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RESEARCH ARTICLE

Genetic Diversity in Cultivated Gene Pool of Indian Barley (*Hordeum vulgare* L.): An Analysis Using SSR Markers

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Genetic diversity in the released varieties and registered germplasm of Indian barley was assessed using Simple sequence repeats (SSR) markers. Twenty five SSR markers representing all the seven barley chromosomes were used to study genetic diversity in 88 barley germplasm lines. The average PIC of these SSR markers was 0.40 and the average number of alleles amplified by these markers was 3.4 with a maximum of 5 alleles and a minimum of 2 alleles Average genetic diversity among the germplasm accessions was 0.45, ranging from as low as 0.05 to as high as 0.91. Results indicated that these barley germplasm accessions were quite diverse in nature. Cluster analysis revealed that the majority of varieties originating from one centre clustered together, which pointed to the use of narrow genetic base for varietal development.

Key Words: Barley, Genetic Diversity, Germplasm, SSR markers

Introduction

Barley improvement efforts in India date back to the 1920s and 1930s. During this time, most of the varieties were derived from pure line selection from indigenous landraces. However, it was only after the inception and initiation of the All India Wheat and Barley Improvement Project (AICW&BIP) in 1997, that the research efforts for barley improvement in the country became streamlined and more target oriented. Concerted efforts under this project, has led to the development of 94 varieties of barley (Kumar *et al*., 2014). Based on their end use, these have been categorized into feed barley, food barley, malt barley and dual purpose barley, the latter serves as a source of both forage crop and feed crop.

The development of new varieties entails a thorough evaluation of germplasm for both morphological and molecular diversity. Diversity analysis is of paramount importance in order to enable a judicious utilization of these genetic resources by breeders in various crop improvement programmes. Many different molecular markers have been used to study genetic diversity in different crops. The choice and the nature of the markers utilized depends on a large number of factors such as purpose of the study, availability of resources, time at hand, ease of applicability of markers etc. In barley, RAPDs (Manjunatha *et al*., 2007), AFLPs (Varshney *et al*., 2007), ISSRs (Wang *et al*., 2009), SSRs (Jaiswal *et al*., 2010; Fu and Horbach, 2012; Naceur *et al*., 2012; Gougerdchi *et al*., 2014; Ferreira *et al*., 2016), DArTs (Lamara *et al*., 2013; Zhou *et al*., 2015), SNPs (Varshney *et al*., 2007) have all been used for diversity analysis. Seed storage proteins, hordeins have also been used to study genetic diversity among barley landraces collected from Lebanon (Mzid *et al*., 2016). Microsatellite markers (SSRs) appear to be the markers of choice for diversity studies and have been most widely used across different plant species. Varshney *et al*., 2007 have conclusively proven that SSRs markers are most useful for diversity analysis and fingerprinting in barley by comparing EST derived SSRs, EST derived SNPs and AFLPs, all of which were used to characterize 43 wild (*Hordeum vulgare* ssp. *spontaneum*), 35 cultivated (H. *vulgare* ssp. *vulgare*) and 12 elite (H. *vulgare* ssp. *vulgare*) barley lines. More recently, temporal changes in diversity of landraces of Jordan collected from the same locations from the period 1981-2012 has been studied using SSR markers (Thormann *et al.,* 2018). The use of SSR markers for these studies will be of

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immense utility in future to understand the climate influenced genotypic and phenotypic variations in plant species.

The objective of the present investigation was to estimate genetic diversity present in the germplasm collection (Table 1) of released varieties and registered germplasm of barley using SSR markers.

Material and Methods

Plant Material and Genomic DNA Extraction

Seeds of 88 barley genotypes were procured from the national genebank at ICAR-NBPGR and grown in pots in greenhouse. The leaf samples were collected at seedling stage and immediately preserved in liquid nitrogen for DNA isolation at later stage. The details of each genotype along with their Indigenous Collection (IC) number, cultivar name, origin, parentage and important characteristics, are given in Table 1 and Supplementary Table 1. Total genomic DNA isolation was carried out from the young leaf samples using modified CTAB based micro-extraction method (Doyle and Doyle Method, 1987). The DNA quality was first checked on 0.8% agarose gel and then quantified using Nanodrop spectrophotometer (Thermo Fisher, USA). Isolated high quality DNA was diluted to a working concentration of 25 ng/ul for further use.

Genotyping with SSR Markers

Genotyping was carried out using 25 genomic SSR markers (Supplementary Table 2). The PCR reaction mixture consisted of a reaction volume of 10 ul, containing 25-30ng of template DNA, 5pmol of each primer (forward and reverse), 0.05 mM dNTPs, 10X PCR buffer with 15mM $MgCl_2$ and 0.5U of Taq DNA polymerase. The thermocycling conditions were standardized at; template DNA denaturation at 94°C for 5 min followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C to 60°C (varying with the primers) and 2 min of primer extension

Table 1. Centre wise details of released varieties and registered germplasm of barley used in the study

S.No.	Developing Centre	State	No. of Accessions
1.	ARS, SKRAU, Durgapura	Rajasthan	Bilara 2, RD2660, RD2849, RD2035, RD2052, RD2503, RD2508, RD2552, RD2624, RD2668, RD2715, RD2786, RD2794, RD31, RD57, RDB1, RS 6 **(17)**
2.	CSAUA&T, Kanpur	Uttar Pradesh	Azad (K125), Jagrati (K287), Jyoti (K141), Lakhan (K226), Manjula (K329), Narmada (K-603), Priti (K409) **(8)**
3.	CCSHAU, Hisar	Haryana	BG105, BG25, BH946, BH959, BH393, *BH462, BH490, BH561, BH562, BH563*, BH75, BH902, *BHMS12A/12B, BHMS25A/25B* **(14)**
4.	CSKHPKV, Bajaura	Himachal Pradesh	Dolma, HBL113, HBL316, HBL391 (Gokul), Sonu (HBL87) **(5)**
5.	IIWBR, Karnal	Haryana	Alfa93, *BCU5762*, *BK1127*, DWR30, *DWR37, DWR38, DWR39, DWR44, DWR47, DWR51*, DWRB101*, DWRB128, DWRB143*, DWRB127, DWRB92, DWRUB52, Karan 16, Rekha (BCU73) **(18)**
6.	GBPUA & T, Pantnagar	Uttarakhand	PRB502, UPB1008 **(2)**
7.	IARI, New Delhi	New Delhi	DL36 (Kedar), DL70 (Ranjit), Ratna **(3)**
8.	IARI, Shimla	Himachal Pradesh	*BHS369*, BHS400, BHS169, Pusa losar (BH380), Himadri (BHS352) **(5)**
9.	JNKV, Rewa	Madhya Pradesh	JB58 **(1)**
10.	NDUA&T, Faizabad	Uttar Pradesh	Narendra Barley-1 (NDB209), Narendra Barley-3 (NDB1020), NDB1445, NDB1465, NDB1544, NDB1173 **(6)**
11.	PAU, Ludhiana	Punjab	PL172, PL419, PL426, PL56, PL751 **(5)**
12.	SKUAST, Leh	Jammu and Kashmir	Sindhu (NBL11) **(1)**
13.	VPKAS, Almora	Uttar Pradesh	VLB1, VLB85 **(2)**
14.	BHU, Varanasi	Uttar Pradesh	Mahamana113 (HUB113) **(1)**

Figure in parenthesis: Number of accessions; registered germplasm in italics; SKRAU: Swami Keshwanand Rajasthan Agricultural University; CSAUA&T: Chandra Shekhar Azad University of Agriculture and Technology; CCSHAU: Chaudhary Charan Singh Haryana Agricultural University; CSKHPKV: Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya; IIWBR: Indian Institute of Wheat and Barley Research; GBPUA&T: G. B. Pant University of Agriculture and Technology; IARI: Indian Agricultural Research Institute; JNKV: Jawaharlal Nehru Krishi Vishwa Vidyalaya; NDUA&T: Narendra Dev University of Agriculture and Technology; PAU: Punjab Agricultural University; SKUAST: Sher-e-Kashmir University of Agricultural Sciences and Technology; VPKAS: Vivekananda Parvatiya Krishi Anusandhan Sansthan; BHU: Banaras Hindu University.

at 72°C, followed by a final extension at 72°C for 10 min. The amplified products or amplicons were separated on 3.5% metaphor agarose gels and run for three hours at 120V in 1X TAE buffer.

Data Analyses

The allelic data obtained after running SSR assay, was scored according to the allele sizes of the amplified PCR products. Each SSR amplified band was scored as present (1) or absent (0) for each genotype. For a given marker, the bands differing by 4–5 base pairs were assigned as different alleles. Null allele was assigned to an accession for a microsatellite locus whenever an amplification product was not detected for a particular genotype marker combination. The data was then analyzed for genetic diversity analysis and for calculation of primer parameters like major allele frequency, gene diversity, heterozygosity and polymorphic information content (PIC) for each locus, using Power Marker 3.5 (Liu and Muse, 2005). The dissimilarity matrix was used for clustering of genotypes and a UPGMA tree was constructed using MEGA software version 6.0 (Tamura *et al*., 2013). A principal component analysis (PCA) based plotting of genotypes was done using the software package PAST (paleontological statistics software package for education and data analysis, Hammer *et al*., 2001).

Results

SSR Marker Polymorphism

The 25 polymorphic SSR markers amplified a total of 86 different alleles (Table 2). The average number of alleles amplified was 3.4, ranging from as high as

Table 2. Summary statistics of SSR markers used in the study

Marker	Annealing temp. (ºC)	Major Allele Frequency	Allele No	Gene Diversity	Heterozygosity	PIC
GBM1324	55	0.65	3.00	0.51	0.00	0.46
EBmac0806	55	0.84	3.00	0.29	0.00	0.27
HVM64	61	0.91	2.00	0.28	0.00	0.20
HVM68	58	0.66	4.00	0.49	0.10	0.43
Ebmac0827	62	0.63	3.00	0.50	0.00	0.42
Ebmac0624	55	0.69	4.00	0.48	0.00	0.43
Ebmac0906	57	0.53	3.00	0.51	0.00	0.39
Ebmac0603	63	0.53	4.00	0.59	0.00	0.52
Bmac0093	57	0.92	3.00	0.15	0.00	0.14
Bmac0040	57	0.63	5.00	0.55	0.04	0.51
Bmag0217	63	0.80	4.00	0.34	0.00	0.31
Bmag0375	55	0.75	3.00	0.41	0.00	0.37
GBM5210	57	0.88	3.00	0.23	0.00	0.21
Bmag0337	55	0.51	4.00	0.65	0.00	0.60
Bmag0378	55	0.71	4.00	0.46	0.00	0.41
Bmag0394	55	0.62	4.00	0.52	0.00	0.44
HVM54	63	0.51	3.00	0.53	0.00	0.43
Bmag0206	57	0.57	4.00	0.56	0.00	0.49
GBM1405	57	0.54	3.00	0.52	0.00	0.41
Bmag0518	55	0.45	4.00	0.62	0.00	0.54
GBM1501	60	0.49	3.00	0.59	0.00	0.51
HVM40	55	0.50	4.00	0.66	0.00	0.62
GBM1482	57	0.50	4.00	0.64	0.02	0.57
SCSSR10559	57	0.50	2.00	0.50	0.00	0.38
SCSSR25538	57	0.92	3.00	0.15	0.00	0.14
Mean		0.65	3.40	0.46	0.01	0.40

5 alleles/ locus for Bmac0040 to 2 alleles/locus for SCSSR10559. The average PIC of these SSR markers was 0.40, with a maximum of 0.62 for HVM40 and a minimum of 0.14 for Scssr25538 and Bmac0093. The mean heterozygosity was 0.006 due to the near absence of any heterozygous banding pattern at any of the marker loci. The average gene diversity at these loci was 0.46.

Genetic Diversity and Cluster Analysis

The barley germplasm accessions clustered into two major groups (group 1 and 2) in the dendrogram (Fig.1). Himadri (BHS 352) released by IARI, Shimla and RD2849 from SKRAU, Durgapura, Rajasthan were most diverse with a genetic distance of 0.91 and the varieties DWRB127 and DWRUB52, both from DWR, Karnal, Haryana were least diverse with a genetic distance of 0.05.

Estimation of genetic diversity among the accessions originating from a common centre, indicated that varieties originating from CCSHAU, Hisar were most diverse. A total of 14 varieties which have been developed at CCSHAU, Hisar formed a part of the germplasm set under study. The genetic divergence between these accessions was 0.43. Many varieties of barley in India have been developed through introductions. These were primarily introduced to strengthen the research on malt breeding. In the present study, we had 9 varieties which were either released directly by using exotic germplasm or had one of the parents as the exotic line. The genetic divergence among these lines was 0.43. The need for using exotic genotypes as the genetic resources for developing new barley cultivars has also been previously established (Elakhdar *et al*., 2018).

Principal Component Analysis

The PCA plotting of genotypes was in concurrence with the UPGMA clustering in the dendrogram. The first eleven principal components with eigen value >1, explained more than 68% of variance. In the two dimensional plot, with the first two principal components, the close clustering of the varieties from a common developing centre could be seen

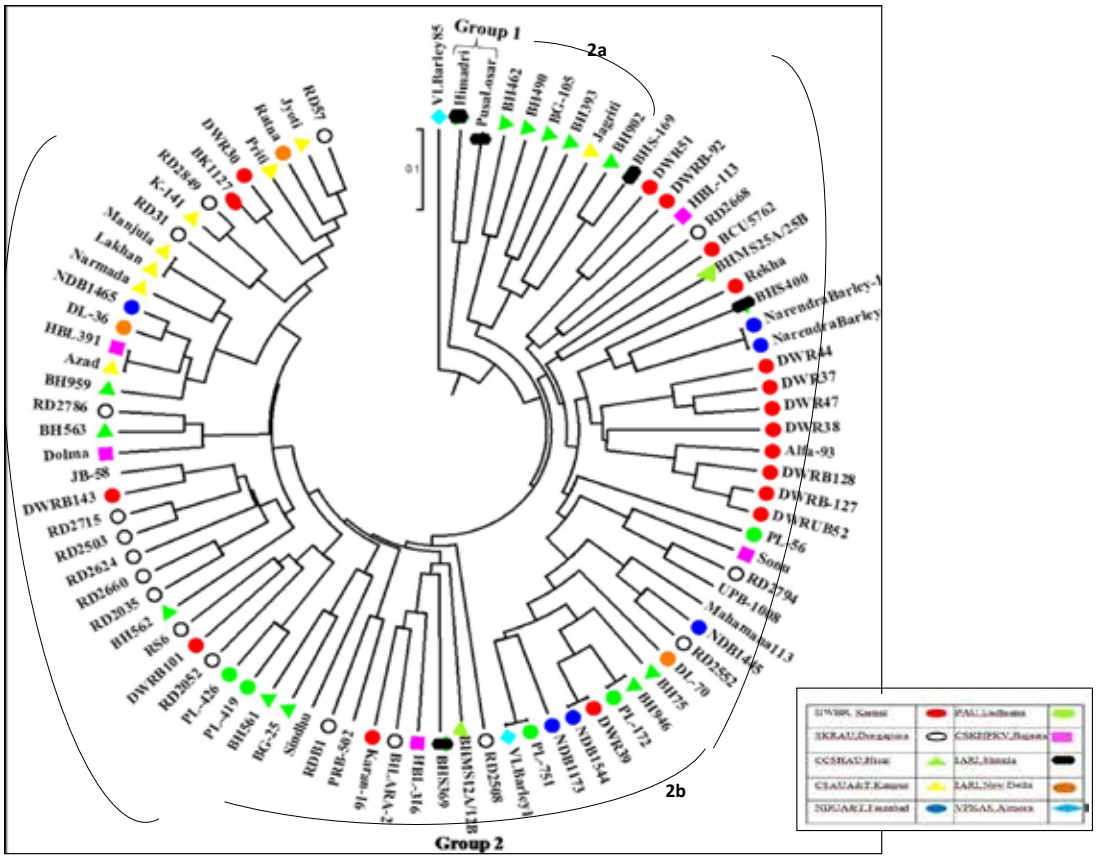

Fig. 1. UPGMA tree depicting the genetic diversity among 88 barley germplasm accessions

(Fig. 2). The varieties from CCSHAU, Hisar were more dispersed as compared to varieties from IIWBR, Karnal and SKRAU, Durgapura, thus substantiating the dissimilarity based clustering in the dendrogram.

Discussion

Genetic variability in the primary gene pool is the most important principle and key determinant for further breeding and cultivar development. It has been known that the lack of sufficient variability in the available genotypes hinders the suitable selection of parents in the breeding programs, thus preventing the development of highly productive cultivars in self pollinated crops like barley. Therefore, estimation of genetic diversity in the primary gene pool of a species is of paramount importance to develop improved cultivars and varieties. The barley germplasm lines in the present study included released varieties and registered germplasm. These varieties have been developed through independent breeding programmes under the All India Wheat and Barley Improvement Project (AICW&BIP). Varieties developed at 14 different centres were included in the study. The maximum number of varieties (18) were from IIWBR, Karnal, Haryana followed closely by SKRAU, Durgapura, Rajasthan (17) and CCSHAU, Hisar, Haryana (14). Amongst these three centres, the varieties developed at CCSHAU, Hisar were the most diverse with an average genetic divergence of 0.43, followed by varieties from IIWBR, Karnal with an average genetic divergence of 0.40 and SKRAU, Durgapura varieties with an average genetic divergence of 0.39. There were 8 varieties from CSAUA&T, Kanpur with an average G.D of 0.24.

There were two major groups in the dendrogram. The first group consisted of only two varieties i.e., Himadri (BHS352) and Pusa Losar (BHS380). Both these varieties have been developed at IARI, Shimla and are recommended for cultivation in the hilly regions of Northern and North-eastern India. It has been reported that while Himadri is tolerant to yellow rust, Pusa Losar is resistant to all the three rusts. Hence, Pusa Losar can serve as a better donor parent in varietal improvement. The second group consisted of the remaining barley varieties and was divided into two smaller subgroups. The first subgroup,2a,

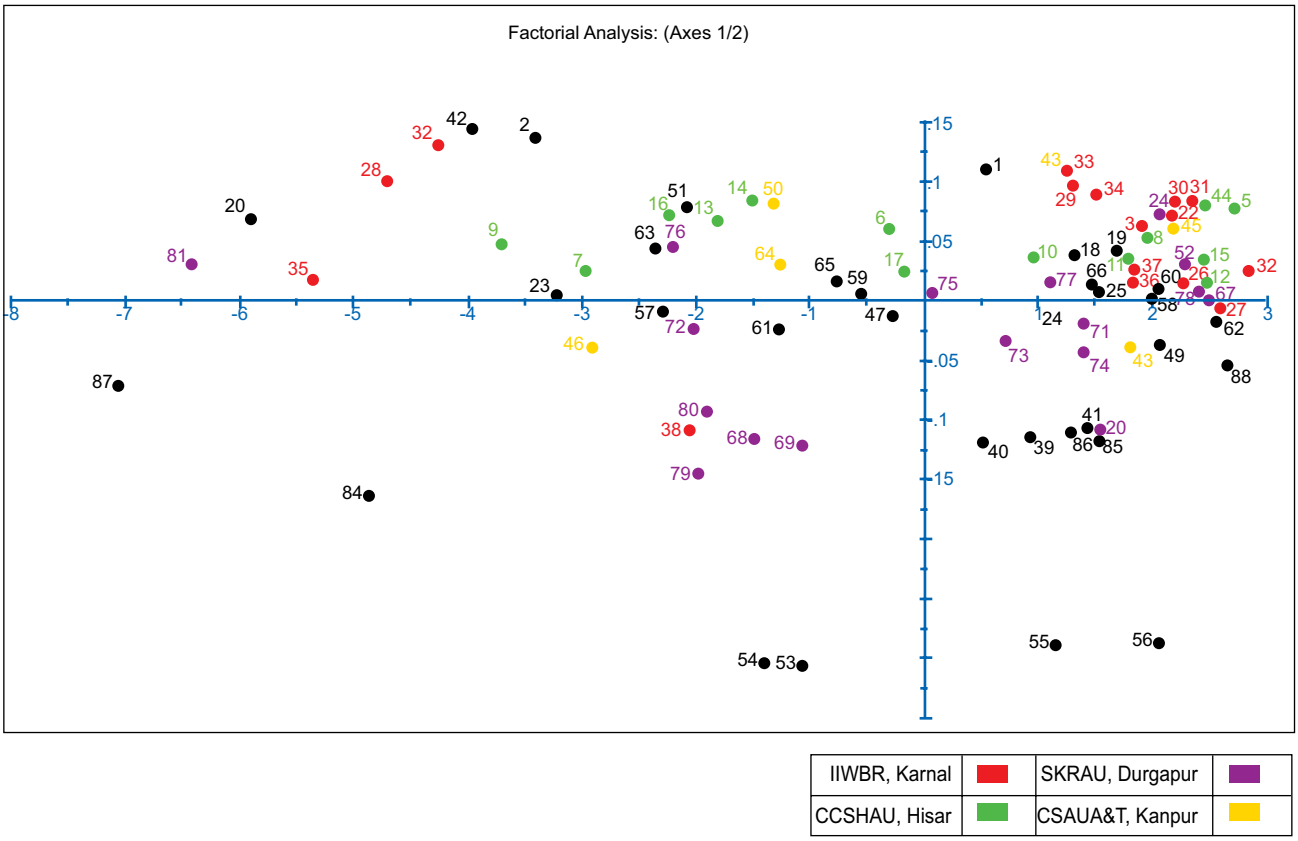

Fig. 2. Two-dimensional scaling of 88 barley genotypes by principal component analysis (PCA)

consisted mostly of varieties from CCSHAU, Hisar i.e., BH462, BH490, BG105, BH393, BH902 except varieties Jagrati and BHS 169. All these varieties are suitable for cultivation in the northern hills of India. The second subgroup,2b, was divided into still smaller clusters. Within the clusters, it was observed that varieties originating from a common centre clustered together with the exception of varieties from VPKAS, Almora. All the varieties from CSAUA&T, Kanpur clustered together in the dendrogram except Jagrati. This observation was in concordance with the breeding history and pedigree data of these lines. It has also been reported earlier that genotypes with the same origin implicate narrow genetic diversity because they may have exchanged genetic material through breeding program (Hamza *et al*., 2004). The overall average genetic diversity in the cultivated gene pool of Indian barley was 0.46. The varieties Himadri (BHS 352) released by IARI, Shimla and RD2849 developed at SKRAU, Durgapura, Rajasthan were most diverse with a genetic distance of 0.91 and the varieties DWRB127 and DWRUB52, both from DWR, Karnal, Haryana were least diverse with a genetic distance of 0.05. While, Himadri is a six row, huskless variety recommended for cultivation in hilly regions of Northern India, RD 2849 is a two rowed malt barley variety recommended for cultivation in the NWPZ. This ecological differentiation with respect to recommended areas for cultivation, might be one of the contributing factors for the underlying genetic diversity. VL Barley 85 and VL Barley 1 both varieties developed at VPKAS, Almora were not placed in the same cluster. The genetic divergence between the two varieties was as high as 0.50. While VL Barley 1 and VL Barley 85 were both released for cultivation for the hilly regions of Uttarakhand, VL Barley 85 exhibits multiple disease resistance and is higher yielding than VL Barley 1 (Kant *et al*., 2008). Due to difference in their pedigrees they were placed in different clusters in the dendrogram.

According to its end use, barley varieties and cultivars have been categorized as feed barley (FB), malt barely (MB) and dual purpose barley which is used both as a feed and fodder crop. In the coming years, barley is increasingly becoming a cash crop. About 20-25 % of barley produced in the country is being used by the malting industries and the malt is utilized for brewing, malt whiskies, energy drinks, baby foods, medicinal syrups and vinegar (Verma *et al*., 2008). Two row barley varieties are usually preferred for malting while six rowed barley varieties are used as food barley. Most of the two row type malt varieties i.e., DWRB92, RD2668, Rekha, Alfa 93, DWRUB52, UPB1008 were differentiated from the six row type malt varieties RD2503 and RS6 with the exception of DWRB101 which is a two row malt variety, which clustered together with RS6, a six row malt variety. Even though DWRB101 is a two row malt variety, it has higher grain yield as compared to DWRB92 and DWRUB52. This differentiation between two and six row malt barley varieties can be of potential use in selection of desirable parents for two x six row malt barley hybridization, in order to improve two rowed malt barley varieties.

A total of 86 different alleles were amplified by the 25 SSR markers used in the present study, with an average allele number of 3.40/locus. The average number of alleles amplified per SSR locus is comparable to many other previously reported studies. This was comparable to the average allele number/locus of 3.25, as reported by Jaiswal *et al*., 2010 while studying the genetic diversity among 69 Indian barley cultivars using 16 SSR markers and Gougordchi *et al*., 2014, who reported 3.26 alleles/ locus while studying genetic diversity of 52 barley lines from Iran, Egypt and China using 68 SSR markers. This indicates that the SSR markers used in the study were highly informative and were based on highly diverse genomic regions of the barley genome. The SSR marker Bmac0040 amplified a total of 5 alleles. The average PIC value of the SSR markers was 0.40, and ranged from a minimum of 0.14 for SSRs Scssr25538 and Bmac0093 and a maximum of 0.62 for the SSR HVM40. The average PIC value of the SSR markers is lesser but still comparable to previously published studies. Hamza *et al*., 2004 used 17 SSR markers to study genetic diversity among 26 Tunisian winter barley cultivars. The average PIC of the SSR markers used was 0.45. Ferreira *et al.,* 2016 used 34 SSR markers to study genetic diversity among 64 barley accessions from Brazil, which also included six wild *H. vulgare* ssp. *spontaneum* genotypes and reported an average PIC of 0.57, which is much higher than the average PIC of 0.40 in our study. This could be due to the inclusion of wild type accessions which are expected to be more diverse in their genetic

background as compared to the cultivated varieties which have been subjected to artificial selection over a period of time resulting in genetic uniformity across the cultivated gene pool. The wild species play an important role in the enrichment of genetic diversity (Nandha and Singh, 2014).

While great progress has been made in India's barley improvement program, a lot more efforts need to be undertaken to diversify and broaden the primary gene pool or genetic base of barley varieties. The development of high yielding varieties which are resistant to biotic and abiotic stresses, varieties serving dual purpose (feed and forage) and varieties with superior malting qualities, are some of the objectives of the barley network programme in the country. In order to achieve these objectives, the genetic diversity of the barley germplasm needs to be estimated, to enable a judicious utilization of the germplasm in the crop improvement program (Elakhdar *et al.,* 2018). Such studies can be applied to carry out association analysis, development of mapping populations and selection of parental lines which can be used in breeding programs.

Supplementary Information

Supplementary Table 1. Details of the barley accessions used in the study

Supplementary Table 2. Details of SSR markers used in the study

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