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Assessing the Genetic Diversity of Squash Genotypes based on Morphological and Molecular Analysis

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ABSTRACT

The genetic diversity increases crop base for selection of varieties, farming yield in agriculture. A study on genetic diversity was conducted for Squash collection (*Cucurbita moschata)* from Indian origin genotypes. The thirty four genotypes were studied with eleven morphological characteristics, which showed 76.4% variation with two different molecular markers analysis, twenty five random amplified decamer and nine Short sequence repeat (RAPD, SSR) showed polymorphism results, the genetic relationship among 34 genotypes were performed by UPGMA cluster analysis, showed three distinct dendogram, each cluster matrix was estimated based on genetic distance, The results showed wide genetic variations among genotypes, taxonomic distance range (0.98-1.68). The associated passport traits with agronomic breeding characters of interest showed significant positive relatedness. The characterization of accessions is important for genetic resource knowledge and crop improvement in vegetable farming.

Keywords: Biodiversity; Cluster Analysis; Fruit Quality; Germplasm; Molecular Markers; Nutrition Value; PCR; Phenotypic Traits; Yield.

1. Introduction

Cucurbits are frost sensitive and tendril bearing plants predominantly found in subtropical and tropical regions of the world (Robinson and Decker-Walters, 1999). Members of Cucurbitaceae are well known for their nutritional and medicinal values, and as potential sources of crop diversity (Ferriol *et al.*, 2000). India is blessed with a rich diversity of cucurbits and is believed to be the primary or secondary center of origin of many of the gourds and melons (Choudhury, 1996). Cucurbit vegetables like pumpkin and squash were introduced in India and now they are well-adapted and become a Squash (*Cucurbita maschata* Duch ex Poir.) is popular vegetable crop in India (Ferriol *et al.*, 2007). The plant is characterized with yield traits like fast growing vines, tendril, fruit size and shape, highly useful in farming. The mature fruits have long shelf-life even at ambient temperatures thus can be stored easily and used whenever other vegetables are not available in the market. The different parts of plants, *viz.*, shoot-tips, vines, male flowers, immature and mature fruits, and seeds are consumed in a variety of ways. Fruit size, flesh color and thickness are some important criteria for consumer preference (Ferriol *et al.*, 2008). Fruits contain good amount of carotene, which is supplement of Vitamin A, seed oil and extracts have been reported to be useful in the prevention and treatment of benign prostate hyperplasia (Garcia *et al.*, 2001). In addition, plants are also used as rootstocks for melon and other cucurbit cultivars (Robinson and Decker-Walters, 2002). In India, it is cultivated on a large-scale in the states of Bihar Uttar Pradesh, West Bengal, and Orissa. Indian accessions have great variability in terms of morphological characters, especially growth habit, fruit size and shape, and skin color (Pandey *et al.*, 2003). The genotype identification based on external morphology only, creates chances of repetition in genotype selection, and unique cultivars may get restricted in local distribution areas. The studies based on genetic resource diversity had been performed on important cucurbit crops like *Cucumis sativus, B. hispida*, *Citrullus vulgaris*, *Cucumis melo*, *Cucurbita moschata C. vulgaris* (Baranek *et al.*, 2000, Brown *et al.*, 2000, Gwanama *et al.*, 2000).

Although there is substantial variation in squash vegetative traits but still there differentiation based on phenotype alone needs support.

1.1. Study Objectives

The main objectives of this present study are as follows:

(1) To find the variations based on agro economic traits in squash accessions available for vegetable farming.

(2) To find the biodiversity present in the collected diversified Germplasm.

(3) To identify the genetic relatedness between the diversity cultivated in different agro climatic zones.

(4) To correlate the genetic relatedness based on phenotype traits and genotypes based on molecular polymorphism.

2. Materials and Methods

2.1. Phenotype evaluation

A total of 34 landraces including 26 indigenous germplasm and 8 elite cultivars were included in the present study. The indigenous germplasm accessions were collected from different sites covering a wide range of agro-climatic zones including hot, semi-arid, lowlands to warm wet highlands. The morphological data of squash accessions were recorded after field evaluation (De *et al.*, 2003). The experiment was laid out in a complete randomized block design (CRBD) with three replications. The morphology was studied and data was recorded on 11 quantitative traits as mentioned in Table1.

2.2. Genotype evaluation

The genomic DNA was extracted using modified protocol (Doyle and Doyle, 1990). The DNA quality was checked on 0.8% agarose gel. The DNA was genotyped by screening 230 random 10-mer primers (Operon Technologies, USA) through PCR amplification, The PCR reaction followed initial denaturation at 94 ^{o}C for 4 min, followed by 40 cycles with 1 min at 94 ^{o}C, 1 min at T_m-5 ^{o}C, and 1 min and 72 ^{o}C; the final extension was allowed for 10 min at 72 ^{o}C. Each 25 µl reaction volume carried 15 ng genomic DNA, 1.5 mM PCR buffer (MBI Fermentas, USA), 400 µM dNTPs (MBI Fermentas), 1.5 U *Taq* DNA polymerase (MBI Fermentas), and 4 µM primer each using a thermal cycler (T1, Biometra, Germany). Later the amplified DNA fragments were resolved through electrophoresis in 1.2% agarose gel electrophoresis (Singh *et al.*, 2015). Similarly the Short Sequence Repeats (SSR) primers were also used to perform PCR analysis, selected from previous reports on *C. maxima*, *C pepo* and other cucurbit crops (Katzir *et al*., 1998, Danin-Poleg *et al*., 2000 and Chiba *et al*., 2003).

2.3. Statistical analysis

Each polymorphic product was scored manually as a binary character for RAPD dominant marker and SSR co dominant marker. All the statistical analysis was performed using the software NTSYS-pc ver. 2.02 (Rohlf, 1998). Pair-wise combinations of genotypes were employed to calculate Jaccord's similarity coefficient (GS): a/(n-d), where a, is the number of positive matches, n, is the total sample size, and d, is the number of negative matches. Genetic distance (GD) between pair of lines was estimated as GD = 1 - GS (Nei, 1972). The dendogram was

prepared based on unweighted pair-group method (UPGMA) using the SAHN module of the software. The correspondence between the matrix of Euclidian distances (average taxonomic distance values derived from 11 quantitative characters) and the matrix of marker-based distances (Jaccard's similarity values) was tested with the MXCOMP module of the software.

3. Results

3.1. Clustering of genotype based on the quantitative traits

The 34 accessions displayed a great diversity for most of the morphological and yield associated traits, especially days to first male flower, fruit weight, flesh thickness and yield per plant (Figure 1 and Table 1). All the accessions differed significantly with respect to different quantitative characters. The genotype SPP-90 was the earliest (45 days after seed sowing) in appearance of male flower, while for female flower appearance, it was only next (48 days) to the genotype, Kashi Harit which showed earliest appearance of female flower (45 days). The genotype Pumpkin-172 was the most late-flowering for male (60 days) as well as female flowers (61 days). Each of the accessions had 3, 4 or 5 primary branches. There was considerable variability in the vine length of different accessions; genotypes like MKV/SP-02 (6.55 meter) and Narendra Amrit (6.23 meter) had significantly higher vine length compared to those of lowest vine length of Tonk Local (3.15 meter). The genotype Narendra Amrit recorded the highest yielder (17.96 kg) while HAPK-4 was the lowest (3.1 kg), this may be attributed to their mean individual fruit weight (1.55 kg for HAPK-4 and 8.98 kg for Narendra Amrit. The genotype, DR/SP-04-07 was the only other genotype to record a higher individual fruit weight (7.05 kg). The mean number of number of fruits per plant ranged between 1.55 (Gujarat-1) and 3.0 (MKV/SP-04). Flesh thickness has a greater influence on consumer preference; in this respect, the genotypes SPP-11 and Gujarat-1 had the highest (5 cm) thickness. The Indian landrace, Hardoi Local was significant in vine length, and number of fruits per vine.

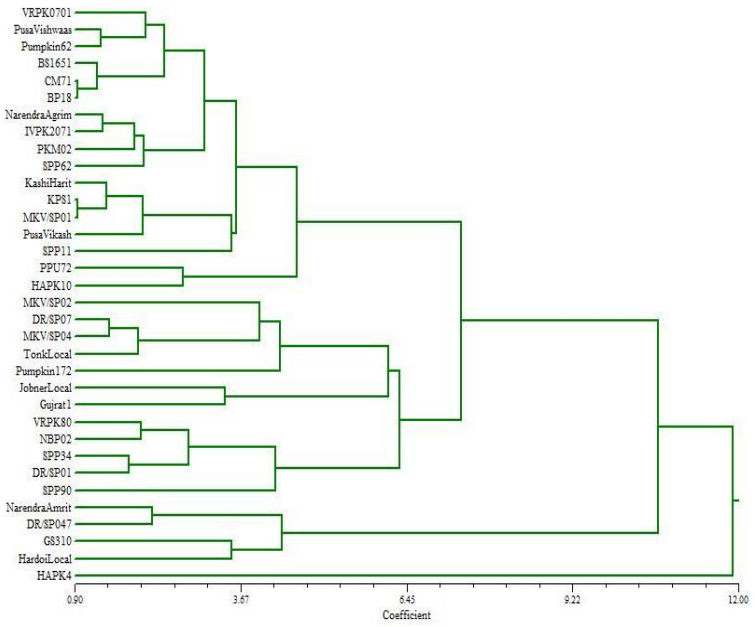

Figure 1. Genetic relationship among the 34 genotypes of pumpkin based on quantitative traits using UPGMA cluster analysis of the distance matrix.

Table 1. Description of morphology

S. No.	Morphological traits observed in the study
(i)	days taken to first male flower
(ii)	days taken to first female flower
(iii)	number of primary branches per plant recorded at 80 days after seed sowing
(iv)	number of node at which first female flower appeared
(v)	vine length (cm)
(vi)	individual fruit weight (kg)
(vii)	polar circumference of fruit (cm)
(viii)	equatorial circumference of fruit (cm)
(ix)	number of fruits per plant
(x)	flesh thickness (cm)
(xi)	fruit yield per plant (kg)

3.2. Clustering of genotypes based on molecular markers

The cluster analysis based on eleven quantitative traits revealed two major clusters, at taxonomic distance of (TD 1.12), (TD 1.16,) for 14 genotypes in cluster 1, and 6 genotypes in cluster 2 (Figure 1 and Table 2). The cultivars VRPK-701, BS-165-1, Pusa Viswash, Punpkin-62, Kashi Harit, Pusa Vikash showed similarity in subgroup Ia, (TD 0.98-1.47), whereas landraces SPP-11, DRSP-01 were grouped together cluster 1b (TD1.68) and six other DRSP-07, KPS-1, SPP-34, Narendra Agrim, NBP-02, Tonk Local grouped in cluster 1c (TD 0.74-1.68). The second major cluster showed similarity on traits like yield and fruits number, between six genotypes CM-71, SPP-62, HAPK-10, BP-18, PKM-02, HAPK-4. The other 14 genotypes were ungrouped based on quantitative characters, the cultivars VRPK-07-01 and BS-1651 showed large taxonomic distance with Hardoi Local and Gujarat-1. Further to understand the genetic association between the different genotypes, molecular study was performed using random amplified and short sequence tags markers (RAPD, SSR), the RAPD markers OPK-9, OPG12, OPA-12 and OPY-19 showed 76.5% polymorphism, with average size of 250 base pair amplified products. In general, the extensive marker assessment proved OPF, OPG and OPY series to be informative producing 100% polymorphism during basic genetic diversity analysis. The Jaccard's similarity coefficient ranged from 0.34 to 0.66. The cluster analysis showed KPS-1 and VRPK-07-01 (TD 0.96) between genotypes. The lowest index (TD 0.02) was found between MKV/SP-01 and PPU-72. The dendogram showed two major clusters; cluster, I with 18 accessions and cluster II had 4 genotypes (Figure 2). The relatedness VRPK-07-01, MKV/SP-02, Jobner Local, VRPK-80, HAPK-4, GS-310, and Pumpkin-62, SPP-62, BP-18, Hardoi Local, SPP-34, SPP-11, DR/SP-04-7 and MKV/SP-01 was highest. The results were compared with 149 microsatellite marker genotyping, were five genotypes BS-165-1, GS-310, Narendra Agrim, Narendra Amrit and VRPK-07- 01 had highest similarity (Figure 3 and Table 3). In this screening only eight primers (5.36%) revealed polymorphic amplification pattern. The study identified five significant SSR markers (CMCT170b, CMMS 2-3, CMGAN62, CMMS 2-3, and CMGAN-12) which showed high polymorphism and suitable for genetic study in *Cucurbita moschata spp*. None of the SSR primers reached 100% level of polymorphism. The UPGMA dendogram revealed closest genetic relatedness

between genotypes VRPK-80 and SPP-11 (TD 0.93), and HAPK-4 and IVPK-207-5-1 (TD 0.06), (fig.3). As defined by SSR markers three major groups at an average coefficient level of 0.51; cluster I having 19 genotypes, cluster II having 12 genotypes, and cluster III with two genotypes (SPP-34 and MKV/SP-04) were diversified. The only genotypes which did not have genetic relatedness were accession IVPK-207-5-1.

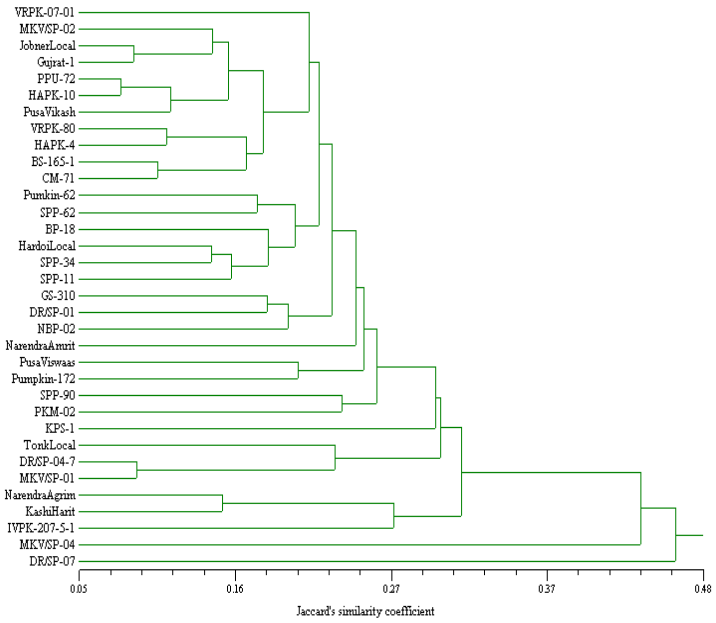

Figure 2. Genetic relationship among the 34 genotypes of pumpkin based on RAPD markers using UPGMA cluster analysis of the distance matrix.

Table 2. Description of Pumpkin Germplasm according to agro climatic zones of India

Zone III	PPU-72
	Pumpkin-172
	Pumpkin-62
Zone IV	KPS-1
	Narendra Agrim
	Narendra Amrit
	Pusa Vikash
	Pusa Vishwaas
	Kashi Harit
Zone V	BP-18
	BS-165-1
	CM-71
	DR/SP-01
	DR/SP-04-07
	DR/SP-07
	VRPK-07-01
	VRPK-80
	Hardoi local

Zone VI	HAPK-10
	HAPK-4
	IVPK-207-5-1
	Jobner Local
	MKV/SP-01
	MKV/SP-02
	MKV/SP-04
	NBP-02
	PKM-02
	SPP -90
	SPP-11
	SPP-34
	SPP-62
	Tonk Local
Zone XIII	GS-310
	Gujrat-1

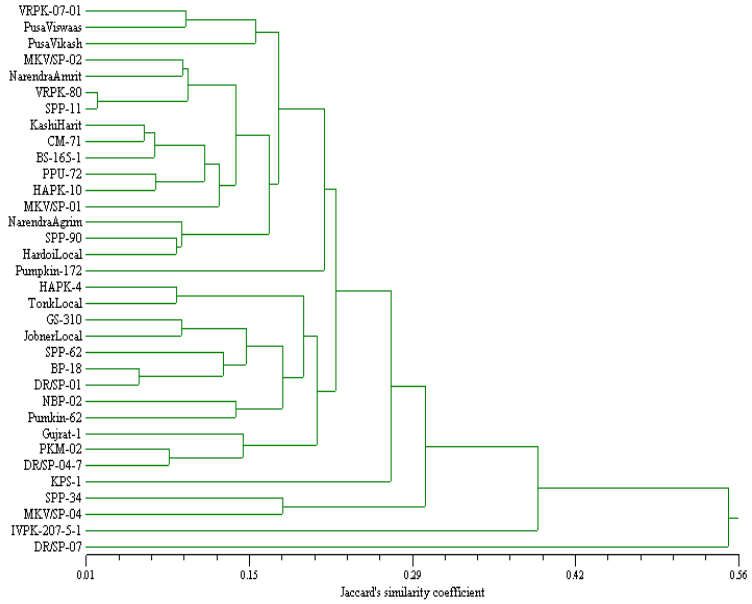

Figure 3. Genetic relationship among the 34 genotypes of pumpkin based on SSR markers using UPGMA cluster analysis of the distance matrix.

Table 3. RAPD primers used in this study, their sequence, number of polymorphic products, percentage of polymorphism and base pair range.

Serial No.	Primer	Sequence(5'- 3')	Total no. of bands	Number of polymorphic bands	Percent of polymorphism	Range of bands(bp)
1.	OPA-9	GGGTAACGCC	5	4	80.0	750-1500
2.	OPA-12	TCGGCGATAG	3	2	66.6	500-1000
3.	OPAF-7	GGAAAGCGTC	6	5	83.3	500-1100
4.	OPAF-13	TGAACCGAGG	5	3	60.0	500-2000
5.	OPAF-15	CACGAACCTC	5	3	60.0	250-1000
6.	OPAG-9	CCGAGGGGTT	4	2	50 .0	500-2000
7.	OPAG-10	ACTGCCCGAC	7	4	56.8	350-1500

8.	OPC-9	CTCACCGTCC	7	5	71.0	275-1300
9.	OPC-10	TGTCTGGGTG	5	4	80.0	500-2500
10.	OPF-8	GGGATATCGG	4	4	100.0	750-1000
11.	OPF-10	GGAAGCTTGG	8	7	87.5	250-1500
12.	OPF-17	AACCCGGGAA	5	5	100.0	1000-1500
13.	OPG-5	CTGAGACGGA	6	5	100.0	500-1500
14.	OPG-12	CAGCTCACGA	3	3	100.0	500-1500
15.	OPG-17	ACGACCGACA	9	8	88.8	150-1000
16.	OPH-9	TGTAGCTGGG	4	3	75.0	600-1000
17.	OPH-12	ACGCGCATGT	4	3	750	200-400
18.	OPK -7	AGCGAGCAAG	6	4	66.6	450-1500
19.	OPK-9	CCCTACCGAC	3	2	66.6	150-1000
20.	OPL-13	ACCGCCTGCT	7	5	71.4	250-3000
21.	OPM-5	GGGAACGTGT	7	3	42.6	500-1600
22.	OPU-7	CCTGCTCATC	5	4	80.0	750-2000
23.	OPY-3	ACAGCCTGCT	4	4	100.0	500-1000
24.	OPY-9	AGCAGCGCAC	4	4	100.0	250-2000
25.	OPY-19	TGAGGGTCCC	10	8	80.0	500-2000
	Mean		**136**	**104**	**76.4**	**150-3000**

Table 4. SSR primers used in this study, their sequence, number of polymorphic products, percentage of polymorphism and base pair range

SSR Primers	**Core motif and number of repeats**	**Sequence (5'-3')**	**Alleles**	**P %**	**Fragment size (base pair)**	**Reference**
CMCTN50	-	TCTACTTCCATGAATCCATC TAGAATGGTTAGGAAACCCT	2	40%	250-600	*Danin-Poleg et al* (2001)
CMGAN12	-	TTTTTGTCGTTATATGAGGG GTTGCATAATGCTAATTT GG	3	60%	200-500	*Danin-Poleg et al* (2001)
CMGAN62	-	AAGATCGCCTCTATTCACAG ATTTGTACTCCCAACGCATC	5	83.3%	250-1300	*Danin-Poleg et al* (2001)
CMATN101	-	GCTTGTCTTTGTGTGTTTGC GAGAACAAGACTCCTTAATCC	3	60**%**	250-1500	*Danin-Poleg et al* (2001)
CMCT170b	(CT)8	ATTGCCCAACTAAACTAAACC CACAACACAATATCATCCTTG	1	33.3%	150-750	*Danin-Poleg et al* (2001)
CMMS1-7	(GT)12	CAAAAGACAAGACGAAGACACC AGACAACTGGTCCACACACACAGT	2	40%	150-1300	*Chiba et al* (2003)
CMMS36-2	(GA)27(GA)26 (GT)12(CAAA)9	CCACACATTACAAACTAAACAAACA GGATTCCGATTTGGTGTGGCGTTT	2	66.6%	200-600	*Chiba et al* (2003)
CMMS2-3	(GA)19	ATCACCCACCCCACCAACATAACA CCTTGGAAACATAACAC	5	71.4%	50-900	*Chiba et al* (2003)
Mean			23	56.8%	50-1500	

4. Discussion

The effective exploitation of the diverse germplasm can be achieved with genetic diversity during parental selections and the introgressions of useful genes into popular cultivars. The squash accessions in India, have great variability in terms of morphological characters, especially growth habit, fruit size and shape, fruit skin color (Pandey *et al.,* 2003), however, it is largely difficult to distinguish a genotype based on external morphology for the purpose of breeding and selection, at traits like yield and yield contributing phenotypes. The genetic resources from different agro-ecological regions present regional market consumption and availability, which affects the farmers economic and growth index. The traits studied days to male and female flower appearance, vine length, number of branches, fruit size and shape, rind pattern and flesh colour are characters for selection to increase market value of the crop. The diversified quantitative characters in the farming pattern increase yield and improve fruit quality, though morphology may diversify, genetic relatedness cannot be estimated unless the presence of variation among the germplasm is confirmed (Katzir et al., 1998, Danin-Poleg *et al.*, 2000). The molecular analysis showed 76.4% polymorphism, this variation was largely expected for the cultivars as selection forces both dominant and recessive alleles are towards fixation (Chiba et al. 2003). Similarities of all 34 genotypes reflected in the 104 RAPD loci and 23 SSR allele specific loci were calculated and clustered into several groups (Figures 2 and 3), and those which were genetically unified grouped together, though grown in different agro climatic zones, this results show synchronization with other studies (Wessel-Beaver L, 2000). No separation in variation was observed between local collections of the Uttar Pradesh and rest of the country, this may be due to initial intermixing in breeding, selection of cultivars of different regions. Though the inter breeding of characters, generate improved traits in cultivated lines, it dissolves the indigenous lines and identification of germplasm origin, a passport data of each germplasm collection based on molecular identification is essential for any further studies in plant pathology, disease resistance and crop improvement programs.

The significance of genotypes HAPK-10 and PPU-72 developed from Ranchi and Punjab may not be separated genetically as consistently grouped together during molecular analysis showing sharing one common ancestral lineage. Two other germplasm MKV/SP-04 and DR/SP-04-07 collected from different agro-climatic zones (Uttar Pradesh and West Bengal) diversified in morphology, clustered together in molecular analysis indicating similar genetic constitution. One genotype KPS-1 obtained from Kanpur district remained invariably distinct in both test may be considered a separate genetic resource in squash. The transferability in the traits showed limitation between muskmelon (*C. melo* L.) and pumpkin (*Cucurbita moschata*), (about 5.36%), which is similar to previous reports (Ferriol et al., 2008), also confirms the unique microsatellite sequence exploitation due to conservation in the flanking regions among the *Cucurbita species*. Other similar studies report utilization of SSR markers, for research on genome structure and evolution of Cucurbitaceae (Chiba *et al.,* 2003). The four varieties of pumpkin Pusa Vishwas, Kashi Harit, Pusa Vikash and Narendra Agrim obtained from local markets of Gorakhpur, Faizabad and Basti district, always grouped together in the study, showing their common genetic resource, a cultivated genotypes for yield, and their commercial demand in North Indian market. We also identified varieties VRPK-701, MKVSP-04 found in similar agro climatic region had no genetic relatedness and remained separated in cluster analysis, further. The genetic studies on Pumpkin genotypes BS165-1, CM-71, PPU-72, HAPK-10, Pusa Vikas,

VRPK-80, Hardoi Local and SPP-11 had no direct correlation in morphology still similar in cluster analysis, were not expected may be due to neutral genetic variation, this results are similar to previous reports (Young *et al.*, 1998) The high genetic variations among the genotypes in the study is an attempt to understand the local germplasm of India and curate a profiling in close agreement with other studies performed in pumpkin accessions other parts of world (Gwanama et al., 2000, Baranek et al., 2000, Ferriol et al., 2008).

5. Conclusion

Germplasm characterization and evaluation generate the information for more efficient utilization of these valuable resources. The study concludes to find the distinct characters in Indian squash germplasm, few of them are described below.

(1) The inter relatedness of genotype though show phenotypic variation may perform similar breeding results as they fall in close genetic proximity.

(2) The information gathered for the germplasm will be aimed to generate new improved cultivars of squash in future.

(3) An unambiguous, reliable, fast and cost-effective assessment of genetic diversity protect breeder's intellectual property rights, time.

(4) The divergent genotypes represent number of heterogeneous group which may be used in future crop improvement.

(5) The dissimilarities shown in the study show rather modest variation in landraces cultivated in India may be considered for future research work of generating recombinant inbred lines, etc.

(6) The molecular markers may be useful for analyzing the genetic studies of squash landraces in future.

Declarations

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This study received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Competing Interests Statement

The authors declare that they have no competing interests related to this work.

Consent for publication

The authors declare that they consented to the publication of this study.

Authors' contributions

All the authors took part in literature review, analysis, and manuscript writing equally.

Availability of data and materials

Supplementary information is available from the authors upon reasonable request.

Institutional Review Board Statement

Not applicable for this study.

Informed Consent

Not applicable for this study.

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