Diversity assessment of hulled barley (*Hordeum vulgare* L.) accessions by agro-morphological traits and SSR markers

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Abstract
Barley accessions from ICARDA, Lebanon were grown in Indian condition at ICARDA-IRP (Indian Research Platform), Amlaha, (M.P.) and analyzed for morphological and genetic variability using nine agro-morphological traits and molecular markers (SSR). Total 94 alleles were scored for 24 genotypes with 37 microsatellite markers. The number of alleles ranged from 2 to 4 with an average of 2.54 alleles per locus. The band fragment size varied from 100 bp to 455 bp with PIC values ranging from 0.153 to 0.707.

On comparing molecular variability across linkage groups, chromosome 3H with PIC and mean allele values 0.577 and 2.67 were observed to be most variable. The clusters obtained from NTSYS grouped ICARDA accessions in three major groups in accordance to their ancestry. These exotic accessions evaluated can be further used for primary or secondary introduction in Indian breeding program.

Keywords: Barley, Genetic Variability, Microsatellite markers, PIC, Agro-Morphological traits.

Introduction

Barley is the fourth most important crop in the world after wheat, rice and maize. Barley grain is used as feed, food and malting purposes while straw provides an important source of roughage for animals particularly in the dry areas.[1] There has been an increasing interest in exploiting barley as a dual purpose cereal as it has high biomass and salt tolerance nature which can permit forage production in early season in addition to the grain yield later on.[2] In India, barley is grown for green forage and grain in semi-arid and arid climatic conditions as it is well adapted to unfavorable environments where no other green forage is available in winter months.[3]

The genetic variation within a species is not uniformly distributed throughout the geographic area where it is growing[4] and populations from area far separated are normally expected to accumulate enormous genetic diversity.[5] This is true for barley also and climatic conditions or epigenetic factors play main role in its evolution by demonstrating significant levels of variation in response to the selection stress in the region.[6] Development of hybrid varieties with desirable traits requires a thorough knowledge about the existing genetic diversity in available germplasm.[7] Morphological characters have been used to evaluate distinctness, uniformity and stability and to establish the description of a genotype in crops including barley. This method is thought to be often influenced by environmental conditions and is labour intensive also.[8]

Molecular markers are advanced and powerful tools in the assessment of genetic diversity within and between genetic populations.[8] They have the advantage of providing thorough genome information that is not influenced by the environmental factors. Molecular markers system provides information for making decisions regarding selection of parental combinations that will maximize gain for selection and maintain genetic diversity.[9] Nowadays the effectiveness and informative value of microsatellite markers in genetic studies has been demonstrated for all the major cereals including barley.[10-12]

ICARDA has set up Indian Research Platform (ICARDA-IRP, Amlaha) in Madhya Pradesh which is the major barley growing region in India. In the present investigation, barley genotypes of ICARDA were evaluated at this station for their agro-morphological and genetic diversity. The purpose of this study is to introduce best performing accessions in Madhya Pradesh barley growing areas to improve production of dual purpose barley. Therefore, the objectives of present study were: (1) to determine the diversity for agro-morphological traits (2) To estimate the genetic distance by molecular means and (3) to classify the genotypes on the basis of agro-morphological and molecular diversity.

Material and Methods

Seeds of 24 barley accessions listed in table 1 were procured from ICARDA, Lebanon and grown under Indian field condition at experimental field of ICARDA- IRP, Amlaha, (27º12' N, 77 º 05' E) in augmented design with 2 replications for 6 lines of 2.5 m with 15 g seeds per line for each entry. Sowing was done in rabi (winter) season of 2014-15 on Nov. 12, 2014. 50% of Nitrogen (30 kg/ha), full dose of Phosphorus (60 kg/ha) and Potassium (40kg/ha) were applied at the sowing as basal dose. Top dressing of remaining nitrogen was done after 30 days of sowing. Water was given at critical stages i.e. crown root initiation stage and flowering stage. First hand weeding was done with the appearance of thick flush of weeds. Second hand weeding was repeated after two weeks.

Agro-morphological screening: Data were recorded taking 10 random plants from the middle row for each accession for nine agro-morphological traits viz. plant height (cm), tillers per plant, days to heading, ear heads per plant, length of ear heads (cm), spikelets per ear head, grains per ear head, yield per plant (gm) and 1000 seeds weight (gm) as given in table 1.

DNA isolation: Equal number of fresh young leaves (two weeks old seedlings) of at least six plants from each genotype was bulked for DNA extraction. Total genomic DNA was isolated using the modified CTAB method.[13] The DNA samples were analyzed both qualitatively and quantitatively using 0.8% agarose gel electrophoresis.

Generation of SSR markers based molecular profiles: Total 70 SSR markers were selected from different locations of each linkage group of barley genome out of which 37 markers showed polymorphism as given in table 2. The sequence of these primer pairs, their Tm and amplified fragment and PCR conditions were obtained from website (www.wheat.pw.usda.gov/cgi-bin/graingenes.com). PCR reaction was conducted in a reaction volume of 10 µl containing 1X PCR buffer, 200 m M dNTPs, 0.25 µM of primer, 2mM $MgCl_2$, 1u Taq polymerase and 50 ng template DNA. PCR amplification was performed using BIORAD S 1000 thermocycler.

PCR products were resolved by electrophoresis on 2.5% agarose gels (HiMedia) at 4v/ cm in 0.5 X TBE buffer. Fragment sizes were approximately calculated by interpolation from the migration distance of marker fragments of 100- bpDNA ladder (NEB, UK) and corroborated with the reported amplified fragment size of respective molecular marker. The occurrence of 'null' alleles was verified by re-amplification under similar PCR conditions. Gels were stained with ethidium bromide (0.5ug/ml) and DNA banding patterns were visualized under UV light (Syngene Synoptics Limited, USA).

Data analysis: The genetic divergence between two genotypes was calculated using the following formula proposed by Mahalanobis:[14]

$$pD^2 = W^{ij}(\bar{X}_i^1 - \bar{X}_i^2)(\bar{X}_j^1 - \bar{X}_j^2)$$

where pD^2 = genetic divergence between two genotypes; W^{ij} = the inverse of estimated variance and co-variance matrix; X_i and X_j = the multiple measurements available on each individual.

Cluster analysis was also done through multivariate analysis under the software NTSYS 2.1 using Euclidean D^2 technique.

Molecular weights for microsatellite products in base pairs were estimated and the summary statistics including the number of alleles per locus and frequency of major alleles were determined. Polymorphic information content (PIC) was calculated for each SSR marker vides estimates of the discriminatory power of locus by taking into account the numbers of alleles that are expressed.[15]PIC value were calculated as:

$$\text{PIC} = 1 - \sum P_{ij}^2$$

where P_{ij}^2 is the frequency of the i^{th} allele.

Allele molecular weight data were also used to export the data in binary format (allele presence = "1" and allele absence = "0") and entered into a matrix. Based on the matrix of (GD) values, NTsys 2.1software was used to obtain the dendrograms depicting genetic relatedness of the cultivars.

Results and Discussion

Agro-morphological traits based genetic variability: Significant variations were observed for nine agro-morphological traits studied indicating sufficient genetic diversity in ICARDA genotypes (Table 1). Similar kind of results were also observed in several previous studies.[16-18] The highest coefficient of variation and as a result highest diversity among the genotypes in this study are found for tiller number per plant (49.77%), earhead per plant (49.02%) and grain yield per plant (23.99%).

On the other hand, length of earhead (16.8%), spikelets per earhead (14.73%), grains per earhead (15.07%) and 1000 seed weight (15.3%) have shown moderate diversity whereas plant height (7.32%) and days to maturity (6.14%) have conributued least to divesity. All these traits could be used to devlop new variety efficiently according to the need of different programes. Average variations for plant height, tiller per plant, spikelets per ear, grains per spike, 1000 seed weight, grain yield per plant were reported during analysis of genetic diversity for barley.[6,19]

Higher variations for spikelet per spike; grains per spike, 1000 seed weight and plant height were reported while analysing diversity in Pakistan's barley genotypes[17] whereas considerable diversity was reported for plant height, days to heading, days to maturity and 1000-grain weight in Ethiopian barley germplasm.[16] Genotypes in present study were selected from single centre of origin but still significant differences were observed among barley accessions for all nine studied morphological traits.

Hierarchical Clustering based on agro-morphological variations: The average of two replications of all the studied characters of the accessions was used to construct a similarity matrix using the NTSYS (version 2.1) software package. The dendrogram clustered 22 accessions into major group 1 and two accessions in single line individualy at G.S. = 0.0 (Fig. 1). The group 1 is further divided into two sub groups A and B containing 10 and 12 accessions respectively. Hierarchical clustering grouped these

genotypes in two major groups at GS=0.17 for phenotypic traits studied. In subgroup A, IBYT-HI 1, IBYT-HI 16 and IBYT-HI 17 are on same node which revealed that in same agronomic conditions, these genotypes perform almost similar.

Similarly, IBYT-HI 4, IBYT-HI 8, IBYT-HI 19, IBYT-HI 22 and IBYT-HI 23 coincided on same fork in subgroup B showing that they may have various or distant parents but in same environment their performance has no significant difference. In earlier studies on hierarchical clustering for morpho-genetic traits in barley germplasm in Pakistan, Ethiopia Syrian, Slovak and Egyptian, accessions from same center of origin or same geographical areas are grouped in same cluster.[16, 17]

Grouping of ICARDA genotypes developed from diverse ancestors in same cluster on basis of their morphological traits in given climate has been done in previous study.[18] The variations observed for most of the traits are sufficient enough to discrimate selected accessions at phenotypic level and contribute for genetic variability in barley germplasm.

Molecular markers based genetic variability: Several studies were conducted in world to evaluate the genetic relationships among different barley genotypes using molecular markers.[8, 20-22] Initially 70 SSR markers were screened and out of these, 37 polymorphic markers equally distributed across seven barley chromosomes were selected. These polymorphic markers amplified 94 bands with an average of 2.54 bands per marker as given in table 2. The band fragment size predominantly ranged between 100-455 bp except for ABG58 (1022 bp). It has been reported that the information of polymorphism would be sufficient if more than 70 alleles were detected.[23]

Earlier research suggested that more than two SSRs from each of seven linkage groups should be selected to ensure the efficiency and representation of the genetic information among accessions.[20] SSR markers screened also were compared for their number of alleles and PIC across seven linkage groups of barley genome as shown in figure 2. Linkage group chromosome 3 (3H) with high PIC (0.577) and mean allele (2.67), was found most variable. The highest frequency allele percentage ranged from 41.67% to 91.67% with an average of 60.47%.

The values for polymorphism information content (PIC) ranged from 0.153 to 0.707 with an average of 0.50 thus indicating sufficient variability in ICARDA accessions. Previous reports also reported similar number of alleles and PIC values in Indian barley during genetic variability studies.[6, 24]

PIC values greater than 0.50 indicate that SSR markers enable sufficient level of polymorphism.[25] These results are comparable with results reported by several previous authors.[6, 21, 24] Accessions screened in this study are from same centre and their pedigree gene pool is also narrow as there were bred for high yield, therefore, chances of getting fewer alleles per locus are expected, however, studies reporting high number of alleles used genotypes of various centers of origin and distinct parents for evaluation.

UPGMA based clustering for molecular variations: Similarity (Sm) matrix was developed using binary (0/1) molecular data for UPGMA based clustering (Figure 1) using NTSYS 2.1 software of selected ICARDA accessions. The genotypes were clustered into two groups at level of GS= 0.52. Group-1 was considered as major group and included 22 accessions whereas minor group (2) got only 2 accessions (IBYT-HI 23 and IBYT-HI 24). At the level of GS=0.57 major group was further subdivided into single node (B) with one accession IBYT-HI 2 and rest of 21 genotypes clustered together as group A. Accessions IBYT-HI 19 and IBYT-HI 23 were found maximum distantly related genotypes with GS=0.415, which are followed by IBYT-HI 14 and IBYT-HI 23 with GS=value 0.425.

It is interesting to observe that the genotypes in the same groups always share one or more common or closely related breeding ancestors according to pedigree information. For example, IBYT-HI 8, IBYT-HI 9, IBYT-HI 10, IBYT-HI 11, IBYT-HI 19 and IBYT-HI 20 are clustered in same subgroup and all these genotypes have one or another ancestor same in their pedigree. Same results of clustering of genotypes of common ancestor in single group were observed in previous report.[17] Similar level of genetic similarity values was reported for Indian barley during UPGMA based clustering.[6,24]

Comparison of the molecular and morphological data: The mantel correspondance test was used to compare the molecular and morphological similarity matrics.[26] Correlation between the distance coefficient was low ($r = 0.01204$, $p = 0.5798$) during mantel correspondance test for molecular and morphological similarity matrics. Although dendrograms clustered the accessions into major and minor groups, UPGMA based clustering is different than hierarchical clustering for morphological traits.

During molecular clustering, IBYT-HI 4 was found geneticaly highly variable from accessions IBYT-HI 8, IBYT-HI 19, IBYT-HI-22 and IBYT-HI 23 but that variability can not be observed by morphological traits of these accessions. Likewise, there have been accessions like IBYT-HI 17, IBYT-HI 18 AND IBYT-HI 19 which have either of the parent same and are grouped into same cluster in UPGMA dendrogram but were grouped into different clusters made by morphological data based dendrogram.

As reported earlier, DNA markers and morpholgical traits may not necessarily gain closely matching result.[27] It was also reported that positive correlation is not necessarily obtained between morphological and molecular markers specialy SSRs because of their genetic nature.[28]

Nevertheless, the genetic relationship observed using molecular markers may provide information on the history and biology of accessions or genotypes but does not necessarily reflect what may be observed with respect to agro-morphological traits.[29] The present study's results were also in agreement with finding of several authors in crops like in wheat and barley.[30,31] In present study, accessions performance evaluated in Indian condition can be further used for primary or secondary introduction in India. More so when markers information was used in conjunction with morphological data, then it classified genotypes better rather that can be done with classifications based on individual data set i.e. morphological or molecular data.

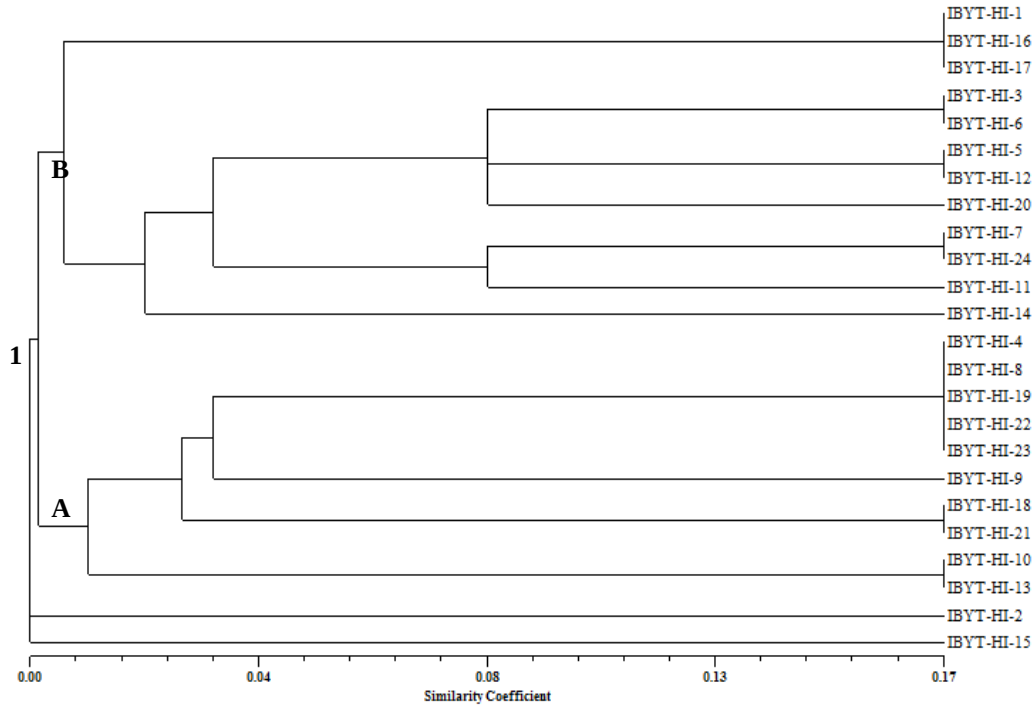

Figure 1: Clustering of ICARDA accessions on the basis of nine agro-morphological data using NTsys2.1 software program

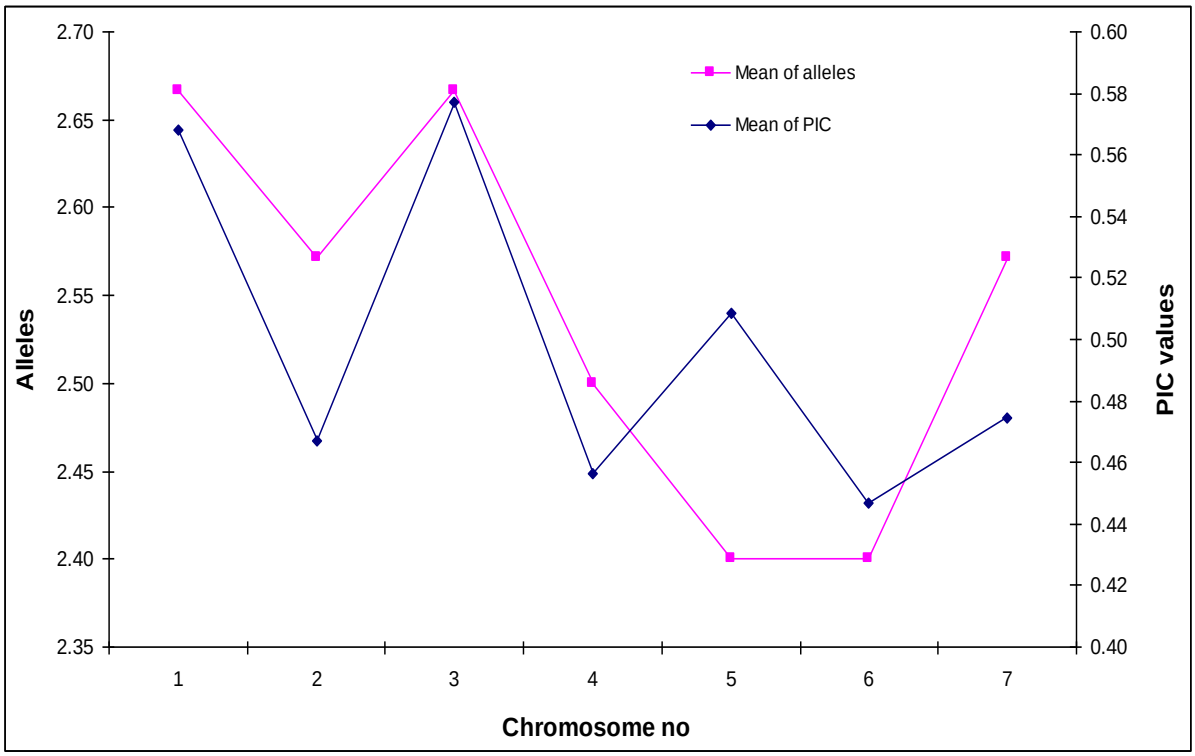

Figure 2: Comparison of genetic diversity (PIC and Highest mean number of allele) among seven chromosomes of Barley on the basis of microsatellite markers.

Table 1
Mean performance of Hulled Barley accessions in Indian conditions and variance estimation for agro-morphological traits at ICARDA- IRP, Amlaha, Madhya Pradesh, India

Accession	PH(cm)	T/P	DH	E/P	EL(cm)	S/E	G/E	TGW (g)	Y/P(g)
IBYT-HI-1	79.2	14	75	14	6.4	58	53	57.2	17.15
IBYT-HI-2	80.6	12	73	12	8.6	80	76	45.7	11.91
IBYT-HI-3	80.9	12	74	11	8.1	68	61	55.9	9.62
IBYT-HI-4	85.8	14	75	14	9.2	76	76	51.0	13.38
IBYT-HI-5	74.5	11	72	11	8.0	71	71	36.7	10.50
IBYT-HI-6	74.2	12	72	12	7.9	72	72	43.3	10.28
IBYT-HI-7	73.7	13	74	13	13.1	84	77	60.4	13.25
IBYT-HI-8	78.3	13	75	13	6.9	66	60	39.0	9.48
IBYT-HI-9	81.8	15	70	15	6.9	67	63	44.3	14.83
IBYT-HI-10	78.3	16	72	16	8.3	32	32	60.9	13.25
IBYT-HI-11	66.8	48	58	47	7.6	73	64	49.8	9.51
IBYT-HI-12	77	12	76	12	8.1	71	69	48.5	10.02
IBYT-HI-13	65.1	11	72	10	8.4	79	73	48.8	7.54
IBYT-HI-14	84.3	16	61	16	8.0	81	71	52.9	17.02
IBYT-HI-15	74.7	15	67	15	7.8	68	61	45.6	10.94
IBYT-HI-16	72.3	14	75	15	7.7	65	63	40.4	13.47
IBYT-HI-17	76.5	11	75	11	6.8	61	55	67.4	9.01
IBYT-HI-18	71.1	9	70	9	8.2	76	65	54.2	14.69
IBYT-HI-19	83.4	15	75	15	6.5	62	59	42.0	8.07
IBYT-HI-20	70.1	11	68	11	8.1	74	68	55.9	10.64
IBYT-HI-21	72.8	12	70	12	7.4	66	66	41.5	15.40
IBYT-HI-22	67.7	19	75	19	7.0	62	50	54.7	7.18
IBYT-HI-23	80	12	75	12	6.1	63	58	48.3	9.76
IBYT-HI-24	70.1	17	74	16	7.6	74	68	43.8	13.44
Mean=	75.80	14.66	71.63	14.53	7.9	68.78	63.73	49.5	11.68
S.D.=	5.55	7.30	4.40	7.12	1.3	10.13	9.60	7.6	2.80
Min	65.10	9.40	58.00	9.40	6.1	32.40	32.40	36.7	7.18
Max	85.80	48.00	76.00	47.00	13.1	84.40	77.00	67.4	17.15
Mean-S.D.=	70.25	7.36	67.23	7.41	6.5	58.64	54.12	41.9	8.88
C.V.=	**7.32**	**49.77**	**6.14**	**49.02**	**16.8**	**14.73**	**15.07**	**15.3**	**23.99**
Mean+S.D.	81.35	21.96	76.02	21.66	9.2	78.91	73.33	57.1	14.48

PH = Plant height, T/P = Tillers number/plant, DH = Days to heading, E/P = Ears number/ plant, EL = Ear Length, S/E = Spikelet number/Ear, G/E = Grain number/ Ear, TGW= 1000 grain weight, Y/P = Yield/ Plant, S.D. = Standard Deviation, C.V. =Coefficient of Variance.

Table 2
Allelic variation, frequency% and PIC of the polymorphic SSR loci in barley accessions.

Marker	Chr	Tm (°C)	No. Of Alleles	Allele size (bp)	High frequency allele	% high frequency allele	PIC
ScSSR10477	1H	60	4	110-140	130	41.67	0.707
ABG500a	1H	58	2	289-300	300	62.50	0.498
Bmag211	1H	58	2	164-174	174	50.00	0.500
Bmag125	2H	55	3	125-145	145	45.83	0.635
ABG58	2H	58	2	1000-1022	1022	54.17	0.497
Bmag749	2H	55	2	155-166	166	87.50	0.219
ABC252	2H	58	3	230-250	250	75.00	0.406
HVM54	2H	55	3	150-170	170	58.33	0.559
EBmac640	2H	55	2	165-176	176	70.83	0.413
Bmag829	2H	55	3	180-200	200	58.33	0.542
Bmac129	3H	58	3	140-160	160	50.00	0.595
EBmac705	3H	55	2	140-150	150	54.17	0.497
Bmag603	3H	55	2	110-120	120	58.33	0.486
Bmag13	3H	58	3	145-165	155	41.67	0.642
Ebmac541	3H	58	3	100-115	115	45.83	0.601
Bmag877	3H	55	3	140-160	160	45.83	0.642
HVM67	4H	55	3	105-120	190	62.50	0.443
HVM40	4H	55	2	150-160	160	54.17	0.497
ABG500b	4H	55	2	180-189	189	83.33	0.278
Ebmac635	4H	58	3	106-126	116	58.33	0.609
Bmag760	5H	55	2	100-110	110	58.33	0.549
Bmag812	5H	55	3	145-165	165	54.17	0.637
GMS61	5H	60	2	135-145	145	70.83	0.413
ABG712	5H	58	2	445-455	455	75.00	0.375
Bmag223	5H	58	3	117-137	127	58.33	0.569
Bmac40	6H	58	3	225-245	236	87.50	0.217
HVM14	6H	55	3	145-168	158	45.83	0.609
ABG387b	6H	60	2	198-210	210	66.67	0.444
MWG798	6H	55	2	350-365	365	66.67	0.444
GBM1275	6H	58	2	110-120	120	58.33	0.519
Bmag110	7H	58	2	130-145	130	62.50	0.469
Bmac162	7H	58	3	187-210	200	54.17	0.601
Bmac167	7H	55	3	170-190	190	66.67	0.469
Bmac64	7H	58	3	140-165	165	66.67	0.486
ABG380	7H	55	2	380-400	380	50.00	0.500
Bmag273	7H	55	2	150-186	186	91.67	0.153
MWG402	7H	55	3	230-250	240	45.83	0.642

Chr= Chromosome number, Tm= Annealing Temperature, PIC= Polymorphism Information Content

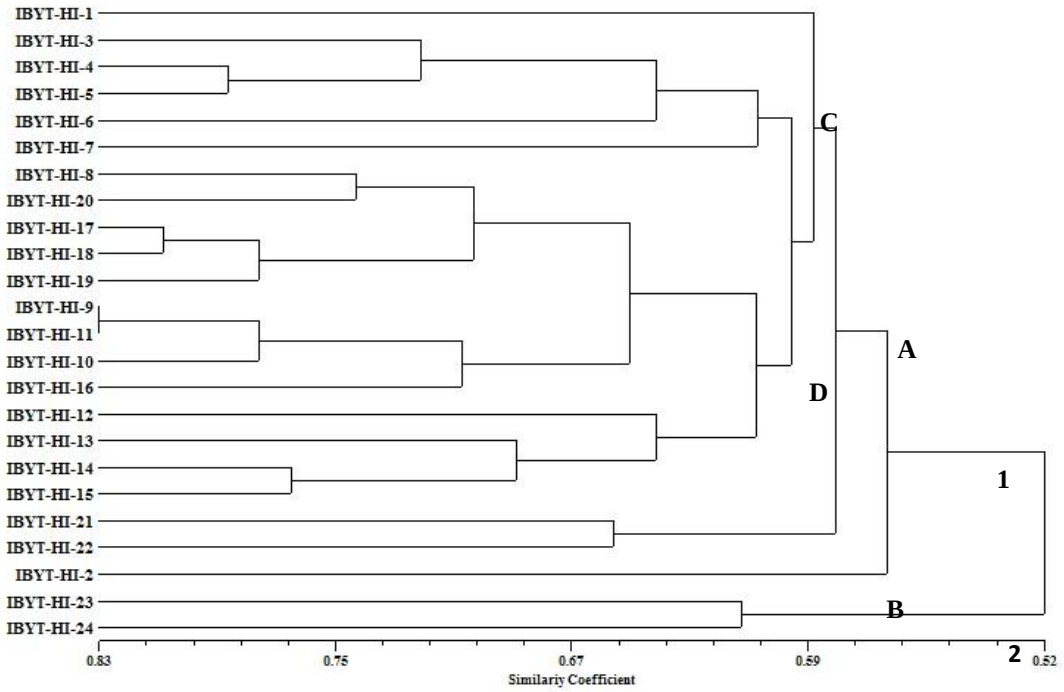

Figure 3: UPGMA based clustering of ICARDA IBYT-HI accessions on the basis of microsatellite markers

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