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Genetic diversity and population structure studies of the wild apple genotypes using RAPD markers

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ABSTRACT

The wild apples (*Malus* sp.) are important genetic resources for apple improvement programmes. In the present investigation, 32 *Malus* genotypes including indigenous Himalayan crab apples and exotic wild apples collected and maintained in the field gene banks at different ICAR institutes, were selected for genetic diversity and population structure analysis using the RAPD markers. The mean diversity indices, *viz*., Rp, PIC, EMR and MI of 18 RAPD markers were 5.829, 0.326, 8.432 and 2.851, respectively among the wild *Malus* genotypes. The cluster analysis segregated the wild *Malus* genotypes into two major clusters and the Jaccard's similarity coefficient ranged from 0.66 to 0.90. The first three axes of PCoA explained 40.26% variation and AMOVA explained 2% between the indigenous and exotic wild apple genotypes. The model based population structure studies revealed three major populations in the present set of genotypes. It could be concluded that there was substantial level of genetic diversity and population differentiation among the wild apple genotypes maintained at different research stations. Thus, the available diversity among the present set of wild apples could be explored for the scion as well as for rootstock breeding.

Key words: *Malus* sp., genetic resources, molecular markers, rootstock breeding, population structure.

INTRODUCTION

The Indian Himalayan regions have great diversity of various fruit crop species including the crab apples. The crab apples distributed over the Indian Himalayan region is collectively called as indigenous Himalayan crab apples and botanically classified in to two *Malus* species, *viz*., *M. baccata* and *M. sikkimensis* (Hooker, 3). These crab apples could be the important genetic resources for any apple improvement programme owing to their resistance for various diseases and pests (Sharma *et al*., 7) as well other important horticultural traits like chilling requirement *etc.* (Kishore *et al*., 4). The apple industry in India immediately requires new gene sources to combat various biotic and abiotic stresses. The various explorations have been made in the Indian Himalayan regions to collect the diverse indigenous Himalayan crab apples and have been collected and conserved in the field *Malus* gene banks (Rana *et al*., 6). As a part of apple germplasm several other exotic wild apples were also introduced and conserved in the field gene banks of *Malus* along with the indigenous Himalayan crab apples. Ascertaining phylogenetic analysis is important for classification of *Malus* genotypes and practical apple breeding (Zhou and Li, 10). Still majority of these indigenous Himalayan crabs and exotic wild apples have not adequately been studied for their phylogenetic relationship, genetic diversity and population structure analysis. Thus, the present investigation was undertaken to decipher the genetic diversity and population structure of the wild apple genotypes collected and conserved in the field gene banks employing the RAPD markers.

MATERIALS AND METHODS

The 32 diverse *Malus* genotypes including 19 exotic wild and 12 Indian Himalayan crab apples (*M. baccata* biotypes and *M. sikkimensis*), and one cultivated *M.* × *domestica* cv. Golden Delicious was undertaken for the genetic diversity and population structure studies. These wild apple genotypes are conserved in the *Malus* Field Gene Banks at three different regional stations of the ICAR institutes, *viz*., Indian Agricultural Research Institute Regional Station (IARI RS), Amartara cottage, Shimla (Himachal Pradesh), National Bureau of Plant Genetic Resources Regional Station (ICAR-NBPGR RS), Phagli (Himachal Pradesh) and Central Institute of Temperate Horticulture Regional Station (CITH RS), Mukteshwar (Uttarakhand). The details of the wild apples along with their source gene bank and introduction or native source are presented in Table 1.

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Table 1. The details of wild *Malus* genotypes selected for the study, respective gene bank and place or country where from they were collected, originated or introduced.

Sl. No.	Genotype	Gene bank	Introduction/ Native source
1.	*M. baccata* (USA)	CITH RS	USA
2.	*M. baccata* var. *mandshurica* (D)	IARI RS	Japan
3.	*M. baccata* var. *mandshurica* (P)	NBPGR RS	Japan
4.	*M.* × *domestica* cv. Golden Delicious	IARI RS	USA
5.	*M. esseltine*	IARI RS	UK
6.	*M. hilleiri*	NBPGR RS	UK
7.	*M. micromalus*	CITH RS	USA
8.	*M. micromalus* (Nagasaki zumi)	IARI RS	Japan
9.	*M. orientalis*	IARI RS	USSR
10.	*M. prunifolia* (Maruba)	IARI RS	USSR
11.	*M. prunifolia* var. *Ringo-Assami*	IARI RS	USSR
12.	*M. pumila*	IARI RS	UK
13.	*M. purpurea*	IARI RS	USSR
14.	*M. sargentii*	IARI RS	USSR
15.	*M. sieboldii*	IARI RS	Japan
16.	*M. sieversii*	NBPGR RS	USA
17.	*M. simcoe*	IARI RS	Canada
18.	*M. species* (Adams)	NBPGR RS	Canada
19.	*M. spectabilis*	IARI RS	USSR
20.	*M. zumi*	IARI RS	USSR
	Indigenous Himalayan crabs		
21.	*M. baccata* (Chamba)	NBPGR RS	Chamba, H.P. (India)
22.	*M.baccata* (Dhak)	IARI RS	Dhak, H.P. (India)
23.	*M. baccata* (Kashmir A)	IARI RS	Srinagar, J&K (India)
24.	*M. baccata* (Kashmir B)	IARI RS	Kashmir Valley, J&K (India)
25.	*M. baccata* (Khrot)	IARI RS	Khrot, H.P. (India)
26.	*M. baccata* (Kinnaur)	IARI RS	Kinnaur, H.P. (India)
27.	*M. baccata* (Ladakh)	NBPGR RS	Ladhak, J&K (India)
28.	*M. baccata* (Pangi)	IARI RS	Pangi, H.P. (India)
29.	*M. baccata* (Rohru)	IARI RS	Rohru, H.P. (India)
30.	*M. baccata* (Shillong)	IARI RS	Shilling, Meghalaya (India)
31.	*M. sikkimensis* (M)	CITH RS	Lachen Valley, Sikkim (India)
32.	*M. sikkimensis* (P)	NBPGR RS	Lachen Valley, Sikkim (India)

M. denotes genus *Malus*; Parentheses of *Malus baccata* represent the name of place where from the particular genotype was collected. (D), (M) and (P) denote the place names *i.e.* Dhanda, Mukteshwar and Phagali, respectively.

The healthy green leaves of the wild apples were collected from their respective field gene banks and stored in the deep freeze (-20°C) for genomic DNA extraction. The genomic DNA from the collected leaf samples were isolated using CTAB method as described by Doyle and Doyle (1) with few modifications. The RNA impurities were removed, while the DNA treated with RNase (2.25 U) and again it was purified. The purified DNA was quantified with 0.8% agarose gel and spectrophotometer (Nanodrop™, Thermo Fisher). The final concentration of purified DNA was maintained 20 ng/ µl in nuclease-free water as working dilution.

Thirty RAPD primers were screened for polymorphism of which 18 were found to be polymorphic and, thus, used for further analysis (Table 2). The total volume of 25 µl PCR reaction mixture contained 60

Table 2. Details of RAPD primers sequence with their per cent GC content, number of polymorphic amplicons, Rp, PIC, EMR and MI values.

Sl. No.	Primer name	Primer sequence	GC content (%)	Size (bp)	No. of polymorphic amplicon	Rp	PIC	EMR	MI
1.	SBSC-08	TGGACCGGTG	70	250-2500	18	9.000	0.320	8.111	2.596
2.	SBSC-11	AAAGCTGCGG	60	100-2500	16	7.000	0.292	7.885	2.302
3.	SBSC-12	TGTCATCCCC	60	400-2800	14	5.625	0.271	7.714	2.090
4.	SBSC-13	AAGCCTCGTC	60	300-3000	17	7.062	0.284	7.000	1.988
5.	SBSC-15	GACGGATCAG	60	400-3200	14	4.680	0.256	5.357	1.371
6.	SBSC-18	TGAGTGGGTG	60	450-2800	13	3.813	0.228	6.077	1.386
7.	SBSC-20	ACTTCGCCAC	60	450-1900	7	2.000	0.219	4.571	1.001
8.	SBSD-16	AGGGCGTAAG	60	300-2400	14	5.680	0.289	7.071	2.044
9.	SBSD-18	GAGAGCCAAC	60	300-1500	12	6.438	0.403	13.700	5.521
10.	SBSD-20	ACCCGGTCAC	70	300-2200	16	8.563	0.376	11.400	4.286
11.	SBSE-01	CCCAAGGTCC	70	300-2500	15	7.312	0.351	10.786	3.786
12.	SBSE-03	CCAGATGCAC	60	300-1800	12	5.375	0.311	15.667	4.872
13.	SBSE-05	TCAGGGAGGT	60	300-2500	16	5.812	0.267	5.813	1.552
14.	SBSE-07	AGATGCAGCC	60	600-3000	10	3.875	0.289	6.200	1.792
15.	SBSE-15	ACGCACAACC	60	550-1200	8	4.188	0.747	8.375	6.256
16.	SBSF-05	CCGAATTCCC	60	300-2400	14	7.375	0.346	9.286	3.213
17.	SBSF-06	GGGAATTCGG	60	400-1800	9	4.375	0.339	9.111	3.089
18.	SBSF-07	CCGATATCCC	60	300-3000	17	6.750	0.283	7.647	2.164
Mean					13.44	5.829	0.326	8.432	2.851

Rp = Resolving Power; PIC = Polymorphic Inf. control; EMR = Effective Multiple Ratia; MI = Marker Index

ng of DNA template, 2.5 µl of 10 mM deoxynucleotide tri-phosphates (dNTPs), 2.5 µl of 25 mM $MgCl_2$, 2.0 µl of 5 pmol primer, 1U *Taq* DNA polymerase (ThermoScientific, USA) and final volume makeup has been done by using nuclease-free water. The PCR reaction was performed in a thermal cycler (G-Storm, UK) and the following conditions were programmed, *i.e.* initial denaturation at 94°C for 4 min. followed by 40 cycles of denaturation at 94°C for 1 min., annealing ranges from 37-40°C for 1 min. and extension at 72°C for 2 min. followed by final extension at 72°C for 7 min. Reactions were stored at 4°C until electrophoresis. The amplified products were separated on 1.4% agarose gel (stained with 0.5 µg/ ml ethidium bromide) at 120 V for 1.5-2.0 h. The PCR products were visualized using gel documentation system (Alpha Innotech Corporation, USA). 1 Kb DNA ladder (Fermentas, USA) was used as standard.

Amplified PCR products were manually scored for the 32 *Malus* genotypes. The clearly distinguishable bands were scored and data analysis was undertaken. Binary data matrix was generated by scoring the presence (1) and absence of (0) of bands from the gel images of all primers. The diversity indices, *viz*., resolving power (Rp), polymorphic information content (PIC), effective multiplex ratio (EMR) and marker index (MI) each primer was calculated to assess their discriminatory power. NTSYS (Numerical taxonomy and multivariate analysis system) software Version 2.1 were used to generate the Distance matrix and cluster among the studied wild apple genotypes. The genetic relationship among the studied wild apple genotypes were analysed using SIMQUAL (Similarity for qualitative data program in NTSYS) module of Jaccard's similarity coefficient. A dendrogram describing the genetic relationship as shown by similarity coefficient was analysed using UPGMA (Un-weighted Pair Group Method with Arithmetic Mean) with Sequential agglomerative hierarchical and nested clustering method programme in NTSYS (SAHN). A model based, Population structure of *Malus* species was constructed using STRUCTURE software version 2.3.4. "Structure harvest" an online available programme (http://taylor0.biology.ucla.edu). Analysis of molecular variance (AMOVA) was used to distinguish the genetic variation between the

indigenous and exotic *Malus* population; and within *Malus* populations using software GenAlEx V6.5 program.

RESULTS AND DISCUSSION

The present investigation on genetic diversity and population structure analysis of 32 *Malus* genotypes belonging to exotic and indigenous Himalayan groups was carried out using RAPD marker system. The results obtained in this study are presented hereunder. The 30 RAPD primers initially screened for their polymorphism of which 18 responded and found polymorphic. The resolving power (Rp), polymorphic information content (PIC), effective multiplex ratio (EMR), marker index (MI) and other diversity indices of each RAPD primer was calculated and is given in the Table 2. The amplicon size ranged from 100 to 3200 bp among the studied RAPD primers. The 18 RAPD primers amplified 242 amplicons among the wild *Malus* genotypes with an average of 13.44 amplicons for each RAPD primer. The RAPD SBSC-08 amplified maximum number of amplicons (18), while minimum (7) in SBSC-20. The Rp values ranged from 2.000 (SBSC-20) to 9.000 (SBSC-08) with an average of 5.829. The highest PIC value (0.403) was obtained with primer SBSD-18 and lowest (0.219) in SBSC-20 primer with an average of 0.326. The EMR of RAPD primers ranged from 4.571 (SBSC-20) to 15.667 (SBSE-03) with an average of 8.432. The MI value among the RAPD primers was 1.001 (SBSC-20) to 6.256 (SBSE-15) with an average of 2.851 among the 18 RAPD primers. In the previous studies, Zhou and Li (10) recorded 41 polymorphic amplicons using 8 primers with an average of 5.13 amplicons for each primer on 14 *Malus* species. Similarly, Erturk and Akcay (2) calculated 180 polymorphic amplicons using 38 RAPD primers with an average of 4.74 amplicons for each primer among the apple accessions. The results of the previous studied illustrated lower number average polymorphic amplicons in comparison to the result of present investigation. Furthermore, PIC and Rp values of the present investigation is similar to that of Tiwari *et al*. (8) working in *Andrographis paniculata* using the RAPD markers. Thus, observing the considerable number of amplicons for each RAPD marker and their considerable diversity indices, it suggests that these RAPD primer sets are suitable for assessing diversity analysis of wild *Malus* genotypes.

To determine the phylogenetic relationship and genetic diversity among the wild *Malus* species, Jaccard's similarity coefficient was calculated. Its values ranged from 0.66 to 0.90 among the studied wild *Malus* genotypes. The UPGMA based dendrogram clustered the *Malus* genotypes in to two major clusters (Fig. 1). The cluster A was further divided into two sub-clusters. The sub-cluster A-I had only the genotype, *Malus zumi*, while sub-cluster A-II contained the genotypes, *M*. *simcoe*, *M*. *sikkimensis* (M), *M*. *sieversii*, *M*. *spectabilis*, *M*. *sieboldii*. The cluster B was divided into two sub-clusters. The sub-cluster B-I constituted by the genotypes, *M*. *sargentii*, *M*. *purpurea, M*. *pumila*, *M*. *prunifolia* (Maruba), *M*. *baccata* (Shillong), *M*. *baccata* var. *mandshurica* (P), *M*. *baccata* (USA), *M*. *baccata* (Rohru), *M*. *esseltine*, *M*. *baccata* (Pangi), *M*. *baccata* (Kinnaur), *M*. *baccata* (Kashmir B), *M*. × *domestica* cv. Golden Delicious, *M*. *baccata* (Kashmir A), *M*. *sikkimensis* (P), *M*. *micromalus* (Nagasaki zumi), *M*. *baccata* var. *mandshurica* (D), *M*. *hilleiri*, *M*. *prunifolia* var. Ringo-Assami, *M*. *orientalis*, *M*. *baccata* (Ladakh), *M*. *baccata* (Khrot), *M*. *baccata* (Dhak), and *M*. *baccata* (Chamba). The sub-cluster B-II contained genotypes, *M*. *micromalus* and *M*. species (Adams). Similarly, based on RAPD data Yan *et al*. (9) and Tiwari *et al*. (8) estimated Jaccard's similarity coefficient ranging from 0.56 to 1.00, 0.26 to 0.83, and 0.59 to 1.00, respectively, while assessing the genetic diversity and phylogenetic relationship among the *Malus*, bamboo and *Andrographis paniculata* genotypes, respectively. Further, UPGMA cluster analysis did not group either the exotic genotype and not the indigenes one. Similarly, Tiwari *et al*. (8) also showed that accessions of *A. paniculata* did not group according to agro-ecological regions, while using the UPGMA dendrogram based on RAPD markers.

The principal coordinate analysis (PCoA) was performed to supplement the results of the cluster analysis (Perrier *et al*., 5). The based on the RAPD data, fist three axes of PCoA explained 40.26% of variations of which first axis accounted for 19.44%, second axis contributed 11.998% and third axis had 8.82% variations (Table 3). The studied *Malus* genotypes including the indigenous and exotic ones did not form any group and they were distributed over the coordinates (Fig. 2), which supplement the results of cluster analysis. AMOVA based on RAPD data revealed that the 2% variation among the *Malus* populations, while 98% variation remained within the population (Table 4, Fig. 3).

The RAPD marker-based population structure analysis differentiated the wild *Malus* genotypes into three genetic populations (Fig. 4). The RAPD based population I, II and III had 16, 9 and 7 wild *Malus* genotypes, respectively (Fig. 5). The mean values of Fsts among the *Malus* population 1 (Fst_1), population 2 (Fst_2) and population 3 (Fst_3) were 0.392, 0.487 and 0.483, respectively; and mean value

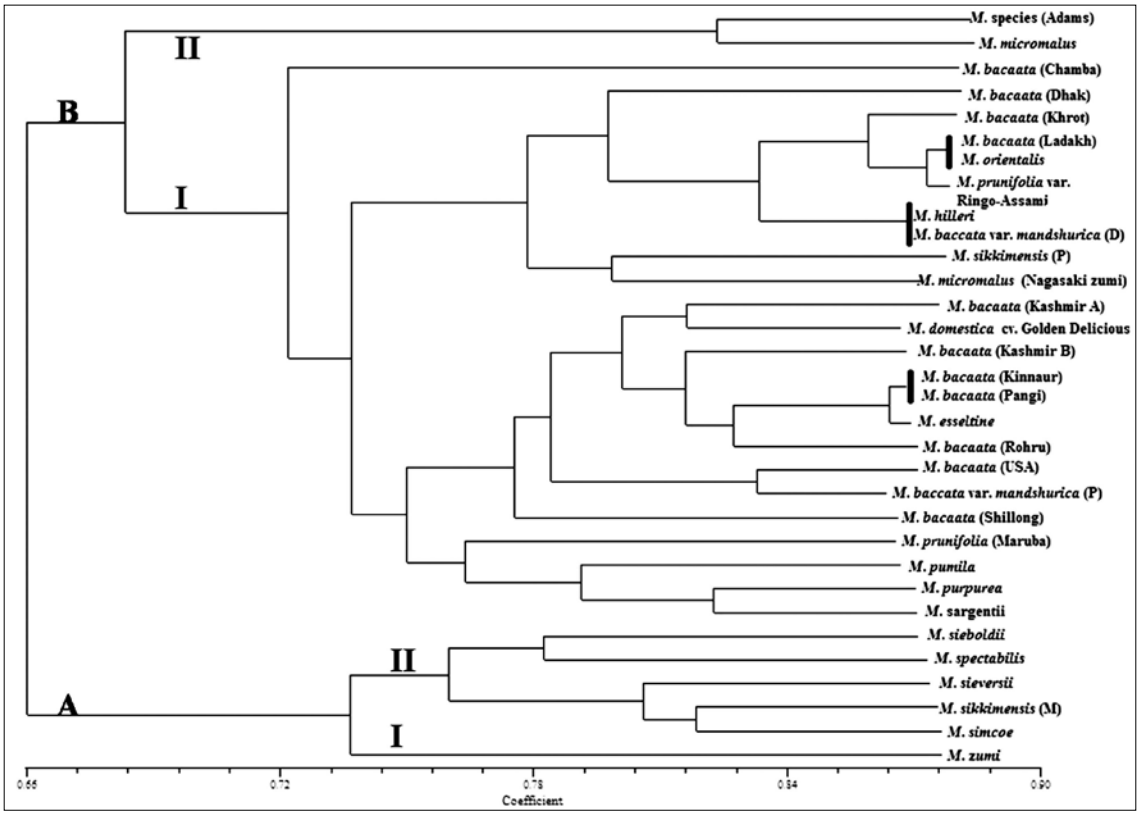

Fig. 1. UPGMA tree based grouping of the 32 *Malus* genotypes using RAPD patterns.

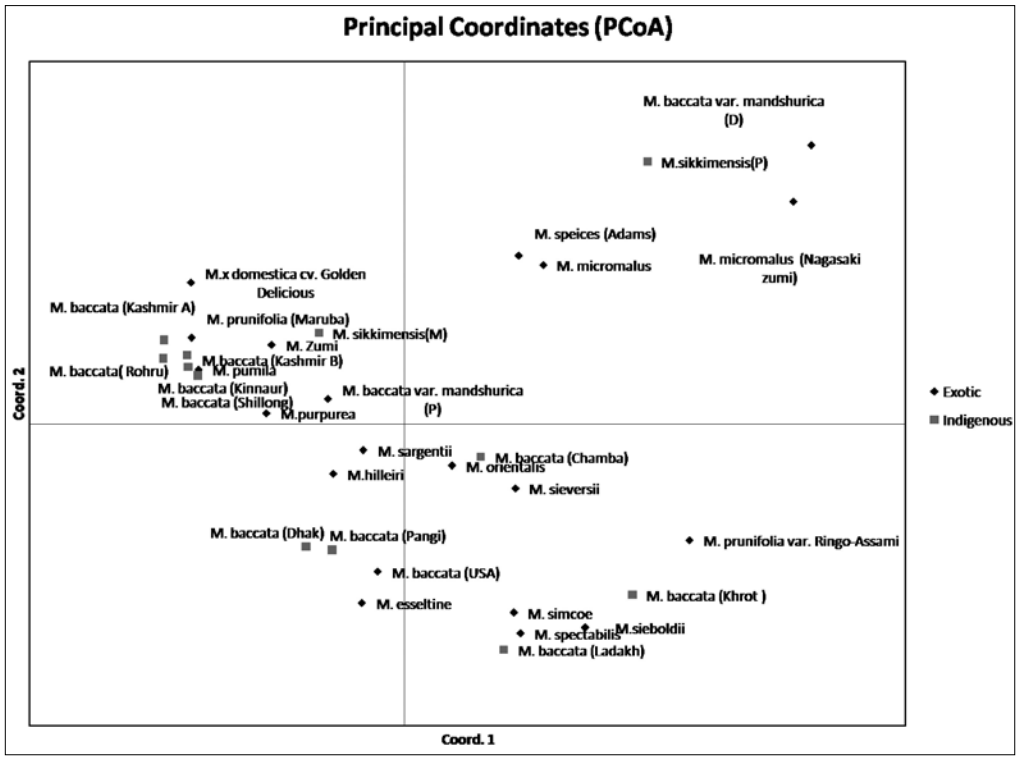

Fig. 2. Principal Coordinate Analysis (PCoA) of *Malus* genotypes based on RAPD patterns.

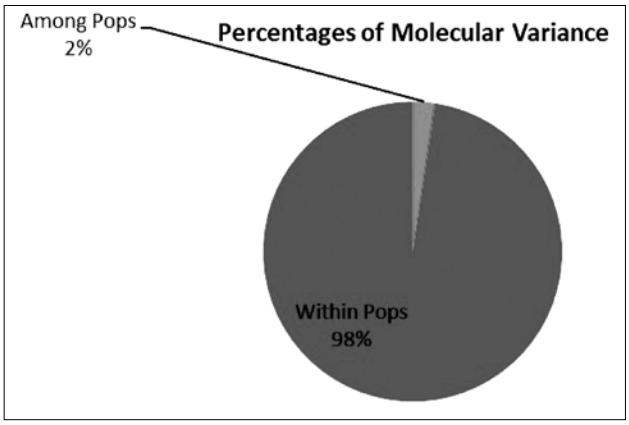

Fig. 3. Analysis of molecular variance (AMOVA) of wild *Malus* genotypes based on RAPD markers.

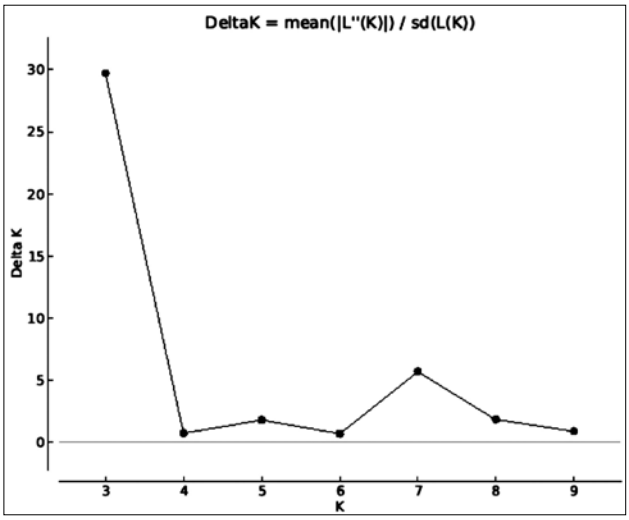

Fig. 4. Estimation of population using LnP(D) derived Δk for k ranging from 2 to 10.

of alpha was 0.097 (Table 5). The allele-frequency divergences among populations of studied *Malus* genotypes were 0.094 between the population I and population II; 0.154 between the population I and population III; 0.141 between the population II and population III (Table 6). In the previous studies, Tiwari *et al*. (8) also differentiated *A. paniculata* population using the model based population structure analysis based on the RAPD data. The present study indicated existence of high allele-frequency divergence (>0.05) between the populations of wild *Malus* genotypes.

It can be concluded that the DNA based RAPD markers could successfully decipher the existing diversity and analyse the population structure in wild *Malus* genotypes. The present study also revealed considerable genetic diversity among

Table 3. Percentage of variation explained by the first three axes using principal coordinate analysis based on RAPD markers.

Particulars	RAPD		
Axis	1	2	3
%	19.441	11.998	8.824
Cum %	19.441	31.440	40.264

Table 4. Analysis of molecular variance (AMOVA) based on RAPD markers among the wild *Malus* genotypes.

Particulars	RAPD	
Source	Among Popln.	Within Popln.
Degree of freedom	1	30
Sum of squares	87.017	1905.983
Est. Var.	1.566	63.533
Total variation (%)	2%	98%

Table 5. Mean value of alpha, Fst1, Fst2 and Fst3 inferred from model based approach using the RAPD data.

Mean value of alpha	0.097
Mean value of Fst_1	0.392
Mean value of Fst_2	0.487
Mean value of Fst_3	0.483

Table 6. Allele-frequency divergence among the wild *Malus* populations computed using estimates of P (Model based approach) based on RAPD data.

Population	Pop1	Pop2	Pop3
Pop1	-	0.094	0.154
Pop2	0.094	-	0.141
Pop3	0.154	0.141	-

the studied wild *Malus* genotypes including both, exotic and indigenous Himalayan crab apples. Thus, the studied *Malus* genotypes could be important genetic resources for the rootstock and scion improvement. Further, the genetic diversity and population structure of the present set of wild apples can be reinvestigated with more reliable and powerful marker system like simple sequence repeats (SSR), which would further make some robust conclusions.

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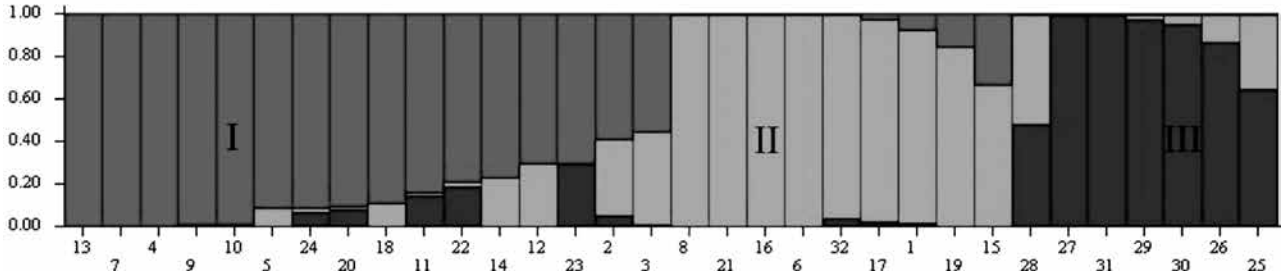

In the bar plot, 1 = *M*. species (Adams), 2 = *M. baccata* (Chamba), 3 = *M. baccata* (Dhak), 4 = *M. baccata* (Kashmir A), 5 = *M. baccata* (Kashmir B), 6 = *M. baccata* (Khrot), 7 = *M. baccata* (Kinnaur), 8 = *M. baccata* (Ladakh), 9 = *M. baccata* (Pangi), 10 = *M. baccata* (Rohru), 11 = *M. baccata* (Shillong), 12 = *M. baccata* (USA), 13 = *M. domestica* cv. Golden Delicious, 14 = *M. esseltine*, 15 = *M. hilleri*, 16 = *M. micromalus*, 17 = *M. baccata* var. *mandshurica* (D), 18 = *M. baccata* var. *mandshurica* (P), 19 = *M. orientalis*, 20 = *M. prunifolia* (Maruba), 21 = *M. prunifolia* var. Ringo-Assami, 22 = *M. pumila*, 23 = *M. purpurea,* 24 = *M*. sargentii , 25 = *M. sieboldii*, 26 = *M. sieversii*, 27 = *M. sikkimensis* (M), 28 = *M. sikkimensis* (P), 29 = *M. simcoe*, 30 = *M. spectabilis*, 31 = *M. zumi*, 32 = *M. micromalus* (Nagasaki zumi).

Fig. 5. Model Based Population Structure Analysis of 32 *Malus* genotypes using RAPD markers.

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