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Comparative assessment of EST-SSR, EST-SNP and AFLP markers for evaluation of genetic diversity and conservation of genetic resources using wild, cultivated and elite barleys

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Abstract

A set of 16 expressed sequence tag (EST)-derived simple sequence repeat (SSR) and 15 EST-derived single nucleotide polymorphism (SNP) markers together with 4 amplified fragment length polymorphism (AFLP) primer combinations were analyzed on 43 wild (*Hordeum vulgare* ssp. s*pontaneum* – HS), 35 cultivated (*H. vulgare* ssp. *vulgare* – HV) and 12 elite (*H. vulgare* ssp. *vulgare* – from EU) barley lines. SSR markers were found most polymorphic with an average PIC value of 0.593 and eight alleles per marker, while AFLP markers showed the highest effective multiplex ratio (26.4) and marker index (5.042). The effective marker index (EMI) was recorded highest (0.468) for AFLP markers and lowest (0.341) for the SNP markers while the SSR markers had an intermediate EMI (0.442). Cluster analysis on combined set of SSR, SNP and AFLP genotyping data classified wild, cultivated and elite barley lines in three distinct groups. The present study suggests the SNP markers as the best class of markers for characterizing and conserving the genebank materials and the AFLP and SSR markers more suitable for diversity analysis and fingerprinting.

Keywords: AFLP; SSR; SNP; Molecular markers; Genetic diversity; Barley

1. Introduction

Genetic diversity in crop plants is continuously being lost in farmers' fields and in nature. In this context, genebanks assume paramount importance as reservoirs of biodiversity and source of alleles that can be relatively easily retrieved for genetic enhancement of crop plants. Increasingly, efforts are being made to collect threatened landraces, cultivars that were obsolete, genetic stocks and wild relatives of cultivated species [1]. All these materials are important for crop improvement because breeding gains rely largely on access to the genetic variation in the respective crop genepools.

Barley (*Hordeum vulgare* L.) is an important staple crop ranking fourth in the world food production. It is grown mainly for animal feed and as a raw material for beer production in a wide range of temperate and semi arid environments with major areas of production in the European Union, Russia, and North America. As ICARDA (Syria) has a mandate of barley improvement in semi arid regions, its genebank has a rich resource of cultivars as well as wild barleys.

For detection of genetic variation in barleys, an array of molecular markers is available [2]. Infact, among different classes of molecular markers available, the simple sequence repeat (SSR) or microsatellite (derived from genomic DNA) and AFLP markers have been used separately as well as in combination in many studies [3–9]. In recent years, with increasing efforts to develop EST (expressed sequence tag) resources for crop plants including barley, a new class of locus-specific DNA markers called 'functional molecular markers' have been developed [10]. These include EST derived SSR

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(EST-SSR) and SNP (EST-SNP) markers which are easy to develop utilizing the EST resources and mirrors the functional genomic component [11–13]. These markers, at present, are gaining momentum for estimating the *functional genetic diversity* in genebank collections, and natural as well as breeding populations of barley [14–16].

The present study attempts to make a critical assessment of the potential of EST-SSR, EST-SNP and AFLP markers for genotyping natural populations, breeding as well as genebank materials. In addition to classifying the examined barleys in different groups, the characteristic properties for all three types of marker assays including polymorphic information content (PIC), multiplex ratio (n), marker index (MI) and two new estimates termed qualitative nature of data (QND) and effective marker index (EMI), are discussed.

2. Material and methods

2.1. Plant materials

A total of 43 wild (*H. vulgare* ssp. *spontaneum* – HS), 35 cultivated (*H. vulgare* ssp. *vulgare* – HV) and 12 European elite (*H. vulgare* ssp. *vulgare* – representing 6 spring and 6 winter types) genotypes, representing 15 countries and European Union (EU) were selected for analysis of genetic diversity. Details on these genotypes including country/province of origin and geographic information system (GIS) data, wherever possible have been provided in Appendix 1. Total DNA was extracted from 20 mg of fresh leaves of each genotype, using the modified CTAB method [17].

2.2. SSR analysis

Amplification of microsatellite loci using fluorescent-dye labeled primer pairs was carried out as given in Thiel et al. [18]. Amplification products were separated on an ABI 377 fragment analyzer and evaluated using the software package Genotyper 3.7 (Applied Biosystems, Foster City, CA, USA).

2.3. SNP analysis

For genotyping the single nucleotide polymorphism in the barley genotypes, 10 single nucleotide polymorphisms (SNPs) were assayed as cleaved amplified polymorphic sequences (CAPS) markers, as described in Varshney et al. [19]. Five additional SNP markers were assayed by Pyrosequencing on PSQ HS96 (Biotage AB, Uppsala, Sweden). Amplification of SNP containing region of genome, optimization of pyrosequencing assay and pyrosequencing were performed as instructed by manufacturer (Biotage AB, Uppsala, Sweden).

2.4. AFLP analysis

The AFLP reactions were carried using out using 250 ng of DNA per genotype as described in Vos et al. [20] with some minor modifications as given in Sasanuma et al. [21]. The following four primer combinations were used: *Pst*I-AAG + *Mse*I-CTG (P-AAG + M-CTG); *Pst*I-AGC + *Mse*I -CAC (P-AGC + M-CAC); *Pst*I-AGG + *Mse*I-CAA (P-AGG + M-CAA); and *Pst*I-AGG + *Mse*I-CTT (P-AGG + M-CTT). The amplified fragments were fractionated on 6% polyacrylamide gel. The fragments in the gel were detected using DNA Silver Staining System (Promega GmbH, Mannheim, Germany).

2.5. Data analysis

Polymorphic information content, effective multiplex ratio (E), marker index, qualitative nature of data and effective marker index were calculated as following:

The PIC values measure the informativeness of a given DNA marker, and these were calculated as follows [22]:

$$\mathrm{PIC} = 1 - \sum_{i=1}^{k} P_i^2$$

where k is the total number of alleles detected for a given marker locus and P_i is the frequency of the ith allele in the set of genotypes investigated.

The average number of DNA fragments amplified/detected per genotype using a marker system is considered as multiplex ratio (n).

In case of AFLP, however, many loci (fragments or bands) are non-polymorphic in the germplasm of interest. The number of loci polymorphic in the germplasm set of interest, analyzed per experiment, called effective multiplex ratio (E) is estimated as:

$$E = n\beta$$

where β is the fraction of polymorphic markers and is estimated after considering the polymorphic loci (n_p) and non-polymorphic loci (n_np) as $\beta = n_\mathrm{p}/(n_\mathrm{p} + n_\mathrm{np})$.

The utility of a given marker system is a balance between the level of polymorphism detected and the extent to which an assay can identify multiple polymorphisms. A product of information content, as measured by PIC, and effective multiplex ratio, called as marker index may provide a convenient estimate of marker utility [23]:

$$\mathrm{MI} = \mathrm{PIC} \times E$$

or

$$\mathrm{MI} = n \times \beta \times \mathrm{PIC}$$

To provide an index for the molecular markers that include additional information on the practical applicability of a marker system to the genebank curators and managers, we propose a term called the qualitative nature of data. The QND depends on many factors such as reproducibility and amenability of peaks/bands for easy documentation (e.g. precise allele sizing and storing in databases) and is defined as:

$$\mathrm{QND} = \mathrm{DC} \times \mathrm{QM} \times \mathrm{PR}$$

where DC is the documentation capability, QM is the quality of marker and PR is the Percent Reproducibility of the fragment(s)/band(s)/peak(s) of the given marker system across the laboratories. DC and PR represent the constant value for

a given marker type, however, QM is a feature of the primer pair for a marker type and it shows a variable value.

The constant values for DC and PR for different markers have been set as follows:

	SNP	SSR	AFLP
DC	1.00	0.75	0.25
PR	1.00	1.00	0.50

DC value for SNP markers has been set 1 as their results can be recorded in the most convenient way e.g. one of the four letters 'A'/'C'/'G'/'T' or '±' form. Results of SSR markers are also easier to record but allele sizes need to be measured. For AFLP results, it is very difficult to record or document as it is not possible to size all the AFLP loci obtained by a primer pair until unless the AFLP experiments are conducted on ABI machines. While conducting AFLP experiments on ABI machines, the DC value for AFLP can be used as 0.50 or between 0.50 and 0.75.

PR value for both SNP and SSR markers is given 1 as SNP and SSR results are most likely to be reproduced on different analysing systems and across the laboratories.

The QM value, however, will vary with the primer pair even for a given marker type. Therefore, the user needs to define the QM value as per the experiments according to following scale:

1.00 good quality marker – single and strong band/peak
0.75 faint band or lower peak
0.50 marker/band with stuttering
0.25 difficult to score (needs special efforts to visualize)

For calculating the QM for a marker system, the average value for the QM should be considered for all the primer pairs/combinations for the given marker system.

Finally, the effective marker index, a possible measure to evaluate the overall utility of a marker system considering all the parameters mentioned above, which can be calculated as follow:

$$\mathrm{EMI} = \mathrm{MI} \times \mathrm{QND}$$

2.6. Phenetic or cluster analysis

The profiles produced by EST-SSR and EST-SNP (including CAPS and Pyrosequencing assays) and AFLP markers were scored manually: each allele was scored as present (1) or absent (0) for each of the SSR, SNP and AFLP loci. The 0/1 matrices for individual marker types were used for the calculation of genetic dissimilarity according to Nei and SAHN clustering (NTSYSpc 2.1) which was evaluated for analyzing the correlation among three marker systems. Finally, individual data obtained with SSR, SNP and AFLP markers were combined to prepare the three-dimensional cluster phenogram by using GelCompar II programme (Applied Biomaths).

3. Results

3.1. Marker analyses

3.1.1. SSR analysis

The 16 EST-SSR markers detected 4 (GBM1047-2H, GBM1075-6H) to 15 (GBM1464-7H) alleles with an average of 8 alleles per marker in all 90 genotypes examined (Table 1). The PIC values for these markers in the examined genotypes ranged from 0.285 (GBM1043-3H) to 0.766 (GBM1064-5H) with an average of 0.593 ± 0.131.

Table 1
Characteristics of SSR loci, including their repeat motif, the number of alleles per locus (A_o) and PIC value in wild (W), cultivated (C) and elite (E) barley germplasm

Linkage group	Marker ID	SSR motif	A_o	PIC			
				W	C	E	Average across germplasm
1H	GBM1002	(CCT)7	9	0.771	0.320	0.420	0.545
	GBM1029	(AG)10	6	0.552	0.531	0.500	0.542
2H	GBM1035	(CT)8	6	0.678	0.580	0.569	0.629
	GBM1047	(AGC)5	4	0.663	0.420	0.486	0.542
3H	GBM1043	(AAC)5	5	0.389	0.180	0.000	0.285
	GBM1059	(GGT)5	14	0.781	0.739	0.753	0.760
	GBM1110	(AAG)6	10	0.751	0.494	0.153	0.622
4H	GBM1003	(CTT)8	7	0.740	0.680	0.740	0.710
	GBM1015	(ACAT)13	13	0.810	0.680	0.716	0.745
	GBM1323	(GCC)8	6	0.610	0.667	0.497	0.638
5H	GBM1064	(AGGG)5	5	0.781	0.750	0.153	0.766
	GBM1483	(GCG)7	7	0.615	0.579	0.615	0.597
6H	GBM1075	(GT)6	4	0.496	0.460	0.625	0.478
7H	GBM1060	(GGT)6	6	0.573	0.420	0.000	0.497
	GBM1464	(CAG)8n(CAG)5	15	0.871	0.000	0.568	0.435
	GBM1516	(CT)9	11	0.740	0.667	0.736	0.703
		Average	8	0.676	0.510	0.471	0.593

3.1.2. SNP analysis

A total of 18 SNP datapoints were assayed in the complete set of germplasm by using 15 SNP markers as 2 SNP markers namely GBS0461 (4H) and GBS0576 (5H) detected two and three datapoints, respectively in the pyrosequencing assay. SNP markers are predominantly bi-allelic markers and therefore as expected, two alleles per SNP locus were observed for all the markers examined in the complete germplasm set. The PIC values ranged from 0.095 (GBS0708-6H) to 0.490 (GBS0526-3H) with an average of 0.341 ± 0.130 (Table 2).

3.1.3. AFLP analysis

Over the 90 genotypes analyzed, the four AFLP primer pair combinations yielded a total of 175 scorable loci, 108 of which (61.7%) were polymorphic (Table 3). The number of loci (or fragments) scored per primer combination ranged from 19 (P-AAG + M-CTG, P-AGC + M-CAC) to 44 (P-AGG + M-CTT), with an average of 27 loci per primer combination. The rate of polymorphism found for different primer combinations ranged from 47.5 (P-AGC + M-CAC) to 86.27% (P-AGG + M-CTT). The PIC values for individual AFLP loci were recorded up to 0.466 (data not shown), however the overall PIC values for individual primer combinations were in the range of 0.122–0.234 with an average of 0.191 ± 0.049 per primer combination (Table 3).

3.2. Comparison of marker systems

Four main aspects of the performance of the examined marker systems were considered (Table 4): overall efficiency of polymorphism detection in the germplasm (i.e. PIC), the number of independent loci assayed simultaneously (called multiplex ratio), the overall utility of a marker system for detecting genetic variation or marker index and the qualitative nature of data obtained by a given marker system (Appendix 1).

3.2.1. Polymorphic information content (PIC)

SSR markers showed highest level of polymorphism in the examined germplasm as the PIC value for the SSR markers was calculated in the range of 0.471(elite) to 0.676 (wild) with an average of 0.593 across the germplasm lines assayed (Table 1). AFLP markers detected lowest level of polymorphism as the PIC value for the AFLP primer pairs was in the range of 0.098 (elite) to 0.285 (wild) with an average of 0.191 across the germplasm analyzed (Table 3). SNP markers showed an intermediate level of polymorphism as the PIC values calculated for these markers were in the range of 0.300 (elite) to 0.351 (wild) with an average of 0.341 across the germplasm collection examined (Table 2).

3.2.2. Multiplex (n) and effective multiplex ratio (E)

Since SSR markers are usually locus specific, the 16 SSR markers analyzed are considered equal to 16 loci (as one locus per primer pair) in the present study. In case of SNP markers also, the 15 SNP markers yielded 15 genetic loci, though 18 SNP datapoints as two SNP markers namely GBS0461 (4H) and GBS0576 (5H), that were assayed on Pyrosequencing, detected two and three datapoints, respectively. Thus the SNP markers also provided the multiplex or effective multiplex ratio as 1.0 per marker (Table 4). As a large number of fragments are

Table 2
Characteristics of SNP markers, including the SNPs assayed and PIC value in wild (W), cultivated (C) and elite (E) barley germplasm

Linkage group	Marker ID	SNP targeted	Assay[a]	PIC				
				W	C	E	Average per SNP across germplasm	Average per marker across germplasm
1H	GBS0554	C/G	CAPS (*Hha*I)	0.215	0.440	0.320	0.343	0.343
2H	GBS0705	A/G	PyroSeq	0.260	0.301	0.486	0.298	0.298
3H	GBS0431	A/G	CAPS (*Rsa*I)	0.370	0.431	0.486	0.430	0.430
	GBS0526	A/T	CAPS (*Psi*I)	0.453	0.500	0.500	0.490	0.490
	GBS0667	A/G	CAPS (*Cac*8I)	0.127	0.202	0.000	0.142	0.142
4H	GBS0288	A/G	CAPS (*Hha*I)	0.444	0.291	0.444	0.399	0.399
	GBS0461	pos1_C/T	PyroSeq	0.229	0.357	0.000	0.265	0.347
		pos2_C/G	PyroSeq	0.397	0.444	0.473	0.428	
5H	GBS0527	C/T	CAPS (*Eco*RV)	0.487	0.488	0.153	0.428	0.428
	GBS0576	pos1_G/T	PyroSeq	0.500	0.500	0.355	0.499	0.488
		pos2_C/T	PyroSeq	0.495	0.499	0.408	0.486	
		pos3_C/G	PyroSeq	0.493	0.496	0.355	0.479	
	GBS0577	A/G	CAPS (*Dde*I)	0.484	0.313	0.375	0.433	0.433
6H	GBS0136	A/G	CAPS (*Taq*I)	0.043	0.229	0.375	0.172	0.172
	GBS0157	C/G	CAPS (*Sal*I)	0.454	0.056	0.000	0.299	0.299
	GBS0369	C/G	CAPS (*Hae*III)	0.498	0.301	0.391	0.459	0.459
	GBS0708	A/G	PyroSeq	0.041	0.142	0.000	0.095	0.095
7H	GBS0591	G/T	PyroSeq	0.330	0.219	0.278	0.297	0.297
			Average	0.351	0.345	0.300	0.358	0.341

[a] CAPS, cleaved amplified polymorphic sequences; PyroSeq, Pyrosequencing.

Table 3
Details on AFLP analysis, including the total number and polymorphic bands obtained, the level of polymorphism and their PIC value in wild (W), cultivated (C), elite (E) germplasm

Primer pairs	Total no. of bands	No. of selected polymorphic fragments	Polymorphism (%)	PIC			
				W	C	E	Average across germplasm
P-AAG + M-CTG	34	19	55.88	0.204	0.151	0.068	0.189
P-AGC + M-CAC	40	19	47.50	0.264	0.207	0.077	0.234
P-AGG + M-CAA	50	26	52.00	0.172	0.150	0.119	0.122
P-AGG + M-CTT	51	44	86.27	0.501	0.276	0.129	0.216
Total	175	108	–	–	–	–	–
Average	43.75	27.00	60.41	0.285	0.196	0.098	0.191

detected in one gel lane, by using one AFLP primer combination, the AFLP markers have a higher multiplex ratio. Effective multiplex ratio of the AFLP, however, depends on the fraction of polymorphic markers (β) as many of the fragments obtained by one primer combination are monomorphic across the examined genotypes. In the present study, the n and E for the AFLP markers were calculated as 44 and 26.4, respectively (Table 4).

3.2.3. Marker index (MI)

For determining the overall utility of a given marker system, the marker index, MI was calculated for all the three marker systems examined. The AFLP markers showed the highest MI as 5.042, which are almost 15 and 8 folds higher than that of the SNPs (0.341) and the SSRs (0.593), respectively (Table 4). This analysis highlights the distinctive nature of the AFLP assay among different marker systems.

3.2.4. Qualitative nature of data (QND)

Another important issue for different marker systems, we propose here, is the qualitative nature of data, produced by a marker. Based on the assumptions and weightages mentioned under materials and methods, the QND for SNP markers was estimated 1 as the highest and 0.093 as the lowest for the AFLP markers. SSR markers showed an intermediate value for the QND (Table 4).

3.2.5. Effective marker index (EMI)

The effective marker index considers all the possible attributes such as information content, fraction of polymorphic fragments, multiplex ratio as well as the qualitative issues for a given marker system. According to our calculations, the EMI was highest (0.468) for the AFLP markers and lowest (0.341) for the SNP markers while it was found to be intermediate (0.445) for the SSR markers (Table 4).

3.3. Genetic diversity in wild, cultivated and elite barleys

SSR markers revealed the highest and AFLP markers the lowest level of polymorphism in the analyzed germplasm (Fig. 1). The highest level of genetic diversity was observed in wild barleys while elite barleys showed the lowest diversity (Fig. 1, Tables 1–3). The cultivated group of genotypes had an intermediate level of genetic diversity. However, a few SSR and SNP markers revealed inverted levels of diversity i.e. higher level of diversity was observed in elite as compared to cultivated and/or cultivated to wild species.

A total of 36 alleles for SNP markers, 128 alleles for SSR markers and 175 fragments/bands for AFLP markers were obtained. Pair-wise comparisons of genetic distance matrices for any two-marker datasets were found to be correlated but at relatively low level of significance. The r (coefficient of correlation) value for the genetic distance matrices for SSR and SNP, SSR and AFLP, and AFLP and SNP data were 0.523 ($P < 0.005$), 0.537 ($P < 0.005$) and 0.553 ($P < 0.005$), respectively. Therefore, to obtain more accurate genetic distance estimates, combined analysis was carried out using all the SSR, SNP and AFLP bands together.

As shown in Fig. 2, all the examined genotypes could be classified in three major clusters. Majority of the genotypes (95%) of wild, cultivated and elite genotypes group separately. Furthermore, under the elite cluster, two sub-clusters containing the spring and winter type of genotypes could also be

Table 4
Comparison of AFLP, SNP and SSR marker systems

Marker system	No. of primer pairs analyzed	No. of genetic loci amplified	Average PIC	Fraction of polymorphic markers (β)	Multiplex ratio (n)	Effective multiplex ratio ($E = n \times \beta$)	Marker index (MI = $E \times$ PIC)	Qualitative nature of data (QND)	Effective marker index (EMI = MI × QND)
AFLP	4[a]	108 (175[b])	0.191	0.60	44	26.4	5.042	0.093	0.468
SNP	15	15 (18[c])	0.341	1	1	1	0.341	1	0.341
SSR	16	16	0.593	1	1	1	0.593	0.75	0.445

[a] Primer combinations.
[b] Total number of genetic loci is 175, however only 108 polymorphic loci were taken in consideration.
[c] SNP datapoints (18) generated by 15 SNP markers used.

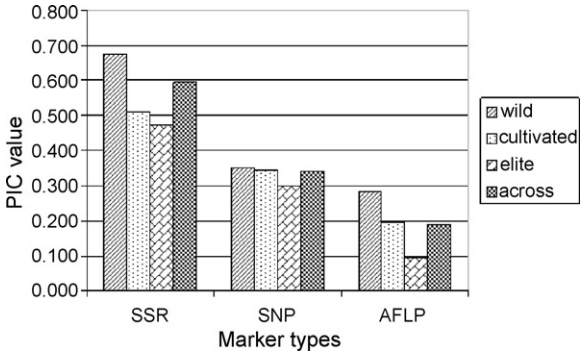

Fig. 1. Comparative level of polymorphism (PIC value) for EST-SSR, EST-SNP and AFLP markers in wild, cultivated, elite and across the germplasm examined.

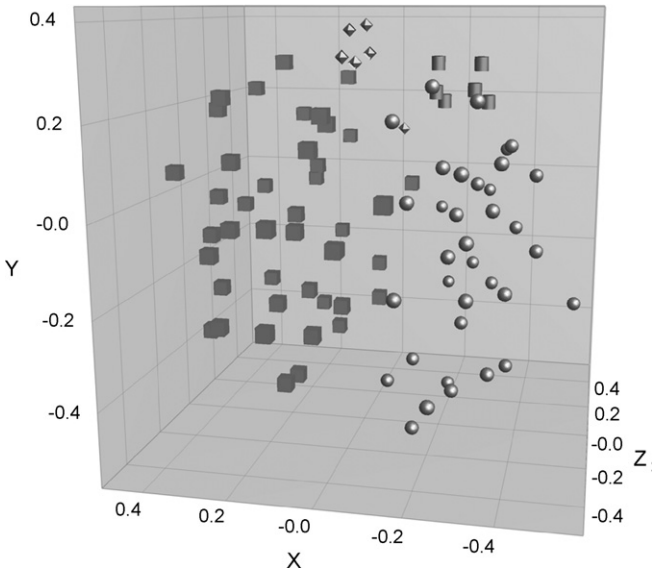

Fig. 2. Three-dimensional cluster phenogram showing the relationships among genotypes examined. Wild and cultivated genotypes have been represented by solid "rectangles" and "circles", respectively; while the EU spring and EU winter type genotypes have been shown by "diamonds" and "cylinders", respectively. The wild, cultivated and elite genotypes are grouped in three different clusters at left, right and top side, respectively.

formed and in the 'winter type' subcluster, 2-rowed and 6-rowed genotypes could be classified further.

4. Discussion

In past, a variety of molecular markers such as RFLPs, RAPDs, SSRs and AFLPs have been used for estimating the genetic diversity in different type of barleys (for a review see [8]. Even recently developed SNP markers have also been used for detection of genetic diversity in barley [24,25]. The use of a particular molecular marker type for estimating the genetic diversity of germplasm collections, however, depends on many factors including costs on genotyping the large population with a marker assay [26]. In recent years, the SSR and SNP markers derived from ESTs, due to their inexpensive developmental costs [11,12], are increasingly being used for genotyping of natural or breeding populations. Together with these markers, AFLP markers are still considered good for fingerprinting or diversity analysis. Therefore, the present study documents the comparative utility of these marker types for genetic diversity studies.

4.1. Marker polymorphism

SSR and SNP markers were found highly polymorphic while AFLP markers showed a lower level of polymorphism in the germplasm examined in the present study. The high level of polymorphism associated with SSR is to be expected because of the unique mechanism responsible for generating SSR allelic diversity by replication slippage (see [12,27]), while the basis of SNP and AFLP polymorphism are single nucleotide mutations and insertions/deletions [28,29]. SNP markers are mainly bi-allelic and therefore a maximum PIC value of 0.50 can be expected for a given SNP locus. In some cases like GBS0526 (3H) and GBS0576 (5H), we have almost reached this threshold as these markers detected a PIC value of 0.490 and 0.488, respectively.

In case of AFLPs, although all primer combinations were polymorphic (60%), these markers showed the least level of polymorphism in the germplasm collection examined. It is important to note here that these four primer combinations were selected randomly. In contrast, the SSR and the SNP markers used in the present study are part of a core set of highly informative genic markers, identified by us in a separate study [Varshney et al. unpublished]. Therefore, also in case of AFLP analysis, it is recommended to pre-select the AFLP primer combinations with a representative set of genotypes of the population to be tested as shown in other studies (e.g. [30]). However, it is recommended to pre-select the markers on the similar population that is planned to use for assessing the genetic diversity so that ascertainment bias can be minimized. In the present study, the portion of AFLP bands/loci that met the quality criteria for scoring was low (61.7%) as compared to some other studies in AFLP [7]. However, the resolution of AFLP fragments also depends on the fragment detection system [28].

4.2. Comparative utility of different marker systems

Assessment of genetic diversity by using molecular markers is important not only for crop improvement efforts but also for efficient management and conservation of plant genetic resources in genebanks [31]. Therefore, the selection of a particular type molecular marker is important and critically depends on the intended use [26].

Regarding the detection of polymorphism SSR markers certainly are better than SNP or AFLP markers as SSR are multiallelic markers in contrast to SNP or AFLP markers. This characteristic attribute of SSR markers together with their co-dominance nature, etc. made them the markers of choice in plant genetics and breeding [12,27]. SNP and AFLP markers are biallelic and less informative than SSRs. However, the abundance of SNPs in the barley genome [15,32] could more than compensate for this deficiency, in the presence of

genotyping platforms that offer a compromise between high throughput and lower costs. Although many assays including Pyrosequencing [33], SNuPE [34], microarray [24], PCR-CTTP [35], tetra-primer ARMS-PCR [25], Illumina Golden Gate assays [36] have been applied for SNP genotyping in barley, the CAPS assay used in the present study is an inexpensive method, in particular, in the laboratories in developing world where majority of times expensive machines are not available. Pyrosequencing based SNP assays, however, can provide more than one datapoint for assaying genetic variation by using a single SNP marker in one reaction. Furthermore, if two or more than two SNPs are in the range of pyrosequencing, assaying of those SNPs at the Pyrosequencing platform facilitates analysis of haplotypes, which are more informative than individual SNPs for genetic diversity studies [15,19,27].

Because of assaying one genetic locus per primer pair, the effective multiplex ratio for SSR and SNP markers was recorded as 1.0. However, as expected, the AFLP markers showed highest effective multiplex ratio as 26.4. Certainly, this is a characteristic feature of AFLP marker system. Other molecular markers like SSR or SNP do not have so high multiplex ratio, though new approaches like Illumina Golden Gate assay or OPA (oligo pool assay) technology (http://genomecenter.ucdavis.edu/dna_technologies/illumina.html, [36]) or detection of SFPs (single feature polymorphism) make it possible to analyze the SNPs in high throughput manner [37], these approaches are not suitable for their use in routine genetic diversity experiments in low-tech labs. Furthermore, the multiplex ratio of the AFLP assays can be adjusted by altering the restriction enzymes chosen and the degree of 3′ – nucleotide extension on the PCR primers, offering a high degree of flexibility [23].

The marker index, which is considered to be an overall measure of the efficiency to detect polymorphism, was highest (5.042) for AFLP-, lowest (0.341) for SNP-, and intermediate (0.593) for SSR-marker systems. Of course, the high MI of the AFLP assay derives from its high effective multiplex ratio rather than from high levels of detected polymorphism. This feature makes the AFLP marker system suitable for fingerprinting or estimating genetic diversity in breeding populations. Infact, in simulation [38] as well as some experimental studies [39,40], the AFLP markers have been shown as possessing the greatest discriminatory power to separate individuals from different groups into distinct clusters.

Genotyping of complete or a significant proportion of the genebank collections provides means to improve the management of plant genetic resources in manifold ways [31]. In this context, storing and managing genotyping data of genebank materials obtained by using molecular marker is important. In view of this, we have introduced the concept of the QND and effective marker index. The QND is the more important measure for the genebank curators and managers as they like to have a genotyping data on their material that can be documented and handled easily in their database as well as can be communicated in terms of 'molecular passport data' among different genebanks across the globe. As the QND for the SNP markers is the highest (1) and lowest (0.093) for AFLP markers, we recommend the utilization of SNP markers for genotyping the genebank materials. SNP genotyping data can be documented in 'digital fashion' or binary format (0–1 matrix) across different genotypes, and thus it is very convenient to store in genebank databases. In contrast, the QND for AFLP markers is the lowest as it is very difficult to interpret and document the AFLP genotyping data in genebanks databases. In this context, *firstly*, one needs to define the accurate sizes for a large number of AFLP fragments per primer combination and *secondly*, the transferability of the presence/absence of the AFLP fragments in different genebank samples across laboratories is impaired as it depends on the visualization system of fragments (e.g. radioactive labelling, silver staining, fluorescence labelling, etc.) and skills and expertise of the laboratory staff. The QND for the SSR markers is intermediate between the SNP and AFLP marker systems. Infact, the SSR markers (like SNP markers) irrespective of their detection platforms (silver staining, radioactive labelling, ABI sequencer, ALF, LICOR, etc.) are considered highly reproducible and reliable. Although the documentation of the SSR data can be digitized, one will have to define different alleles in terms of accurate size for a given SSR locus.

4.3. Relationships in examined germplasm

As expected, the wild genotypes generally showed more polymorphism than cultivated or elite genotypes [15]. This may be due to the presence of unique alleles present in wild genotypes, which have been lost during the cultivation or adoption, etc. in case of cultivated and elite genotypes. These results, like earlier studies [9,15], demonstrate the utility of wild germplasm for exploiting the unique and favourable alleles present therein for crop improvement programmes.

In the present study, genetic distance values were only moderately correlated between marker types. Comparison of different marker systems (especially AFLP and SSR) for diversity and population structure in several plant species frequently revealed incongruent diversity estimates for different types of markers ([40,41] and references cited therein). As speculated earlier [38,41], our results also suggest that analysing a higher number of genotypes (90) with a comparatively low number of markers may be one of the reasons for observing a low correlation among genetic distance values. This is further supported by the observation that analysis of 6 barley cultivars with a much larger number of EST-derived RFLP (253), SSR (632) and SNP (508) markers showed a high correlation (in the range of 0.87–0.93) among the corresponding genetic distance matrices [31].

In summary, the present study highlights the advantages and disadvantages of different marker systems for diversity analyses in breeding or natural populations or genebank materials to exploit the genotyping data for crop improvement as well as *ex-situ* conservation of plant genetic resources. For estimating the diversity of germplasm collections, AFLP or SSR markers (especially, if more than one marker can be used by mutilplexing) are more suitable as they have higher EMI.

Because of their high QND, SNP markers are considered the best type of markers for combining genotyping data from several labs as is frequently the case with decentralized genebank collections. Moreover, the corresponding SNP data can be easily stored in alphanumeric form in databases. Nevertheless, SSR or SNP genotyping should be the markers of choice for major crop species where genome/EST sequence data or primer pairs for such markers are available in sufficient amount. On the other hand, the marker-assisted management of less important species with dearth of genomic resources may still be studied, using relatively conventional marker systems like AFLPs that provide higher EMI and can easily be adapted for genotyping.

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Appendix 1

Table A.1
Details on the genotypes used for assessing molecular diversity

No	IG number of ICARDA Genebank	Taxonomical species names	Country of origin[a]	Province	Collection site or pedigree[b]	Longitude	Latitude	Altitude
Wild								
W01	38693	*Hordeum vulgare* subsp. *spontaneum*	PAK	Baluchistan	Baleli 13 km N Quetta	E66 54	N30 18	1400
W02	38826	*H. vulgare* subsp. *spontaneum*	TJK	Kurgan Tyube	Teshrabad, Tash Rabat	E069 05 53	N37 39	
W03	38840	*H. vulgare* subsp. *spontaneum*	PAL	Hamerkaz	Ben Shemen	E34 56	N31 57	
W04	38912	*H. vulgare* subsp. *spontaneum*	PAL		Tel Gezer			
W05	38981	*H. vulgare* subsp. *spontaneum*	PAL		Yaar Hanassi			
W06	39082	*H. vulgare* subsp. *spontaneum*	PAL	Hazafon	Biriyya, Upper Galilee, site 2	E35 30	N32 59	
W07	39117	*H. vulgare* subsp. *spontaneum*	PAL	Jerusalem	Judean Foothills region, Emek Haela, site 6	E35 12 50	N31 46 20	
W08	39591	*H. vulgare* subsp. *spontaneum*	PAL	Haifa	Atlit	E34 57 20	N32 42 50	
W09	39821	*H. vulgare* subsp. *spontaneum*	JOR	Amman	Madaba; Jiza 44 km S of Amman	E35 57	N31 42	710
W10	39847	*H. vulgare* subsp. *spontaneum*	SYR	Homs	Palmyra; 21 km SE Sukhnah	E39 02 00	N34 45 20	530
W11	39852	*H. vulgare* subsp. *spontaneum*	PAK	Baluchistan	Baleli 13 km N Quetta	E66 54	N30 18	1400
W12	39857	*H. vulgare* subsp. *spontaneum*	SYR	Homs	8 km N Palmyra; on Palmyra - Deir-ez-Zor highway	E38 21 22	N34 34 10	410

Appendix 1 (*Continued*)

No	IG number of ICARDA Genebank	Taxonomical species names	Country of origin[a]	Province	Collection site or pedigree[b]	Longitude	Latitude	Altitude
W13	39880	*H. vulgare* subsp. *spontaneum*	SYR	Damascus	Awajan; 13 km N Domeir	E36 50 10	N33 39 15	655
W14	39885	*H. vulgare* subsp. *spontaneum*	CYP	Famagusta	Cabo Greco	E34 01	N34 59 20	60
W15	39891	*H. vulgare* subsp. *spontaneum*	EGY	Marsa Matruh	Wadi El Habs	E27 10	N31 21	10
W16	39914	*H. vulgare* subsp. *spontaneum*	SYR	Idlib	El Aliye	E36 14 40	N35 47 55	330
W17	39996	*H. vulgare* subsp. *spontaneum*	JOR	Irbid	Kufr Yuba; 4 km W Irbid	E35 47	N32 32	570
W18	40002	*H. vulgare* subsp. *spontaneum*	JOR	Irbid	Deir Abi Said Al Goura district	E35 39	N32 29	300
W19	40014	*H. vulgare* subsp. *spontaneum*	JOR	Al Balqa	Al Yazidieh	E35 44	N32 01	955
W20	40059	*H. vulgare* subsp. *spontaneum*	JOR	Zarqa	Al Azraq Al Janoubi	E36 48	N31 47	560
W21	40063	*H. vulgare* subsp. *spontaneum*	JOR	At Tafilah	Al Rashadieh; S of Tafila	E35 34	N30 42	1450
W22	40064	*H. vulgare* subsp. *spontaneum*	JOR	Ma'an	Al Kadisieh village; S of Tafila	E35 34	N30 35	1600
W23	40072	*H. vulgare* subsp. *spontaneum*	JOR	Karak	Petra-Wadi Musa Road Al Tour	E35 37	N31 11	640
W24	40078	*H. vulgare* subsp. *spontaneum*	SYR	Homs	Homs; 200 m from junction with Lattakia highway towards Damascus	E36 43 30	N34 45 09	605
W25	40090	*H. vulgare* subsp. *spontaneum*	SYR	Sweida	6 km E Quanawat to Taima	E36 43 22	N32 46 03	1500
W26	40109	*H. vulgare* subsp. *spontaneum*	TJK	Khudzhand	W Pendzhikent	E67 30	N39 28	940
W27	40150	*H. vulgare* subsp. *spontaneum*	PAL		(Selected from Cambridge Acc # 13978)			225
W28	40171	*H. vulgare* subsp. *spontaneum*	SYR	Sweida	Aldor; 2 km SE on road to Sweida	E36 25 25	N32 48 20	
W29	40174	*H. vulgare* subsp. *spontaneum*	SYR	Dar'a	2 km SW of Numer	E36 02 30	N33 00 50	
W30	40181	*H. vulgare* subsp. *spontaneum*	LBN	Rachaiya	Rachaiya; 1 km before Kantaba; on the road from Sahmor	E35 46	N33 31	1050
W31	40197	*H. vulgare* subsp. *spontaneum*	IRN	West Azerbaijan	Just S Urumiyeh to Oshnaviyeh	E45 10	N37 30	1300
W32	40198	*H. vulgare* subsp. *spontaneum*	IRN	West Azerbaijan	Naqadeh to Haydar Abad; close to Lake Urumiyeh	E45 28	N37 04	1300
W33	107424	*H. vulgare* subsp. *spontaneum*	IRQ	Ninawa	Tell Afar to Sinjar; 60 km W Mosul	E42 10	N36 20	440
W34	107427	*H. vulgare* subsp. *spontaneum*	IRQ	Ninawa	Salah ed Din; 20 km Achur Ash Shergat on Jebel Makhuf	E43 12	N35 22	260
W35	110739	*H. vulgare* subsp. *spontaneum*	SYR	Sweida	E Busan edge of village, road to Shikka	E36 47 25	N32 41 04	1475
W36	110771	*H. vulgare* subsp. *spontaneum*	SYR	Al Hasakah	Aleppo road from Kamishli; junction with road to Tall Faris	E41 12 56	N37 01 56	480
W37	110798	*H. vulgare* subsp. *spontaneum*	SYR	Al Hasakah	3 km S of Masawieh	E40 27 33	N36 27 08	500
W38	110816	*H. vulgare* subsp. *spontaneum*	LBN	Biqaa Al Gharbi	Karaoun, 1 km from the main road to the lake	E35 43	N33 34	1010
W39	110833	*H. vulgare* subsp. *spontaneum*	LBN	Rachaiya	2 km before Ain Arab; road from Yanta	E35 51	N33 35	1310
W40	112846	*H. vulgare* subsp. *spontaneum*	LBY	Al Marj	Al Marj, occasionally in city area	E20 54	N32 30	
W41	115781	*H. vulgare* subsp. *spontaneum*	JOR	Al Mafraq	Al Mniusa	E36 43	N32 18	960
W42	119424	*H. vulgare* subsp. *spontaneum*	SYR	Homs	4 km W Kafr Na'am	E36 38 07	N34 54 23	360
W43	124017	*H. vulgare* subsp. *spontaneum*	UZB	Dzhizak		E67 05.15	N40 00.62	830
Cultivated								
C01	31396	*H. vulgare* subsp. *vulgare* convar. *distichon*	SYR	Homs	Altaibe	E38 55	N35 05	470

C02	31416	*H. vulgare* subsp. *vulgare* convar. *vulgare*	SYR	Raqqa	Dukhan	E39 12 30	N36 24 45	320
C03	31510	*H. vulgare* subsp. *vulgare* convar. *distichon*	SYR	Hama	Kasr Ibn Wardan	E37 15 20	N35 22 05	400
C04	31891	*H. vulgare* subsp. *vulgare* convar. *vulgare*	MAR	Sud	Tagounite; Oued Drea	W05 36	N29 58	600
C05	31925	*H. vulgare* subsp. *vulgare* convar. *vulgare*	MAR	Nord Ouest	Near Tedders	W006 15 55	N33 35 03	550
C06	31933	*H. vulgare* subsp. *vulgare* convar. *vulgare*	MAR	Oriental	Bouanane Oasis	W03 03	N32 03	800
C07	31938	*H. vulgare* subsp. *vulgare* convar. *vulgare*	MAR	Oriental	Figuig oasis	W01 15	N32 10	800
C08	31958	*H. vulgare* subsp. *vulgare* convar. *vulgare*	MAR	Sud	Near Tizi-en-Test	W08 18	N30 51	1800
C09	31965	*H. vulgare* subsp. *vulgare* convar. *vulgare*	MAR	Nord Ouest	Tizouggart; 10 km N of Tedders	W06 17	N33 40	1500
C10	31968	*H. vulgare* subsp. *vulgare* convar. *vulgare*	MAR	Sud	Isfoutelil Oasis; 7 km NW of Ourzazat	W06 51	N30 58	1300
C11	31995	*H. vulgare* subsp. *vulgare* convar. *vulgare*	MAR	Tensift	Ouriki; Marrakech oasis	W08 00	N31 49	900
C12	32000	*H. vulgare* subsp. *vulgare* convar. *vulgare*	MAR	Centre Sud	ca. 25 km E of Khenifra	W05 58	N33 00	1400
C13	32037	*H. vulgare* subsp. *vulgare* convar. *vulgare*	MAR	Centre Nord	9 km from Fes to Sefrou	W004 54 21	N33 51 24	1150
C14	32039	*H. vulgare* subsp. *vulgare* convar. *vulgare*	MAR	Centre Nord	Sefrou; 2 km from Sefrou to Boulmane	W04 51	N33 50	1525
C15	32062	*H. vulgare* subsp. *vulgare* convar. *vulgare*	MAR	Centre Nord	Kassetta; 10 km from Kassetta to Al Hoceima	W03 55 37	N34 57 35	1550
C16	32066	*H. vulgare* subsp. *vulgare* convar. *vulgare*	MAR	Nord Ouest	El Zeibe; 18 km from Bab Berret to Tetouan	W05 01	N35 03	1200
C17	32080	*H. vulgare* subsp. *vulgare* convar. *vulgare*	MAR	Centre Nord	Dauar Kalxa; Dulode Aissa 8 km S of Karia Ba Mohammed	W005 19 34	N34 19 28	500
C18	32469	*H. vulgare* subsp. *vulgare* convar. *vulgare*	EGY	North Sinai	Al Salam; 5 km S of El Arish	E33 50	N31 03	20
C19	32598	*H. vulgare* subsp. *vulgare* convar. *distichon*	SYR	Hama	Zaagba	E36 57 50	N35 22 40	430
C20	32733	*H. vulgare* subsp. *vulgare* convar. *vulgare*	SYR	Tartous	Ayn Khalifa; 12 km E Draikeesh	E36 13 00	N34 50 00	725
C21	32803	*H. vulgare* subsp. *vulgare* convar. *distichon*	SYR	Sweida	Sahwat Al Khidr	E36 45 55	N32 36 05	1480
C22	32973	*H. vulgare* subsp. *vulgare* convar. *vulgare*	OMN	Ad Dakhiliyah	35 km S Jabrin	E57 20	N22 40	400
C23	33029	*H. vulgare* subsp. *vulgare* convar. *vulgare*	DZA	Constantine	Constantine/ ITGC	E06 35	N36 13	620
C24	33088	*H. vulgare* subsp. *vulgare* convar. *distichon*	DZA	Medea	Djelfa	E03 13 48	N34 44 05	1060
C25	35377	*H. vulgare* subsp. *vulgare* convar. *vulgare*	DZA	Mostaganem	Ould Ali SW Relizane; 7 km on the way to Mascara	E00 30	N35 50	170
C26	35386	*H. vulgare* subsp. *vulgare* convar. *vulgare*	DZA	Saoura	Abadla; 50 km W of Bechar	W02 43	N31 02	540
C27	35398	*H. vulgare* subsp. *vulgare* convar. *vulgare*	DZA	Oran	Ain El Bared; 22 km E Sidi El Abbes	W00 30	N35 22	510
C28	39117	*H. vulgare* subsp. *vulgare* convar. *vulgare*	PAL	Jerusalem	Judean Foothills region, Emek Haela, site 6	E35 12 50	N31 46 20	
C29	108944	*H. vulgare* subsp. *vulgare* convar. *vulgare*	IRQ					
C30	120631	*H. vulgare* subsp. *vulgare* convar. *vulgare*	TJK					
C31	123980	*H. vulgare* subsp. *vulgare* convar. *vulgare*	UZB	Dzhizak	Dzhizak	E067 58 03	N39 55 48	570
C32	128127	*H. vulgare* subsp. *vulgare* convar. *distichon*	IRN	East Azerbaijan	Tabriz (selected from IG 27894)	E46 18	N38 05	
C33	128160	*H. vulgare* subsp. *vulgare* convar. *vulgare*	PAK	Baluchistan	Warchum 24 km W Ziarat (selected from IG 32621)	E67 30	N30 25	1810
C34	128184	*H. vulgare* subsp. *vulgare* convar. *distichon*	IRN		(Selected from IG 35550)			
C35	128219	*H. vulgare* subsp. *vulgare* convar. *vulgare*	LBY	Ghat	El Feowt (selected from IG 37592)	E10 11	N24 58	640
Elite: Spring type (two-rowed)								
ES01	Alexis	*H. vulgare* subsp. *vulgare* convar. *vulgare*	EU					
ES02	Aramir	*H. vulgare* subsp. *vulgare* convar. *vulgare*	EU					
ES03	Berolina	*H. vulgare* subsp. *vulgare* convar. *vulgare*	EU					
ES04	Grit	*H. vulgare* subsp. *vulgare* convar. *vulgare*	EU					
ES05	Koral	*H. vulgare* subsp. *vulgare* convar. *vulgare*	EU					
ES06	Toga	*H. vulgare* subsp. *vulgare* convar. *vulgare*	EU					

Appendix 1 (*Continued*)

No	IG number of ICARDA Genebank	Taxonomical species names	Country of origin[a]	Province	Collection site or pedigree[b]	Longitude	Latitude	Altitude
Elite: Winter type (two-rowed)								
EW01	Danillo	*H. vulgare* subsp. *vulgare* convar. *vulgare*	EU					
EW02	Malta	*H. vulgare* subsp. *vulgare* convar. *vulgare*	EU					
EW03	Sonate	*H. vulgare* subsp. *vulgare* convar. *vulgare*	EU					
Elite: Winter type (six-rowed)								
EW04	Adonia	*H. vulgare* subsp. *vulgare* convar. *vulgare*	EU					
EW05	Catinka	*H. vulgare* subsp. *vulgare* convar. *vulgare*	EU					
EW06	Ginso	*H. vulgare* subsp. *vulgare* convar. *vulgare*	EU					

[a] Standarad code for country of origin, e.g. CYP, Cyprus; DZA, Algeria; EGY, Egypt; EU, European Union; IRN, Iran; IRQ, Iraq; JOR, Jordan; LBN, Lebanon; LBY, Libya; MAR, Morocco; OMN, Oman; PAK, Pakistan; PAL, Palestine; SYR, Syria; TJK, Tajikistan; UZB, Uzbekistan.
[b] Pedigree/selection shown in parenthesis, wherever possible.

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