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GENETIC ANALYSIS OF FERTILITY RESTORATION SYSTEMS AND CYTOPLASMIC TYPES IN ONION (*ALLIUM CEPA*) GENOTYPES USING DNA MARKERS

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Abstract

Comparing the yield of open-pollinated varieties (OPVs) and hybrids, signifies the importance of hybrid breeding. Hybrid development in onion relies on cytoplasmic genic male sterility (CGMS) system. But identification and maintenance of cytoplasmic genic male sterile (A) and maintainer (B) lines in biennial crop takes four to eight years through conventional breeding approach. However, DNA marker-based characterization of cytotype and restoration of fertility (*Rf*) gene reduces the time span to speed up hybrid development process. Present study was designed for morphological and molecular characterization of cytotype and *Rf* locus. For this purpose, twenty-one onion genotypes were characterized for bulb weight, bulb diameter, number of rings, plant height, and neck diameter during field experimentation. Likewise, four DNA markers i.e., 5Cob marker, OrfA501, orf725MFKR1 and orf725MFKR2 were used for cytotype and three markers i.e., AcSKP1FU, AcSKP1SU and AcPMS1 for *Rf* locus characterization. Molecular characterization classified genotypes into three groups: group 1 (including Phulkara, White-Pearl, Sultan, Yellow-S_3, Desi-Red-S_4, MirpurKhas-S_3, Nasarpuri-S_4, VRIO-02, Desi-Red, VRIO-10, VRI-06, Golden-ORB and T.E.G.) suited for deriving B lines; group 2 (featuring White-Pearl-V, 1122, Sultan-F1, HNO300A and Kessar) with potential for A line derivation; and group 3 (comprising of Hike and Glory) suitable for both A and B line derivation. These findings will help in faster derivation of A and B lines, consequently accelerating onion hybrid development.

Key words: Cytoplasmic genic male sterility, Cytotype, Hybrid breeding, Heterosis, DNA markers, nuclear genes.

Introduction

Onion (*Allium cepa* L.) holds a significant position in global agriculture and food production due to its multifaceted importance. It is a staple vegetable consumed in various forms worldwide and is used as a primary ingredient in countless cuisines, imparting flavor, aroma, and nutritional value to dishes. Beyond its culinary significance, it is valued for nutritional attributes i.e., rich in essential vitamins, minerals, and antioxidants, hence, contributing to human health and well-being (Arshad *et al*., 2017). There is need to establish hybrid system in onion due to its cross-pollination mode of reproduction, which results in increased genetic diversity within varieties allowing increased adaptability to agro-ecological conditions. However, prevalence of cross-pollination in onion still varies, and certain varieties may exhibit some degree of self-pollination. Developing onion hybrids allows for controlled crossbreeding between genetically distinct parent lines, resulting in offspring with desirable traits and more heterosis (Khar & Singh, 2020).

Moreover, it is crucial for exploitation of heterosis for better yield. Male sterility, a trait often associated with certain onion genotypes, is desirable for hybrid seed production as it prevents self-pollination, promoting the purity and consistency of hybrid seed (Ahmad *et al*., 2020). Understanding the cytotype and genetic basis of fertility restoration in onions is of primary importance for establishment of AB system. Cytoplasmic male sterility (CMS) is a phenomenon where the inability to produce functional pollen is controlled by mitochondrial genes in the cytoplasm (CMS genes), rather than nuclear genes. CMS in onions is often classified into two major types, CMS-S and CMS-T, each of which interacts differently with nuclear genes to regulate fertility (Kim *et al*., 2009).

On the other hand, fertility restoration is a process by which male fertility is regained in plants being cytoplasmic male sterile (Ferreira *et al*., 2017). Fertility restoration system in onions is primarily associated with specific genes i.e. *Rf* locus characterized by ACPMS1 marker (Kim, 2014). *Rf* gene is typically dominant, and its presence ensures that male-sterile plants produce functional pollen, enabling them to set seeds on crossing with male-fertile lines (Manjunathagowda & Selvakumar, 2021).

Different DNA marker systems are available for cytotype (Kim *et al*., 2009; Ahn & Kim, 2023; Khrustaleva *et al*., 2023) and *Rf* locus (Kim *et al*., 2015) characterization. Specifically, codominant marker orf725 characterizes genetic variation in cytoplasm type, discriminating between CMS-S (628 bp allele), CMS-T (628 and 833 bp alleles), and fertile cytoplasm N (833 bp allele) (Khrustaleva *et al*., 2023). Likewise, ACPMS1 marker classifies genotypes based on fertility restoration locus being dominant (242 bp allele) which carry a dominant restoration allele, the recessive (e.g., 276 bp allele) which does not exhibit ability to restore fertility on its own and results in male sterility in homozygous condition and the heterozygous (both 242 and 276 bp alleles), capable of restoring fertility (Kim *et al*., 2015).

In this study, we employed molecular markers, Orf725 and ACPMS1 to unravel genetic control of different cytotype and fertility restoration system in 21 onion genotypes. Furthermore, genotypes were characterized based on different morphological characters such as bulb weight, bulb diameter, number of rings, neck diameter and

plant height. These findings will be valuable for onion breeders, enabling them to make informed decisions in developing onion hybrids at rapid pace to deliver more gains not only to farmers but also ensures a consistent and high-quality supply of onions to consumers.

Table 1. List of genotypes used for morphological and molecular characterization of cytotype and *Rf* locus.

Sr. No.	Genotype name	Source of origin
1.	Phulkara	Vegetable Research Institute, Ayub Agricultural Research Institute, Faisalabad-Pakistan
2.	White Pearl-V	
3.	Sultan	
4.	Yellow S_3	
5.	Desi Red S_4	
6.	Mirpurkhas S_3	
7.	Nasarpuri S_4	
8.	Red Imposta S_3	
11.	VRIO-2	
12.	Desi Red	
13.	VRIO-10	
14.	VRIO-6	
20.	White Pearl	
9.	1122	Rashid Seeds Pakistan
10.	Golden ORB	Magnus Kahl Seeds Netherland
15.	Sultan F1	Rashid Seeds Pakistan
16.	T.E.G	Green Gold Agri Seeds (Pvt) Ltd. – Pakistan
17.	HIKE	National Trade Links seed corporation-Pakistan
18.	Glory	Klash Seed Company Pvt. Ltd.-India
19.	HON300A	Certus Seeds Kanzo Agpharma Combagro Evyol Group-Pakistan
21.	Kessar	National Trade Links seed corporation-Pakistan

Material and Method

Present study was conducted at Vegetable Research Institute, and Agricultural Biotechnology Research Institute, both located at Ayub Agricultural Research Institute, Faisalabad. Plant material was comprised of 21 onion genotypes (Table 1). The genotypes were sown in experimental area of Vegetable Research Institute, Faisalabad, Pakistan (between longitude 73º74 East, latitude 30º31. 5 North, with an elevation of 184m (604 ft.) for morphological characterization in the third week of October 2021.

Subsequently, seedlings were transplanted on both sides of 75 cm apart ridge with 10 cm plant to plant distance, in first week of December. The experiment was arranged in a randomized complete block design with three replications. The well-decomposed farmyard manure was applied at a rate of 10 tons per acre during land preparation one month prior to sowing. Chemical fertilizers were used at the recommended dosage of 50:35:25 kg per acre for nitrogen, phosphorus, and potassium, respectively. During land preparation, all the phosphorus, potassium, was applied into the soil. Half of the nitrogen was applied 30 days after the transplantation stage and remaining half was applied in two splits at 20 days interval. Moreover, recommended agronomic practices and plant protection measures were carried out throughout the growing season. Genotypes were characterized, for bulb weight (g), bulb diameter (cm), neck diameter (cm), plant height (cm), and number of rings per bulb. Ten plants were tagged, and data was also recorded regarding fertile/ sterile nature of plants.

During early growth stage, after germination and seedling establishment till 3-4 leaves a composite sample was drawn comprising of 02-03 leaves from selected 10 plants of each genotype. DNA was extracted using a modified CTAB (cetyltrimethyl ammonium bromide) protocol (Iqbal *et al*., 2021a; Jamil *et al*., 2021; Rahman *et al*., 2022). Similarly, DNA quality and quantity was checked using Nano Drop spectrophotometer (ND, 2000 Thermo fisher Scientific) and agarose gel electrophoresis. Molecular markers i.e. 5Cob marker S, 5Cob marker N, orfA501, orf725 MFKR1 and orf725 MFKR2 distinguishing onion S cytoplasm from N and T cytoplasm (Kim *et al*., 2009; Ahn and Kim, 2023; Khrustaleva *et al*., 2023); and nuclear AcPMS1 and AcSKP1 markers that distinguishes alleles at the Ms locus for fertility restoration (Kim *et al*., 2015) were searched and got synthesized from GeneLink (https://www.genelink.com/) Company, USA (Table 2).

Table 2. List of DNA markers and primer sets used for molecular characterization.

Marker name	Forward primer	Character	Reference
5Cob marker S	F-GTCCAGTTCCTATAGAACCTATCACT R-CTTTTCTATGGTGACAACTCCTCTT	Cytoplasm type	(Khrustaleva *et al*., 2023)
5Cob marker N	F-TCTAGATGTCGCATCAGTGGAATCC R-CTTTTCTATGGTGACAACTCCTCTT		(Khrustaleva *et al*., 2023)
orfA501	F-ATGGCTCGCCTTGAAAGAGAGC R-CCAAGCATTTGGCGCTGAC		(Ahn & Kim, 2023)
orf725 MFKR1	F-CATAGGCGGGCTCACAGGAATA R-AATCCTAGTGTCCGGGGTTTCT		(Kim *et al*., 2009)
orf725 MFKR2	F-CATAGGCGGGCTCACAGGAATA R-CAGCGAACTTTCATTCTTTCGC		(Kim *et al*., 2009)
AcSKP1FU	F-GCAATACACAGCTTCTAGCTGAATT R-AACACACACACAGAGTGAGAAATTTTATAT	Fertility restoration locus	(Kim *et al*., 2015)
AcSKP1SU	F-TCTGTGTGTGTGTGTAATTTCTCTG R-CGGAAGATTAATATTTTGCGTATACAT		(Kim *et al*., 2015)
AcPMS1	F-GGTCACCAGGTGGAGAGAGAA R-TCATTGAGCTGCATCCAAAA		(Kim *et al*., 2015)

Polymerase Chain Reaction (PCR) was performed to amplify genes linked DNA markers. PCR products were separated by electrophoresis on 1.5% agarose gels using 1X TAE buffer. Further, gels were stained with ethidium bromide and visualized under ultraviolet light using gel documentation system as described by Jamil *et al*., (2020, 2020a) and Iqbal *et al*., (2021b). Gels were scored for presence or absence of amplicons in binary form. Mean data over replications of morphological characters were used to draw biplot to determine the genotypic variation and for identification of best genotype for each trait using Gen5 software. Moreover, binary data was used to perform cluster analysis and to determine the type of cytoplasm and fertility restorer genes. Correlation analysis was carried out using R software packages i.e. corr_mat, reshape2, ggplot2 and heatmaply.

Results

Morphological characterization: Morphological characterization for economical important traits i.e. bulb weight (BW), bulb diameter (BD), number of rings (NOR), neck diameter (ND) and plant height (PH) depicted significant variation (Fig. 1). The highest bulb weight (358 g) was recorded for 1122 genotype followed by Sultan F1 (316 g) and Kessar (291 g) whereas lowest bulb weight was recorded for NasarpuriS_4 (80.23 g) followed by Red ImpostaS_3 (82.55 g) and MirpurkhasS_3 (84.01 g). Similarly, highest bulb diameter was recorded for Glory (9.25 cm), White-Pearl-V (9.0125 cm) and Kessar 8.88 cm) with the lowest bulb diameter observed in Red ImpostaS_3 (4.96 cm), MirpurkhasS_3 (5.0 cm) and Desi Red S_4 (5.1 cm).

The neck diameter ranged between 0.93 and 1.3 cm with VRI-10 (1.3 cm), Desi Red (1.22 cm) & White-Pearl-V (1.12 cm) exhibiting the highest values, while Sultan F1 (0.93 cm), Golden ORB (0.94 cm) & Kessar (0.97 cm) showed the lowest values (Fig. 1). The number of rings varied from six to nine, with Sultan-F1, Kessar, HIKE, 1122, Phulkara and VRIO-10 displaying the highest number (09) and YellowS_3, Red ImpostaS_3, Desi RedS_4 exhibiting the lowest number (06). Minimal variation in plant height (ranging from 50-65.23 cm) was observed among genotypes with VRI0-10 (65.23 cm), Glory (63.44 cm), Phulkara (63.43 cm) recording the highest height and Desi RedS_4 (50 cm), YellowS_3 (50.11 cm), Red ImpostaS_3 (50.5 cm) the lowest (Fig. 1).

Likewise, correlation analysis showed positive association of plant height with bulb weight (0.13), bulb diameter (0.45), neck diameter (0.53) and number of rings (0.47). Bulb weight also exhibited a positive association with bulb diameter (0.89), number of rings (0.48) and negative association with neck diameter (-0.31). In the same way, bulb diameter displayed positive association with number of rings (0.67) and negative with neck diameter (-0.16). Moreover, neck diameter exhibited positive association with number of rings (0.08) (Fig. 2).

Molecular characterization: Different DNA markers were employed for characterization of cytotype and allelic composition at *Rf* locus (Table 2) but only two marker system i.e. Orf725MFKR1 and Orf725MFKR2 for cytotype and one marker system ACPMS1 for *Rf* locus were optimized. Orf725MFKR1 and Orf725MFKR2 when applied in multiplex PCR amplified two alleles i.e. 628 and 833 bp. These alleles identified different cytotype as CMS-S (628 bp), CMS-T (both 628 and 833 bp) and N (833 bp alleles) (Fig. 3).

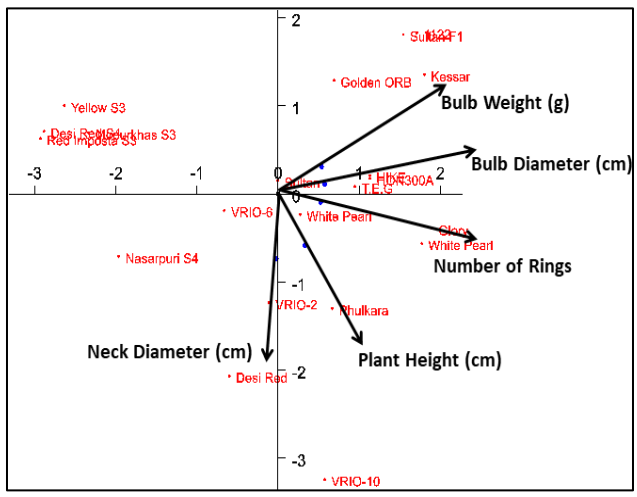

Fig. 1. Biplot analysis demonstrating morphological variability among twenty-one onion genotypes.

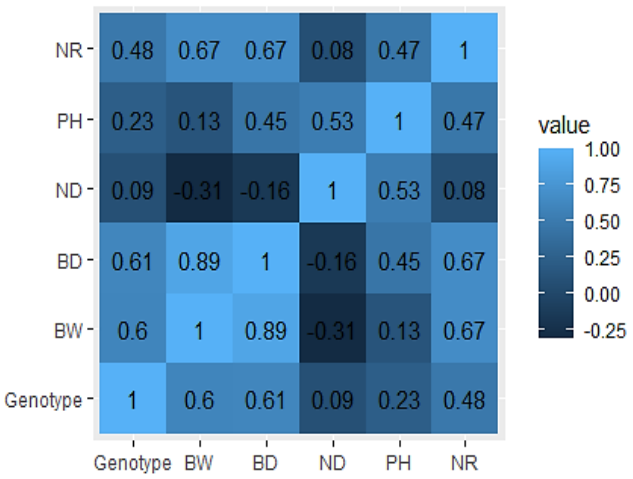

Fig. 2. Correlation heat map depicting the interplay among morphological traits.

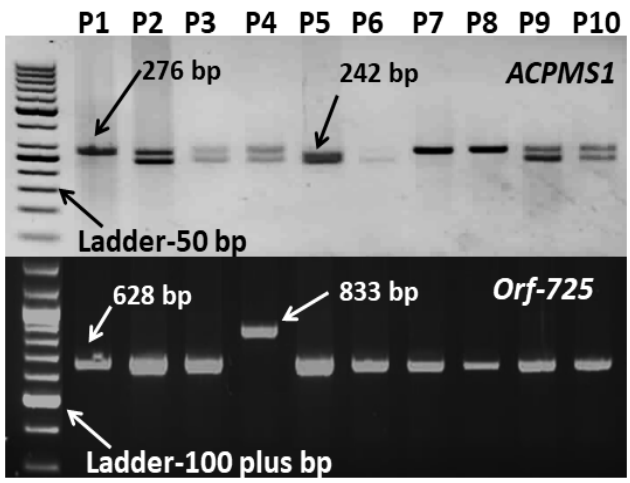

Fig. 3. Identifying cytoplasm types and fertility restoration mechanisms in VRIO-2 through molecular characterization.

On the other hand, *Rf* locus characterized by ACPMS1 marker amplified two alleles 242 bp, 276 bp and both for dominant, recessive and heterozygote phenotype respectively (Fig. 3). Dominant allele restores the fertility whereas recessive does not and heterozygote is also fertile. The Phulkara genotype had N cytoplasm (100%) and all plants were recessive (msms) at *Rf* locus hence all plants were fertile in the field observations as well. Similarly, White-Pearl genotype had N (90%) and T (10%) cytotype and all plants were recessive at *Rf* locus, therefore, 90% fertile and 10% sterile plants were observed in the field. The genotypes Sultan, Yellow-S_3, Desi-Red-S_4, Mirphurkhas-S_3 and Nasarpuri-S_4 had N cytoplasm (100%) and plants were fully fertile in field as well. The detailed characterization of all 21 genotypes elucidating cytotype, genetic composition at *Rf* locus and field observations are provided in Table 3 for in depth understanding.

The composition of A-line for hybrid development needs to be sterile (S, T) cytotype and recessive alleles at *Rf* locus whereas that of B-line is N cytoplasm and recessive alleles at *Rf* locus. Therefore, taking into consideration the above facts, the genotypes Phulkara, White-Pearl-V, Sultan, Yellow-S_3, Desi-Red-S_4, MirpurKhas-S_3, Nasarpuri-S_4, VRIO-02, Desi-Red, VRIO-10, VRI-06, Golden-ORB and T.E.G. were suitable for development of B-line. Similarly, White-Pearl, 1122, Sultan-F1, HNO300A and Kessar might be used for derivation of A-line and Hike and Glory for derivation of both A & B lines (Table 3).

Genetic similarity among genotypes: Binary data of 21 genotypes from *Orf725* and *Rf* loci was used to construct dendrogram based on Jaccords similarity coefficients following Unweighted Pair Group Method of Arithmetic Means (UPGMA) and SAHN clustering. Genetic similarity coefficients among genotypes varied from 0.47 to 1.00 and genotypes were largely classified into two groups. Cluster-I was comprised of Golden-ORB, Desi-Red-S_4, Yellow-S_3, White-Pearl-V, Phulkara, MirpurKhas-S_3, Nasarpuri-S_4, Sultan, VRI0-10, Desi-Red, VRIO-06, Red-Imposta-S_3, T.E.G. and VRIO-02 genotypes. The cluster-II was comprised of Glory, Kessar, 1122, HIKE, HON300A, White-Pearl and Sultan-Ad genotypes. Two genotypes VRIO-10 and Desi-Red were exactly alike, similarly Sultan and Nasarpuri-S_4 were identical to each other. Golden-ORB and Sultan-Ad were distantly related genotypes (Fig. 4).

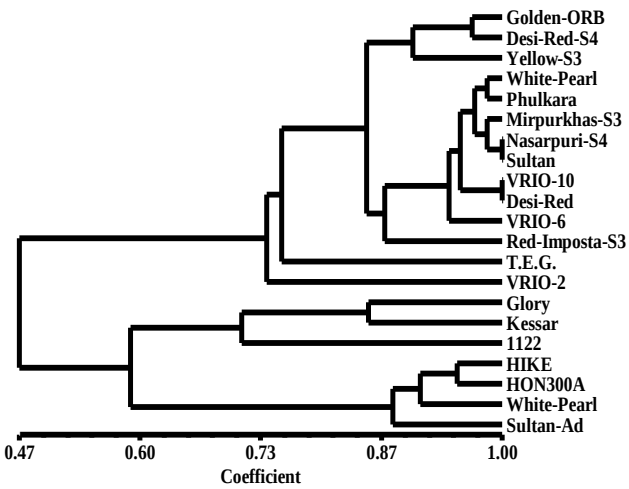

Fig. 4. Dendrogram of twenty-one onion genotypes constructed via UPGMA clustering using jaccard similarity coefficients based on binary data from orf725 and ACPMS1 loci.

Table 3. Molecular characterization of cytotype and *Rf* locus in twenty-one onion genotypes using specific DNA markers.

Genotype name	Cytoplasm type (Orf-725)			Nuclear genes (Rf locus/ ACPMS1)			Field observation	
	N (%)	T (%)	S (%)	MsMs (%)	msms (%)	Msms (%)	Fertile (%)	Sterile (%)
Phulkara	100	0	0	0	100	0	100	0
White-Pearl-V	90	10	0	0	100	0	90	10
Sultan	100	0	0	0	100	0	100	0
Yellow-S_3	100	0	0	10	40	50	100	0
Desi-Red-S_4	100	0	0	60	0	40	100	0
Mirpurkhas-S_3	100	0	0	0	100	0	100	0
Nasarpuri-S_4	100	0	0	0	100	0	100	0
Red Imposta-S_3	80	0	20	10	80	10	100	0
VRIO-2	90	0	10	20	30	50	90	10
Desi-Red	100	0	0	0	10	90	100	0
VRIO-10	100	0	0	0	90	10	100	0
VRIO-6	90	10	0	0	90	10	90	10
White-Pearl	0	100	0	0	10	90	90	10
1122	10	10	80	50	20	30	80	20
Golden-ORB	100	0	0	10	70	20	100	0
Sultan-F1	0	100	0	0	0	100	100	0
T.E.G	100	0	0	0	30	70	100	0
HIKE	10	90	0	0	10	90	90	10
Glory	10	10	80	0	60	40	60	40
HON300A	0	80	20	0	10	90	10	90
Kessar	0	30	70	10	50	40	50	50

Discussion

The average onion yield in top producing countries i.e., China (22 tones/ha) and India (16.18 tones/ha) is low in comparison to Netherland (49.70 tones/ha), United States (56.40 tones/ha) and Iran (37.95 tonnes/ha) because of cultivation of open pollinated varieties in former and hybrids in later countries (Manjunathagowda *et al*., 2021). However, hybrid breeding in onion being hermaphrodite flowers and highly cross-pollinated crop is a tedious job and physical emasculation and cross pollination is very difficult rather impossible (Manjunathagowda & Anjanappa, 2020). Therefore, male sterility-based hybrid breeding program is inevitable; however, it is difficult to deal with onion male sterility in the field being biennial crop and take usually 4-6 years to identify maintainer lines through progeny test (Manjunathagowda, 2021). Therefore, one should look around for other options around to cut short the breeding time span.

Male sterility/fertility system in onion is cytoplasmic genic male sterility system (CGMS). The cytoplasm induces sterility which is restored by corresponding nuclear genes and hybrids are developed through A, B and R/C line system (Manjunathagowda, 2021). Different available markers system for cytotype (Kim *et al*., 2009; Ahn and Kim, 2023; Khrustaleva *et al*., 2023) and *Rf* locus (Kim *et al*., 2015) were used for molecular characterization (Table 2). Only two marker systems i.e., Orf725 for cytotype and ACPMS1 for *Rf* locus were optimized as also reported by previous authors (Kim *et al*., 2009; Khar & Saini, 2016; Yu & Kim, 2021) whereas remaining markers i.e. cob marker and OrfA501 linked with cytotype and AcKP1SU and AcKP1FU linked to *Rf* locus were not amplified which brings into question the reliability of these markers. The limitations of PCR markers for accurate prediction of type of cytoplasm and nuclear composition at *Rf* locus was earlier highlighted by (Khar & Saini, 2016) and reason behind this was explained (Khrustaleva *et al*., 2016). Sometime markers work well in one population do not work in other populations. Therefore, relying on a marker requires its validation on contrasting parents and segregating populations (Khar & Saini, 2016; Khrustaleva *et al*., 2016).

Orf725 and ACPMS1 markers (Fig. 3) identified cytotype and *Rf* locus composition in first year of breeding cycle rather spending 04-08 years. Moreover, three groups of genotypes could be used for derivation of A and B lines (Manjunathagowda, 2021). Further, type of sterile cytoplasm either CMS-S or CMS-T was also explored as it was also useful because, CMS-S cytoplasm was preferable over CMS-T due to stability in different environmental conditions (Havey, 2000). Our study also provided evidence that Orf725 and ACPMS1 were reliable markers for identification of cytotype and genetic composition at *Rf* locus for local germplasm as confirmed through markers results and field validation (Khar *et al*., 2022).

Furthermore, field study suggested that bulb diameter, plant height and number of rings had positive correlation with bulb weight whereas neck diameter showed negative association (Fig. 2). For better yield indirect selection for these traits will help in improvement of bulb weight (Chalbi *et al*., 2023). The biplot analysis identified Sutan-F1, 1122, Kessar, Glory, White-Pearl-V, Phulkara and VRIO-10 as best genotypes based on growth parameters (Fig. 1). These genotypes should preferably be used for derivation of A-line and B-line due to better growth and heterosis. Additionally, it was proved that biplot was still a better tool for exploitation of variation among genotypes and to identify best genotypes (Tahir *et al*., 2020). Furthermore, genetic diversity was studied among onion genotypes for Orf725 and *Rf* locus using cluster analysis, with genetic similarity coefficient ranging from 0.47 to 1.0. The genotype VRIO-10 and Desi-Red were exactly alike similarly; Sultan and Nasarpuri-S_4 were identical (Fig. 4) which indicated that these genotypes would express less heterosis if used as parents in development of a particular hybrid hence, their crossing be avoided for exploitation of better heterosis as crossing of distant parents yields better heterosis (Tahir *et al*., 2018).

Conclusion

Twenty-one onion genotypes were characterized morphologically for identification of best genotype for derivation of A and B lines. Further, characterization for cytotype, CMS-S, CMS-T or N and genetic composition at *Rf* locus whether dominant (*MsMs*), recessive (*msms*) or heterozygote (*Msms*) was also carried out and it will be useful for derivation of A-line, B-lines in one year rather four to six years for utilization of CGMS system in onion for fast-track establishment of hybrid breeding program.

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