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# Assessing genetic diversity among 'Bhagwa' like pomegranate (*Punica granatum* L.) genotypes by Inter Simple Sequence Repeat (ISSR) Markers

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## Abstract

In the present study, molecular characterization of pomegranate cultivar 'Bhagwa' and 'Bhagwa' like genotypes available in Maharashtra of India was undertaken along with two other cultivars, Phule Arakta and Mridula to assess their authenticity and diversity. All the 14 ISSR primers used were found polymorphic producing a total of 171 reproducible amplicons with an average of 12.5 amplicons per primer, out of which 144 amplicons (90.28 %) were polymorphic. The similarity coefficient between the genotypes varied from 0.00 to 0.94. Maximum similarity coefficient (0.94) was observed among four different genotypic combinations and high similarity coefficient (≥ 0.90) was observed among 18 different genotype combinations indicating that these genotypes are least divergent from each other. The UPGMA based cluster analysis using dice similarity coefficient grouped 19 pomegranate genotypes into two major clusters. The closely related genotypes of 'Bhagwa' cultivar of pomegranate could be clearly differentiated from cultivars Phule Arakta and Mridula.

**Key words:** *Punica granatum*, pomegranate, Bhagwa, ISSR, genetic diversity, molecular characterization

## Introduction

Pomegranate (*Punica granatum* L.) belongs to the family Punicaceae and is considered to be the native to Iran. India is the world's largest producer of pomegranate fruits followed by Iran. It is cultivated in other countries like Turkey, Spain, Tunisia, Morocco, Afghanistan, China, Greece, Japan and France.

In India, Maharashtra is the leading producer of pomegranate followed by Karnataka, Andhra Pradesh, Gujarat and Tamil Nadu. In Maharashtra, it is cultivated commercially in Solapur, Satara, Sangli, Nashik, Ahmednagar, Malegaon, Dhule, Pune, and Aurangabad. In fact these are the important pockets where pomegranate is grown on large scale. The cultivars *viz*., 'Ganesh', 'Mridula', 'Phule Arakta' and 'Bhagwa' are the main cultivars grown in Maharashtra. 'Bhagwa' is a leading cultivar having high acceptance in European market. 'Bhagwa' cultivar is originally a selection from segregating progeny of the crosses between 'Ganesh' and 'Gul Shah Red' (Russian cultivar) made at Horticultural Farm, Mahatma Phule Krishi Vidyapeeth, Rahuri. It was recommended for cultivation in 2005.

Since then, many progressive farmers from commercial pomegranate growing pockets of Maharashtra, came up with 'Bhagwa' like genotypes showing minute morphological differences with the 'Bhagwa' cultivar. These differences were like larger leaf and fruit size, variation in leaf shape and colour, stigma colour, fruit shape and colour. Accordingly, different progressive farmers presented their cultivar with different names like Ashtagandha, Raktagandha, Jai Maharashtra, Mastani, Shendri and Red Diana, etc. There had been lot of debate over similarity or dissimilarity of these 'Bhagwa' clones presented by different progressive farmers under different names.

Molecular characterization is considered most reliable compared to physico-chemical or morphological characterization. Earlier, various studies have been successfully employed to characterize genetic diversity in pomegranate using molecular markers such as inter simple sequence repeats (ISSR) by Narzary *et al*. (2010), Bedaf *et al*. (2011), Zhao *et al*. (2011), Noormohammadi *et al*. (2012), Ghorbani, *et al*. (2013), Ismail *et al*. (2014), Li *et al*. (2015) and Attanayake *et al*. (2017).

The present study was undertaken with the objective to estimate the diversity or similarity among the various closely related genotypes of 'Bhagwa' cultivar of pomegranate. For this, sixteen 'Bhagwa' cultivar like genotypes available in Maharashtra were identified and subjected to molecular characterization with ISSR primers and thereby proving their least divergence.

## Materials and methods

**Plant material:** In present study, nineteen pomegranate cultivars were used (Table 1). The young and fully expanded greenish leaves of 19 genotypes were used for the extraction of genomic DNA. Leaf samples were collected in aluminium foil cover and immediately immersed in liquid nitrogen ($N_2$). Such samples were brought to laboratory and stored at -78 $^{o}$C. The molecular analyses of above nineteen genotypes were carried out by ISSR markers, at State Level Biotechnology Centre, Mahatma Phule Krishi Vidyapeeth, Rahuri, District Ahmednagar.

**Isolation of genomic DNA:** The isolation of genomic DNA was carried out as per the protocol given by Porebski *et al.* (1997) and CTAB method (Dellaporta *et al*., 1983) with some modifications. The DNAs were detected by electrophoretic separation using 0.8 % (w/v) agarose gel in 1X TAE buffer for comparison of mobility

Table 1. Source of 19 pomegranate genotypes studied

| S.No. | Genotype | Source of collection |
|---|---|---|
| 1 | Bhagwa -1 | Satana, District - Nashik |
| 2 | Bhagwa -2 | Dabhadi, Taluka - Malegaon, District - Nashik |
| 3 | Bhagwa -3 | Ugav, Taluka - Niphad, District - Nashik |
| 4 | Bhagwa -4 | Taharabad, Taluka -Satana, District - Nashik |
| 5 | Bhagwa -5 | Satana, District - Nashik |
| 6 | Bhagwa -6 | Vadnerkhakurdi, Taluka - Malegaon, Nashik |
| 7 | Bhagwa -7 | Deola, Taluka - Satana, District - Nashik |
| 8 | Bhagwa -8 | Solapur, District - Solapur |
| 9 | Bhagwa -9 | Ajanale, Taluka - Sangola, District - Solapur |
| 10 | Bhagwa-10 | Wakishiva, Taluka - Sangola, District - Solapur |
| 11 | Bhagwa-11 | Javalekadlag, Taluka - Sangamner, District - Ahmednagar |
| 12 | Bhagwa-12 | Dhandarphal, Taluka - Sangamner, Ahmednagar |
| 13 | Bhagwa-13 | Jejuri, Taluka - Purandar, District - Pune |
| 14 | Bhagwa-14 | Phadtarewadi, Taluka - Phaltan, District - Satara |
| 15 | Bhagwa-15 | Girvi, Taluka - Phaltan, District - Satara |
| 16 | Bhagwa-16 | Atpadi, District - Sangli |
| 17 | Selection-4 | Selection from Bhagwa like types collected from all over Maharashtra state and maintained at All India Coordinated Research Project on Arid Zone Fruits, MPKV, Rahuri, India |
| 18 | Phule Arakta | Commercial pomegranate cultivar pre-released by MPKV, Rahuri in 1989 and maintained at All India Coordinated Research Project on Arid Zone Fruits, MPKV, Rahuri, India |
| 19 | Mridula | Commercial pomegranate cultivar released by MPKV, Rahuri in 1994 and maintained at All India Coordinated Research Project on Arid Zone Fruits, MPKV, Rahuri, India |

relative to that of known concentrations of calf thymus standard DNA. The quality and quantity of DNA was determined by measuring optical density (OD) at 260 nm and 280 nm wavelength using spectrophotometer (Nanodrop, ND-1000 USA). The ratio of absorbance 260/280 was calculated. The DNA was then diluted to 50 ng $\mu L^{-1.}$ for the further amplification.

**ISSR assays:** ISSR primers were designed randomly with repeat sequences and synthesized. Total 14 ISSR primers (Table 2) were used for screening.

ISSR amplification was performed in a 0.2 mL PCR tubes having 20 µL reaction volume containing 1.0 µL (50 ng) of pomegranate DNA, 2.0 µL of 1 X of Genei*Taq* DNA Polymerase Buffer F, 2.0 µL of 1.50 mM$MgCl_2$, 2.0 µL of 0.25 mMdNTPs, 0.33 µL of Genei*Taq* DNA polymerase (3 U/µL), 1.0 µL each of primer and 11.67 µL of sterile deionized water.

All PCR reactions were performed using Thermal Cycler (Eppendorf, Master Cycler Gradient, Germany). ISSR amplification was performed by following the conditions and thermal profile of PCR as: an initial denaturation at 94 °C for 2 min, followed by 40 cycles at 94 °C for 30 s, 1 min at optimal annealing temperature (Table 2) which was determined by testing in the range of ± 5 °C from the theoretical annealing temperature and 72 °C for 1 min. Then a final extension reaction was allowed to proceed at 72 °C for 7 min. Amplification PCR reaction products were separated using 1.5 % denaturing agarose gel electrophoresis in 1X TAE buffer at a constant current of 80 volts. Medium Range DNA Ruler (100 bp-5 kb) was used as a molecular size standard. The separated amplification reaction products were visualized for band intensity of genomic DNA and photographed on gel documentation unit (Flour Chem™ Alpha Innotech, USA) and compared to that of Medium Range DNA Ruler (100 bp-5 kb).

**ISSR Data Analysis:** In order to evaluate the informativeness of the ISSR markers, the clearly resolved PCR amplified bands were scored manually for their presence (1) and absence (0) in the data sheet. The band size was estimated by using medium range DNA ruler (100 bp-5 kb) which was run along with the amplified products. The number of alleles per locus and the Polymorphism Information Content (PIC) were calculated.

Data were analysed and similarity matrix was constructed from binary data with Dice Similarity Coefficients (DSC) which were calculated as per model suggested by Nei and Li (1979). Unweighted Pair Group Method Using Arithmetic Averages (UPGMA) was employed for cluster analysis. The analyses were carried out using the computer package NTSYSpc 2.02i (Rohlf, 1998).

The PIC value was calculated as PIC = $1-\sum P^2_{ij}$ where $P_{ij}$ is the frequency of the $j^{th}$ allele for the $i^{th}$ marker locus and summation extends over n alleles. The ability of the primers to distinguish between accessions was assessed by calculating their resolving power (Rp) as $Rp = \sum Ib$, where Ib is band informativeness, Ib=1-[2x(0.5-*pi*)] and *pi* is the proportion of accessions containing band *i* (Soriano *et al*., 2011).

## Results and discussion

**Polymorphism of ISSR markers:** A total of 175 amplicons were generated by amplification of 14 ISSR primers out of which 144 amplicons were polymorphic with polymorphism of 90.28 %. Each primer thus produced on an average 12.5 amplicons. The size of amplification product ranged from 0.14 kb to 2.54 kb.

Maximum numbers of amplicons *i.e.* 19 were produced by primer ISSR 8932805, whereas least number of amplicons *i.e.* 7 were generated by primers IS12 and IS13. Similar results were reported by other researchers. Narzary *et al*. (2010) reported seventeen ISSR primers producing 268 polymorphic bands, with 87.01 % polymorphism. Bedaf *et al*. (2011) reported 173 ISSR amplification products, out of which 64 were polymorphic (37 %). Zhao *et al*. (2011) reported six polymorphic primers amplifying 120 bands in which 109 bands were polymorphic with 90.83 % polymorphism. Ajal *et al*. (2014) also observed that eight ISSR primers generated 70 bands of which 61 were polymorphic (87.14 %). Madadi *et al*. (2017) observed 83.23 % polymorphism while analyzing four commercial cultivars in Iran.

The primer ISSR-834 showed the highest per cent polymorphism (100.00 %) while the least per cent polymorphism (42.10 %) was shown by ISSR 8932805. The primer ISSR 8932798 amplified

Table 2. ISSR primer sequences, repeat types, allele size ranges and number of alleles in 19 pomegranate genotypes

| S. No. | Primers | Repeat motif | Allele size range (bp) | Number of alleles | Annealing temperature $T_m$ (°C) | PIC |
|---|---|---|---|---|---|---|
| 1 | ISSR 8932798 | $(AG)_4AGA$ | 0.14 - 2.54 | 14 | 25 | 14.8 |
| 2 | ISSR 8932798 | $(AG)_6GC$ | 0.19 - 2.25 | 18 | 55.4 | 20.8 |
| 3 | ISSR 8932804 | $(CA)_6GT$ | 0.14 - 1.68 | 15 | 42 | 15.6 |
| 4 | ISSR8932805 | $(CA)_6GC$ | 1.9 - 2.07 | 19 | 44 | 32.1 |
| 5 | ISSR8932806 | $(CA)_6GA$ | 0.27 - 1.17 | 8 | 44 | 13.2 |
| 6 | ISSR 8932807 | $(CA)_6AA$ | 0.32 - 1.65 | 13 | 42 | 18.3 |
| 7 | ISSR 8932809 | $(GT)_6TG$ | 0.23 - 1.63 | 13 | 42 | 15.7 |
| 8 | ISSR 8932811 | $(GT)_6CT$ | 0.28 - 1.75 | 10 | 44 | 11.2 |
| 9 | ISSR 8932812 | $(GT)_6AT$ | 0.42 - 1.74 | 12 | 39 | 14.5 |
| 10 | ISSR 834 | $(AG)_8YT$ | 0.31 - 1.78 | 13 | 52 | 18.6 |
| 11 | ISSR 857 | $(AC)_7YC$ | 0.17 - 1.26 | 13 | 52 | 20.1 |
| 12 | IS 8 | $(CA)_7GC$ | 0.30 - 1.39 | 13 | 47 | 11.2 |
| 13 | IS 12 | $(GT)_7TG$ | 0.39 - 1.39 | 7 | 48 | 8.8 |
| 14 | IS 13 | $(GT)_8CA$ | 0.43 - 1.62 | 7 | 52 | 7.5 |

14 amplicons out of which 10 were polymorphic and 2 were monomorphic while two amplicons were unique. One unique amplicon having size 1.95 kb and 0.14 kb were present in Bhagwa-6 and Mridula, respectively. The size of amplified primer ISSR 8932798 ranged from 0.14 kb to 2.54 kb.

The ISSR 8932799 primer amplified 18 amplicons out of which 16 were polymorphic, one was monomorphic and one was unique of size 2.25 kb in Bhagwa-7. The size of this primer ranged from 0.19 kb - 2.25 kb with 94.44 % polymorphism.

The primer ISSR 8932804 produced 15 amplicons out of which 13 were polymorphic, one was monomorphic and one was unique of size 0.73 kb in Bhagwa-5. The 93.33 per cent polymorphism was generated with fragment size 0.14 kb to 1.68 kb by this primer.

The primer ISSR 8932805 produced highest number of amplicons (19) among amplified primers out of which 6 were polymorphic, 11 were monomorphic and 2 were unique. The lowest (42.10 %) polymorphism was produced with size of fragment 10.9 kb to 2.07 kb. The unique amplicons present in Mridula were of size 2.07 kb and 1.51 kb.

The highest PIC value was observed with primer IS8 (0.72) and lowest with ISSR 8932805 (0.20). The primer ISSR 8932804 (0.68), ISSR 8932798 (0.63), IS13 (0.62), ISSR 8932811 (0.59) and ISSR 8932799 (0.59) showed high PIC values indicating that these primers were more informative. Further, it was observed that there was no correlation between PIC value and per-cent polymorphism.

**Divergence analysis:** The Dice similarity coefficient ranged from 0.00 to 0.94. Maximum similarity coefficient (0.94) was observed in between Bhagwa-8 (Solapur): Bhagwa-10 (Wakishiva) followed by Bhagwa-16 (Atpadi): Selection-4, Bhagwa-8 (Solapur): Selection-4 as well as Bhagwa-16 (Atpadi): Bhagwa-10 (Wakishiva) indicating that these genotypes are least divergent from each other.

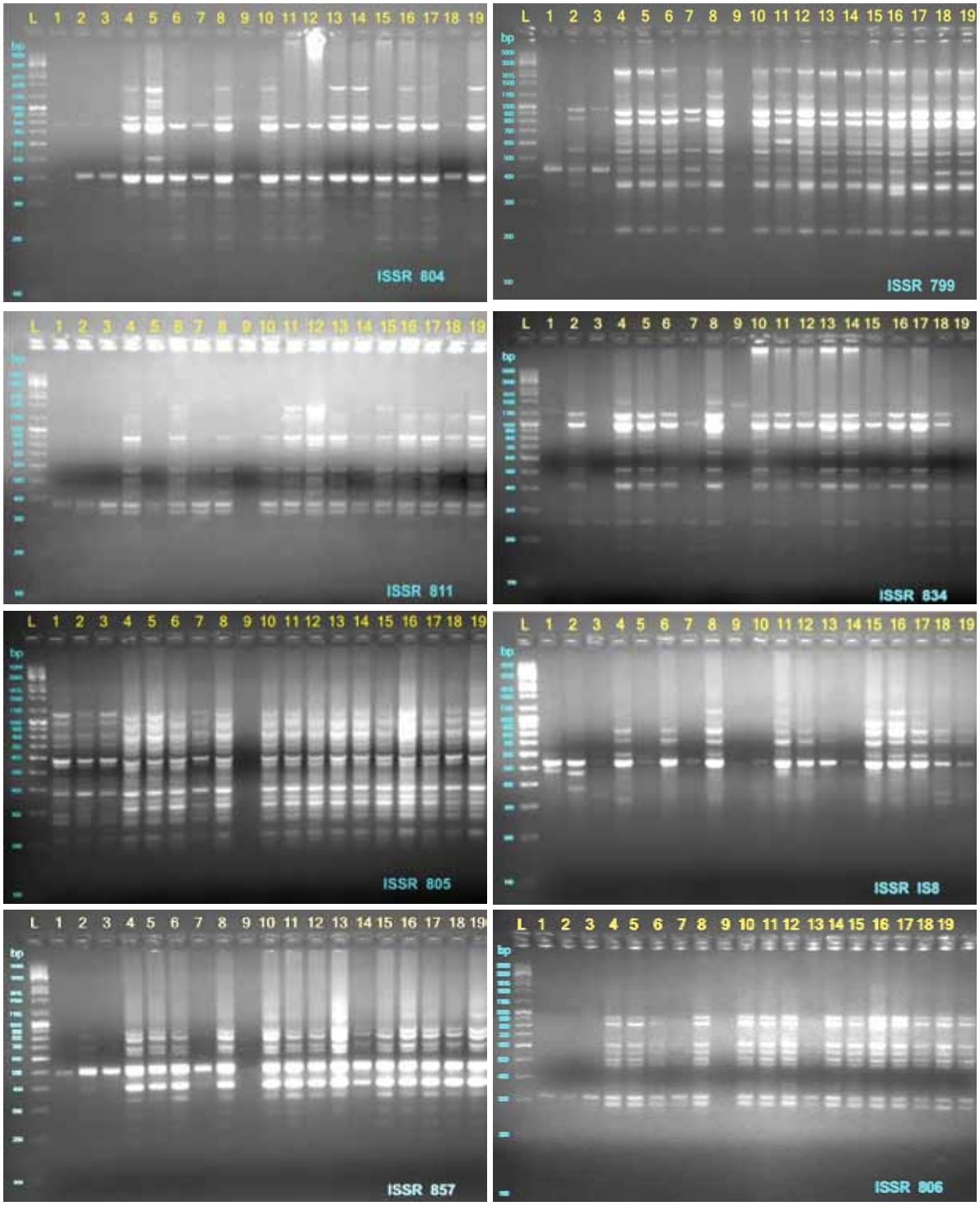
High similarity (≥ 0.90) was observed in between 18 different genotype combinations with Bhagwa-10 (Wakishiva) sharing 8 of such combinations. Five such high similarity indices were shared by Bhagwa-13 (Jejuri) while four such high similarity indices were shared by Bhagwa-11 (Javalekadlag) whereas 3 such high similarity indices were shared by Selection-4, Bhagwa-8 (Solapur), Bhagwa-4 (Taharabad) and Mridula while 2 such high similarity indices were shared by Bhagwa-16 (Atpadi), Bhagwa-6 (Vadnerkhakurdi). Bhagwa-5 (SatanaD) and Bhagwa-14 (Phaltan) shared single such high similarity index while 10 genotypes did not share any such combination.

Selection-4 showed maximum similarity with Bhagwa-16 (Atpadi) and Bhagwa-8 (Solapur) genotypes. The similarity was not observed between Bhagwa-9 (Ajnale) with Bhagwa-10 (Wakishiva), Bhagwa-13 (Jejuri) and Bhagwa-14 (Phaltan) indicating that these genotypes are not similar to each other. Very low (≤ 0.20) similarity coefficient was observed in 11 genotype combinations all of which were shared by Bhagwa-9 (Ajnale) indicating that Bhagwa-9 (Ajnale) is a very distinct genotype. In case of Bhagwa-9 (Ajnale) genotype amplification was observed only with 3 primers that to amplifying only single band each. This could be the reason for non-similarity of Bhagwa-9 (Ajnale) genotype.

The UPGMA dendrogram was generated from ISSR genetic similarity coefficients (Fig.1) to elucidate genetic relationships among the 19 pomegranate genotypes. In the present study, all 19 genotypes of pomegranate were grouped in two broad clusters *i.e.,* Cluster A and Cluster B.

Cluster A comprised of only a single distinct genotype Bhagwa-9 (Ajnale) which was well separated from other clusters of pomegranate genotypes at the coefficient of similarity level of 0.15. The genotype Bhagwa-9 (Ajnale) was the most diverse and variable among all other genotypes used in the present study.

In case of Bhagwa-9 (Ajnale) genotype, amplification was observed only with 3 primers that too amplifying only single band




| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| L | DNA Ladder | 3 | Bhagwa -3 | 6 | Bhagwa -6 | 9 | Bhagwa -9 | 12 | Bhagwa -12 | 15 | Bhagwa -15 | 18 | Arakta |
| 1 | Bhagwa -1 | 4 | Bhagwa -4 | 7 | Bhagwa -7 | 10 | Bhagwa -10 | 13 | Bhagwa -13 | 16 | Bhagwa -16 | 19 | Mridula |
| 2 | Bhagwa -2 | 5 | Bhagwa -5 | 8 | Bhagwa -8 | 11 | Bhagwa -11 | 14 | Bhagwa -14 | 17 | Selection – 4 | | |

Plate 1. PCR profile of 19 pomegranate genotypes using ISSR primers

each. The failure of amplification with ISSR primers would have contributed to the divergence observed in Bhagwa-9 (Ajnale) genotype.

The cluster B could be grouped into two sub-clusters *viz*., B1 and B2. Sub-cluster B1 was the largest cluster showing high similarity coefficient level *i.e.* more than 0.85 comprising 14 genotypes. A higher level of genetic relationship was observed among these genotypes. The sub-cluster B1 comprising of 14 genotypes could be clearly distinguished from sub-cluster B2 at similarity coefficient level of 0.62. The sub-cluster B2 was divided into two sub-clusters separated at 0.75 similarity coefficient level.

In 2D scattered plot derived from principle co-ordinate analysis of 19 pomegranate genotypes based on ISSR primers (Fig. 2), highly divergent genotypes of sub-cluster B1 and B2 in dendrogram tree were separated by first component (axis). All the 14 genotypes of sub-cluster B1 were present on the other half of the first component. Arakta which was most divergent of sub-cluster B1.1 was placed exactly in the middle of first axis. Divergence pattern in dendrogram tree was also reflected at subcluster level.

Overall, it could be concluded that almost all the Bhagwa like

Table 3. Similarity Coefficient values for pairwise comparison of 19 Pomegranate genotypes based on ISSR analysis

| | Bhagwa-1 | Bhagwa-2 | Bhagwa-3 | Bhagwa-4 | Bhagwa-5 | Bhagwa-6 | Bhagwa-7 | Bhagwa-8 | Bhagwa-9 | Bhagwa-10 | Bhagwa-11 | Bhagwa-12 | Bhagwa-13 | Bhagwa-14 | Bhagwa-15 | Bhagwa-16 | Selection-4 | Phule Arakta | Mridula |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bhagwa-1 | 1.00 | | | | | | | | | | | | | | | | | | |
| Bhagwa-2 | 0.83 | 1.00 | | | | | | | | | | | | | | | | | |
| Bhagwa-3 | 0.82 | 0.84 | 1.00 | | | | | | | | | | | | | | | | |
| Bhagwa-4 | 0.54 | 0.63 | 0.61 | 1.00 | | | | | | | | | | | | | | | |
| Bhagwa-5 | 0.50 | 0.58 | 0.56 | 0.88 | 1.00 | | | | | | | | | | | | | | |
| Bhagwa-6 | 0.55 | 0.62 | 0.57 | 0.86 | 0.82 | 1.00 | | | | | | | | | | | | | |
| Bhagwa-7 | 0.68 | 0.82 | 0.78 | 0.74 | 0.70 | 0.73 | 1.00 | | | | | | | | | | | | |
| Bhagwa-8 | 0.54 | 0.62 | 0.57 | 0.91 | 0.85 | 0.87 | 0.71 | 1.00 | | | | | | | | | | | |
| Bhagwa-9 | 0.67 | 0.30 | 0.38 | 0.11 | 0.11 | 0.11 | 0.27 | 0.10 | 1.00 | | | | | | | | | | |
| Bhagwa-10 | 0.51 | 0.62 | 0.64 | 0.91 | 0.92 | 0.91 | 0.78 | 0.94 | 0.00 | 1.00 | | | | | | | | | |
| Bhagwa-11 | 0.55 | 0.64 | 0.58 | 0.88 | 0.80 | 0.90 | 0.74 | 0.92 | 0.11 | 0.91 | 1.00 | | | | | | | | |
| Bhagwa-12 | 0.52 | 0.60 | 0.58 | 0.80 | 0.76 | 0.86 | 0.74 | 0.83 | 0.12 | 0.88 | 0.89 | 1.00 | | | | | | | |
| Bhagwa-13 | 0.51 | 0.65 | 0.68 | 0.90 | 0.86 | 0.87 | 0.78 | 0.87 | 0.00 | 0.91 | 0.84 | 0.85 | 1.00 | | | | | | |
| Bhagwa-14 | 0.49 | 0.66 | 0.67 | 0.88 | 0.89 | 0.85 | 0.85 | 0.89 | 0.00 | 0.92 | 0.86 | 0.84 | 0.86 | 1.00 | | | | | |
| Bhagwa-15 | 0.55 | 0.61 | 0.59 | 0.86 | 0.81 | 0.88 | 0.71 | 0.87 | 0.12 | 0.89 | 0.91 | 0.85 | 0.84 | 0.80 | 1.00 | | | | |
| Bhagwa-16 | 0.51 | 0.57 | 0.55 | 0.87 | 0.85 | 0.85 | 0.69 | 0.91 | 0.09 | 0.93 | 0.89 | 0.83 | 0.90 | 0.85 | 0.87 | 1.00 | | | |
| Selection-4 | 0.52 | 0.60 | 0.57 | 0.89 | 0.85 | 0.85 | 0.69 | 0.93 | 0.10 | 0.92 | 0.87 | 0.82 | 0.90 | 0.85 | 0.88 | 0.93 | 1.00 | | |
| Phule Arakta | 0.54 | 0.66 | 0.67 | 0.85 | 0.78 | 0.81 | 0.76 | 0.80 | 0.20 | 0.87 | 0.81 | 0.80 | 0.86 | 0.86 | 0.80 | 0.78 | 0.85 | 1.00 | |
| Mridula | 0.49 | 0.58 | 0.57 | 0.84 | 0.83 | 0.79 | 0.72 | 0.87 | 0.10 | 0.91 | 0.81 | 0.81 | 0.91 | 0.86 | 0.79 | 0.87 | 0.90 | 0.84 | 1.00 |

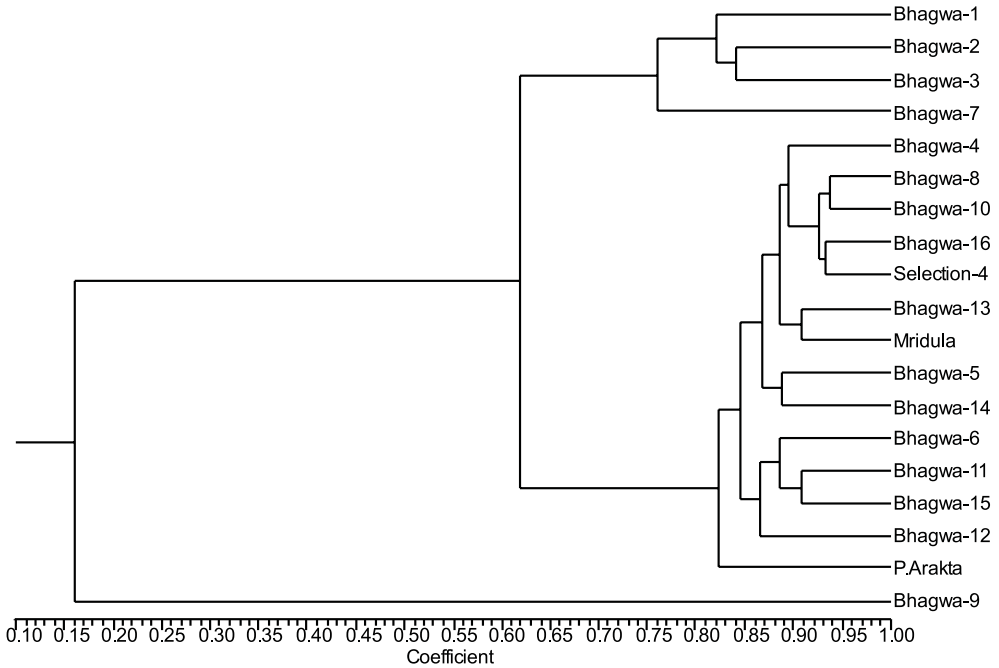



Fig. 1. UPGMA based dendrogram of 19 pomegranate genotypes by ISSR primers

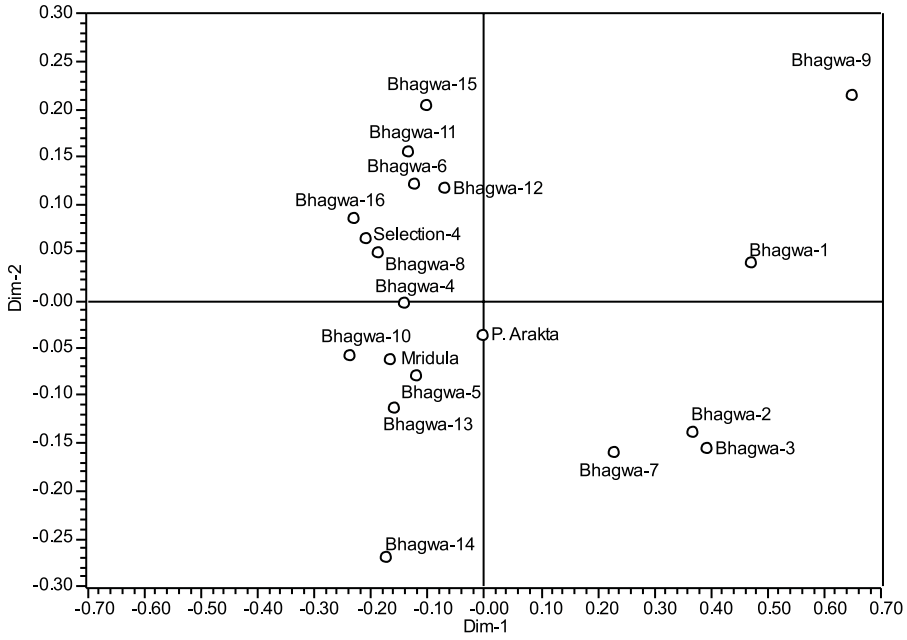



Fig. 2. 2D scattered plot derived from principle co-ordinate analysis of 19 pomegranate genotypes based on ISSR primers

genotypes being present in single large cluster B, are sharing a common genetic basis except Bhagwa 9 owing to its non-amplification with ISSR primers that has contributed to its divergence. Therefore, the claims of Bhagwa like genotypes being independent identities by various farmers and nurseries in Maharashtra State of India were rejected. This conclusion will definitely help further breeding programme at university levels in India.

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