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Analysis of genetic diversity and population structure of the indigenous and exotic wild *Malus* species using ISSR markers

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ABSTRACT

The genetic diversity and population structure studies on 32 wild *Malus* species were conducted using the nine inter simple sequence repeat (ISSR) markers. The average value of diversity indices, viz. resolving power (Rp), polymorphic information content (PIC), effective multiplex ratio (EMR) and marker index (MI) of ISSR markers were 2.389, 0.388, 16.429 and 6.228, respectively, while the Jaccard's similarity coefficient ranged from 0.46 to 0.97. The cluster analysis divided the selected *Malus* species into two major clusters and principal coordinate analysis (PCoA) further reconfirmed the result of the cluster analysis. The first three axes of PCoA explained 43.49% variation and analysis of molecular variance (AMOVA) explained 16% variation between the indigenous and exotic *Malus* populations. The studied *Malus* species were genetically differentiated into four distinct populations which were revealed through the model based population structure analysis. Thus, the present investigation revealed substantial genetic diversity among the studied wild *Malus* species and existing genetic diversity could be valuable genetic resources for future apple improvement programme.

Key words: Crabapples, Genetic diversity, Indigenous, ISSR markers, Wild *Malus* species

The Indian Himalayan range has the rich diversity of the wild *Malus* species (Kishore *et al.* 2005, Rana *et al.* 2007). The wild *Malus* distributed over the Indian Himalayan region is collectively known as indigenous Himalayan crabapples. These crabapples are botanically classified into two *Malus* species, viz., *M. baccata* and *M. sikkimensis* (Hooker 1879, Anon 1962). The crabapples have the resistance for various diseases and pests (Sharma *et al.* 2006) and posses important horticultural traits like low to moderate tree vigour, variation in chilling requirements etc. (Kishor and Randhwa 1993, Kishore *et al.* 2015).

The apple industry in India immediately requires desired gene sources to combat the various biotic and abiotic stresses and thus, indigenous Himalayan crabapples might turn out to be a valuable genetic resource. Various explorations have been undertaken to collect diverse indigenous Himalayan crabapples and number of crabapples had been collected and conserved in the field gene banks of *Malus* germplasm (Randhawa 1987, Dhillon and Rana 2004, Rana *et al.* 2007). A good number of apple germplasm of several exotic *Malus* species have been introduced in India and conserved in different field gene banks along with the indigenous Himalayan crabapples. Still these indigenous and exotic wild *Malus* species have not been adequately studied for their genetic diversity, phylogenetic relationship and population structure. The assessment of genetic diversity can be performed by various means like morphological, agronomical as well as application of biochemical and molecular markers (Höfer *et al.* 2014). The morphological and agronomical parameters are mostly influenced by the environmental variables, while molecular markers are free from the influence of environmental variables (Belaj *et al.* 2007). Among the developed genetic marker systems, inter simple sequence repeat (ISSR) is one of the important marker system for assessing the genetic diversity and study the phylogenetic relationship among the plant genotypes (Thimmappaiah *et al.* 2009). ISSR markers are very efficient and economical in terms of genetic variability analysis since they could identify closely related plant species/genotypes too (Lin *et al.* 2008). Thus, the aim of this study was to assess genetic diversity and population structure of the selected wild *Malus* species using ISSR markers.

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MATERIALS AND METHODS

Plant materials: Thirty-two *Malus* species including 19 exotic wild *Malus* species, 12 indigenous Himalayan wild *Malus* species (*M. baccata* biotypes and *M. sikkimensis*) and one cultivated *Malus* species, i.e. *M.* × *domestica* cv. Golden Delicious were selected for the genetic diversity and population structure analysis. These selected wild *Malus* species are conserved in the *Malus* field gene banks at three different regional stations of the ICAR institutes, viz., ICAR-Indian Agricultural Research Institute Regional Station (ICAR-IARI RS), Amartara cottage, Shimla (Himachal Pradesh), ICAR-National Bureau of Plant Genetic Resources Regional Station (ICAR-NBPGR RS), Phagli (Himachal Pradesh) and ICAR-Central Institute of Temperate Horticulture Regional Station (ICAR- CITH RS), Mukteshwar (Uttarakhand). The details of the 32 *Malus* genotypes along with their source gene bank and introduction or native source are shown in Table 1. The present study was conducted during the 2015-16 and all the laboratory work was done at Division of Fruits and Horticultural Technology, ICAR-Indian Agricultural Research Institute, New Delhi and Div. of Genomic Resources, ICAR-National Bureau of Plant Genetic Resources, New Delhi.

DNA extraction and quantification: The leaves of *Malus* species were collected from their respective field gene banks and stored in the deep freeze (-20ºC) for further extraction of genomic DNA. The genomic DNA from *Malus* species was isolated using CTAB method as described by Doyle and Doyle (1990) with minor modifications. The DNA pellets were air-dried and dissolved in 150 µl of nuclease-free water. To remove the RNA impurities, the diluted DNA was treated with RNase (2.25 U) and then purified using standard procedure. The purified DNA was quantified using spectrophotometer and quality was checked on 0.8% agarose gel. The final concentration of purified DNA was maintained at 20 ng/µl in nuclease-free water as working dilution.

Genotyping with ISSR markers: A total of 25 ISSR primers were screened for their polymorphism, of which nine were found polymorphic for the present set of *Malus* species (Table 2). A 12.5 µl PCR reaction volume was prepared containing 60 ng of DNA template, 1.5 µl of 10 mM deoxynucleotide tri-phosphates (dNTPs), 1.5 µl of 25 mM $MgCl_2$, 1.5 µl of 10 × PCR Buffer 1.5 µl of 5 pmol primer, 1U *Taq*DNA polymerase (Thermo Scientific, USA) and final volume makeup by using nuclease-free water. The PCR cycle was performed with following conditions: initial denaturation at 94ºC for 4 min followed by 36 cycles of denaturation at 94ºC for 1 min, annealing ranges from 45-62ºC for 1 min and extension at 72ºC for 2 min followed by final extension at 72ºC for 7 min. Reactions were stored at 4ºC until electrophoresis and PCR products were visualized using gel documentation system (Alpha Innotech Corporation, USA). 1 Kb DNA ladder (Fermentas, USA) was used as standard.

Statistical analyses: The ISSR marker based amplified PCR products were scored manually for the 32 *Malus* species. Binary data matrix was generated by scoring the

Table 1 The details of *Malus* species, name of the gene bank where this particular *Malus* species were maintained and place or country name from where these were collected, originated or introduced

Species – Name	Gene bank	Introduction/ Native source
M. baccata (USA)	CITH RS	USA
M. baccata var. *mandshurica* (D)	IARI RS	Japan
M. baccata var. *mandshurica* (P)	NBPGR RS	Japan
M. x *domestica* cv. Golden Delicious	IARI RS	USA
M. esseltine	IARI RS	UK
M. hilleiri	NBPGR RS	UK
M. micromalus	CITH RS	USA
M. micromalus (Nagasaki zumi)	IARI RS	Japan
M. orientalis	IARI RS	USSR
M. prunifolia (Maruba)	IARI RS	USSR
M. prunifolia var. Ringo-Assami	IARI RS	USSR
M. pumila	IARI RS	UK
M. purpurea	IARI RS	USSR
M. sargentii	IARI RS	USSR
M. sieboldii	IARI RS	Japan
M. sieversii	NBPGR RS	USA
M. simcoe	IARI RS	Canada
M. species (Adams)	NBPGR RS	Canada
M. spectabilis	IARI RS	USSR
M. zumi	IARI RS	USSR
Species – Name	Gene bank	Place of collection
M. baccata (Chamba)	NBPGR RS	Chamba, H.P. (India)
M. baccata (Dhak)	IARI RS	Dhak, H.P. (India)
M. baccata (Kashmir A)	IARI RS	Srinagar, J&K (India)
M. baccata (Kashmir B)	IARI RS	Kashmir Valley, J&K (India)
M. baccata (Khrot)	IARI RS	Khrot, H.P. (India)
M. baccata (Kinnaur)	IARI RS	Kinnaur, H.P. (India)
M. baccata (Ladakh)	NBPGR RS	Ladhak, J &K (India)
M. baccata (Pangi)	IARI RS	Pangi, H.P. (India)
M. baccata (Rohru)	IARI RS	Rohru, H.P. (India)
M. baccata (Shillong)	IARI RS	Shilling, Meghalaya (India)
M. sikkimensis (M)	CITH RS	Lachen Valley, Sikkim (India)
M. sikkimensis (P)	NBPGR RS	Lachen Valley, Sikkim (India)

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

Table 2. Details of ISSR primers sequences their GC %, number of polymorphic amplicon, Rp, PIC, EMR and MI value among the studied *Malus* species

Primer name	Primer sequence (5'-3')	GC %	Size range	Polymorphic band	Rp	PIC	EMR	MI
UBC807	AGAGAGAGAGAGAGAGT	47.06	250-850	7	3.875	0.357	22.857	8.151
UBC808	AGAGAGAGAGAGAGAGC	52.94	400-750	3	2.688	0.494	17.667	8.730
UBC809	AGAGAGAGAGAGAGAGG	52.94	250-500	3	1.438	0.352	15.000	5.283
UBC 859	TGTGTGTGTGTGTGTGRC	50.00	500-550	2	1.250	0.398	10.000	3.984
UBC 860	TGTGTGTGTGTGTGTGRA	44.44	400-500	2	1.250	0.359	10.000	3.594
UBC 861	ACCACCACCACCACCACC	66.67	200-650	7	3.938	0.398	9.000	3.583
UBC 862	AGCAGCAGCAGCAGCAGC	66.67	400-550	3	2.563	0.482	17.000	8.201
UBC 864	ATGATGATGATGATGATG	33.33	300-800	3	1.000	0.255	26.667	6.806
UBC 865	CCGCCGCCGCCGCCGCCG	100	250-1500	6	3.500	0.393	19.667	7.721
			Mean	4.00	2.389	0.388	16.429	6.228

Where, RP = resolving power, PIC= polymorphic information content, EMR= effective multiplex ratio, MI = markers index.

presence (1) and absence of (0) bands from the gel images induced by the selected primers. The Resolving power (Rp) was estimated as described by Prevost and Wilkinson (1999); Polymorphic information content (PIC) (Lynch and Walsh, 1998); effective multiplex ratio (EMR) and marker index (MI) (Powell *et al.* 1996) were also estimated. NTSYS (Numerical taxonomy and multivariate analysis system) software Version 2.1 (Rohlf 2000) was used to generate the Distance matrix and cluster among the studied *Malus* species. The genetic relationship was analysed by using SIMQUAL (Similarity for qualitative data program in NTSYS) module of Jaccard's similarity coefficient (Jaccard 1908). A dendrogram was generated using UPGMA (Un-weighted Pair Group Method with Arithmetic Mean) with Sequential agglomerative hierarchical and nested clustering method program in NTSYS (SAHN). A model based, population structure was constructed using STRUCTURE software version 2.3.4 (Pritchard *et al.* 2000). The membership of individual species was estimated for range of genetic clusters from value of K = 2-10 using correlated allele frequency and admixture model (Evanno *et al.* 2005). "Structure harvest" -an online available programme (http://taylor0.biology.ucla.edu) was used to study the final population structure. Analysis of Molecular variance (AMOVA) was calculated using the software GenAlEx V6.5 program (Peakall and Smouse 2012).

RESULTS AND DISCUSSION

Descriptive diversity statistics: The 25 ISSR primers were tried initially for confirming their polymorphism among the selected *Malus* species of which nine were polymorphic. The resolving power (Rp), Polymorphic information content (PIC), effective multiplex ratio (EMR), marker index (MI) and other diversity indices of each ISSR primers were calculated and presented in the Table 2. The amplicon size ranged from 200 to 1500 bp among the nine ISSR primers. A total of 36 amplicons were amplified by these nine ISSR primers with maximum (7) in UBC807 and UBC861, while minimum (2) in UBC859 and UBC860 with an average of 4.0 per marker. The Rp value was obtained maximum (3.938) in ISSR primer UBC861, while minimum (1.000) in UBC864 with an average of 2.389 among the nine ISSR primers. The PIC value ranged from 0.255 (UBC864) to 0.494 (UBC808) with an average of 0.388 among the nine ISSRs. The EMR of studied ISSRs ranged from 9.000 (UBC861) to 26.667 (UBC864) with an average of 16.429. The maximum MI value (8.730) recorded in ISSR primer UBC808, while minimum (3.583) was in UBC 861 with an average of 6.228 among the studied ISSRs. In the previous studies, Smolik and Krzysztoszek (2010) reported 128 polymorphic amplicons using the 17 ISSRs with an average of 7.53 amplicons of each primer among the apple cultivars. Dhyani *et al.* (2015) recorded a total of 127 polymorphic amplicons using the 14 ISSRs with an average of 9.07 amplicons of each primer among the delicious group of apple cultivars. The previous studies recorded more number of polymorphic amplicons in comparison to the number of polymorphic amplicons in this study. In contrast, Verma *et al.* (2007) recorded 2.8 polymorphic amplicon for each primer among the *Benincasa hispida* accessions using ISSR markers, which indicated less number of polymorphic amplicons. Further, PIC and Rp values obtained by Tiwari *et al.* (2016) in *Andrographis paniculata* using the ISSR markers were comparable to those obtained in this study. Furthermore, an average of marker index of ISSR primers of this study is congruent with the average marker index obtained by Verma *et al.* (2017) in *Citrullus colocynthis* using the ISSR markers. However, Ariffin *et al.* (2015) recorded an average of PIC, EMR and MI value of 0.27, 4.32 and 1.19 per primer, respectively in diversity studies on *Mangifera indica* using ISSR markers, which was lower than the PIC, EMR and MI values estimated in the present investigation. Thus, the substantial number of polymorphic amplicons and other diversity indices were observed for the tested set of ISSR primers indicate their suitability for the determination of genetic diversity among the wild

Malus species.

Phylogenetic relationship and genetic diversity: The Jaccard's similarity coefficient ranged from 0.46 to 0.97 among the studied *Malus* species. The UPGMA based cluster analysis divided the *Malus* species into two distinct clusters (Fig. 1). Cluster A comprised the species *M. sieboldii* and *M. baccata* (Pangi), which remained separated from other *Malus* species. Cluster B further divided into two sub-clusters; sub-cluster B-I had the species, namely, *M. simcoe*, *M. sikkimensis* (M), *M. zumi*, *M. sieversii*, *M. sargentii*, *M. purpurea* and *M. prunifolia* (Maruba). Cluster B-II contained the species like *M. micomalus* (Nagasaki zumi), *M. pumila*, *M. baccata* var. *mandshurica* (P), *M. baccata* var. *mandshurica* (D), *M. spectabilis*, *M. sikkimensis* (P), *M. Prunifolia* var. Ringo-Assami, *M. micomalus*, *M. orientalis, M. hilleiri*, *M. esseltine*, *M.* × *domestica* cv. Golden Delicious. *M. baccata* (USA), *M. baccata* (Ladakh), *M. baccata* (USA), *M. baccata* (Shillong), *M. baccata* (Rohru), *M. baccata* (Khrot), *M. baccata* (Kashmir B), *M. baccata* (Dhak), *M. baccata* (Kinnaur), *M. baccata* (Kashmir A), *M. baccata* (Chamba) and *M.* species (Adams). Earlier, He *et al.* (2011), Patel *et al.* (2015) and Verma *et al.* (2017) recorded Jaccard's similarity coefficient ranging from 0.70 to 0.94, 0.13 to 0.96, and 0.67 to 0.97 among different accessions of wild apple, *Ocimum* species, *Citrullus colocynthis*, respectively using ISSR markers. Further, the UPGMA cluster analysis based on the ISSR markers divided the *Malus* species into two major clusters. The indigenous wild *Malus baccata* ecotypes got separated into the same clad of the sub-cluster indicating their genetic similarity. Cluster analysis further suggested that the *Malus sikkimensis* (M) was closely related with *M. sieversii*. *Malus sikkimensis* (M) and *M. sikkimensis* (P) were found to be the accessions of the same species. Similar observations were recorded for both the individuals of *M. baccata* var. *mandshurica*. Interestingly, *M. sieversii* did not group with the cultivated apple cultivar Golden Delicious, which might be due to the high genetic diversity within the *Malus sieversii* population (Volk *et al.* 2013).

Principal coordinate analysis (PCoA) and analysis of molecular variance (AMOVA): The first three axes of PCoA explained 43.49% of variation of which first axis accounted 17.46%, second axis contributed 13.18% and third axis had 12.85% variation (Table 3). The exotic and indigenous wild *Malus* species were distributed all over the coordinate, while indigenous *M. baccata* ecotypes formed a group, which indicated their genetic relatedness as well as the genetic distinctness from other *Malus* species (Fig. 2). The analysis of molecular variance was performed to estimate the genetic variation between the indigenous and exotic *Malus*

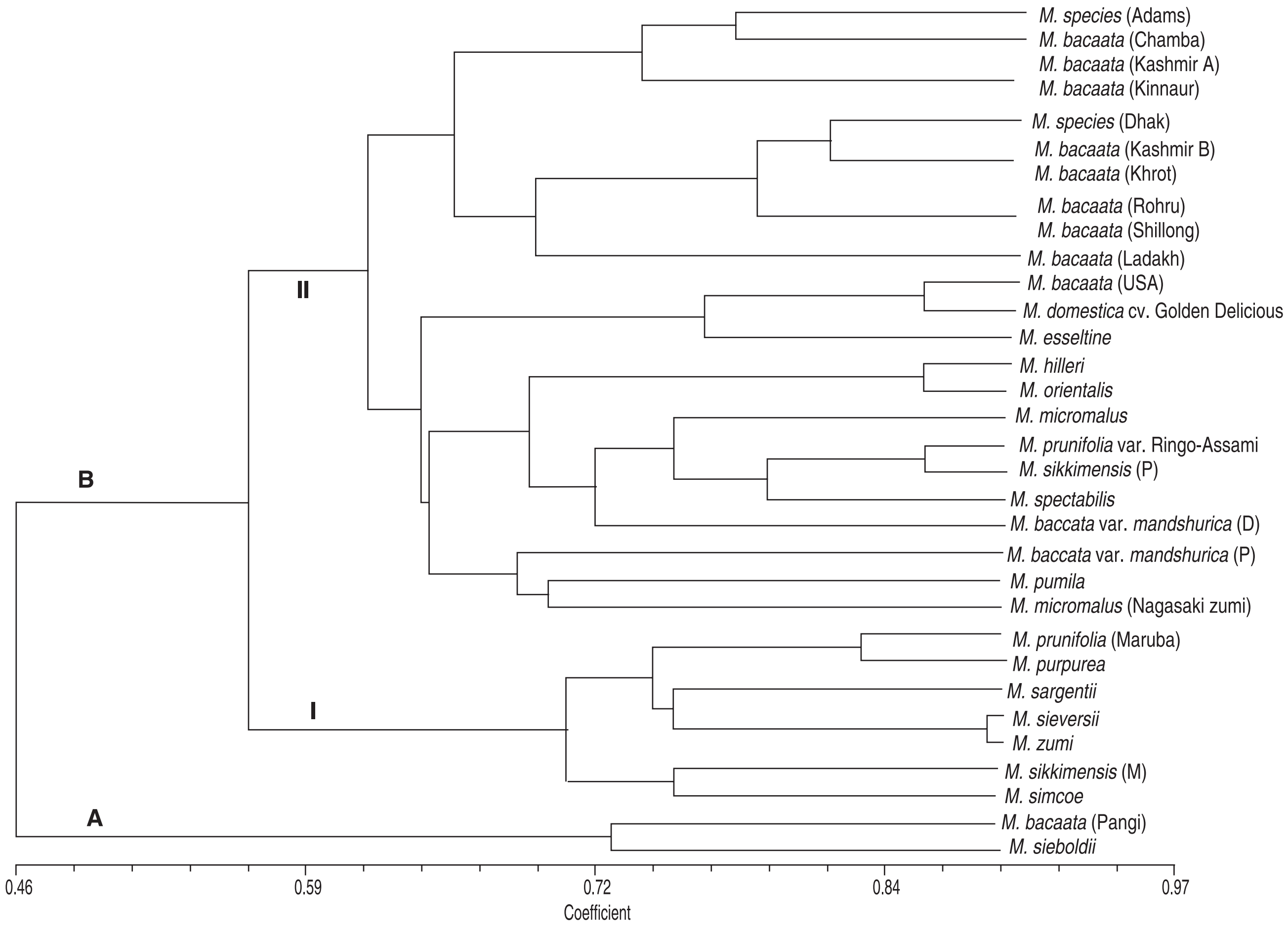

Fig 1. UPGMA tree based grouping of the 32 *Malus* species based on ISSR markers

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

Axis	1	2	3
%	17.455	13.179	12.851
Cum %	17.455	30.634	43.486

populations, which revealed 16% variation among the wild *Malus* population, while 84% variation was present within the population. The PCoA was performed to supplement the results obtained through cluster analysis (Perrier *et al.* 2003). The separation of indigenous wild *Malus* species further indicated that they are genetically unique from other exotic wild *Malus* species. Furthermore, the three axes of PCoA explained the substantial genetic variation existing among the studied *Malus* species. Furthermore, AMOVA also explained the substantial molecular variance existing between the indigenous and exotic wild *Malus* species.

Model based population structure analysis: The Evanno method (ΔK value) was used for the determination of the number of population among the wild *Malus* species using the ISSR markers. The ISSR markers based population structure analysis differentiated the wild *Malus* species in to four genetic populations. The populations, namely, I, II, III and IV contained 10, 8, 10 and 4 *Malus* species respectively (Fig. 3). The mean values of Fsts among the *Malus* population I (Fst_1), population II (Fst_2), population III (Fst_3) and population IV (Fst_4) were 0.626, 0.561, 0.411 and 0.769 respectively; and mean value of alpha was 0.095. The allele-frequency divergence among populations of studied *Malus* species were 0.268 between the population I and II; 0.178 between the population I and III; 0.341 between the population I and IV; 0.195 between the population II and III; 0.372 between the population II and IV; and 0.317 between the population III and IV. Similarly, Wang *et al.* (2012) used population structure analysis and genetically differentiated the *Rheum officinale* accessions into three populations using ISSR markers. Tiwari *et al.* (2016) also differentiated the *Andrographis paniculata* population using the model based population structure using ISSR data, while Nilkanta *et al.* (2017) differentiated the *Melocanna baccifera* populations based on structure analysis using ISSR markers.

The DNA based ISSR markers were successfully explored for the diversity assessment and population structure analysis of wild *Malus* species. The present study deciphered that the

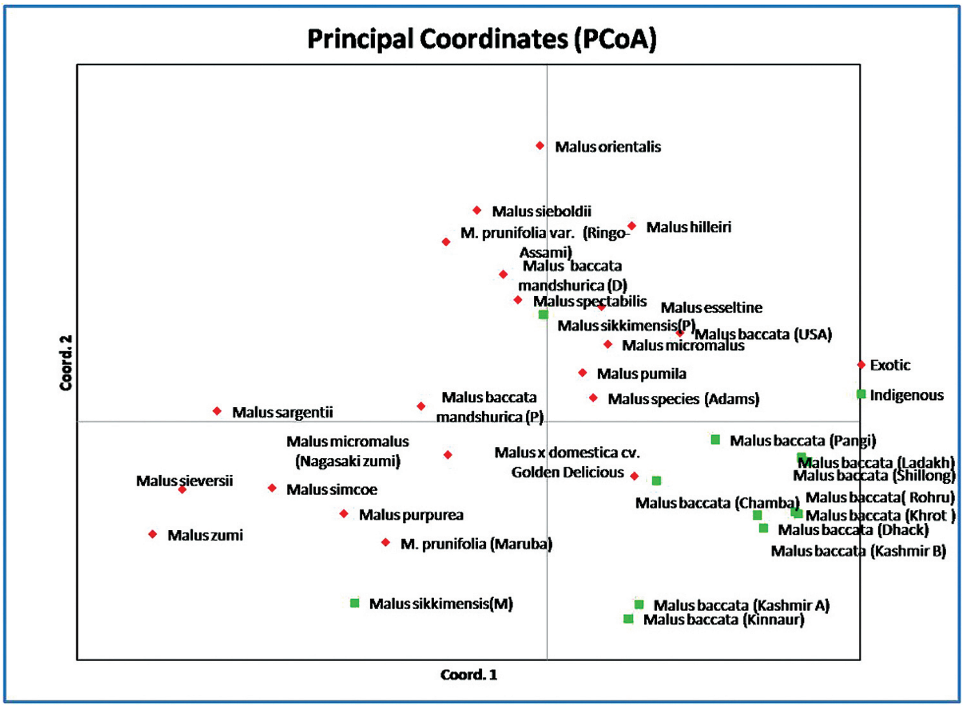

Fig 2. Principal Coordinate Analysis (PCoA) of 32 *Malus* species based on the based on ISSR

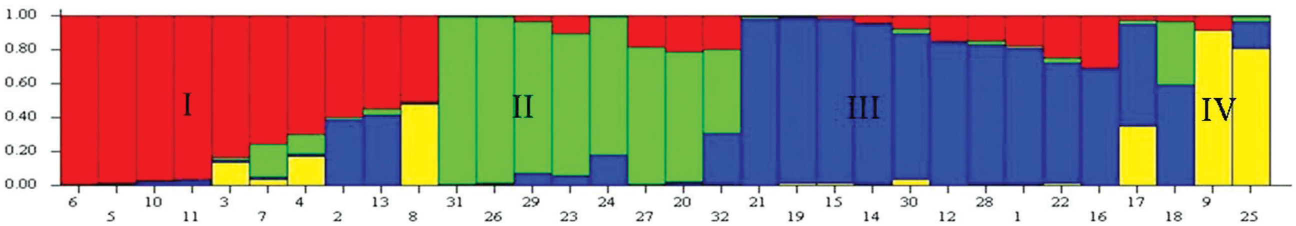

Fig 3. Model based population structure analysis of 32 *Malus* species based on ISSR markers Red = Popln. I, green = Popln. II, blue = Popln. III, Yellow= Popln. IV. 1= *Malus* species (Adams), 2= *M. baccata* (Chamba), 3= *M. baccata* (Dhak), 4= *M. baccata* (Kashmir A), 5 = *M. baccata* (Kashmir B), 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).

indigenous *Malus baccata* genetically separated from other *Malus* species owing to their uniqueness. Thus, the studied wild *Malus* species could be an important genetic resource for the apple improvement programmes.

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