Molecular markers based genetic relatedness studies in tissue culture propagated japanese plum cultivars Santa Rosa and Frontier

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Abstract  Santa Rosa and Frontier are the major Japanese plum (*Prunus salicina* Lindl.) cultivars grown throughout the world. The present investigation was performed to understand the genetic relatedness among in vitro propagated plum cultivars Santa Rosa and Frontier using PCR based molecular markers. For the study, three arbitrary markers viz. RAPD (Random amplified Polymorphic DNA), ISSR (Inter-Simple Sequence Repeats) and SCoT (Start Codon Targeted) were used. In RAPD analysis, 18 primers out of 28 amplified and generated 33 scorable bands. The allelic variations when analysed, revealed 84% similarity between these two cultivars with highest polymorphic information content of 0.78. Similarly, 15 ISSR primers produced 73 amplicons with an average of 4.86 amplicon per primer and similarity coefficient ranging from 62 to 67%. Seven SCoT primers out of 26 resulted in a total of twenty-six scorable bands with 24 polymorphic bands. Cluster analysis from all the three markers used broadly divided plum cultivars Santa Rosa and Frontier into two major clusters containing in vitro shoots, their progenies and mother trees of respective genotypes. The study concluded that these three marker systems were found to be effective in revealing genetic relationship of these two commercially important plum cultivars.

Introduction

Plums are widely grown in most of the temperate regions of the world. Plum belongs to family *Rosaceae*, genus *Prunus* and subgenus *Prunophora*. Most of the commercial varieties of plum are divided into European (*Prunus domestica*; hexaploid) and Japanese (*Prunus salicina*; diploid) type (Okie and Weinberger 1996). Japanese plum is a dominant species with broad adaptation from temperate to subtropical regions of the world. Though it is indigenous to China but was refined in Japan and United states (Okie and Hancock 2008). All the cultivars of plum planted today were developed by Luther Burbank during 19th century by crossing *P. salicina* Lindl. with *P. simonii* Carriere, *P. cerasifera* Ehrh., *P. americana* Marshall and many American species of plum (Howard 1945). The great variability among plum cultivars is due to poly-specific breeding and natural outcrossing. Plums are self-incompatible hence it is difficult to elucidate if cultivars are pure species or hybrids. Santa Rosa and Frontier (Fig. 1) are two prime cultivars of Japanese plum planted commercially in India (Thakur et al. 2020) whose genetic relationship is not known.

Awareness of genetic diversity and relatedness is the key in designing methods for conservation of species for the use in breeding programmes. Molecular
markers have proved to be a productive tool in deciphering genetic relatedness in plants (Agarwal et al. 2008). All the data obtained from these markers provide details on population structure, relation and inbreeding (Dobson et al. 1998; Surridge et al. 1999). Recently, we revealed the genetic fidelity of tissue culture propagated plum cvs. Santa Rosa and Frontier using RAPD, ISSR & SCoT markers (Thakur et al. 2018, 2020, 2021). The outcome of these studies was used to elucidate genetic relatedness between these two commercial plum cultivars for their future use in breeding and development of new cultivars.

Materials and methods

Fresh leaves were collected randomly from axenic shoot cultures of Santa Rosa and Frontier, two years old hardened plants and their mother trees maintained in the Department of Biotechnology and in the fields of Horticultural Research and Training Station & Krishi Vigyan Kendra, Kendaghat, Solan (HP) India for DNA extraction (Fig. 1). Leaves were macerated in 1.2 ml extraction buffer (65°C) and DNA was isolated following Doyle and Doyle (1987) protocol. Purification of DNA was carried out by successive RNase treatment followed by treatment of phenol: chloroform: Isoamyl alcohol (25:24:1) and centrifugation at 12,000 rpm for 10 min. The DNA pellet formed was mixed in TE buffer for storage till further use. Quantification of DNA was performed using nanodrop spectrophotometer (BioPhotometer) at 260 nm. Each sample was diluted to 12.5 ng/μl in 1X TE buffer and stored at 4°C.

PCR amplifications were executed in 10 μl PCR reaction containing 1X PCR buffer, 1.5 mM MgCl₂, 200 μM dNTPs, 0.3 U Taq DNA polymerase, 1.0 μM primer and 50ng DNA. DNA was amplified in MJ Mini Personal Thermal cycler (BioRAD, Singapore). PCR amplification included initial denaturation at 94°C for 3-4 min followed by 35 cycles of initial denaturation at 94°C, for 1 min, annealing depending upon Tm of the primer for 1 min, extension at 72°C for 2 min and final extension at 72°C for 5-7 min. The amplified products were mixed with 6X loading dye and then electrophoresed at 1.5% agarose gel and stained with ethidium bromide using 1X TAE buffer. Each reaction was repeated twice. Electrophoretic separations were conducted at 80 mA (70 V) for 1-2 h and gels were photographed using gel documentation system (Alpha Imager, CA).

Reproducible and well-defined amplicons were scored for their presence (1) or absence (0) through visual observations. All the diversity parameters i.e. number of effective alleles (Ne), Shanon’s information index (I), total genetic diversity (Ht) and Nei’s expected heterozygosity (h) were determined using POPGENE version 1.31 (Yeh et al. 1999). Dendrograms were constructed by Jaccard’s genetic similarity matrix to display cultivars relationship using the unweighted pair group method with arithmetic mean (UPGMA) from data generated using NTSYSpc 2.2 software.

Results and discussion

There is very limited genetic information on plum species (Esmenjaud and Dirlewanger 2007) required for plum breeding which happens to be slow due to high degree of heterozygosity, self-incompatibility and long juvenile phase (Guerra and Rodrigo 2015). In this study, resolving power of RAPD, ISSR and SCoT was used to confirm the genetic relatedness in plum cvs. Santa Rosa and Frontier. The relationship within and between two cultivars was drawn by cluster analysis from binary data generated through these marker systems. For RAPD analysis eighteen RAPD primers out of 28 resulted in 33 scorable bands ranging from minimum of 1 in primers OPA 08, OPB 01, OPB 06, OPB 07, OPB 08, OPB 10, OPE 04 and OPE 06 to a maximum of 5 in OPC 02. All the amplified products ranged from 100 to 1500 bp. Genetic similarity between the two cultivars ranged from 0.74 to 0.94 having an average of 0.84 indicating very low level of polymorphism. Similarly, the Nei’s gene diversity ranged from 0 to 0.37 depicting very high similarity between the two cultivars. The highest PIC value of
Fig. 1 Fruits, in vitro multiplying shoots and hardened plants of Plum cvs. Santa Rosa (a–c) and Frontier (d–f)
RAPD primers was 0.78, indicating the efficiency of these primers in genetic diversity studies (Table 1). Shimada et al. (1999) reported a polymorphism of 24% while studying the genetic variations in plum commercial genotypes through RAPD markers, which is nearly similar to our studies. In the past RAPD analysis has proved useful for classification (Ortiz et al. 1997; Casas et al. 1999), origin and parentage analysis of many plum cultivars (Heinkel et al. 2000).

ISSR markers are effective in distinguishing DNA polymorphism and for identification and conservation of genetic resources in plum (Goulao et al. 2001; Liu et al. 2007; Carrasco et al. 2012). During ISSR analysis, all of 15 ISSR primers used for the study amplified the genomic DNA generating 73 detectable bands which ranged from 3 to 8. Minimum of 3 bands were amplified by ISSR 825, ISSR 841, ISSR 845 and ISSR 873 whereas, maximum of 8 bands was produced through ISSR 807, having an average of 4.86 bands per primer ranging from 100 to 3000 bp size. The similarity coefficient between the two cultivars ranged from 0.62 to 0.67 and Shannon’s information index was between 0 and 0.67 depicting low genetic diversity. ISSR markers have also been utilized for studying the genetic variability of many plum varieties of Southern China (Wu et al. 2019). In SCoT analysis seven out of 26 primers resulted in amplification of genomic DNA ranging from 200 to 2000 bp having genetic similarity of 0.67 to 0.89 and Nei’s gene diversity index ranging from 0 to 0.25.

All the three markers were effective in distinguishing the two genotypes as majority of the amplicons generated were polymorphic (0.75%) amongst both the genotypes. However, the dendrogram pertaining to the above data broadly divided these two cultivars into two major clusters containing in vitro shoots, their progenies and mother trees of respective cultivars (Fig. 2) which depicted a genetic similarity of 0.65 to 0.84 between plum cvs. Santa Rosa and Frontier. Amongst all the three markers used for the study, SCoT were found to be more efficient having highest PIC (0.88) and Rp value (15.09) followed by ISSR having highest PIC value of 0.86. Many RAPD primers had the lowest PIC and Rp values. On the contrary low polymorphism has been reported by SCoT markers in date palm (Qurainy et al. 2015).

Plums are self-incompatible, therefore in many cases it becomes tough to determine if cultivars are pure species. Many of the varieties cultivated today are hybrids with unknown parents. Previously, a close genetic relationship between Plum cvs. Golden Japan and Santa Rosa was derived by Hend et al. (2009) with the aid of RAPD markers, the information regarding which was useful for the improvement of this crop. Santa Rosa is a complex hybrid developed by Luther Burbank (Athanasiadis et al. 2013) and is a parent to many other varieties of plum including Frontier (Jonathan, 1993) that is justified by our studies showing a molecular relatedness between them indicating a common lineage.

Conclusions

The present investigation concluded that Japanese plum cultivars Santa Rosa and Frontier are related to each other in their genetic makeup which was revealed using RAPD, ISSR and SCoT markers. The outcome of the results suggests that both of these plum cultivars have a common ancestry.
| Primer | Primer code | Primer sequence | Tm (°C) | No. of amplicons | No. of polymorphic bands | Fragment size range (bp) | PIC | Rp | E | MI | ne | h | I | Ht |
|--------|-------------|-----------------|---------|-----------------|--------------------------|--------------------------|-----|----|---|----|----|----|----|----|
| RAPD   | OPA 03      | AGTCAGCCAC      | 28.90   | 2               | 2                        | 100–400                  | 0.50 | 1.57 | 0.043 | 0.015 | 1.00 | 0.00 | 0.00 | 0.00 |
|        | OPA 08      | GTGACGTAGG      | 28.90   | 1               | 0                        | 600                      | 0.00 | 0.00 | 0.000 | 0.000 | 1.00 | 0.00 | 0.00 | 0.00 |
|        | OPA 13      | CAGCAACCAC      | 33.00   | 2               | 2                        | 400–600                  | 0.50 | 3.13 | 0.043 | 0.015 | 1.00 | 0.00 | 0.00 | 0.00 |
|        | OPA 14      | TCTGTGCTGG      | 28.90   | 3               | 3                        | 300–1000                 | 0.67 | 3.00 | 0.065 | 0.023 | 1.00 | 0.00 | 0.00 | 0.00 |
|        | OPA 15      | TCCGAACCCC      | 28.90   | 3               | 3                        | 600–1500                 | 0.67 | 5.74 | 0.065 | 0.023 | 1.00 | 0.00 | 0.00 | 0.00 |
|        | OPA 18      | AGGTGACCCTT     | 28.90   | 2               | 2                        | 200–300                  | 0.50 | 3.83 | 0.043 | 0.015 | 1.00 | 0.00 | 0.00 | 0.00 |
|        | OPB 01      | GTTTTCGCTCC     | 28.90   | 1               | 1                        | 100                      | 0.00 | 1.00 | 0.022 | 0.008 | 1.00 | 0.00 | 0.00 | 0.00 |
|        | OPB 03      | CATCCCCCTG      | 33.00   | 2               | 0                        | 100–300                  | 0.50 | 0.00 | 0.000 | 0.000 | 1.00 | 0.00 | 0.00 | 0.00 |
|        | OPB 06      | TGCTCTGCCC      | 33.00   | 1               | 0                        | 400                      | 0.00 | 0.00 | 0.000 | 0.000 | 1.00 | 0.00 | 0.00 | 0.00 |
|        | OPB 07      | GTGTGACCGAG     | 33.00   | 1               | 0                        | 500                      | 0.00 | 0.00 | 0.000 | 0.000 | 1.00 | 0.00 | 0.00 | 0.00 |
|        | OPB 08      | GTCACACCGG      | 33.00   | 1               | 0                        | 300                      | 0.00 | 0.00 | 0.000 | 0.000 | 1.00 | 0.00 | 0.00 | 0.00 |
| ISSR   | ISSR 807    | AGAGAGAGAGAGAGAGT | 50.36   | 8               | 3                        | 100–800                  | 0.86 | 3.00 | 0.065 | 0.049 | 1.44 | 0.30 | 0.48 | 0.30 |
| Primer code | Primer sequence                  | Tm (°C) | No. of amplicons | No. of polymorphic bands | Fragment size range (bp) | PIC | Rp  | E   | MI  | ne  | h   | I   | Ht  |
|-------------|----------------------------------|---------|------------------|--------------------------|--------------------------|-----|-----|-----|-----|-----|-----|-----|-----|
| ISSR 812    | GAGAGAGAGAGAGAGAA                | 50.36   | 7                | 4                        | 100–800                  | 0.84| 4.96| 0.087| 0.066| 1.51| 0.34| 0.52| 0.34|
| ISSR 817    | CACACACACACACACAA                | 50.36   | 6                | 1                        | 200–900                  | 0.83| 1.00| 0.022| 0.016| 1.18| 0.15| 0.29| 0.15|
| ISSR 818    | CACACACACACACACAG                | 52.77   | 6                | 2                        | 100–800                  | 0.83| 3.83| 0.043| 0.033| 1.03| 0.03| 0.08| 0.03|
| ISSR 825    | ACACACACACACACACT                | 50.36   | 3                | 3                        | 900–3000                 | 0.64| 4.74| 0.065| 0.049| 1.45| 0.31| 0.49| 0.31|
| ISSR 827    | ACACACACACACACAGG               | 52.77   | 4                | 0                        | 200–1000                 | 0.75| 0.00| 0.000| 0.000| 1.00| 0.00| 0.00| 0.00|
| ISSR 835    | AGAGAGAGAGAGAGAGCTC             | 56.67   | 7                | 5                        | 200–1000                 | 0.84| 5.91| 0.109| 0.082| 1.70| 0.41| 0.60| 0.41|
| ISSR 840    | GAGAGAGAGAGAGAGACTT             | 54.51   | 4                | 4                        | 300–700                  | 0.72| 5.83| 0.087| 0.066| 1.61| 0.38| 0.57| 0.38|
| ISSR 841    | GAGAGAGAGAGAGAGACTC             | 56.67   | 3                | 0                        | 300–700                  | 0.67| 0.00| 0.000| 0.000| 1.00| 0.00| 0.00| 0.00|
| ISSR 845    | CTCTCTCTCTCTCTCTAGG             | 56.67   | 3                | 1                        | 300–700                  | 0.64| 1.00| 0.022| 0.016| 1.38| 0.28| 0.45| 0.28|
| ISSR 847    | CACACACACACACACAAGC             | 56.67   | 4                | 3                        | 200–500                  | 0.72| 2.87| 0.065| 0.049| 1.91| 0.48| 0.67| 0.48|
| ISSR 868    | GAGAGAGAGAGAGAGAA               | 46.85   | 5                | 4                        | 300–700                  | 0.78| 4.00| 0.087| 0.066| 1.92| 0.48| 0.67| 0.48|
| ISSR 873    | GACAGACAGACAGACA                | 58.39   | 3                | 3                        | 300–1000                 | 0.67| 5.87| 0.065| 0.049| 1.00| 0.00| 0.00| 0.00|
| ISSR 880    | GGAGAGAGAGAGAGA                | 50.57   | 6                | 2                        | 200–700                  | 0.82| 2.00| 0.043| 0.033| 1.38| 0.28| 0.45| 0.28|
| ISSR 888    | CGTATCGTCACACACACACACA          | 62.43   | 4                | 4                        | 300–800                  | 0.73| 6.83| 0.087| 0.066| 1.29| 0.22| 0.38| 0.22|
| SCoT S1     | CAACAATGCGTACACCACCA            | 60.6    | 2                | 2                        | 800–1200                 | 0.50| 3.30| 0.043| 0.025| 1.00| 0.00| 0.00| 0.00|
| SCoT S3     | CAACAATGCGTACACCACCG           | 62.7    | 9                | 9                        | 200–2000                 | 0.88| 15.09| 0.196| 0.111| 1.33| 0.25| 0.41| 0.25|
| SCoT S4     | CAACAATGCGTACACCACCT           | 58.5    | 5                | 3                        | 200–1200                 | 0.79| 4.65| 0.065| 0.037| 1.30| 0.23| 0.39| 0.23|
| SCoT S7     | CAACAATGCGTACACCAGG           | 62.7    | 2                | 2                        | 500–1200                 | 0.50| 3.30| 0.043| 0.025| 1.00| 0.00| 0.00| 0.00|
| SCoT S11    | AAGCAATGCGTACACCA              | 61.8    | 2                | 2                        | 600–2000                 | 0.50| 3.39| 0.043| 0.025| 1.00| 0.00| 0.00| 0.00|
| SCoT S12    | AAGCAATGCGTACACCG              | 63.5    | 5                | 5                        | 300–1200                 | 0.80| 8.48| 0.109| 0.062| 1.15| 0.13| 0.25| 0.13|
Declarations

Conflict of interest Authors declare no conflict of interest.

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Table 1 continued

| Primer sequence code | SSS | Tm (°C) | Primer sequence | Primer code | Fragment size range (bp) | No. of polymorphic bands | No. of amplicons | PIC | Rp | E | MI | Ne | h | I | Rp | Em | Ht | Rp | E | MI | Ne | h | I | Rp | Em | Ht |
|----------------------|-----|---------|-----------------|--------------|-------------------------|--------------------------|---------------------|-----|-----|---|----|----|---|---|----|----|----|---|----|----|---|---|----|----|----|---|----|----|----|
| CATG CACCACGCCACCCOGG | 71.0 | 3.30 | 500 | 1 | 1 | 1 | 2.20 | 0.00 | 1.78 | 0.0022 | 0.0912 | 1.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Mean | 3.30 | 2.20 | 310.00 | 1 | 1 | 1 | 2.20 | 0.00 | 3.15 | 0.0384 | 0.0282 | 1.19 | 0.13 | 0.20 | 0.20 | 0.13 |

PIC = polymorphic information content; Rp: resolving power; E: effective multiplex ratio; MI: marker index; h = Nei’s gene diversity; I = Shannon’s information index; ne = effective number of alleles; Ht = variability within treatments.
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