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

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Received: 13 November 2018; Accepted: 21 January 2019

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 possess 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 (Thimmappiah 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.
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 MgCl₂, 1.5 µl of 10 × PCR Buffer 1.5 µl of 5 pmo1 primer, 1U TaqDNA 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

| Species – Name | Gene bank | Place of collection |
|----------------|-----------|---------------------|
| M. baccata (Chamba) | IARI 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 (Kinnaul) | IARI RS | Kinnaul, H.P. (India) |
| M. baccata (Ladakh) | NBPGR RS | Ladakh, 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 | Shillong, 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      | AGAGAGAGAGAGAGAGAGT     | 47.06| 250-850    | 7                | 3.875 | 0.357 | 22.857| 8.151 |
| UBC808      | AGAGAGAGAGAGAGAGAGC     | 52.94| 400-750    | 3                | 2.688 | 0.494 | 17.667| 8.730 |
| UBC809      | AGAGAGAGAGAGAGAGGAGG    | 52.94| 250-500    | 3                | 1.438 | 0.352 | 15.000| 5.283 |
| UBC 859     | TGTGTGTGTGTGTGTRG       | 50.00| 500-550    | 2                | 1.250 | 0.398 | 10.000| 3.984 |
| UBC 860     | TGTGTGTGTGTGTGTRA       | 44.44| 400-500    | 2                | 1.250 | 0.359 | 10.000| 3.594 |
| UBC 861     | ACCACACACACACACACC      | 66.67| 200-650    | 7                | 3.938 | 0.398 | 9.000 | 3.583 |
| UBC 862     | AGCACGCACGACGCACGC      | 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     | CCGCCGCGCCGCCGCCCGCGC   | 100  | 250-1500   | 6                | 3.500 | 0.393 | 19.667| 7.721 |

Mean = 4.00

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

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 minimum (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 Krzysztofszek (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 *Citrus cololychnis* 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
ANALYSIS OF *MALUS* SPECIES USING ISSR MARKERS

July 2019

**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*
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.
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.

ACKNOWLEDGEMENTS

The senior author duly acknowledges the Post Graduate School, Indian Agricultural Research Institute, New Delhi for providing facilities and Department of Science and Technology, Govt. of India for providing INSPIRE Fellowship. Authors duly acknowledge to Scientists In-charge, ICAR-IARI Regional Station, Shimla, ICAR-NBPGR Regional Station, Phagli, Shimla and ICAR-Central Institute of Temperate Horticulture Regional Station, Mukteshwar, Uttarakhand, for sparing the germplasm.

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