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Assessment of genetic divergence among Indian genotypes of pomegranate for economic traits

Nusrat Perveen, Sarvamangala S. Cholin*, Kulapati Hipparagi, BNS Murthy, Dadapeer Peerjade**

University of Horticultural Sciences, Bagalakot 587104, Karnataka

ABSTRACT

The efficacy of pomegranate improvement for higher productivity as well as fruit quality can be achieved by selecting superior parental combinations made between divergent clones. The nature and magnitude of genetic divergence was assessed for 35 quantitative traits using Mahalanobis D^2 analysis in 23 popular Indian pomegranate genotypes. Interestingly, peel weight followed by seed width had higher contribution to the diversity among the selected genotypes. Tocher's method of cluster analysis grouped all the cultivars into four distinct clusters. Genotypes falling in clusters II and III were highly diverse from each other. Mean value for most of the fruits and aril parameters were highest in cluster III, a solitary cluster comprising of the most popular cultivar Ganesh followed by cluster I consisting of a commercial pomegranate cultivars like Bhagwa and its clones. The wider variations for different traits among the clusters in the selected pomegranate cultivars shows that genotypes from desirable clusters could be directly used in breeding experiments for the desirable traits of interest depending upon the breeding objectives.

Key words: *Punica granatum*, genetic divergence, Mahalanobis D^2, clusters, quantitative traits.

INTRODUCTION

Pomegranate (*Punica granatum* L.), favourite table fruit in the tropical and sub-tropical regions, is a predominant member of family Lytheraceae, comprising only two species, *Punica granatum* L. and *P. protopunica* Balf. f. 1882. *Punica protopunica* is endemic to Socotra Island (Yemen) and is considered to be the only congeneric relative of *P. granatum* species currently in cultivation (Zukovski, 19; Mars, 11; Levin, 10) and has been suggested as the ancestor of this genus based on its xylem anatomy (Shilkina, 18). The chromosome number differs among the cultivars and haploid chromosome number of eight (Sheidai and Noormohammadi, 17) or nine (Darlington and JanakiAmmal, 3) has been reported. Pomegranate and its usage are an integral part of human history, with its utilization spreading across many ancient human cultures as food as well as a medical remedy. Pomegranate fruits are widely consumed as fresh or processed into juice, syrup, jams and wine (Poyrazoglu *et al*., 14). Dried pomegranate arils known as anardana are used as acidulant for culinary purposes. A recent upsurge witnessed in the demand for pomegranate products is mainly attributed to its nutritional and medicinal properties including anti-oxidant anti-carcinogenic, anti-microbial, anti-viral and anti-atherosclerotic activities (Gil *et al*., 5; Seeram *et al*., 16).

*Corresponding author's E-mail: sarugpb@gmail.com

**Indian Institute of Horticulture Research, Hesaraghatta, Bengaluru, Karnataka

Pomegranate being an out crossing species possesses a huge diversity in pomological traits (Patil and Sanghavi, 13). In spite of the presence of significant amount of variability in pomegranate germplasm, its utilization in breeding programs has been meagre till date. Being a perennial species, introgression of desirable traits in to cultivated varieties is laborious and time consuming. Understanding the diversity, superiority for multiple traits and also the lacunas among the already existing popular cultivars would help a breeder to improve the quality and productivity of otherwise superior cultivar through hybridization. Being a clonally propagated crop, the identified superior segregants can be directly fixed by vegetative propagation. In this regard, prior quantitative assessment of genetic divergence of the popular cultivars is of prime importance. With the increase in the magnitude of divergence in the parents, the chances of achieving heterotic F1 with wide spectrum of recombination or transgressive segregants in the segregating generations, also increases. The genetic divergence between the population can effectively be quantified by using appropriate statistical analysis, among which, multivariate analysis has been reported to be the most effective one (Joshi and Dhawan, 7; Kumar, 9). Hence, in the present investigation, an effort has been made to assess the genetic divergence among 23 popular Indian pomegranate genotypes by using Mahalanobis D^2 analysis in order to find out the

most diverse parents to be used in the hybridization programme.

MATERIALS AND METHODS

The present investigation has been carried out in University of Horticultural Sciences, Bagalkot, Karnataka during the year 2016-2017 in a Randomized Block Design with three replications. Each plant selected randomly from each genotype was considered as one replication. Twenty three pomegranate genotypes were studied for 35 quantitative traits including fruit and aril parameters and biochemical parameters, details of which have been presented in Table 1 and 2, respectively. For recording the observations, fruits of Hasta-bahar flowering (October-November, 2016) were retained and harvested during February-April, 2017. For the estimation of physio-chemical parameters of fruits, three randomly selected fruits from each replication, after harvesting were selected. The details of the methods followed for recording observations is given below.

For recording the physio-chemical characteristics, fruits after harvesting were transferred to laboratory and observations were made on three randomly selected fruits from each replication. Weight of the fruit was taken using a precision balance with an accuracy of 0.001g. Fruit volume was calculated by liquid displacement method. The length and diameter of the fruit and calyx were measured using digital Vernier callipers with 0.001 mm accuracy. The measurement of fruit length (mm) was done in the polar axis *i.e.*, between the apex and the end of stem. The maximum width of the fruit (mm), measured in the direction perpendicular to the polar axis, was taken as diameter. After recording the external morphological traits, the arils were separated manually from the fruits, and total number of arils was counted. Peel thickness and other parameters like length and breadth of arils and seeds were measured using the digital Vernier callipers. The measurement of rind thickness (mm) was performed on two opposite faces of the fruit in equatorial zone. Moisture percent of arils was determined by drying the arils at 60°C in hot air oven until reaching a constant weight. The juice of arils were analysed for biochemical parameters.

Colour measurements were performed using a colorimeter (Hunter lab Colorflex EZ). Skin colour measurements were taken along the equatorial axis of each fruit. Three readings of each colour index in the Hunter scale (L, a, b) were taken per fruit, making a total of 27 measurements per cultivar. Similarly, nine measurements of aril colour were also taken per cultivar. The instrument was standardized during each sample measurement with a black and a white tile, and the colour values represented whiteness or brightness/darkness (L), redness/greenness (a) and yellowness/blueness (b).

Table 1. Indian Pomegranate cultivars and their source of collections.

Sl. No.	Cultivar	Source of collection
1	Amlidana	IIHR, BENGALURU, Karnataka
2	Bhagwa	UHS, BAGALKOT, Karnataka
3	CO-1	HRES,TIDAGUNDI, Karnataka
4	Dholka	HRES, TIDAGUNDI, Karnataka
5	Early Bhagwa	UHS, BAGALKOT, Karnataka
6	G-137	HRES, TIDAGUNDI, Karnataka
7	Ganesh	UHS, BAGALKO, Karnataka
8	Kabul Yellow	IIHR, BENGALURU, Karnataka
9	Kaladagi Local	UHS, BAGALKOT, Karnataka
10	KRS	HRES, TIDAGUNDI, Karnataka
11	Mridula	HRES, TIDAGUNDI, Karnataka
12	P-23	HRES, TIDAGUNDI, Karnataka
13	P-26	HRES, TIDAGUNDI, Karnataka
14	PhuleArakta	UHS, BAGALKOT, Karnataka
15	Ruby	HRES, TIDAGUNDI, Karnataka
16	Super Bhagwa	UHS, BAGALKOT, Karnataka
17	Tobesto	HRES, TIDAGUNDI, Karnataka
18	UHSP 23	HRES, TIDAGUNDI, Karnataka
19	UHSP 57	HRES, TIDAGUNDI, Karnataka
20	UHSP 81	HRES, TIDAGUNDI, Karnataka
21	UHSP 125	HRES, TIDAGUNDI, Karnataka
22	Wonderful	UHS, BAGALKOT, Karnataka
23	Yercaud	HRES, TIDAGUNDI, Karnataka

Fruit juiciness percentage was determined by extracting the juice of 100 g of arils in six replicates per genotype using an electric juice extractor. Titratable acidity (TA), pH, total soluble solids (TSS) and ascorbic acid were evaluated as juice quality indices. The pH values were measured using a pH-meter. The TA was determined by titrating 10 ml of juice with 0.1 N NaOH (pH 8.1). Results were expressed as g citric acid per 100 ml of sample, in accordance with AOAC (2). The TSS contents were recorded in an HI9680I refractometer (0-85%) at 26.5°C with values being expressed as °Brix. Ascorbic acid was estimated using dye (dichloro phenol indophenol) binding method using oxalic acid as the titrating medium. The results were expressed as mg per 100 g of juice.

Table 2. Observations recorded for morphological and biochemical characters in pomegranate cultivars.

Sl. No.	Characters	Particulars
		Morphological Parameters
1	Fruit weight (g)	Precision balance
2	Fruit length (mm)	Digital vernier calipers with 0.001mm accuracy
3	Fruit diameter (mm)	Digital vernier calipers with 0.001mm accuracy
4	Fruit length/width	Ratio calculated
5	Fruit volume (cm^3)	Liquid displacement methods
6	Fresh wt. of 100 arils (g)	Precision balance
7	Dry wt. of 100 arils (g)	Precision balance after drying at hotair oven at 50^0 for 36 hrs
8	Moisture %	Oven drying arils at 60°C until constant weight
9	Crown length (mm)	Digital vernier calipers with 0.001mm accuracy
10	Peel weight (g)	Precision balance
11	Aril weight (g)	Precision balance
12	Seed %	Percent ratio calculated
13	Skin %	Percent ratio calculated
14	Total No. of Arils/fruit	Manual counting
15	Aril length (mm)	Digital verniercallipers with 0.001 mm accuracy
16	Aril width (mm)	Digital verniercallipers with 0.001 mm accuracy
17	Seed length (mm)	Digital vernier calipers with 0.001 mm accuracy
18	Seed width (mm)	Digital verniercallipers with 0.001 mm accuracy
19	Rind thickness (mm)	Digital vernier calipers with 0.001 mm accuracy
20	Red coverage of Peel (%)	Visual observation
21-23	Fruit Colour (L, a, b)	Hunter's colour Lab, Colorimeter- L a b
24-26	Aril Colour (L, a, b)	Hunter's colour Lab, Colorimeter- L a b
27	Days to flowering	Days counted from pruning to flowering
28	Days to maturity	Days counted from flowering to harvesting
		Biochemical Parameters
29	Anthocyanin content (mg/L)	pH differential method
30	Ascorbic Acid (mg/100gm)	Dye (dichlorophenol indophenol) binding method
31	Titratable Acidity (%)	Titration method with 0.1 N NaOH (pH 8.1)
32	pH of the Juice	pH-meter
33	Fruit Juiciness % (per 100gm aril wt.)	Extracted juice from 100 arils and measured as weight/weight with aril wt.
34	Total Sugars (%)	Phenol Sulphuric Acid method
35	Reducing Sugars (%)	Dinitrosalicylic acid (DNS) method
36	Non-Reducing Sugars (%)	(Total Sugars - Non-Reducing Sugars)
37	TSS (°Brix)	Refractometer

Total anthocyanins were estimated by pH differential method using two buffer systems; potassium chloride buffer, pH 1.0 (25 mM) and sodium acetate buffer, pH 4.5 (0.4 M) (Giusti and Wrolstad, 6). The samples were diluted by a potassium chloride buffer until the absorbance of the sample at 510 nm wavelength was within the linear range of the spectrophotometer (Cecil 2010 UV–visible). This dilution factor was later used to dilute the sample with the sodium acetate buffer. The wavelength reading was performed after 15 min of incubation, four times per sample, diluted in two different buffers and at two wavelengths viz. 510 nm and 700 nm.

The total anthocyanins content was calculated by using following formula:
Total anthocyanins = [(A × MW × DF × 100)/MA]
Where, A = (A510 - A700) pH1.0 - (A510 - A700) pH4.5; MW: molecular weight (449.2); DF: dilution factor; MA: molar absorptive coefficient of cyaniding-3-glucoside (26.900). Results were expressed as total anthocyanin mg/L.

Total sugars were estimated using phenol sulphuric acid method (Dubois *et al*., 4; Krishnaveni *et al*., 8) and dinitrosalicylic acid method (Miller, 12) was used to determine reducing sugars in the pomegranate samples, the difference between the above two was used to estimate the amount of non-reducing sugars.

The replicated mean data of all the 35 traits were Mahalanobi's D^2 – statistics was performed to assess the genetic divergence between 23 pomegranate cultivars by using the software Window stat version 5.1.

RESULTS AND DISCUSSION

Pomegranate is mainly cultivated for its arils, and its related traits like total number of arils per fruit, aril weight, 100-arils fresh weight are among the major yield attributing traits along with fruit weight, peel weight *etc*. In the present study, a total of 35 quantitative phenotypic traits including 26 morphological and 9 biochemical parameters were subjected to diversity analysis (Table 2). The performance of these cultivars for the above quantitative parameters has been discussed elsewhere (data not shown).

Very interestingly, the fruit parameter, peel weight (79%) followed by seed width (54.94%), contributed maximum to the diversity. However, the contribution of biochemical traits such as non-reducing sugars (7.91%), anthocyanin content (6.72 %) and titratable acidity (5.93%) indicates the effectiveness of the plant material for diversity analysis for both productivity as well as quality traits (Table 3). The diverse parents identified in the present study can be utilized for selection of superior progenies for both yield and nutritional quality in pomegranate breeding program.

Twenty three genotypes were classified into four clusters on the basis of D^2 value (Table 4; Fig. 1 and 2). Among these four clusters, the maximum numbers of cultivars (14) were comprised in cluster I, which included Bhagwa, Super Bhagwa, Early Bhagwa, Phule Arakta, Kabul Yellow, CO-1, P-26, Wonderful, P-23, Tobesto, Kaladagi Local, G-137, Dholka, Mridula. The grouping of the Bhagwa and its superior clones and other cultivars in a single cluster indicates that, these cultivars would have been the result of

Table 3. Percent contribution to diversity from 35 quantitative traits in Mahalanobis D^2 analysis.

Sl. No.	Characters	Times ranked 1st	% Contribution
1	Fruit length (mm)	-	-
2	Fruit diameter (mm)	-	-
3	Fruit shape	-	-
4	Fruit volume (cm^3)	-	-
5	Fresh wt. of 100 arils	-	-
6	Dry wt. of 100 arils	4	1.58%
7	Moisture %	-	-
8	Crown length (mm)	-	-
9	Peel weight	2	79.00%
10	Aril wt	-	-
11	Seed%	-	-
12	Skin%	-	-
13	Total No. of Arils/fruit	-	-
14	aril length (mm)	-	-
15	aril width (mm)	-	-
16	seed length (mm)	-	-
17	seed width (mm)	139	54.94%
18	rind thickness (mm)	-	-
19	Red coverage%	-	-
20	FC(L)	-	-
21	FC(a)	-	-
22	FC(b)	6	2.37%
23	AC(L)	19	7.51%
24	AC(a)	-	-
25	AC(b)	-	-
26	fruit weight (g)	9	3.56%
27	Anthocyanin estimation (mg/L)	17	6.72
28	Ascorbic Acid (mg/100gm)	2	0.79
29	Titratable Acidity (%)	15	5.93%
30	pH of the Juice	3	1.19%
31	Fruit Juiciness % (per 100 gm aril wt.)	2	0.79%
32	Total Sugars (%)	6	2.37%
33	Reducing Sugars (%)	8	3.16%
34	Non Reducing Sugars (%)	20	7.91%
35	TSS (°Brix)	1	0.40%

Table 4. Cluster composition of pomegranate cultivars based on 35 quantitative traits by Mahalanobis D^2 analysis.

Sl. No.	Cluster No.	No. of Cultivars	Name of the cultivars
1	Cluster I	14	Bhagwa, Super Bhagwa, Early Bhagwa, PhuleArakta, Kabul Yellow, CO-1, P-26, Wonderful, P-23, Tobesto, Kaladagi Local, G-137, Dholka, Mridula
2	Cluster II	7	UHSP 23, UHSP 81, UHSP 125, Ruby, UHSP 57, KRS, Yearcaud
3	Cluster III	1	Ganesh
4	Cluster IV	1	Amlidana

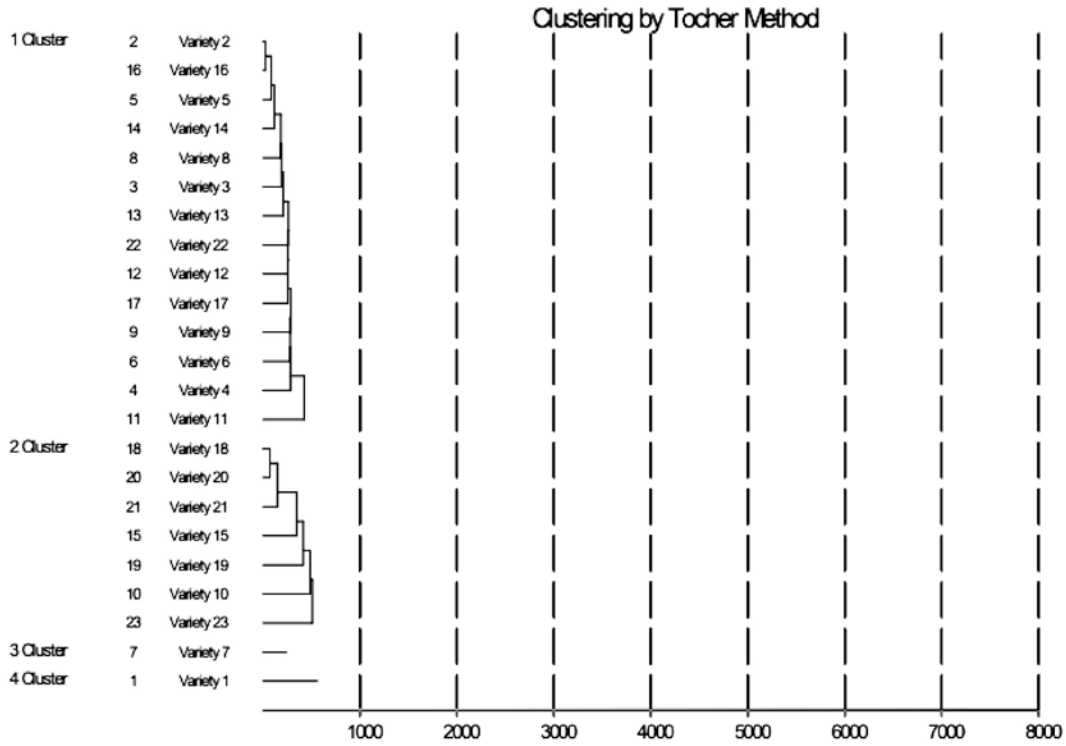

Fig. 1. Dendogram showing number of clusters with the composition of pomegranate cultivars with genetic divergence

*The variety number is depicted in Table 1 with respective serial no.

clonal selections from the single ancestor which could be further confirmed by molecular phylogeny.

Seven genotypes *viz*. UHSP 23, UHSP 81, UHSP 125, Ruby, UHSP 57, KRS, Yercaud formed the second cluster indicating a different ancestor. The presence of all the mutants of Bhagwa in to a single cluster but the differentiation from its parent indicates that the mutation created greater variation among these mutants from its parent. Remaining two clusters (III and IV) consisted of single cultivars each, namely Ganesh and Amlidana respectively representing the solitary clusters (Table 4) indicating their diverse from the other two clusters. The formation of different clusters thus indicates the presence of diversity among different genotypes.

The estimation of inter and intra-cluster distances by D^2 for phenotypic traits revealed that the range of intra-cluster distance varied from a minimum of 0.00 (in solitary clusters) to a maximum of 1334.10 in the second cluster (Table 5; Fig. 2). This emphasizes that cluster II has genotypes which are relatively distant from each other as compared to the cluster I which has lower D^2 distance (805.00). Although lesser number of cultivars are present in cluster II than in cluster I, their higher intra-cluster distance necessitates further evaluation by molecular markers to clearly understand the diversity between the cultivars present within this cluster.

The maximum inter-cluster distance was observed between cluster II and III (8169.86) suggesting wider

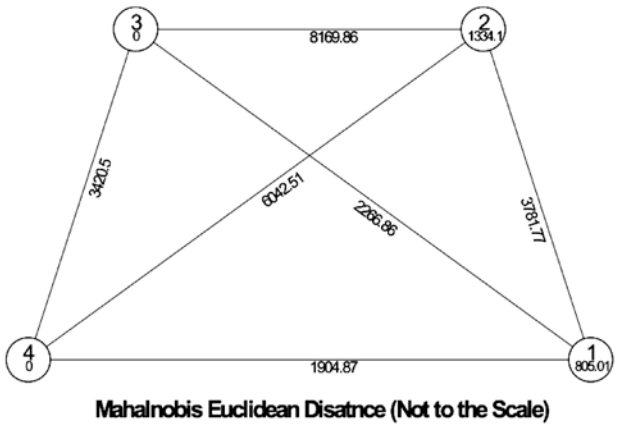

Fig. 2. Mahalanobis Euclidean Distance (Not to scale) showing inter and intra cluster ditncaces among teh four clusters.

*1, 2, 3 and 4 are the cluster numbers and the values in circle indicates intra-cluster distance and the values on joining lines indicates the inter-cluster distance with the respective clusters

Table 5. Inter- and intra-cluster distance among four clusters obtained from 35 quantitative traits among 23 pomegranate cultivars.

Clusters	Cluster I	Cluster II	Cluster III	Cluster IV
Cluster I	805.0	3781.77	2266.86	1904.87
Cluster II		1334.10	8169.86	6042.51
Cluster III			0.00	3420.50
Cluster IV				0.00

genetic differences among the genotypes comprised in these clusters. Furthermore, the minimum inter-cluster distance of 1904.87 was found between cluster I and IV indicating significantly lesser genetic divergence among the genotypes of these clusters and the single cultivar (Amlidana) present in the IV cluster would have been diverged because of its more acidic nature. Larger inter-cluster distance is an indicative that the genotypes comprised in these clusters are genetically diverse and can be employed in hybridization programme for getting better recombinants in the segregating generations. Similarly, lower intra-cluster distances demonstrated the narrow genetic base of the clusters and hence, selection of parents from these clusters should be avoided. Larger inter-cluster distances as compared to the intra- cluster distances have also been reported by Raina *et al.* (15) in pomegranate genotypes indicating a wider genetic diversity between genotypes of the clusters with respect to trait considered. Different intra- and inter-cluster distances have previously been recorded in various fruit crops like pomegranate, walnut, almond, and pecan cultivars (Akbarpour *et al.*, 1).

Selection of diverse varieties is of paramount importance in breeding program, however, the *per se* performance of the genotypes as well as clusters is equally important to end up with the superior variety. Hence, the cluster means for all the 35 quantitative traits were evaluated to identify the superior clusters for various fruit, aril and biochemical parameters. Cluster means obtained from D^2 analysis demonstrated wide variation among the clusters for morphological traits. Cluster III performed better than other clusters in context of fruit length (112.35 mm), fruit diameter (95.87mm), fruit volume (527.78cm^3) and fruit length by width ratio (1.17) followed by cluster I for fruit length (72.43 mm) and volume (215.94 cm^3) and cluster IV for fruit diameter (72.80 mm). Fresh weight of 100 arils, dry weight of 100 arils, peel weight, aril weight, total no. of arils per fruit, aril length, aril width, rind thickness and fruit weight were also highest in cluster III *viz*. 46. 89 g, 9.62 g, 192.44 g, 312.55 g, 1077.89 , 11.48 mm, 8.63 mm, 4.19 mm and 505.00 g respectively. While lowest fruit weight and fruit volume was recorded in cluster II being 120.72 g and 123.54 cm^3 respectively (Table 6).

With regard to biochemical parameters anthocyanin content was highest in cluster II (31.18 mg/L) while it was found to be lowest in cluster III (5.20 mg/L). Cluster IV performed best in terms of ascorbic acid (50 mg/100gm) followed by cluster I (27.40 mg/100gm). Highest titratable acidity (1.09%) along with lowest pH (2.53) was recorded for cluster IV, while pH was found to be the highest in cluster III (3.99). Furthermore, fruit juiciness and reducing sugars and total sugars were observed to be the highest in cluster I, 60.72%, 17.04 % and 33.93 % respectively while, cluster IV exhibited highest TSS (15.56 °Brix) closely followed by cluster III (15.53 °Brix). Cluster based estimation of means proves to be very useful in selecting the genotypes for breeding programme, as the requirement of tedious efforts of screening the inferior germplasm lines can be ruled out.

In conclusion, the genetic divergence of 23 pomegranate genotypes assessed using Mahalanobis D^2 revealed larger inter-cluster distances than the intra-cluster distances demonstrating a wider genetic divergence between the genotypes of the cluster with respect to the traits under consideration. The wide variations for different traits and their *per se* performance for economic traits of pomegranate suggest that, genotypes from desirable clusters could be directly used for final field evaluation in advanced breeding experiments depending upon the breeding objectives. As all these cultivars are

popular cultivars (except the mutants) accepted by farmers, hence, the present study can be helpful for pomegranate breeders selecting superior parents for hybridization as well as for the development of mapping population by selecting multiple diverse parents so that the progeny would be effectively utilized for mapping and locating QTL for number of economic traits rather than only few traits with

Table 6. Cluster means of four clusters for 35 quantitative (morphological and biochemical) traits in D^2 analysis.

Sl. No.	Characters	Cluster I	Cluster II	Cluster III	Cluster IV
1	Fruit length (mm)	72.43	56.43	112.35	70.57
2	Fruit diameter (mm)	72.28	56.87	95.87	72.8
3	Fruit shape	1.00	1.00	1.17	0.97
4	fruit volume (cm^3)	215.94	123.54	527.78	189.44
5	Fresh wt. of 100 arils	27.48	17.58	46.89	32.55
6	Dry wt. of 100 arils	5.86	2.51	9.62	8.03
7	Moisture %	21.65	14.51	20.53	24.66
8	Crown length (mm)	14.3	13.71	12.83	20.05
9	Peel weight	76.03	40.87	192.44	34.00
10	Aril wt	136	79.84	312.55	153.56
11	Seed%	63.52	64.06	61.89	81.4
12	skin%	36.48	35.94	38.11	18.6
13	Total No. of Arils/fruit	494.18	303.06	1077.89	317.44
14	aril length (mm)	8.86	7.79	11.48	9.63
15	aril width (mm)	5.91	4.68	8.63	6.72
16	seed length (mm)	6.9	5.97	7.28	5.89
17	seed width (mm)	2.93	2.88	2.89	3.04
18	rind thickness (mm)	3.55	3.25	4.19	1.44
19	Red coverage%	68.39	70.19	56.67	47.5
20	FC(L)	56.36	53.61	57.02	48.96
21	FC(a)	26.45	27.45	38.29	21.17
22	FC(b)	32.04	30.36	36.52	17.88
23	AC(L)	42.22	34	43.54	29.02
24	AC(a)	40.48	22.91	17.29	20.19
25	AC(b)	17.12	14.98	16.96	8.14
26	fruit weight (g)	212.03	120.72	505	187.56
27	Anthocyanin estimation (mg/L)	13.97	31.18	5.2	7.31
28	Ascorbic Acid (mg/100 gm)	27.4	24.24	25.6	50.00
29	Titratable Acidity (%)	0.11	0.33	0.19	1.09
30	pH of the Juice	3.49	3.41	3.99	2.53
31	Fruit Juiciness % (/100 gm aril wt.)	60.72	53.12	53.12	54.89
32	Total Sugars (%)	33.93	22.25	12.46	14.2
33	Reducing Sugars (%)	17.04	15.51	9.95	6.29
34	Non Reducing Sugars (%)	16.89	6.74	2.51	7.91
35	TSS (°Brix)	13.87	11.8	15.53	15.56

narrow genetic base. Most promising among them would be Ganesh and the genotypes of cluster I for the improvement in fruit morphological traits especially the most important one with bearing on total yeild like fruit weight, aril weight, peel weight and total number of arils.

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