Assessment of genetic diversity in *Lepidium sativum* L. using inter simple sequence repeat (ISSR) marker

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Abstract *Lepidium sativum* L. is a fast-growing, edible and medicinal plant that belongs to the family Brassicaceae. Indian system of medicinal and health (ISHM) recognizes this plant as a source of several medicinal and nutraceutical factors. Ninety-four accessions collected from 19 states of India were assessed for genetic diversity using inter-simple sequence repeat (ISSR) marker. Ten ISSR primers amplified a total of 172 bands across the 94 accessions and out of these, 139 bands were found to be polymorphic and 33 as monomorphic. The percentage polymorphism varied from 60.00 to 91.30% with an average of 80.10%. The polymorphism information content (PIC) varied from 0.14 to 0.39 with an average of 0.27. The Jaccard similarity coefficient ranged from 0.11 to 0.89 with minimum between accession LS61 and LS60 and maximum between accession LS95 and LS81. Cluster analysis based on UPGMA grouped all the 94 accessions into three major clusters with accessions per cluster ranging from 12 to 45. Similar to UPGMA clustering, PCA also differentiated all the accessions into three major groups. Model-based clustering determined three sub-populations ($K = 3$). Further, analysis of molecular variance showed that 67% of allelic diversity was attributed to individual accessions

within populations while 33% was distributed among populations. This preliminary study shows that significant variability exists in the collected accessions.

Keywords AMOVA · Population structure · PIC · UPGMA · Linkage disequilibrium

Introduction

L. sativum is an erect, fast-growing annual plant that belongs to the family Brassicaceae. Although the origin of *L. sativum* is not clearly known, it is believed to have its origin in North-East Africa (Asfaw and Demissew 2009; Datta et al. 2011). It is known as garden peppercress, peppergrass, pepperwort and Chandrasur. Its main character is that it can grow in any type of climate and soil condition and its ability to tolerate slight acidity. *L. sativum* is considered as under-utilized horticultural crop (Bermejo and León 1994) and possesses a significant amount of essential oil with high medicinal values (Asfaw and Demissew 2009). Its seed contains 25–39% protein, 33% sugar, 2.4% rough fat, 7.6% unrefined fiber and 6.4% minerals which can be utilized as raw material for preparation of functional foods (Patel et al. 2010). The seeds of *L. sativum* have medicinal properties and are used as tonic, carminative, diuretic, aphrodisiac, laxative etc. (Yadav et al. 2010). The “lepidine” is one of the major imidazole alkaloids found in *L. sativum* seeds (Maier et al. 1998) which are mainly used for treating bacterial and contagious diseases (Mukhopadhyay et al. 2010). Furthermore, the sprouts are an exceptionally well known component of servings of mixed greens and sandwiches in a few areas of the world (Michalczyk et al. 2011).

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In recent years, various types of molecular markers such as AFLP, ISSR, SSR, SNP etc. have been extensively utilized to assess the genetic variability in large group of crop plants. The ISSRs markers have been widely used for assessing genetic diversity and variability because they are highly polymorphic, don't require sequence information, and are quick and cost-effective. It is PCR based technique that uses primers (repeat anchored or non-anchored) to amplify DNA sequences among two inverted SSRs (Reddy et al. 2002). ISSR markers have been effectively utilized for the evaluation of genetic diversity of different therapeutic plants like *Tribulus Terrestris* (Sarwat et al. 2008), *Anoectochilus formosanus* (Zhang et al. 2010), *Rheum Officinale* (Wang et al. 2012), *Costus pictus* (Naik et al. 2017) and *Ocimum* species (Patel et al. 2015). Previously, few marker based genetic studies has been reported in *L. sativum*. Sharma et al. (2015) carried out polymorphism and diversity analysis deploying 10 RAPD primers over 15 *L. sativum* genotypes and reported 86.27% polymorphism. Bansal et al. (2012) reported 82.59% of polymorphism using 32 RAPD primers over 18 genotypes. Kaur et al. (2015) evaluated genetic variability among 15 genotypes with ISSRs and; Kumar et al. (2012) used a combination of RAPD and ISSR markers to assess the genetic diversity in 19 genotypes of *L. sativum*. Said and Kassahun (2015) evaluated 85 *L. sativum* genotypes from Ethiopia using 4 ISSR markers. The previous marker based studies on *L. sativum* are restricted to a limited number of markers and accessions and thus it might not capture the actual genetic diversity and variability available in the genetic materials. Therefore, in the present investigation, a larger accession of *L. sativum* collected from the different eco-geographical region of India has been evaluated to measure the extent of genetic variability and also identify potential accessions for further genetic improvement.

Materials and methods

Plant materials and isolation of genomic DNA

Ninety-four accessions of *L. sativum* used in the present investigation were collected from 19 states of India representing different eco-geographical region. The collection site, accessions code and state wise number of accessions is provided in supplementary table S1. All the accessions were grown in pot during October 2015–January 2016. Fresh and young leaves was collected from 21 days old seedlings and total genomic DNA was extracted following the CTAB method (Murray and Thompson 1980) with slight modifications. The isolated genomic DNA was quantified using spectrophotometer (NanoDrop ND 1000) and quality was checked by electrophoresis on 0.8% (w/v)

agarose gel. Finally, the DNA was diluted to 10 ng/μl for PCR amplification.

ISSR primer screening and genotyping

A set of 35 UBC series ISSR primers (Bangalore Genei, Bangalore, India) were initially used to amplify four genomic DNA comprising three of *L. sativum* and one outgroup *Raphanus sativus* to determine the appropriateness of each primer for further genotyping of remaining accessions. Finally, a total of 10 ISSR primers were selected based on their distinct, clearly resolved and polymorphic amplified products. The polymerase chain reaction (PCR) reaction consists of 10 ng DNA, 10 pmol primers, 1X PCR buffer, 0.2 mM of each dNTP and 1U/μl Taq DNA Polymerase (Qiagen, India) in a final volume of 25 μl. The PCR amplification was performed in a Proflex Thermal Cycler (Life Technologies, USA) under the following conditions: pre-denaturation at 94 °C for 5 min, followed by 45 cycles of denaturation at 94 °C for 45 s, annealing at an optimized temperature for 45 s and extension at 72 °C for 2 min. Finally, an additional extension cycle at 72 °C for 5 min was performed. The amplified PCR products was separated by electrophoresis in 1.2% agarose gel containing ethidium bromide (0.5 μg/ml) in 0.5X TBE buffer at constant 75 V. Low range ruler (Bangalore Genei, Bangalore, India) was used as a size marker. The gel image was captured with Alpha Imager™ gel documentation system.

Data scoring and statistical analysis

The ISSR band was scored as binary data with presence of band indicated by 1 and its absence by 0. The polymorphic information content (PIC) was calculated as: $PIC = 2f_i(1 - f_i)$, where f_i is the frequency of the amplified allele. The binary data matrix was subjected to calculate distance matrix following Jaccard similarity coefficient using NTSYS-pc software (Rohlf 1988). The resultant distance matrix was then employed to construct a UPGMA based dendrogram. The principal component analysis was also performed to construct plots using Eigenvalues and vectors. Further, to determine the number of population among accessions, a simulation was carried out using model-based Bayesian clustering deployed in the STRUCTURE program (ver. 2.3.4) (Pritchard et al. 2000). The membership of each accession was tested for the range of genetic clusters (K) from 2 to 10 with the admixture model. Three independent runs were performed for each K and each run consisted of a burn-in period of 30,000 and 100,000 iterations. The optimal value of K was determined by ΔK statistic and L (K) using the program Structure Harvester (Earl 2012; Evanno et al. 2005). Based on dendrogram

clustering, three population was considered and analysis of molecular variance (AMOVA) was carried out using the program GenAlEx (ver. 6.5) (Peakall and Smouse 2006). To determine state wise genetic diversity estimates, individual cluster analysis was performed for accessions of six states which had ≥ 5 accessions (Supplementary Table 2). Based on these state wise cluster analysis, further individual AMOVA was also carried out to determine state wise diversity estimates. The linkage disequilibrium (LD) between the loci and distance (in base pair) was estimated using recombination coefficient (r^2 value) calculated using TASSEL v3.0 (Bradbury et al. 2007). The percentage of the marker combinations at significant ($p < 0.01$) LD was calculated by estimating the number of marker combinations at different r^2 values (> 0.1).

Results

ISSR polymorphism

A total of 172 bands were amplified with 10 ISSR primers and of which 139 were found to be polymorphic and 33 monomorphic (Table 1). The total number of bands produced by ISSR markers varied from 12 (UBC810) to 23 (UBC881) with an average of 17.5 ± 2.9 band per ISSR markers. The maximum level of polymorphism was produced by primer UBC881 (92.0%), followed by primer UBC827 (90.0%) and UBC823 (88.8%). However, the primer UBC809 and UBC808 showed a minimum level of polymorphism of 60.0% and 70.6% respectively. The average percentage of polymorphism was observed as 80.16%. The polymorphism information content (PIC) of markers varied from 0.14 to 0.39 with an average of 0.27 ± 0.07 . The maximum PIC was shown by primer

UBC817 (0.39), followed by UBC878 (0.32) and UBC823 (0.31).

Genetic diversity and population structure

The binary data of 10 ISSR primers generated across 94 accessions of *L. sativum* was used to calculate genetic distance following Jaccards similarity coefficient. The Jaccard similarity coefficient ranged from 0.11 to 0.89 with an average of 0.78 ± 0.07 . The minimum similarity was noticed between accession LS88 and LS23 and maximum between accession LS64 and LS63. The UPGMA clustering grouped all the 94 accessions into three major clusters namely cluster I, II and III consisting of 45, 33 and 12 accessions respectively (Fig. 1). The *Raphanus sativus* which was taken as out-group clearly separated from all the *L. sativum* accessions. Further, three accessions namely LS25, LS22 and LS23 could not clearly be grouped in any of the cluster. The cluster I could be further divided into two sub-clusters Ia and Ib consisting 21 and 24 accessions respectively. Likewise, the cluster II could also be divided into three sub-clusters i.e. IIa, IIb, IIc consisting of 20, 8 and 5 accessions respectively. The cluster III contains two sub-clusters IIIa and IIIb, each consisting of 9 and 3 accessions. The principal component analysis (PCA) also differentiated all the accessions into three major groups, almost with similar clustering pattern of UPGMA dendrogram (Fig. 2). In order to understand the population stratification among the collected accessions, the model based simulation of population structure was calculated to investigate the subpopulations among the accessions. It was noticed that there were three subpopulations based on ΔK value (Supplementary Fig. S1). The bar plot of model-based clustering showed that 47 accessions (50.00%) were assigned to sub-population 1, among which 20 accessions

Table 1 List of ISSR primers used for amplification, amplified bands, percent polymorphism and PIC value

S.No.	ISSR	Sequence(5'-3')	Total band	Monomorphic band	Polymorphic band	Polymorphic (%)	PIC
1	808	AGA GAG AGA GAG AGA GC	17	5	12	70.58	0.14
2	809	AGA GAG AGA GAG AGA GG	15	6	9	60.00	0.19
3	810	GAG AGA GAG AGA GAG AT	12	2	10	83.33	0.28
4	817	CAC ACA CAC ACA CAC AA	17	3	14	82.35	0.39
5	822	TCT CTC TCT CTC TCT CA	17	4	13	76.47	0.2
6	823	TCT CTC TCT CTC TCT CC	18	2	16	88.88	0.31
7	826	ACA CAC ACA CAC ACA CC	17	5	12	70.58	0.27
8	827	ACA CAC ACA CAC ACA CG	20	2	18	90.00	0.3
9	878	GGA TGG ATG GAT GGA T	16	2	14	87.50	0.32
10	881	GGG TGG GGT GGG GTG	23	2	21	92.00	0.3
	Range		12–23	2–6	9–18	–	–
	Average		17.5	4	13.5	–	–

Fig. 1 UPGMA based dendrogram showing the genetic relationship among 94 accession of *Lepidium sativum* L. using 10 ISSR primer

(42.55%) were pure lines and rest 27 admixture types (Fig. 3). The sub-population 2 comprised of 24 accessions (25.53%) with 13 accessions (54.16%) pure lines and 11 admixture types. The sub-population 3 had 23 accessions (24.46%) in which 17 accessions (73.91%) were pure lines. The structure analysis revealed that sub-population 1 had maximum number of admixture accessions (27), followed by sub-population 2 (11) and sub-population 3 (6). Accession composition of dendrogram and structure analysis showed that 87% accessions of sub-population 3 fall into the cluster I of the dendrogram. Likewise, 57% accessions of subpopulation 1 shared with cluster IIb, IIc and cluster III of the dendrogram. Similarly, 54% accessions of sub-population 2 shared with cluster II. The accessions showing admixture nature were also randomly distributed in the dendrogram.

Analysis of molecular variance (AMOVA)

Based on UPGMA dendrogram, all the 94 accessions were divided into 3 populations and AMOVA was carried out to test the significance of genetic structure. It was noticed that

67% of the total genetic variation was within the population and 33% was among the population (Table 2). The average pairwise Φ_{PT} (similar to F_{ST}) was 0.329 supporting the strong population structure. Different genetic diversity estimates for three populations of *L. sativum* was calculated in which the maximum gene diversity (H_e) was recorded for population 1 (0.20 ± 0.01), followed by population 2 (0.14 ± 0.01) and population 3 (0.10 ± 0.01) with an average of 0.15 ± 0.01 (Table 3). Likewise, the maximum number of alleles per locus (N_a), effective number of alleles per locus (N_e) and Shannon's information index (I) was noticed for population 1 except N_a for population 2 (Table 3). In order to understand the state-wise extent of genetic diversity in the accessions, six individual cluster analyses was carried out for accessions of six states in which the number of accessions was ≥ 5 (data not shown). Based on state-wise cluster, AMOVA was performed to determine the diversity estimates which showed that the accessions collected from Rajasthan had lower estimates for N_a , N_e , I , and H_e (Supplementary Table S3). On the other hand, the accessions collected from Uttar Pradesh showed higher values for N_a , N_e , I , H_e and moderate

Linkage disequilibrium estimation

accessions. The recombination coefficient (r^2) values ranged from 0.0651 to 1.000 with an average of 0.20. Across all the 147 loci, 6075 locus pairs were detected in the 94 *L. sativum* accessions (Fig. 4). Among all of the locus pairs, 1020 (16.74%) pair were found to be in significant LD ($P < 0.01$) and among which 793 loci-pair exhibited $r^2 > 0.1$ up to 50 bp (Fig. 4). The LD analysis indicated no LD decay up to 50 bp in the accessions used in the present investigation.

Fig. 3 Estimated genetic structure $K = 3$ obtained with the STRUCTURE program for three sub-populations of 94 accessions of *Lepidium sativum* L

Table 2 Analysis of molecular variance (AMOVA) for 94 accessions from three populations

Source	Degree of freedom	Sum of squares	Variance	Variance (%)
Among population	2	445.17	7.25	33
Within population	91	1346.48	14.80	67
Total	93	1791.67	22.04	100

$F_{ST} = 0.329$; $P < 0.010$

Table 3 Different genetic diversity estimates for three populations of *Lepidium sativum* L

Population	N_a	N_e	I	H_e
Pop1	1.51 ± 0.06	1.33 ± 0.02	0.31 ± 0.02	0.20 ± 0.01
Pop2	1.91 ± 0.07	1.23 ± 0.02	0.22 ± 0.02	0.14 ± 0.01
Pop3	0.94 ± 0.07	1.15 ± 0.02	0.16 ± 0.02	0.10 ± 0.01
Mean	1.21 ± 0.04	1.24 ± 0.01	0.23 ± 0.01	0.15 ± 0.01

N_a number of different alleles; N_e effective number of alleles; H_e Nei's (1973) gene diversity; I Shannon's information index

Discussion

The existence and evaluation of variability in the genetic resources of any crop plant pave a suitable platform for strategic breeding program towards its genetic improvement. Various types of molecular markers have extensively been used to assess the genetic variability in crop plants (Nadeem et al. 2018). In the present investigation, 10 ISSR markers have been used to assess and evaluate the extent of genetic variability among the 94 accessions of *L. sativum* collected from the different eco-geographical regions of India. The ISSRs offer great potential to determine inter- and intra-genomic diversity compared to other random primers, since they reveal variation within unique regions of the genome at several loci concurrently. Previous reports

Fig. 4 An LD plot showing distribution of ISSR loci at different distance (in bp) for different level of recombination coefficient (r^2) in *Lepidium sativum* L

of molecular marker based genetic diversity in *L. sativum* are very few and based on a limited number of markers and germplasm (Kaur et al. 2015; Kumar et al. 2012; Said and Kassahun 2015). A total of 139 polymorphic bands were produced by 10 ISSR across the 94 accessions which showed the comparatively high level of polymorphism (80.16%). Similar level of polymorphism was also reported previously by Bansal et al. (2012) with RAPD markers (82.0%), Kaur et al. (2015) with ISSR markers (82.0%) and Sharma et al. (2015) with RAPD markers (86.27%).

However, the study by Kumar et al. (2012) using RAPD and ISSR markers showed lower polymorphism (~ 66%) with 19 genotypes of *L. sativum*. The discrepancies in degree of percentage polymorphism might be due to the marker types, number of markers, number of accessions and extent of genetic diversity in the genetic materials used for the study. The ISSR primers showing higher level of polymorphism such as UBC881, UBC827, UBC823 and UBC878 could be considered as potential marker for future application in further evaluating other set of genetic material of *L. sativum*.

The ISSR marker data based similarity coefficient value among the 94 accessions enables to identify diverse and potential accessions which could be utilized in breeding program to create further genetic variability. It is assumed that crossing distantly related accessions produced more potential transgressive segregants. The accessions LS23, LS88, LS94, LS92, LS86 and LS27 were identified as more diverse based on their similarity coefficient value. To understand the level of genetic divergence and population structure among the collected accessions, UPGMA based dendrogram and model-based structure plot was created. The UPGMA cluster and population structure analysis revealed three major cluster and three sub-populations respectively. The genetic distance based clustering and population structure showed an almost similar pattern of grouping of accessions which indicates robustness of the clustering of accessions. Further, principal component analysis (PCA) also differentiated all the accessions into three major groups clearly indicating diverse nature of accessions. The AMOVA analysis showed significance of the genetic structure which indicates 67% of the genetic variation found within the populations and 33% variation due to differences among the three populations. The accessions from Rajasthan showed higher estimates of diversity parameters and it was also evident that out of six accessions identified as diverse based on genetic distance, two (LS-92 and LS-94) was from Rajasthan. The observed F_{st} value (0.329) supported the strong population structure among the collected accessions. Similarly, Said and Kassahun (2015) also reported higher genetic variation (94%) within population among 85 accessions of *L. sativum* collected from Ethiopia. Further, the calculated genetic diversity estimates as gene diversity (H_e), Shannon index (I), number of alleles per locus (N_a) and effective number of alleles (N_e) was found to be higher for population 1 while these parameters was lowest in population 3 indicating that population 3 contains comparatively more variable accessions with less abundant and low uniform distribution. Almost, similar magnitude of H_e and I was reported by Said and Kassahun (2015) in Ethiopian population of *L. sativum*. The LD analysis revealed that the average recombination coefficient (r^2) was found to be

0.2010 which was higher than that reported by Zhang et al. (2016) in Switchgrass using ISSR markers. Further, the percentage of linkage decay was found to be 16.74% ($P < 0.01$). Vaishnav et al. (2018) reported ~ 10% LD decay in case of teak analysed with AFLP marker. No LD decay up to 50 pb in the present investigation indicated that these accessions could of potential value for association mapping with precise phenotyping for important traits and use of advance markers systems such as SNPs in future studies.

Conclusion

The present investigation identified potential ISSR markers and diverse accessions of *L. sativum*. The ISSR markers such as UBC881, UBC827 and UBC823 with high percentage of polymorphism and PIC value could be used for genetic characterization of new set of accessions. Likewise, the accessions LS23, LS88, LS94, LS92, LS86 and LS27 with diverse genetic makeup and variability could be exploited in the future breeding program for creating further genetic variability and genetic improvement. The LD analysis revealed that the accessions could also be utilized for association mapping to identify marker trait relationship with application of advance markers system and precise phenotyping.

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Author contributions HKY conceived and designed the research. VK carried out the experiments. VK and HKY wrote the manuscript. All authors read and approved the manuscript

Compliance with ethical standards

Conflict of interest The authors declare that they have no compete of interest.

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