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Genetic diversity estimates in landraces of pumpkin (*Cucurbita moschata*) from North Eastern Himalayan region of India using agro-morphological and molecular traits

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ABSTRACT

The present investigation was carried out to assess the diversity existing among the landraces of pumpkin (*Cucurbita moschata* Duch.) from North Eastern Himalayan region of India. Clustering analysis based on morphological and molecular traits exhibited high level of genetic diversity and grouped the pumpkin landraces into 5 major clusters. Maximum and minimum inter-cluster average D^2 values obtained were 442.27 and 86.29, respectively. High degree of correlation was found between diversity obtained by morphological and molecular traits. The highest and lowest PIC values of SSR markers observed was 0.91 (OCM2) and 0.30 (OCM9) respectively and the Jaccard's similarity coefficient ranged from 0.51 to 0.92. The most divergent landraces obtained (GHP 1, GHP 26, GHP 43, GHP 51, GHP 52 GHP 56) from this study can be effectively utilized in future pumpkin breeding programme for development of new hybrid combinations.

Key words: Clustering, Genetic divergence, Pumpkin landrace, SSR

Cucurbita moschata Duch. (2n=2x=40) commonly known as pumpkin is a high value vegetable due to its long shelf life, long period of availability, high nutritive estimates in mature and immature stages, and better transport qualities (Muralidhara and Narasegowda 2014). Pumpkin is a potential crop to improve the nutritional status of the people, particularly with respect to vitamin A (Satkar *et al*. 2013).

Analysis of extent of genetic variability for desirable traits is an important aspect for any crop improvement and variety development programme (Devi *et al*. 2016). Characterization and classification of the existing landraces morphologically is helpful for preliminary evaluation and classification of germplasm (Barboza *et al*. 2012). On the other hand, molecular markers such as simple sequence repeats (SSR) provide a quick and reliable method to estimate the genetic relationships among germplasm (Lakhani *et al*. 2015).

The loss of traditional landraces is a major concern in any breeding programme and it needs collection and variability analysis prior to their use in breeding program (Oliveira *et al*. 2016). Genetic diversity observed across the local races of pumpkin from north east India represents an important fraction of the genetic variability exhibited by this genus. Our hypothesis is based on information which reveals unscientific management and less availability of distinguishing morphological features among the landraces of pumpkin from North Eastern India. In this context, the study was aimed to measure the extent of genetic diversity among the pumpkin landraces based on morphological traits using multivariate analysis and also using molecular markers to identify parents of diverse group that could be recommended for crossing in pumpkin breeding program.

MATERIALS AND METHODS

A total of 60 pumpkin landraces were collected from different geographical locations of North Eastern India during 2014-2016 (Supplementary Table 1). Landraces were raised systematically during the research period in randomized block design (RBD) with 3 replications at spacing of 2 m × 1 m. Recommended cultural practices were followed during crop growing period and to control red pumpkin beetle at seedling stage and fruit fly at fruiting stage, control measures were taken according to Muralidhara and Narasegowda (2014).

Observation on 11 quantitative characters like vine length (m), number of leaves/plant, days to first female flower, fruit weight/plant (kg), fruits/plant, fruit diameter (cm), fruit skin toughness (g/mm^2), fruit skin thickness (mm), flesh thickness (cm), TSS (%) and fruit yield (tonnes/ha) were recorded using 5 randomly selected plants of each landrace at the vegetative, flowering, fruiting, and harvesting stages. The mean values of the observed data

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for each landrace in each replication were calculated and used for multivariate statistical analysis.

The genomic DNA of the collected landraces was isolated using our earlier standardized CTAB DNA extraction protocol (Barman *et al*. 2017). The concentration and quality of total genomic DNA was detected by spectrophotometer.

DNA fingerprinting of pumpkin landraces was carried out using 20 SSR markers (Table 1). Polymerase chain reactions (PCR) were carried out in 15 µl reaction volume which includes 2 µl template DNA (25 ng/µl); 1.5 µl 10 × buffer; 1.0 µl $MgCl_2$ (2.5 mM); 0.15 µl forward primer (10 µM); 0.15 µl reverse primer (10 µM); 0.15 µl Taq DNA polymerase (0.5 U); 0.6 µl dNTP (10 mM) and 9.45 µl double distilled water. SSR amplification was performed using a Biorad thermocycler (Biorad, C1000). The PCR profile starts with 94°C for 2 minutes followed by 35 cycles of denaturation at 94°C for 30 sec, primer annealing (specific for each primer, Table 2) for 1 min extension at

Table 1 SSR markers used for diversity analysis

Primer	Sequence (5’ - 3’)	Annealing temp (°C)	Expected band size (bp)	PIC value	Allelic frequency
OCM1	F: GGTCCCAATAATAGCAACCAA R: GTGGGACACATCTTGAGCA	52.8	273	0.88	0.35
OCM2	F: GAAGTTCGTGGTCTGTGCAAGTC R: CCTGAGTAACCTCCGTGCTTCC	57.3	300	0.91	0.30
OCM3	F: GTTGCAGCGACCGTTCTTCTTC R: GCATCTGAAGACGATGCGTCGT	57.2	189	0.77	0.48
OCM4	F: CTAAACAGGATGCCTCTGACAC R: CGGGATTTCCGAAACAACGT	57	211	0.45	0.74
OCM5	F: TCGGAATCGTCTTCAGCAATAGTC R: TCCTCTTCCATTCCACTTTCTCCT	55.6	238	0.59	0.64
OCM6	F: TGTGGGGTTTTGCTTTTAGG R: ATCCAAAATGGTGGTGCATT	56.1	133	0.68	0.57
OCM7	F: TGACATCTGAGACGAGTGCC R: GCAACAATGGACCAAGA	52.4	211	0.85	0.39
OCM8	F: GCAAGCCCTAGCTGATTTTT R: GGGCGAAAACAGAGTGAGAG	51.1	127	0.33	0.82
OCM9	F: TGATTGTTTCAGGGTGTCAA R: AGCCTCTCAGAATCACCTGC	53.4	129	0.31	0.83
OCM16	F: GGATTGCCTTGCTGGAGAG R: CAAATCCAGGTGGAAGGCTA	53.4	279	0.80	0.45
OCM17	F: AGGGATGAAGGTGATCGTTG R: GAATTGGAGGAACGAACGAA	55.1	108	0.39	0.78
OCM18	F: GGCAGCACAGTGTAGGTGAA R: AGTGAGGCACTCGGAGAAAA	53.2	187	0.55	0.67
OCM19	F: AAACTCCTTTCACCACCACG R:TGTAGCAGGAGCCATGTTTG	57	111	0.84	0.40
OCM20	F: AACCAACGTGTAACTCTATTGCC R: GGAAGCAGCAACAACAACAA	58	100	0.40	0.77
OCM21	F: TCACCAACTTGCCATAGACG R: CGCGCAACTGATAAGGATTT	57.5	206	0.39	0.78
OCM22	F: TGATCTGACAGCAACCGAAG R:CCATTCCCTTAGTTTCTAAACCACT	58	190	0.84	0.40
OCM23	F: AAAAATGGAGAACCCGGTG R: CACACCACATTCCTCAAACG	57	190	0.40	0.77
OCM24	F: TGATGACAAAGAAGCCATCG R: ATCTTATGCCGGAGCAGATG	56.7	256	0.40	0.77
OCM25	F: TTTCGTTGCAGAGAATGGTG R: CCCATTTCTTTCTCGCTCAA	57	180	0.85	0.39
OCM26	F: GAAGTGATAGGGTTCATGCGA R: CAGTCCATGTGAAGCCATTG	55.7	205	0.83	0.41

Table 2 Cluster means for important yield contributing characters in 60 pumpkin landraces

Character	Cluster mean					% Contribution towards divergence	SEd	CD (P=0.05)	CV (%)	P value	Range	Superior landraces (GHP)
	I	II	III	IV	V							
Vine length (m)	3.32	10.12	7.23	11.13	5.22	0.79	0.44	0.88	9.98	<.001	2.32-12.50	1,26,43,56
Leaves/plant	92.42	111.98	162.73	164.27	121.12	3.26	5.98	9.72	12.24	<.001	42.80-176.00	1,51,52,56
Days to first female flower	38.45	62.78	55.77	58.27	44.29	0.17	2.67	4.74	11.23	<.001	29.23-72.28	1,26,43,51,52,56
Fruit weight/plant (kg)	2.78	1.82	2.28	2.94	2.33	31.23	0.19	0.32	9.77	<.001	0.78-8.23	1,26,43,51,52
Fruits/plant	4.22	5.02	5.55	2.66	3.11	0.72	0.63	0.95	8.57	<.001	1.08-7.12	1,26,43,51,52
Fruit diameter (cm)	17.88	16.33	14.24	13.12	15.19	11.90	0.62	1.46	6.78	<.001	9.98-34.26	1,26,43,51,52,56
Fruit skin toughness (g/mm^2)	3231.18	3572.21	3892.24	3232.18	4001.22	2.01	257.55	523.11	13.26	<.001	1398.67-5423.60	43,51,52,56
Fruit skin thickness (mm)	3.09	3.89	4.26	3.28	3.21	3.12	0.31	0.66	10.24	<.001	2.10-5.23	1,51,52,56
Flesh thickness (cm)	3.19	3.11	4.01	3.58	3.44	3.98	0.38	0.81	7.24	<.001	1.23-5.98	1,26,43,52,56
TSS (%)	8.52	9.78	6.99	8.82	8.11	3.05	1.96	2.43	7.67	<.001	3.57-16.34	1,43,51,56
Fruit yield (t/ha)	13.41	14.77	22.12	10.95	17.19	39.77	2.01	4.81	11.38	<.001	5.78-52.17	1,26,43,51,52,56

72°C for 1 min. A final extension at 72°C for 7 min was included. The PCR products were electrophoresed in a 1.5% agarose gels and then photographed using Bio-Rad gel doc XR documentation system.

Allelic polymorphic information content (PIC) of each SSR primer was calculated using the following formula: PIC = 1- $\Sigma(P_i)^2$, where P_i is the proportion of the population carrying ith allele (Botstein *et al*. 1980). NTSYS-PC V2.02 was used for computation of UPGMA based dendogram (Rohlf 2000), similarity coefficients (Jaccard 1908) and principal coordinate analysis (PCoA). Population genetic structure and analysis of molecular variance was calculated using STRUCTURE Version 2.2 (Pritchard *et al*. 2000) and AMOVA Version 1.55 (Excoffier *et al*. 1992) software, respectively. Genetic divergence was calculated using Mahalonabis (1936) generalized distances (D^2), as extended by Rao (1952) using OPSTAT statistical software.

RESULTS AND DISCUSSION

SSR profiling

Twenty SSR markers used in the study amplified a total of 323 amplicons with size range varying from 100-300 bp. Allelic frequency ranged from 0.30 for marker OCM2 to 0.83 for marker OCM9 with an average of 0.59 (Table 1). On average, 55% of the total pumpkin landraces shared a common major allele at any given locus. The more numbers of alleles detected in this study may be attributed to the analyzed populations which were geographically apart from each other (Kaur *et al*. 2016). PIC value of SSR markers ranged from 0.31 for marker OCM 9 to 0.91 for marker OCM 2 with an average of 0.62 and the markers of the most interest were those having high PIC values, such as OCM1, OCM2, OCM3, OCM7, OCM16, OCM19, OCM22, OCM25 and OCM26. These 9 SSR markers exhibited allelic frequency of less than 5% thus it comes under the rare allele group and these were also found to be highly polymorphic. These highly informative and polymorphic markers can be utilized for genetic purity analysis of pumpkin landraces. Similar results had also been reported by Sim *et al*. (2015) in pumpkin.

SSR cluster analysis

Distance-based dendrogram using UPGMA method grouped all the 60 pumpkin landraces into 5 clusters (Cluster I to V) based on source of origin (Fig 1a). Clusters I, II, IV and V were divided into 2 sub clusters at the Jaccard's coefficients of 0.53-0.65. The largest clusters (Cluster II) comprised 32 landraces. The clusters I, III IV and V comprised 20, 1, 3 and 4 landraces respectively. Cluster III landrace (GHP 56) was totally different from the landraces of other clusters and having oval fruit shape. Dendrogram revealed that not only the landraces that were derivatives of genetically similar type clustered more together, but it was also observed that different fruit shape landrace were also grouped into the same cluster due to the existence of

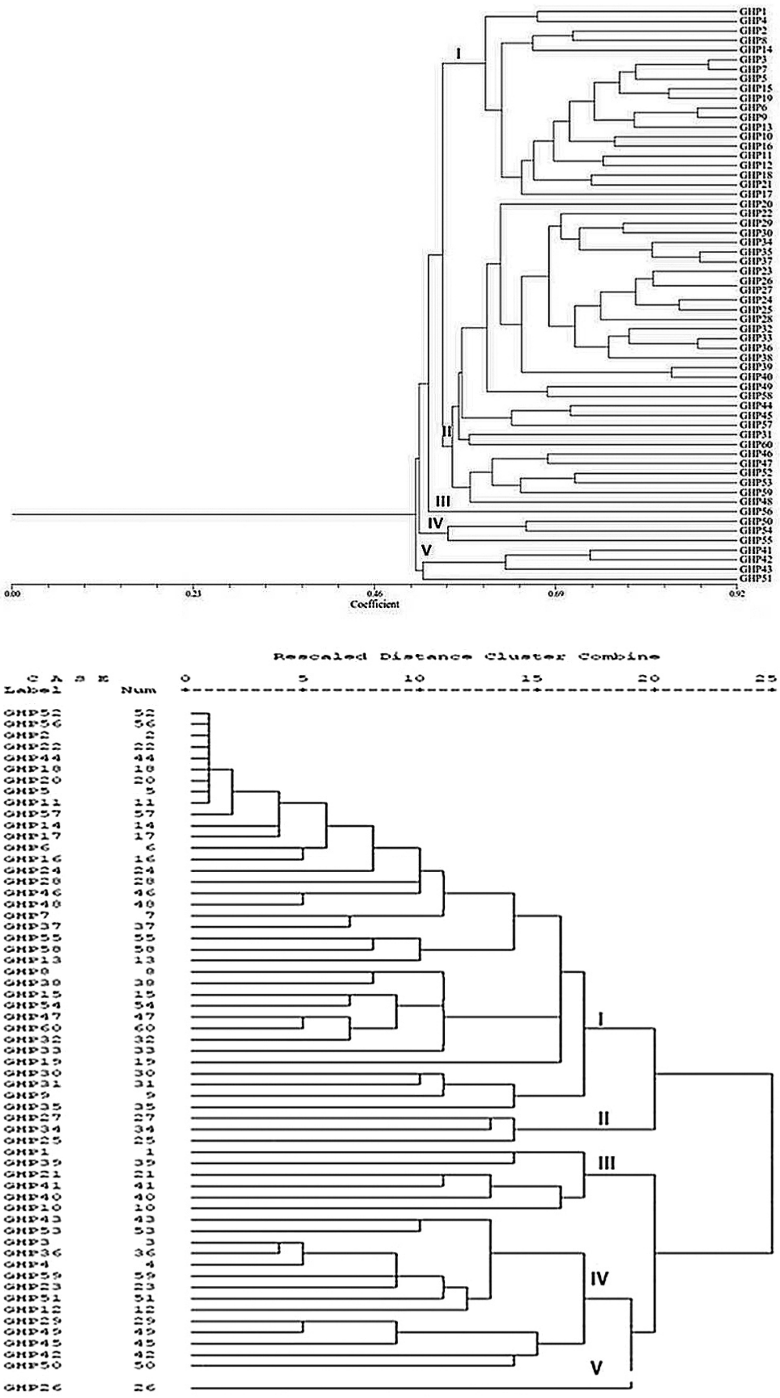


Fig 1 (a) UPGMA based cluster analysis using SSR data; (b) Dendrogram showing the morphogenetic relationships among 60 landraces of pumpkin based on Mahalonabis D^2 statistics.

considerable genetic diversity among the landraces (Zhang *et al*. 2013).

In the present study, the Jaccard's similarity coefficient values ranged from 0.51 to 0.92. The maximum similarity value was 0.91 between GHP35 and GHP 7 showing them the most similar accessions, whereas GHP1 and GHP51, GHP1 and GHP52, GHP1 and GHP56 were genetically most diverse with the minimum similarity index value of 0.51. Most of the genetic similarity values lie between 0.55-0.65 with an average of 0.53, which indicated considerable high degree of genetic diversity among the landraces under study (Riatova *et al*. 2010). In the present study, both phylogenetic and PCoA analysis was found in agreement with each other and grouped the pumpkin landraces into 5 clusters (Fig 1a and Fig 2). Further, AMOVA analysis revealed that the major (85%) portion of genetic variation resided within different populations while 15% genetic variance resided among populations, which implied that allele sharing was more frequent within the 60 pumpkin populations than in between populations due to the diverse genetic base.

Genetic structure analysis

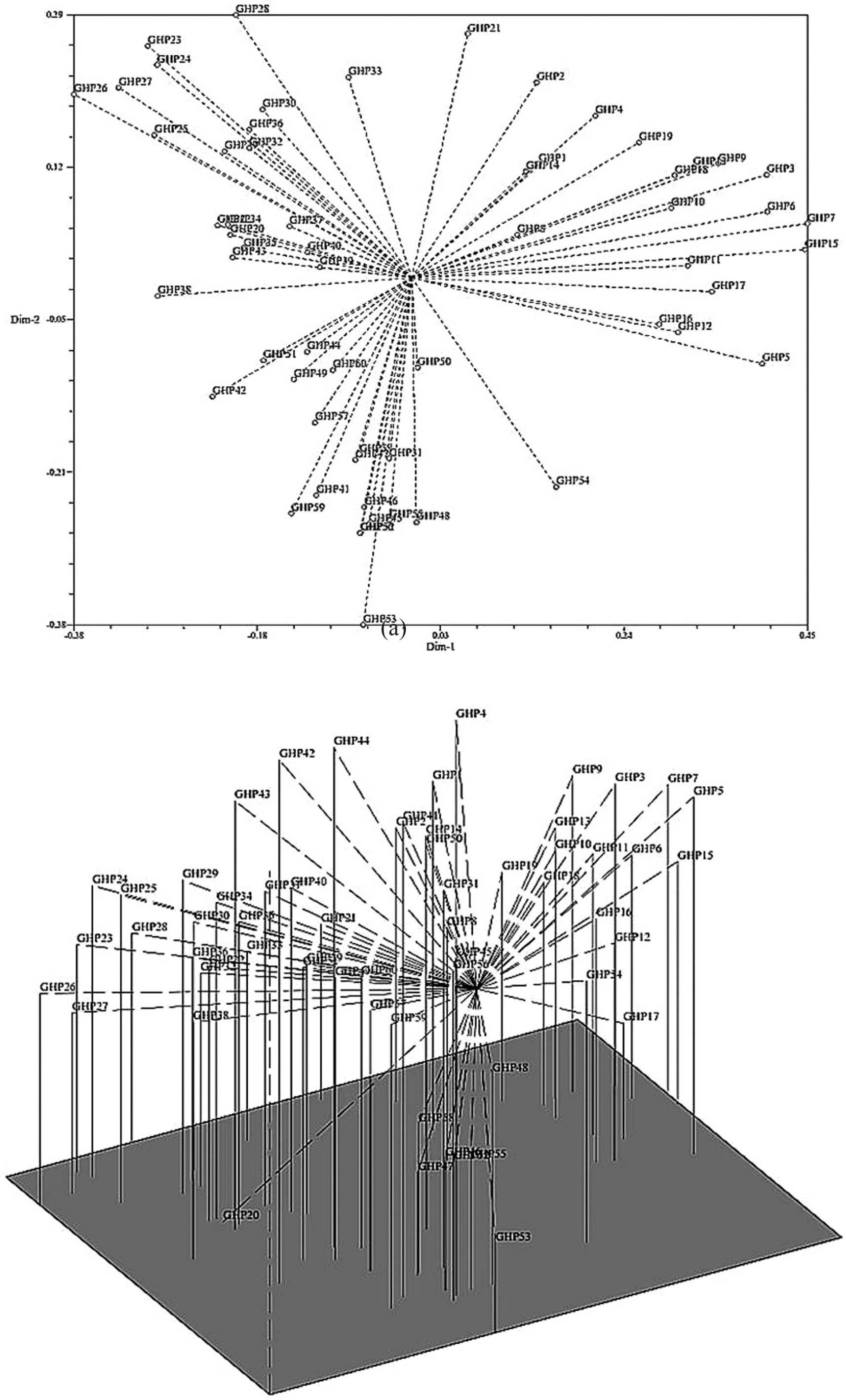
Analysis of population structure identified the highest log likelihood with the number of populations set K=4 and separated pumpkin landraces into four population or clusters, where cluster I, Cluster II, cluster III and cluster IV comprised 22, 14, 12 and 12 landraces of pumpkin, respectively (Fig 3). Average distance between individuals in same cluster was 0.1562 for cluster I, 0.2301 for cluster II, 0.2192 for cluster III and 0.3694 for cluster IV. Allelic frequency distribution was highest between the population of cluster I and II (0.3185) and lowest between population of cluster II and IV (0.0427) (Table 3). At these level individual proportions of membership in each group estimated using the multi-allele data set suggested the existence of some population structure. Mean values of F_{st} for cluster-I, cluster-II, cluster III and cluster IV were 0.6722, 0.3057, 0.4221 and 0.0002 respectively. Percentage of pure accession in cluster I, cluster II, cluster III and cluster IV were 36.67, 23.33, 20 and 20%, respectively. The values of structure analysis showed that genetic differentiation was high in analyzed landraces of pumpkin and the occurrence of relatively high percentages of pure accessions in all the four clusters indicated low admixture of pumpkin landraces, where geographical distance and environmental factors have a great effect in shaping the populations structure (Fuchs and Hamrick 2010).

D^2 cluster analysis

Highly significant variation was found among all the 11 quantitative morphological traits studied, where coefficient of variation ranged from 13.26 (fruit skin toughness) to 6.78 (fruit diameter) (Supplementary Table 2). The use of Mahalonabis D^2 statistics to estimate genetic divergence grouped all the pumpkin landraces into 5 highly divergent clusters (Supplementary Table 2; Fig 1b) and it is more or less in conformity with the SSR cluster analysis. Unlike the SSR cluster pattern, in divergence analysis GHP26 was occurred in totally different cluster. The distribution of pattern of landraces in different clusters revealed that landraces were not distributed according to their geographical origin, as landraces of same geographical region were grouped into different cluster and vice-versa (Babu and Patil 2004). Different factors like the exchange of germplasm, genetic drift, grower selection, and differential gene expression due to genotype × environment interactions could be responsible for the generation of genetic diversity in crop population (Devi *et al*. 2016).

Inter- and intra- cluster distance

The highest and lowest intra-cluster distance was observed in cluster I (171.60) with 36 landraces, and cluster V (0.00) with one landrace, respectively (Supplementary Table 2). Intra-cluster distance of cluster V was zero due to the occurrence of only one landrace in this cluster. Lower intra-cluster distances than inter-cluster distance in all the 4 clusters indicated that landraces of pumpkin within the same cluster were closely related. Inter-cluster average D^2 values was maximum (442.27) between cluster I and cluster IV, followed by between cluster I and cluster III (357.88) and between cluster I and cluster II (339.91). This indicated that landraces belonging to cluster I and Cluster IV were highly divergent from the others, followed by landraces of cluster I and cluster III, then landraces of cluster I and cluster II. Minimum inter-cluster average D^2 value (86.29) between cluster IV and cluster V indicated that landraces included in this clusters were very close to each other. Parents should be selected from the landraces present in clusters with maximum inter-cluster distance to obtain highly heterotic progeny in hybridization programme (Mehta *et al*. 2004). Considering the average D^2 distances and other morphological traits, it can be concluded that the landraces of cluster I and cluster IV would expected to exhibit high heterosis and produce new recombinants with more transgressive segregants in inter-cluster crosses followed by between the landraces of cluster I and Cluster



(b)

Fig 2 2D (a) and 3D (b) pictorial representation of Principle coordinate analysis of based on SSR data

Table 3 Allelic frequency distribution based on divergence among pumpkin population using structure analysis

Cluster	I	II	III	IV
I	0.0000			
II	0.3185	0.0000		
III	0.1147	0.1958	0.0000	
IV	0.1684	0.0427	0.0764	0.0000

III, then landraces of cluster I and cluster II.

Mean cluster values for different traits

A perusal of the cluster means for different morphological traits indicated considerable difference between the clusters for all the traits under study (Table 2). Cluster I landraces exhibited earliness in days to first female flower production (38.45) and maximum mean values for fruit diameter (17.88). Cluster II landraces exhibited maximum mean values for days to first female flower production (62.78) and TSS (9.78). Cluster III landraces exhibited maximum mean values for fruits per plant (5.55), fruit skin thickness (4.26), flesh thickness (4.01) and fruit yield (22.12). Cluster IV landraces exhibited maximum mean values for vine length (11.13), leaves per plant (164.27) and fruit weight per plant (2.94). Cluster V landrace exhibited maximum mean values for fruit skin toughness (3801.04). All the maximum cluster mean values were distributed in relatively distant clusters. None of the cluster contained landraces with all the desirable traits, which could be directly selected and utilized in breeding programme. Landraces present in all the 5 clusters for above mentioned traits will be much in use and would offer a good scope of crop improvement through rational selection for hybrid breeding program. Results of the study indicated that landraces could be selected from cluster I in breeding programme for earliness in female flower production and to increase fruit diameter. Cluster II landraces could be selected to increase TSS. Cluster III landraces could be selected to increase number of fruits/plant, fruit skin thickness, flesh thickness and fruit yield. Cluster IV landraces could be selected to increase vine length, leaves per plant and fruit weight per plant. Cluster IV landraces could be selected for fruit skin toughness. The selection of parents for hybridization programme should be done based on their genetic distances to obtain a wide spectrum of variation among segregants and hybridization between genotypes of different cluster is necessary for the development of new forms (Singh *et al.* 2013).

Contribution of different traits towards genetic divergence

The contribution range of different traits towards divergence was 0.17 to 39.77 (Table 2). Among the 11 quantitative traits studied, fruit yield contributed maximum towards divergence (39.77) followed by fruit weight per plant (31.23) and fruit diameter (11.90). The lowest contribution towards divergence of 0.17 was observed for days to first female flower production. The traits *viz.* fruit yield, fruit weight/plant and fruit diameter contributed 82.90% towards total divergence. Hence, these traits should be given importance during hybridization and selection in the segregating population. Based on the per se performance of the best landraces within the clusters, selection of potential parents should be done in hybridization programme. Similar results were also reported by Blessing *et al.* (2012) in pumpkin.

Table 4 Eigen values and proportions of the variance in quantitative traits

Principal components (PC)	Eigen value	Percentage of variability (%)	Cumulative variability (%)
PC1	6.68	36.43	99.79
PC2	4.67	23.22	99.67
PC3	4.11	12.53	71.16
PC4	2.51	7.68	99.96
PC5	2.42	6.49	71.73
PC6	1.71	4.30	81.78
PC7	1.59	3.91	99.37
PC8	1.12	2.99	99.91
PC9	0.89	2.34	81.58
PC10	0.09	0.08	100
PC11	0.03	0.03	100

Principle component analysis (PCA)

The result showed that 5 out of 11 principle component (PC) axes had eigen values greater than two and altogether accounted for 86.36% of the total variation (Table 4). PC1 accounting for 36.43% of the total variation followed by PC2 (23.22%), PC3 (12.53%), PC4 (7.68%), and PC5 (6.49). The output of the PCA analysis confirmed the individual contributions of different quantitative traits to the total variations observed as well as to the fruit yield in pumpkin landraces. This agrees with the report of Nwangburuka *et al.* (2011) working with okra. The traits that contributed most positively to PC1 were vine length, leaves/plant, days to first female flower production, fruit weight/plant, fruit diameter, fruit skin toughness, fruit skin thickness, flesh thickness, TSS and fruit yield. PC2 was positively related to leaves/plant, fruit diameter, fruit skin toughness, fruit skin thickness, flesh thickness, TSS and fruit yield (Supplementary Table 3). From the present study it can be reported that best landraces of pumpkin will have to be selected for the traits that contributed positively to first five PCs without considering the yield loss potential.

Genetic similarity distribution, PCoA, dendrogram, population structure and genetic divergence analysis revealed that the studied pumpkin landraces have considerable diverse genetic base. Hence the most divergent landraces obtained (GHP 1, GHP 26, GHP 43, GHP 51, GHP 52 GHP 56) from this study can be effectively utilized in future pumpkin breeding programme for the development of new hybrid combinations. The SSR markers along with the diverse parents identified in this study can be used in pumpkin breeding programme for tagging and mapping of various genes/QTLs for various biotic and abiotic stresses affecting pumpkin and also in quality breeding. This will form a strong beginning for future molecular characterization and genetic improvement works in this promising cucurbitaceous plant and also since SSR analysis is a very fast, rapid, economic method of genotyping it will be an useful tool for cultivar registration, certification and identification to address the IPR issues in better way.

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