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Microsatellite-based diversity analysis and the development of core-set germplasm in Pakistani barley lines

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ABSTRACT

Illustrating the population structure and genetic diversity in selected germplasm resources (after three-year multi locations trials) plays a key role that directly utilizes the selection of lines in a population for accumulative trait breeding in crops. In order to further understand, the structure of population and genetic variability, this study explored 100 selected lines, conducted for three consecutive years (2016-2019) in swat, University of Malakand, Khyber Pakhtunkhwa Pakistan and Provinces of China (Chongqing and Beijing) with 33 mapped SSR markers. Through an integrated population structure analysis, the study examined a core set of one hundred germplasm of Pakistani origin, along with three approved commercial barley cultivars. The analysis revealed a robust stratification within the population, resulting in the classification of the germplasm into four major subpopulations (PI, PII, PIII, and PIV), as well as an admixture subpopulation consisting of 52, 9, 15, and 27 germplasm, respectively. A total of 133 alleles were identified with a mean value of 0.80 Polymorphic information content. The number of alleles detected by the system varied from two alleles amplified to six with an average of 4.03 per SSR marker pair. The gene diversity ranged from 0.56 to 0.98 with an average of 0.82 in selected germplasm resources. Based on the SSR data, the 100 selected germplasm with three cultivars were classified into four main phylogenetic lineages (LI, LII, LIII and LIV) which corresponded to the phylogenic grouping in genotypes. We assembled a core set of 20 barley genotypes (~1/5 of original population size) to sustain sufficient mapping of SSR marker with Phenotype, in which we proposed four SSR markers, Bmac0040, Bmac0134, Bmag0125 and Bmag0211 for malt gene and marker (Bmac0399) for tolerance to salinity gene, which will be applicable for marker-assisted breeding in barley gene resources.

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Introduction

Barley is economically significant and the fourth most important cereal in the world. The value of this crop is increased due to its consumption as a functional food and having an array of beneficial impacts on human health (1, 2). Application of barley such as malt in brewing, animal feeding, human consumption and distilling industry makes it an important crop plant of the world (2, 3). Cultivator chooses the best variety of barley according to the speci-

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fic climate and the genetic diversity of the material also plays a significant role in the selection of parent species for a specific location (4-6). In plant research and breeding programs, molecular markers are essential for the identification of genetic variation (7-9). In barley, the assessment of genetic diversity through molecular markers had been reported previously by many researchers (10-18). The SSR markers provide significant information about a species, as they have a high polymorphism rate. They have the advantage of being a PCR marker i.e. they are fast and moderately inexpensive to analyze. They are abundant in quality and highly polymorphic, locus particular. Simple sequence repeats (SSRs) also called (di-or tri-nucleotide repeat sequences) which are extremely consistent, co-dominant markers (19-21). For identification of variety and breeding program microsatellite are used widely due to their high level of polymorphism and easy use, in a wide range of crops as well as barley and wheat SSRs have now become vital genetic markers (22-24). In different countries SSR markers are used to demonstrate barley (25-32). Barley cultivation is not widely practiced in Pakistan and is primarily restricted to specific villages where it is mainly used for animal feeding purposes. Barley has a limited presence in the human diet in Pakistan and its production is constrained by the absence of a malting industry. This is evident from the fact that in 2020, Pakistan's barley production was a modest 70,000 metric thousand, highlighting its restricted scale.

Considering the situation, our experiment was centered around this ancient crop with the goal of developing improved cultivars that can thrive in the diverse environmental conditions of the country. In our recent study, we utilized SSR markers to assess the molecular assortment of Pakistani barley genotypes. This pioneering attempt signifies the first-ever characterization of carefully selected genotypes using SSR markers, providing invaluable insights into the genetic relationships among different barley genotypes.

Materials and Methods

The present molecular marker experiment was operated in the laboratory of the barley gene resources Institute of Crop Sciences Graduate School of Chinese Academy of Agriculture Sciences Beijing China.

Plant Materials

Genotypes

Barley (*Hordium vulgari* L.), consisting of 600 genotypes collected from the Gene Bank of Plant Genetic Resources Institute /National Agriculture Research Center Islamabad Pakistan. The Catalogue information data assort these genotypes included various parts of the world including Pakistan, Japan, China, Iran, Cyprus, Syria and the USA, that represent a wide range of ecological zones from dry highlands to wetted plains.

Field Experiment

A total of 600 genotypes were collected from the Gene Bank of National Agriculture Research Center Islamabad Pakistan planted in October 2016 at the Botanical Garden, University of Malakand. Block field design was used for the experiment. The length of the row was 2m and the space between the rows are 20cm. Every single genotype was planted in contiguous rows where the distance between varieties (between the seed) remained 12cm. To obtain healthy and vigorous plants, Irrigation was applied during and after sowing. Other agronomic practices were kept uniform.

Plant Materials for DNA Extraction

In this study, our main objective was to identify superior genotypes from a pool of 600 genotypes. After a thorough screening, we selected 100 genotypes that exhibited desirable traits, including high yield, plant height, seed weight, and number of seeds per spike. We also took into consideration qualitative characteristics such as seed colour

To evaluate the genomes of these selected genotypes, we conducted three-year consecutive field trials using three commercial cultivars: Barke, Cap, and IG. Through this process, we aimed to gain insights into their genetic makeup using 33 mapped SSR markers. The chosen genotypes represent a diverse range of origins. They cover the eco-geography of all provinces in Pakistan, including genotypes from unknown localities within the country. Additionally, some genotypes were sourced from exotic origins such as Syria, Japan, and the USA.

For DNA extraction and analysis, we collected leaf samples from 69 genotypes that were grown under field conditions in Chongqing, China. Furthermore, we collected 31 leaf samples from the greenhouse of the Barley Gene Resources Institute at the Crop Sciences Graduate School of the Chinese Academy of Agricultural Sciences.

DNA Extraction

The extraction and quantification of genomic DNAs were conducted following the previous description (33, 34). In brief, the seedling leaves from every single plant of each accession from the glasshouse planting in 2018 were harvested for DNA miniprep. DNA samples were quantified and qualified with NanoDrop 2000c ™ Spectrophotometer (Thermo Fisher Scientific, US). The stock solution was diluted to 40 ng/µl (working solution) and ready for PCR reactions. The Genomic DNA was further run on 1.5% agarose gel. The gel was prepared by taking 1.5g agarose powder and dissolved in 150 ml of 1x TBE. The 33 mapped SSR markers (Table 1) from the linkage map previously detected by scientists (4, 35, 36) were used in the study with their high PIC values (<0.70).

PCR Cocktail and PCR Reaction

For SSR markers (36), polymerase chain reaction (PCR) was performed in a 10 µl PCR mixture which contained 40 ng genomic DNA (2µl), 100 µM of each dNTP (0.4 µl), 10x mM Taq buffer (2 µl), (0.4 µl) of both forward and reverse primer, Taq polymerase (0.15 µl) and water (4.65µl). The PCR reaction was performed in a T100- Thermal cycler from Bio-Rad PCR System. DNA of all selected accessions was run independently, with each primer for PCR amplification. Denaturation was done at 95 °C for 5 minutes, followed by 95°C for 15 seconds, annealing at 65°C for 20 seconds, extension at 72°C for 1 minute and a final extension of 5 minutes at 72 °C with 4°C infinite hold (37). Polyacrylamide gel electrophoresis separation technique was used for SSR markers and visualize samples through Monarch gel visualizer.

Data Analysis

The allelic data matrix of "1" or "0" was used to calculate the population genetic analysis using POPGENE version 1.3242, including the number of observed alleles (Na), and the number of effective alleles (Ne). Nei" s and Shannon's index (I) were computed for each genotype based on the obtained allele frequencies. The allelic data matrix of "A/A" or "-/-" was used to perform phylogenetic analysis. Phylogenetic tree was calculated under Roger"s Distance 1973 under power marker and constructed with MEGA-X version using UPGMA statistical method with substitution model of Maximum Composite Likelihood45. The robustness of the node of the phylogenetic tree was assessed from 1000 bootstrap replicates. To find out the core of SSR mapped markers. The Structure software version 2.2 software was used to construct the structure algorithm, using the Bayesian model to explore the subpopulation in Pakistani genotypes. We simulated the number of subpopulations (k) from one to ten, with ten replications. For each replication, the posterior probability, LnP(D), of the model was estimated based on 10,000 MCMC (Markov Chain Monte Carlo) iterations before using a burn-in length of 10,000 times with the admixture and related frequency model. The optimal k value was determined by the LnP(D) value with an ad-hoc statistic delta k based on the rate of change in the LnP(D) between successive k. The polymorphic information content (PIC) value for each marker was determined using the following equation:

$$PIC = 1 - \sum_{i=1}^{n} p_i^2 - 2\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} p_i^2 p_j^2$$

Where p_i is the frequency of the i^{th} allele, and n is the number of alleles (38).

Results

Evaluation and assessment of genetic diversity through molecular markers is considered one of the prestigious steps in barley breeding programs. A total of 133 polymorphic SSR alleles were identified through this electrophoresis with a mean value of polymorphic information

Table 1. List of 33 SSR primer pairs for estimation of Population structure and genetic diversity.

S.no	*SSR*	*F- primer*	*R- primer*	*Repeats*	*Size*	*Chr*	*DI*
1	Bmac0030	CCCAATCGGAGTTACAGATG	GCCTCTCTGAGAATGGATC	(AC)22	155	4H	0.77
2	Bmac0040	AGCCCGATCAGATTTACG	TTCTCCCTTTGGTCCTTG	(AC)20	236	6H	0.89
3	Bmac0067	AACGTACGAGCTCTTTTTCTA	ATGCCAACTGCTTGTTTAG	(AC)18	171	3H	0.82
4	Bmac0093	CGTTTGGGACGTATCAAT	GGGAGTCTTGAGCCTACTG	(AC)24	151	2H	0.81
5	Bmac0127	AACTATGTCCAGTCGTTTCC	CTTGTCGTATCATCTTATTCAGA	(AC)26	118	6H/3H	0.83
6	Bmac0129	ACTGCATGATAGTATATGGAACA	AATCACTAAGGGCACTAGATG	(AC)28	145	3H/2H	0.72
7	Bmag0013	AAGGGGAATCAAAATGGGAG	TCGAATAGGTCTCCGAAGAAA	(CT)21	155	3H	0.72
8	Bmac0181	ATAGATCACCAAGTGAACCAC	GGTTATCACTGAGGCAAATAC	(AC)20	177	4H	0.75
9	Bmac0134	CCAACTGAGTCGATCTCG	CTTCGTTGCTTCTCTACCTT	(AC)28	148	2H	0.76
10	Bmac0399	CGATGCTTTACTATGAGAGGT	GGGTCTGAAGCCTGAAC	(AC)21	145	1H	0.72
11	Bmag0120	ATTTCATCCCAAAGGAGAC	GTCACATAGACAGTTGTCTTCC	(AG)15	230	7H	0.86
12	Bmag0125	AATTAGCGAGAACAAAATCAC	AGATAACGATGCACCACC	(AG)19	134	2H	0.76
13	Bmag0173	CATTTTTGTTGGTGACGG	ATAATGGCGGGAGAGACA	(CT)29	150	6H	0.72
14	Bmac0096	GCTATGGCGTACTATGTATGGTTG	TCACGATGAGGTATGATCAAAGA	(AT)6(AC)16	173	5H	0.74
15	Bmag0740	ATTGTCATGGAGGTGAGTG	AAGAACACCATATGACTCGAC	(CT)28	150	4H	0.78
16	EBmac0755	AGCCTTGTGTATCAGGACA	CTGCTGGTGTTCTCTAAAAGT	(AC)16	143	7H	0.72
17	Bmag0603	ATACCATGATACATCACATCG	GGGGGTATGTACGACTAACTA	(AG)24	120	3H	0.78
18	Bmag0013	AAGGGGAATCAAAATGGGAG	TCGAATAGGTCTCCGAAGAAA	(CT)21	155	3H	0.72
19	Bmag0606	CTATTTGTAATGTATGTATGTCCC	TCATTGGTCCAGATAATACAA	(CT)22	140	3H	0.87
20	Bmac0156	AACCGAATGTATTCCTCTGTA	GCCAAACAACTATCGTGTAC	(AC)22(AT)5	139	7H	0.89
21	Bmag0125	AATTAGCGAGAACAAAATCAC	AGATAACGATGCACCACC	(AG)19	134	2H	0.76
22	Bmag0211	ATTCATCGATCTTGTATTAGTCC	ACATCATGTCGATCAAAGC	(CT)16	174	1H	0.83
23	Bmag0223	TTAGTCACCCTCAACGGT	CCCCTAACTGCTGTGATG	(AG)16	127	5H	0.82
24	Bmag0720	AAACCGTTTGTATAGCACG	ATAAGTGAATGCTTCTGAGGA	(GT)6,(AG)49	171	2H	0.84
25	EBmac0501	ACTTAAGTGCCATGCAAAG	AGGGACAAAAATGGCTAAG	(AC)13	151	1H	0.93
26	EBmac0764	AGAATCAAGATCGACCAAAC	AAAAACATGAACCGATGAA	(GT)16	124	7H	0.84
27	EBmac0603	ACCGAAACTAAATGAACTACTTCG	TGCAAACTGTGCTATTAAGGG	(CA)10	149	7H	0.74
28	Bmag0900	AGCCTGTGATACATCAAGATC	AGGATGAGGGTATGTAGACG	(GA)25	122	7H	0.79
29	Bmag0812	ATAGTTCTTTCAGGACCAATG	GTCATATGGATCTCCAAAGAG	(CT)26	157	5H	0.79
30	Bmag0808	TCATAGACTACGACGAAGATG	TCTTTGGATGTGTGTTTACTG	(GA)16	177	4H	0.84
31	Bmag0872	ATGTACCATTACGCATCCA	GAAATGTAGAGATGGCACTTG	(AG)27	125	1H	0.81
32	Bmag0829	TAAAAGCCAAACTCGATAATC	TTGTTGATGAGAAGTTTTGTG	(GA)28	189	2H	0.81
33	Bmag0711	GGAGAGTCACATATCAAGGAC	CCACTCCTTCTCATACCTTTA	(GA)21	192	2H	0.84

content (PIC) of 0.80. Genetic diversity parameters among these selected genotypes revealed that these genotypes are diverse based on SSR marker data.

Development of Core Set Collection in Pakistani Barley Varieties

In the present study, we selected 100 genotypes out of 600 barley genotypes based on the breeder's interest traits after three years of morphometric multi locations trials. The 100 core collection is assembled through phenotypic traits variations in their plant height which is 17.12%, followed by 100 seed weight at 52.12%, spike type 2, 4 and 6-row type, no of seeds per spike at 51.13%, grain color (black-brown), kernel covering, stem pigmentation and growth habit to capture the genetic diversity and facilitate the selection of suitable candidate alleles related to these traits.

Population Structure and Genetic Diversity in the Pakistani Barley Genotypes

The population structure of Pakistani hundred selected genotypes and three approved cultivars (Barke, IG and CAP) was evaluated using 33 mapped SSR markers. The structure of the genotype population was investigated by structure software using the Bayesian model the maximal posterior probability (LnP (D) with the value of 65.2 and (Var. LnP (D) 0.0023 of the Bayesian model was in which we estimated the subpopulation in genotypes based on (k) from 1 to 9 and observed the subpopulation in genotypes. We found that the most apparent change in the population appeared when k increased from 1 to 4 (Table I). In addition, a sharp peak in delta k appeared at k = 4. Accordingly, the barley genotypes and three approved cultivars were classified into four major subpopulations represented by POPI, POPII, POPIII and POPIV in "Figure 1". The POPI subpopulation contained 52 genotypes which mostly belong to unknown localities in Pakistan while some of them belong to Baluchistan province with three approved cultivars i.e. Barke, IG and CAP while this POPI population of genotype also represents one genotype from the USA and Syria. The POPII contains 9 genotypes which majority of which belong to the Northern areas of Pakistan. The POPIII subpopulation contained 15 genotypes while POPIV Subpopulation consist of 27 genotypes which are clustered with each other based on provinces in Pakistan. There clustering catalog representation and subpopulation

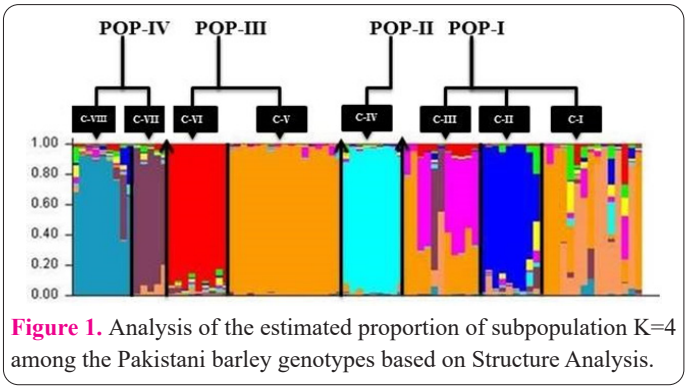

Figure 1. Analysis of the estimated proportion of subpopulation K=4 among the Pakistani barley genotypes based on Structure Analysis.

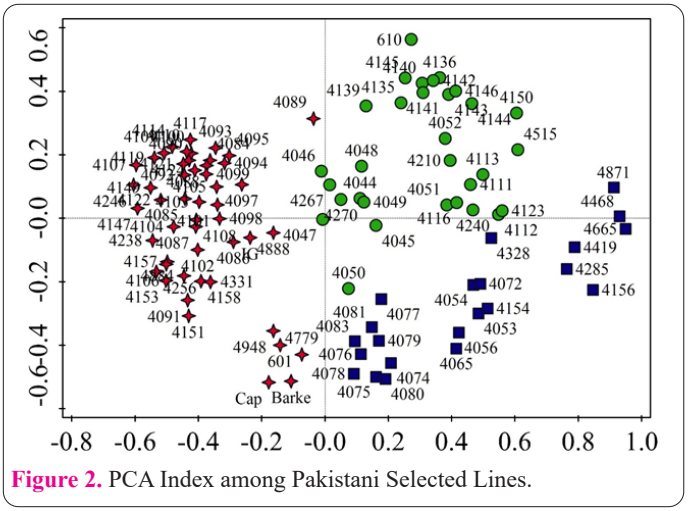

Figure 2. PCA Index among Pakistani Selected Lines.

details are presented in Table 3. We further employed principal component analysis (PCA) for further verification of the subpopulation of genotypes as determined by structure analysis. All genotypes were clustered into four apparent plots (Plot-I, Plot-II, Plot-III and Plot-IV) corresponding to the subpopulation determined by structural analysis consisting of the similar structure of genotypes as we illustrated in Table 2, in which the top two plots accounted for over 18% and 6.5% of the molecular variance while Plot-III and Plot- IV account for 5.4% and 4.4% respectively "Figure 2".

Total Alleles Amplification of 33 SSR Markers

A total of 133 SSR alleles were amplified from the DNA of 100 selected genotypes and 3 approved cultivars using 33 mapped SSR markers The number of alleles detected by the system varied from two alleles amplified

Table 2. Population derivatives among Pakistani barley genotypes.

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	3	-4330.06667	0.208167	—	—	—
2	3	-3636.93333	8.288747	693.133333	77.133333	9.305789
3	3	-3020.93333	0.23094	616	368.833333	1597.09518
4	3	-2773.76667	0.404145	247.166667	90.666667	224.341819
5	3	-2617.26667	16.214294	156.5	26.266667	1.61997
6	3	-2487.03333	24.73951	130.233333	35.966667	1.453815
7	3	-2392.76667	9.148406	94.266667	53.966667	5.899024
8	3	-2352.46667	6.585843	40.3	752.433333	114.25011
9	3	-3064.6	1265.18288	-712.133333	480.333333	0.379655
10	3	-3296.4	980.734154	-231.8	455.933333	0.46489
11	3	-3984.13333	1421.36232	-687.733333	1709.56667	1.202766
12	3	-2962.3	797.811087	1021.83333	—	—

by markers (Bmac0399, Bmag0740) to six alleles which are amplified by markers such as (Bmac0040, Bmac0127, Bmag0120) with an average of 4.03 per SSR marker pair. Nine SSR pairs, namely Bmac0040, Bmac0127, Bmac0181, Bmag0120, Bmag0125, Bmag0603, Bmac0156, Bmag0900 and Bmag0872, were highly polymorphic, each producing 6 to 5 alleles. Other fifteen SSR, namely, Bmac0030, Bmac0067, Bmac0093, Bmac0129, Bmag0173, Bmac0096, Bmag0013, Bmag0606, Bmag0125, Bmag0720, EBmac0501, EBmac0764, EBmac0603, Bmag0812 and Bmag0829 was moderately polymorphic, each producing four to three alleles. The remaining nine SSR, Bmag0013, Bmac0134, Bmac0399, Bmag0740, EBmac0755, Bmag0211, Bmag0223, Bmag0808 and Bmag0711, were less polymorphic by producing less than three to two alleles (Table 4). The PIC values of these markers ranged from 0.95 (Bmac0067) to 0.52 (Bmag0872) with a mean value of 0.80 (Table 4). Furthermore, indicators of locus diversity (polymorphism information content - PIC) were calculated in which large variation was observed in three markers (Bmag0013, Bmag0720 and Bmag0872) with PIC values below 0.60, therefore the majority of the markers were highly informative, according to the criteria proposed by Botstein et al. (1980) in which they determine the range of PIC <0.50. While we assorted our markers PIC range <0.60, therefore

Table 3. Illustration of genotypes in subpopulation through Structure and PCA Analysis.

Subpopulation	***Genotypes***
Subpopulation 1 Genotypes	*4210, 4153,4157, 4158 4151, 4149, (Baluchistan) 4779, 4948, 4888,4884, Syria, 4267 USA, 601 Dir Lower, Barke, Cap, 1G, 4089, 4095, 4121, 4117,4110, 4107, 4119, 4122, 4147, 4134, 4109, 4102,4106, 4091, 4098, 4101,4105 4108, 4100, 4085, 4092, 4088, 4093,4096, 4090, 4084, 4897, 4087, 4228, 4104,4103,4124,4099, (unknown Locality), 4331, 4056 (Gilgit), 4246, 4256 (Punjab).*
Subpopulation 2 Genotypes	*4419, 4665, 4515 (Japan), 610 (Dir Lower), 4468 (KPK), 4156 (Baluchistan), 4285 (Punjab), 4371 (unknown), 4328 (Gilgit).*
subpopulation 3 Genotypes	*4081, 4076, 4077, 4075, 4074, 4078, 4079, 4080, 4083, 4154 (Unknown), 4065 (Sindh), 4154 (Baluchistan), 4054, 4053 (Skardu), 4056 (Gilgit).*
subpopulation 4 Genotypes	*4146, 4141, 4136, 4143,4135, 4140, 4145, 4139, 4142, 4116, 4112, 4123, 4144, 4113, 4111, 4250, 4340 (Unknown), 4046, 4047, 4045, 4150 (Baluchistan), 4051, 4052, 4050, 4049, 4048 (Skardu), 4044 (Punjab)*

Table 4. Statistical analysis of 33 SSR markers used in the present study.

NO	***Markers***	***Bands***	***Exp_ Hom****	***Exp_Het****	***Avg_Het***	***ne****	***I****	***Allele freq***	***Gene Div***	***PIC***
1	Bmac0030	4	0.5704	0.4296	0.4273	1.75	0.61	0.54	0.94	0.92
2	Bmac0040	6	0.5906	0.4094	0.4072	1.70	0.59	0.51	0.86	0.84
3	Bmac0067	4	0.5736	0.4264	0.4241	1.76	0.61	0.52	0.98	0.95
4	Bmac0093	4	0.5644	0.4356	0.4332	1.76	0.62	0.53	0.74	0.71
5	Bmac0127	6	0.5943	0.4057	0.4035	1.69	0.59	0.59	0.92	0.89
6	Bmac0129	4	0.5475	0.4525	0.4501	1.81	0.64	0.52	0.96	0.94
7	Bmag0013	3	0.5205	0.4795	0.4769	1.91	0.66	0.57	0.56	0.54
8	Bmac0181	5	0.6025	0.3975	0.3954	1.67	0.58	0.64	0.9	0.88
9	Bmac0134	3	0.5185	0.4815	0.479	1.92	0.67	0.62	0.84	0.81
10	Bmac0399	2	0.4994	0.5006	0.498	1.99	0.69	0.54	0.84	0.82
11	Bmag0120	6	0.5166	0.4834	0.4807	1.92	0.67	0.54	0.94	0.92
12	Bmag0125	5	0.5134	0.4866	0.4866	1.93	0.67	0.52	0.74	0.71
13	Bmag0173	4	0.5136	0.4864	0.4838	1.93	0.67	0.58	0.66	0.64
14	Bmac0096	4	0.5149	0.4851	0.4825	1.93	0.67	0.56	0.76	0.74
15	Bmag0740	2	0.5121	0.4879	0.4853	1.94	0.67	0.52	0.7	0.67
16	EBmac0755	3	0.5372	0.4628	0.4603	1.85	0.65	0.54	0.9	0.87
17	Bmag0603	5	0.5498	0.4502	0.4478	1.81	0.63	0.57	0.76	0.74
18	Bmag0013	4	0.5811	0.4189	0.4167	1.71	0.6	0.54	0.94	0.92
19	Bmag0606	4	0.5219	0.4781	0.4755	1.9	0.66	0.57	0.88	0.85
20	Bmac0156	5	0.5195	0.4805	0.4779	1.7	0.6	0.59	0.9	0.87
21	Bmag0125	4	0.5448	0.4552	0.4528	1.86	0.64	0.52	0.88	0.86
22	Bmag0211	3	0.5187	0.4813	0.4787	1.91	0.67	0.53	0.82	0.79
23	Bmag0223	3	0.5026	0.4974	0.4947	1.97	0.68	0.52	0.96	0.94
24	Bmag0720	4	0.5022	0.4978	0.4951	1.98	0.68	0.51	0.61	0.57
25	EBmac0501	4	0.5012	0.4988	0.4961	1.98	0.68	0.54	0.94	0.92
26	EBmac0764	4	0.524	0.476	0.4735	1.9	0.66	0.59	0.84	0.81
27	EBmac0603	4	0.5077	0.4923	0.4897	1.95	0.68	0.61	0.72	0.69
28	Bmag0900	5	0.5546	0.4454	0.443	1.8	0.63	0.61	0.91	0.88
29	Bmag0812	4	0.5592	0.4408	0.4384	1.78	0.63	0.64	0.88	0.86
30	Bmag0808	3	0.6512	0.3488	0.3469	1.58	0.52	0.52	0.96	0.94
31	Bmag0872	5	0.5221	0.4779	0.4753	1.9	0.66	0.65	0.54	0.52
32	Bmag0829	4	0.5092	0.4908	0.4881	1.95	0.68	0.51	0.76	0.74
33	Bmag0711	3	0.4982	0.5018	0.4992	1.99	0.69	0.54	0.81	0.78
Avg		*4.03*	*0.53*	*0.46*	*0.45*	*1.85*	*0.64*	*0.55*	*0.82*	*0.803*

these three markers are located on chromosomes three, two and one and these three chromosomes showed lower PIC values as compared with other markers located on other chromosomes.

Genetic Variability

Using the SSR marker-based diversity detection system, an average of 133 polymorphic SSR bands were observed in 100 selected lines and three approved cultivars. The number of observed alleles detected by 33 mapped SSR markers in all selected lines is (Na = 2), while the highest number of effective alleles (Ne = 1.99) was detected by Bmac0399 and Bmag0711 markers followed by Bmag0720 and EBmac0501 with (Ne = 1.98) while the lowest number of effective alleles was detected in these lines by markers Bmag0808 with (Ne = 1.58) with average number of effective alleles noted in these Lines as 0.64 (table 4). Shannon's index information based on marker allele detection in these lines ranged from 0. 52 to 0.69 with an average of 0.64. Analysis of Shannon's index (I) showed that the highest Shannon's index was scored in these lines by Bmac0399 and Bmag0711 marker with (I) value of 0.69, while The lowest Shannon's diversity index value of 0.52 was observed in these lines. The gene diversity of these lines ranged from 0.56 to 0.98 with an average value of 0.82. The highest genetic diversity was recorded by marker Bmac0067 which is 0.98 followed by Bmac0129 and Bmag0223 which is 0.96 while the lowest Gene Diversity was noted in marker Bmag0013 which is 0.56.

Phylogenetic Cluster Analysis

A phylogenetic tree was constructed in selected lines and approved three cultivars based on Rogers distance 1983 bootstrap using the UPGMA method as shown in "Figure 3". Based on the phylogenetic analysis, the 100 selected genotypes and three approved cultivars were clustered into four major lineages (L-I, L-II, L-III and L-IV). These linages consist similar pattern of subpopulation as derived by structure software based on a subpopulation of genotypes as occurred in structure "Figure 1". L-I consists of 52 genotypes which occupy three clusters in the L-I, Cluster I, II and III "Figure 3" Cluster-I consist of only one genotype (4210) which belong to Baluchistan province, Cluster-II also consist of one genotype (4779) which belong to SYRIA country while cluster-III consist of 50 genotypes in which majority of them belong to unknown localities while on the base of core collection from provinces of Pakistan while some belong to Punjab province and one genotype was from USA and SYRIA respectively. Linage-II consists of a total of 9 genotypes which were further divided into one cluster, CL-IV which consists of nine genotypes (4328, 4156, 4285, 4371, 4419, 4468, 4665, 4515 and 610). Genotypes 4328 and 4285 belong to the Gilgat region, 4156 Baluchistan while the details of other genotypes are mentioned in Table 3. L-III consists of one cluster CL-V and six genotypes (4065, 4072, 4154, 4054, 4053 and 4056) which separately belong to Sindh, unknown Locality, Baluchistan, Gilgat and Skardu. L-IV consists of 36 genotypes and three clusters CL-VI, CL-VII and CL-VIII. CL-VI and CL-VII consist of 9 genotypes (4081, 4076, 4077, 4075, 4074, 4078, 4079, 4080 4083,). While CL-VIII contains 18 genotypes (4146, 4141, 4136, 4143, 4135, 4140, 4145, 4139, 4142, 4116, 4112, 4123, 4144, 4113, 4111, 4150, 4250 and 4340), detail description base on their Locality are available in Table 3.

PCR Product Variability

The PCR products were compared with 5000 bp DNA ladder "Figure 4". Twenty-three out of 33 markers were found to have allele size within the reported range. It was observed that four mar-kers (Bmac0040, Bmac0134, Bmag0125 and Bmag0211) showed different PCR products from the reported range (180-100bp Size) while our genotypes (4051, 610, 4048,4948,4779,4267, 4046, 4056, 4065, 4089, 4090) have size in range from 250 to 120 bp. The percentage of mar-kers within the reported size range from 65.4%, while 35.6% of markers were found to have different ranges in size than that of the reported range. According to Ramsey et al. (2011) these markers displaying sizes outside the expected range indicate that the respective barley genotypes are purebred and possess malt genes within their genome (39).

Characteristics of Pakistani Genotypes as Genetic Resource

Considering the need for genotyping in Pakistani barley, we assembled a core set of 20 barley genotypes (~1/5 of the original population size) to sustain sufficient mapping of SSR markers with Phenotype. We found that the

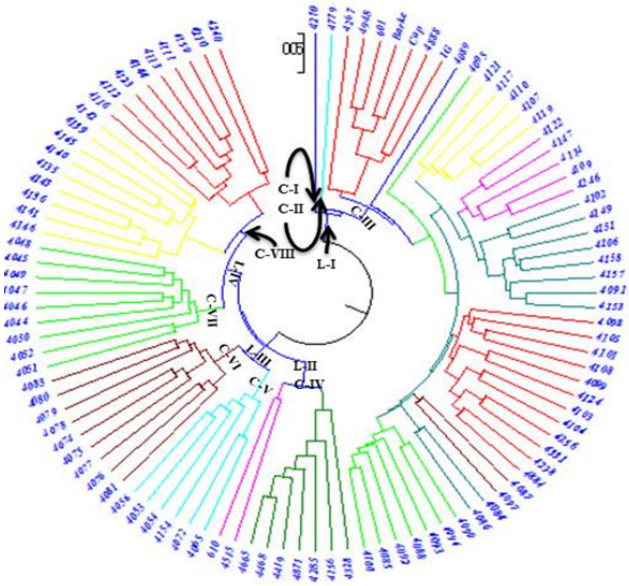

Figure 3. Phylogenetic trees of Pakistani Barley selected Genotypes based on SSR data. A distance tree was constructed in MEGA 6 using the UPGMA method. The robustness of the node of the phylogenetic tree was assessed from 1000 bootstraps as shown.

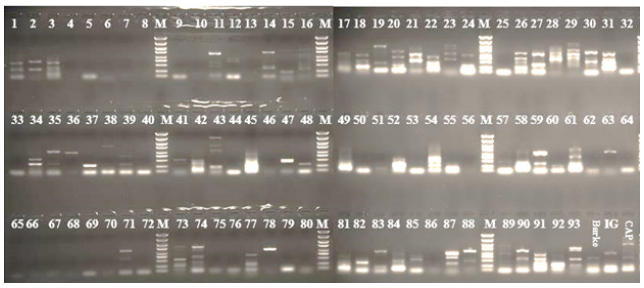

Figure 4. PCR amplification profile generated from genomic DNA of selected genotypes with SSR marker, Bmac0040 and Bmag0211. M-marker = 5000bp and digits represent genotypes.

core genotypes were evenly distributed in 100 genotypes that were consistently revealed by the structure analysis, UPGMA phylogenic and PCA Plotting. Specifically, also the selection criteria of genotypes by the number of alleles specifically detected the loci for malt phenotype in 20 genotypes by markers (Bmac0040, Bmac0134, Bmag0125 and Bmag0211) these markers are also previously screened for identification of malt genotypes in barley through SSR marker by Quain et al., 2018 (35) which further illustrates our result as we screened the 33 mapped SSR markers with phenotypic traits. While marker (Bmac0399) were predicted to amplify two alleles responsible for salinity tolerance in genotypes (4044, 4046,4056,4065,4089,4090) while, other mapped SSR markers (Bmag0900, Bmac0156, Bmag0603, Bmag0120, Bmac0181) showed their relationship with high yielding Genotypes (4044,4046,4056, 4065,4089,4090,4051,4048,4267,4331,4888,610,4100,4 089,4076,4267,4948,4267,4779) which we derived from these genotypes in three years consecutive field trials (2016-2017,2017-2018, 2018-2019) in multi-locations of Pakistan (Herbarium & Botanical garden of university of Ma-lakand, Glass House of Botanical Garden UOM, Agriculture Research Centre of Swat, Glass House of Graduate School of Chinese Academy of Agriculture Sciences Beijing China, Chongqing province of China).

Discussion

Microsatellites, also known as SSRs (Simple Sequence Repeats), are extensively utilized for breeding and variety identification purposes. These markers are highly polymorphic and offer ease of use. Notably, SSRs have gained significant importance as genetic markers in various crops, including barley and wheat. Data obtained from many research show that SSR markers are noble implements for the determination of genetic changeability in barley crops. SSRs are informative, co-dominant and abundant and are very simple in detection (12). Until the present study conducted in 2019, no previous research had assessed the molecular diversity of Pakistani landraces using SSR markers or any other marker. Therefore, this study represents the first-ever attempt to characterize 100 selected lines and three approved cultivars through the application of SSR markers. The primary aim was to estimate the genetic relationships among these lines, providing crucial insights into the genetic diversity of Pakistani landraces. Understanding of the genetic diversity and population structure provides an opportunity to evaluate the application potential of a new germplasm resource in the breeding aims and fundamental studies.

In this study, we employed 33 mapped SSR markers to evaluate the structure of the population and genetic diversity in 100 selected Pakistani genotypes with approved cultivars of barley for the core relationship of these genotypes with each other and approved cultivars. On average, the SSR mapped markers amplified 4 alleles per SSR, with a range from 2 to 6 alleles per SSR, which was comparable to the value (4 on average) of the 33 SSR which is similar to the study of Khodayari et al., 2012 (30) on Iranian barley landraces. Our study shares some similarities with the previously published report by Jaiswal et al. in 2010 (40) as they also focused on barley accessions but specifically from India. However, our findings differ from those of other researchers, as our study yields different results (4, 25, 28, 31, 41). A total of 133 alleles were detected by 33 mapped SSR markers. The Polymorphic Information Content (PIC) value of SSRs loci ranged from 0.52 to 0.95, with a mean value of 0.80 which show deviation from the study of Hasan et al., 2018 (42) through Polymorphic information content (PIC) value which is ranged from 0.29 to 0.89, with a mean value of 0.65. The conducted experiment on 140 accessions of Jordanian barley landraces and five barley cultivars using 28 mapped SSR markers while we use 33 mapped SSR markers on 100 selected Pakistani Lines and three cultivars. The number of alleles detected by the system varied from two alleles amplified by markers (Bmac0399, Bmag0740) to six which are amplified by markers such as (Bmac0040, Bmac0127, Bmag0120) with an average of 4.03 per SSR marker pair. Nine SSR primer pairs, namely Bmac0040, Bmac0127, Bmac0181, Bmag0120, Bmag0125, Bmag0603, Bmac0156, Bmag0900 and Bmag0872, were highly polymorphic, each producing 6 to 5 alleles. Other fifteen SSR primer pairs, namely, Bmac0030, Bmac0067, Bmac0093, Bmac0129, Bmag0173, Bmac0096, Bmag0013, Bmag0606, Bmag0125, Bmag0720, EBmac0501, EBmac0764, EBmac0603, Bmag0812 and Bmag0829 was moderately polymorphic, each producing four to three alleles. The remaining nine SSR markers namely, Bmag0013, Bmac0134, Bmac0399, Bmag0740, EBmac0755, Bmag0211, Bmag0223, Bmag0808 and Bmag0711, were less polymorphic by producing less than three to two alleles. A similar study was also conducted by Ferreira et al., 2016 (4) on 34 markers loci in Brazilian 64 two and six-row wild and domesticated barley genotypes in which the detected 280 alleles with an average of eight alleles per locus while in our study we detected 133 alleles with an average of four alleles per locus. In this regard of alleles detection assay the Pakistani barley, genotypes show deviation from Brazilian barley genotypes, while the PIC value of our Lines is in a range from 0.95 to 0.52 with a mean value of 0.80 which are different from the study conducted by Ferreira et al., 2016 (4) in which their PIC value is in the range of 0.07 to 0.86 with an average of 0.57, our lines show their clustering assistance with approved cultivars of Barley Like Barker and IG while the clearly show deviation base on SSR mapping from Brazilian barley which indicates us that population of Pakistani barley genotypes are different from Brazilian barley.

The Bayesian-model-based structure analysis is widely used a method for the inference of hidden population structure in human and plant species. In this study, four major subpopulations (PI, PII, PIII and PIV) were identified in Pakistani barley lines with three approved cultivars, the structural pattern of which was verified by the UPGMA phylogenic and PCA analyses. Interestingly, we found that the four subpopulations of genotypes show similar relationships and clustering also in UPGMA tree and PCA analyses. The structure of the genotype population was investigated by structure software using the Bayesian model the maximal posterior probability (LnP (D) with the value of 65.2 and (Var. LnP (D) 0.0023 of the Bayesian model was in which we estimated the subpopulation in genotypes based on (k) from 1 to 9 and observe the subpopulation in genotypes. We found that the most apparent change in the population appeared when k increased from 1 to 4. In addition, a sharp peak in delta k appeared at k = 4. Accordingly, the barley genotypes and

three approved cultivars were classified into four major subpopulations represented by POPI, POPII, POPIII and POPIV. The POPI subpopulation contained 52 Genotypes which belong mostly to unknown localities in Pakistan while some of them belong to Baluchistan province with three approved cultivars Barke, IG and CAP this POPI population of genotype also represents one genotype from USA and Syria. The POPII contains 9 genotypes in which majority of them belong to the Northern areas of Pakistan. The POPIII subpopulation contained 15 genotypes while POPIV Subpopulation consist of 27 genotypes which are clustered with each other based on provinces in Pakistan. There clustering catalog representing subpopulation. There are two main ways of analyzing the resulting distance (or similarity) matrix, namely, principal coordinate analysis (PCA) and dendrogram (or clustering, tree diagram). PCA is used to produce a two or three-dimensional scatter plot of the samples such that the distances among the samples in the plot reflect the genetic distances among them with a minimum of distortion. Another approach is to produce a dendrogram (or tree diagram), that is, grouping o samples together in clusters that are more genetically similar to each other than to samples in other clusters (43). We further employed principal component analysis (PCA) additionally to verify the subpopulation of genotypes as determined by structure analysis. All genotypes were clustered into four apparent plots (Plot-I, Plot-II, Plot-III and Plot-IV) corresponding to the subpopulation determined by structural analysis consist similar structures of genotypes in which the top two plots accounted for over 18% and 6.56% of the molecular variance while Plot-III and Plot- IV account for 5.38% and 4.38% respectively. A similar investigation was also done by Xu et al., 2017 (44) on 562 hulls accessions of barley while the population was divided into two major subpopulations while in our study the population is divided into four subpopulations which shows deviation from our result. A phylogenetic tree was constructed in selected lines and approved three cultivars based on Rogers distance 1983 bootstrap using the UPGMA method, based on phylogenetic analysis, the 100 selected genotypes and three approved cultivars was clearly clustered into four major Linage (L-I, L-II, L-III and L-IV). These linages consist similar pattern of subpopulation as derived by structure software base on subpopulation of genotypes.

Conclusions

The main aim of this study was to evaluate the population structure and genetic diversity of 100 selected barley lines using PCR-based mapped SSR markers. Our findings validate the effectiveness of SSR markers in exploring genetic diversity among barley lines and their suitability for marker-trait selection in barley. Moreover, we propose utilizing SNP-derived caps markers to investigate trait-based variations in barley. Our research findings reveal substantial heterogeneity among Pakistani barley genotypes, as demonstrated by their classification into four distinct subpopulations based on SSR marker data. This diversity holds significant implications for barley breeders, as it indicates the presence of unique genes within these genotypes, presenting potential opportunities for genetic improvement. These genes, such as malting genes, yield genes, and seed color alleles, can be harnessed by breeders to improve breeding programs and develop enhanced barley varieties.

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