A	1	56	56
	2	41	71
	3	48	64
	4	49	63
B	5	94	18
	6	93	19
	7	97	15
C	8	88	24
	9	107	5
	10	104	8
	11	99	13
	12	108	4
D	13	101	11
	14	65	47
	15	101	11
	16	90	22
E	17	106	6
	18	103	9
	19	112	0
	20	112	0
Total		1774	
Mean per genotypes		15.84	

to develop desirable recombinant breeding lines and cultivars for future breeding programs.

Cluster III was the largest among all five clusters and consisted of 62 most similar genotypes (55% of the total), revealing low genetic diversity at genomic level (Table 3). However, the results might indicate the limitation on the number of markers used in the current study. In this connection, Opond-Konadu et al. (2005) reported the absence of large genetic difference among cowpea genotypes which hindered the use of protein electrophoresis to investigate diversity. Hence, genotypes in cluster III need to be further investigated in combination with 2D electrophoresis to minimize the lower variability detection efficiency of SDS-PAGE (Javaid et al., 2004; Jan et al., 2016). In this case, the integration of the usual electrophoresis separation with isoelectric focusing point electrophoresis to maximize the resolving power of seed storage protein markers due to amphoteric nature of amino acids is useful.

The cluster analysis also revealed that genotypes from different zones were observed to be closely related and genotypes from the same zone had different genetic background. This suggests that different selection pressures have been applied to yield and other biochemical properties in different genotypes. The high diversity among the genotypes from same region also shows the high exchange of germplasm among garden cress farmers although the exact mechanism of seed exchange among farmers from different regions has not yet been reported. According to Sihag et al. (2004) and Faisal et al. (2009) the cluster pattern for soybean genotypes showed that genetic diversity and geographic distribution were independent of each other and there was no definite relationship existed between them.

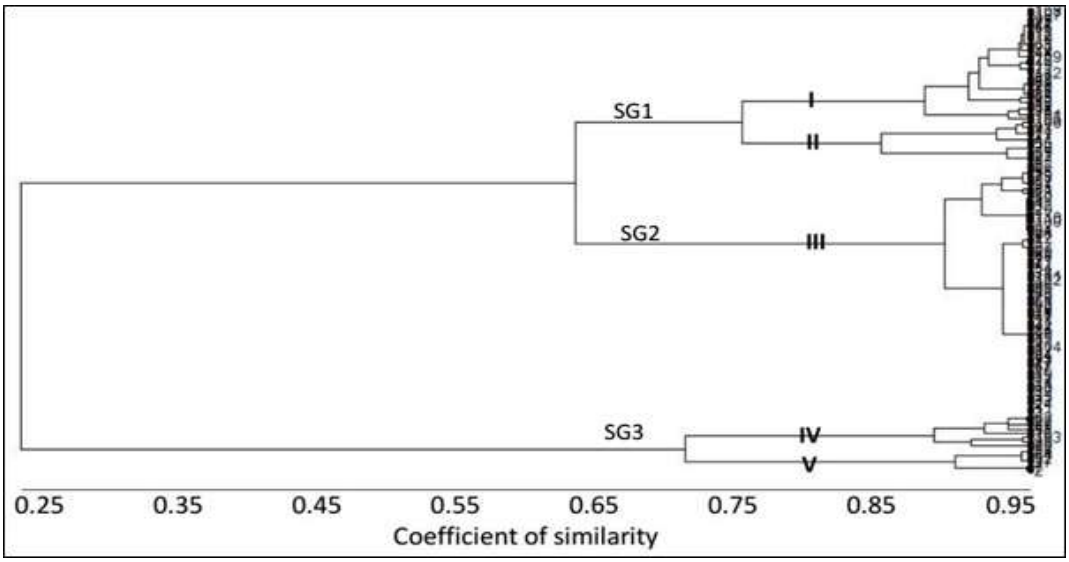

Figure 2. Dendrogram showing the relationships among genotypes based on SDS-PAGE and Ward method. Names of accessions under the five groups are shown in Table 3.

Table 3. Grouping of tested genotypes using data derived from SDS-PAGE analysis.

Cluster[1]	Accession ID	Origin of genotype[2]
I (26)	241777, 225725, 219962, 212852, 240396, 229203, 237991, 229205, 90022, 212628, 90010, 90009, 229201, 240397, 231 210' 229204, 208667, 215713, 90004, 18843, CG10, CG12, G16, CG18, CG19, CG20	1, 2, 3, 4, 5, 6
II(10)	233984, 237512, 229798, CG2, CG17, 219960, 233982, 90016, 240578, 242609	1, 2, 3, 5
III (62)	207542, 208693, 8604, 205141, 202116, 238273, 90006, 230831, 219959, 235892, 234355, 233983, 229202, 233370, 216885, 219958, 214243, 216816, 230829, 229200, 242916, 240579, 90002, 205163, 215714, 233985, 215807, 216886, 19001, 19000, 219961, 208030, 90021, 230523, 234828, 208666, 240808, 229199, 90018, 90014, 19002, 207910, 216815, 90005, 212853, 230830, 208669, 90007, 230524, 205162, 233981, CG1, CG4, CG5, CG6, CG7, CG8, CG11, CG13, CG15, CG21, CG22	1, 2, 3, 4, 5, 6
IV(8)	90012, 233679, 225799, 229799, 90017, 18841, 90008, CG14	1, 2, 3, 6
V(6)	208769, 215808, 233986, 90020, CG3,CG9	1, 2, 5

[1]Values in parenthesis indicate number of genotypes.
[2]Origin of genotype. 1, Amhara; 2, Oromia; 3, Southern Nation, Nationalities and of People Regional State (SNNP); 4, Somali; 5, Tigray; 6, unknown source.

In general, the seed storage protein profiling generates wide array of polymorphism, hence could serve as a valuable tool in determining the extent of genetic diversity. Thus, SDS-PAGE marker data provided more sub-groupings and revealed considerable amount of genetic diversity.

Conclusions

Based on similarity indices, the dendrogram divided the genotypes into three groups and five clusters, indicating the genetic relationships among genotypes. The grouping of genotypes into clusters did not associate with their geographic distribution. Seed storage protein profile could be economically useful marker to assess genetic diversity in garden cress germplasm. Predominately polymorphic proteins were noted in SDS-PAGE analysis used for selection of desirable genotypes in the garden cress improvement programs. However, the study revealed that nearly half of the genotypes were grouped into similar clusters requiring further analysis with a combination of 2D electrophoresis. The hybridization among the genotypes from distantly related groups is

suggested in order to enhance future breeding programs towards the development of desirable varieties.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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