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Research Article

Seed Protein Profiling, An Efficient Method in Diversity Analysis of Pumpkin (*Cucurbita moschata* Duch. Ex. Poir.) From Northeast India

Md. Ramjan[1]* Chandra Deo[1], Mohd Talha Ansari[1] and M. Chandrakumar Singh[2]

[1]Department of Vegetable Science, College of Horticulture and Forestry, Central Agricultural University, Pasighat-791102, Arunachal Pradesh. India
[2]Department of Basic Sciences and Humanities, College of Horticulture and Forestry, Central Agricultural University, Pasighat-791102, Arunachal Pradesh. India

Abstract
For the present study, 25 diverse genotype of pumpkin were evaluated at Vegetable Research Farm, College of Horticulture and Forestry, Central Agricultural University, Pasighat Arunachal Pradesh India for genetic diversity using the total seed proteins using SDS-PAGE and morphological characteristics. All the germplasm clearly revealed remarkable polymorphism from their protein banding patterns. On the basis of banding patterns,the 89 numbers of protein bands were observed in all the genotypes. Total protein band which ranged from 13 to26 with Relative Mobility (Rm) values 0.10 to 0.98. The result showed that, among the genotypes, CHFPUM-6, CHFPUM-18 and CHFPUM-25 showed maximum numbers (26) of protein bands while the minimum numbers (13) of bands were present in genotypes CHFPUM-13. The study concluded that, the genotype CHFPUM-1 with CHFPUM-21, CHFPUM-3 with CHFUM-13 and CHFPUM-20 were more distantly related to each other. Hence these genotypes could be utilized for breeding programme.

Keywords: Cluster analysis, genetic diversity, Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

***Correspondence**
Author: Md. Ramjan
Email:mohammadramjan165@gmail.com

Introduction

The North-eastern region (NEH) comprising the states of Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim and Tripura which is located between 22–29.3° N and 89.7–97.8° E. Pumpkin (*Cucurbita moschata*Duch. ex. Poir.) is a most important crop of Cucurbitaceae family and is known for high productivity, excellent storability and rich source of carotene. Almost all the parts of NEH region and most of them are local cultivars or landraces. Landraces are variable plant populations adapted to local agro climatic conditions, which are locally named, selected and maintained by the traditional farmers to meet their social, economic, cultural and ecological needs [1].

The pumpkin landraces have been selected by farmers for agronomic and horticultural traits important to them (*e.g*., fruit size, shape, colour, maturity and storability) [2].Any crop improvement programme depends on the amount of genetic variability present in the population of the crop [3]. Morphological and physiological plant traits are generally used to estimate the magnitude of genetic diversity present in the germplasm. Traditional technique does not provide an accurate indication of genetic diversity because of environmental influences [4].

Biochemical techniques like Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) has been helpful in inducing systematic relationships between groups where morphological and cytological data are not corollary [4]. Stability is one of the important features of the seed protein and the composition of seed protein is highly stable in nature and is affected negligible by environmental conditions or seasonal fluctuations. Seed proteins are mainly storage proteins and are not likely to be changed in dry mature seed [5]. Storage proteins play important role that includes nitrogen and energy source and defence against insect and pathogens [6].

Diversity band provides information on the relationship among the genotypes of seeds collected from various geographic regions. Indeed plant breeding provide an opportunities for selection and cultivar identification to enhance the production and productivity of the within genetic diversity [7].

In gel electrophoresis, an electric field is used to move charged molecules through a matrix of a polymerized substance such as agarose or polyacrylamide. The rate at which individual molecules move through the gel depend on the properties of both the separation system and the molecules themselves. Gel matrices are permeated with networks

of pores through which the molecules move. The amount of resistance that the matrix presents to the movement of a molecule depends on the diameter of the pore as well as the size and geometry of the molecule. In general, smaller, more highly charged molecules migrate fast through gels than larger or less charged molecules. The mobility of a molecule is also affected by the buffer system and the strength of the electrophoretic field used for the separation.

Genetic resource of pumpkin landraces in North eastern region of India have not been well familiar, however, a wide range of variability for several attributes *viz.*, fruit shape, size, colour, bearing habit, perennial and sweetness. Therefore, a need exists for proper documentation and analysis of pumpkin genotypes from different geographical locations. Keeping these points in mind, the present investigation was undertaken to evaluate the variability in 25 germplasm of North-eastern region of India, through morphometric and seed protein analysis to provide a scientific basis for future selection and crop improvement programme.

Materials and Method

Plant materials

For the present study, 25 diverse genotypes of pumpkin (*Cucurbita moschata*Duch. ex. Poir.) were collected from different location of north eastern region, India. The list of genotypes along with their source of origin and morphological traits are given in (**Table1**). The experimental materials for the present study were evaluated at Vegetable Research Farm, College of Horticulture and Forestry, Central Agricultural University, Pasighat Arunachal Pradesh India.

Table 1 Pumpkin genotypes with their sources of collection and morphological characters

S. N.	Genotype	Source	Coordinates of the places	Fruit colour	Fruit shape	Fruit ribs	Flesh colour
1.	CHFPUM-1	East Siang, Arunachal Pradesh	28.07˚ N, 95.33˚ E	Yellow	Elongated	Intermediate	Yellow
2.	CHFPUM-2	East Siang, Arunachal Pradesh	28.07˚ N, 95.33˚ E	Brown	Round	Superficial	Yellow
3.	CHFPUM-3	East Siang, Arunachal Pradesh	28.07˚ N, 95.33˚ E	Yellow	Round	Intermediate	Yellow
4.	CHFPUM-4	East Siang, Arunachal Pradesh	28.07˚ N, 95.33˚ E	Green	Round	Superficial	Yellow
5.	CHFPUM-5	East Siang, Arunachal Pradesh	28.07˚ N, 95.33˚ E	Yellow	Round	Superficial	Yellow
6.	CHFPUM-6	East Siang, Arunachal Pradesh	28.07˚ N, 95.33˚ E	Green	Elongated	Deep	Cream
7.	CHFPUM-7	IIVR, Varanasi (U.P.)	25.28˚ N, 82.96˚ E	Green	Flattened	Intermediate	Yellow
8.	CHFPUM-8	Pasighat, Arunachal Pradesh	28.07˚ N, 95.33˚ E	Green	Round	Intermediate	Yellow
9.	CHFPUM-9	Pasighat, Arunachal Pradesh	28.07˚ N, 95.33˚ E	Green	Round	Absent	Yellow
10.	CHFPUM-10	Ziro, Arunachal Pradesh	27.56˚ N, 93.83˚ E	Green	Pyriform	Absent	Yellow
11.	CHFPUM-11	Aizawal, Mizoram	23˚43’38” N, 92˚43’4” E	Yellow	Oblong	Superficial	Yellow
12.	CHFPUM-12	Aizawal, Mizoram	23˚43’38” N, 92˚43’4” E	Cream	Oval	Absent	Yellow
13.	CHFPUM-13	Aizawl, Mizoram	23˚43’38” N, 92˚43’4” E	Green	Round	Intermediate	Yellow
14.	CHFPUM-14	Aizawl, Mizoram	23˚43’38” N, 92˚43’4” E	Green	Flattened	Deep	Yellow
15.	CHFPUM-15	Imphal, Manipur	24.80˚ N, 93.93˚ E	Brown	Round	Intermediate	Orange
16.	CHFPUM-16	Imphal, Manipur	24.80˚ N, 93.93˚ E	Brown	Elongated	Absent	Orange
17.	CHFPUM-17	Imphal, Manipur	24.80˚ N, 93.93˚ E	Green	Oblong	Superficial	Orange
18.	CHFPUM-18	Gangtok, Sikkim	27.33˚ N, 88.62˚ E	Grey	Round	Intermediate	Yellow
19.	CHFPUM-19	Gangtok, Sikkim	27.33˚ N, 88.62˚ E	Green	Round	Deep	Yellow
20.	CHFPUM-20	Kohima, Nagaland	25.67˚ N, 94.10˚ E	Yellow	Elongated	Absent	Yellow
21.	CHFPUM-21	Kohima,Nagaland	25.67˚ N, 94.10˚ E	Green	Round	Deep	Yellow
22.	CHFPUM-22	Kohima, Nagaland	25.67˚ N, 94.10˚ E	Yellow	Flattened	Superficial	Orange
23.	CHFPUM-23	NDUAT, Faizabad (U.P.)	26.77˚ N, 82.14˚ E	Green	Round	Superficial	Yellow
24.	CHFPUM-24	Agartala, Tripura	23.84˚ N, 88.62˚ E	Cream	Oval	Absent	Cream
25.	CHFPUM-25	Agartala, Tripura	23.84˚ N, 88.62˚ E	Green	Flattened	Deep	Orange

Estimation of protein

Estimation of protein was done as per procedure described by Lowry’s method [8]. From each genotypes 1 g of seed sample were macerated in mortar and pestle with 10 ml of buffer (0.06 M Tris-HCl, 2.5% Glycerol, 0.5% SDS, 1.25% β-mercaptoethanol, 0.1% TCA, 10 mM urea, 1 mM EDTA) and transferred to centrifuge tubes then centrifuged at 8000 rpm for 20 min. The supernatants were mixed and volume made up to 50 ml with phosphate buffer. 1 ml of 20% TCA was added to 1 ml of the extract and the mixture was kept for 30 min. The mixture was then centrifuged at 8000

rpm for 20 min. The resultant pellets were washed twice with acetone and again centrifuged. The supernatant was then discarded. The pellet was collected and dissolved in 5 ml of 0.1N NaOH till it had dissolved. 1 ml of the aliquot was taken in which 5 ml of freshly prepared alkaline copper sulphate reagent were added and mixed properly. After 10 min, 0.5 ml of Folin's reagent was added and mixed instantaneously and allowed to develop colour for 30 min. Absorbance at 660 nm was recorded after setting the instrument (UV-VIS Spectrophotometer, Hitachi U-1900) with reagent blank which contained 1 ml of 0.1 N NaOH instead of the sample aliquot. In another set of tubes, suitable aliquots of BSA solution (in the range of 0-100 µl) were taken and volume made up to 1 ml with 0.1 N NaOH and allowed to develop colour as described above. A standard curve of absorbance at 660 nm versus µg of Bovine serum albumin (BSA) was drawn and from this standard curve, the amount of protein in the sample tube was determined as protein per gram of the sample.

Extraction of total seed proteins for gel electrophoresis

Polyacrylamide gel electrophoresis in presence of denaturing agent (SDS) was carried out as per procedure described by Laemmli (1970) [9] with some modifications. Acrylamide Solution: 29.2 g acrylamide and 0.8 g bisacrylamide were dissolved in water and the final volume was made up to 100 ml and stored at 4^0C, Separating Gel Buffer: 1.5M Tris-HCl of pH 8.8 was prepared and stored at 4^0C, Stacking Gel Buffer: 1M Tris-HCl of pH 6.8 was prepared and stored at 4^0C, Sodium Dodecyl Sulphate solution: 2% aqueous solution of SDS was prepared, Ammonium per sulphate solution: 10% aqueous solution of ammonium persulphate was prepared, Bromophenol blue solution: 0.1% aqueous solution of bromophenol blue was prepared, Electrophoresis buffer: 3.0 g of Tris base and 14.4 g of glycine was dissolved in water and the final volume was made up to 1 L. The final pH was adjusted to 8.3 with glycine solution. The gel was then subjected to silver staining and the protein bands on the gel were visualized by silver staining.The protocol followed in the present study was in accordance with some modifications silver staining was performed as method described [10].

SDS-PAGE

The soluble seed proteins were subjected to SDS-PAGE in gel slabs of 1 mm thickness (5% stacking and 10% resolving gels). Electrophoresis was performed with a discontinuous buffer system in a vertical electrophoresis unit. Cathode and anode terminals were connected to the electrophoretic power supply and the SDS- PAGE was started by applying a voltage of 80 V which was increased to 100V when the dye font has moved into the separating gel. The gel was run until the bromophenol blue reaches the bottom of separating gel. The power supply was switched off when the tracking dye approached the bottom of the gel. The system was disconnected and the gel was taken out from the slab. The gel was then immersed in silver staining solution and kept on a gel rocker for 4 hours.

Statistical data analysis of protein profiling

The gels were scored as presence (+) or absence (−) of protein polypeptide bands. Depending upon the presence or absence of polypeptide bands, similarity index (SI) [11] between the genotypes was calculated by the following formula:

$$SI = \left(\frac{2Z}{X+Y}\right) X\ 100$$

Where, Z= Number of similar bands between the genotypes, and X+Y =Total number of bands in the two genotypes compared [12] and distance matrix of dissimilarity was produced for a set of individuals. Dendrogram was generated using an unweighted pair group method with arithmetic mean analysis (UPGMA) by use of statistical software SPSS (Version 14).

Result and Discussion

The morphological variations under this study are summarized in (Table 1). SDS-PAGE is one of the most widely used techniques to separate and characterizes the proteins and used to estimate the extent of genetic diversity in the present set of pumpkin germplasm. Seed storage proteins profiling provides an aid in identification and characterization of diversity among the landraces, and prevailing their phylogenetic relationships [13].

Morphological and molecular characterization is important tools for study the genetic variations of genotypes. The electrophoretic seed protein profiles of the same have been outlined in the form of electrophorograms based on UPGMA resulting in distinct clusters (**Figure 1**) and were studied and summarized in (**Table 2**). Cluster analysis of banding pattern of 25 genotypes based on similarity and UPGMA resulted in distinct clusters (**Figure 2**). A total of 89 protein bands as per Rm values were identified by silver staining. The genotypes exhibited considerable variation in

protein band number ranging from 13-26. Among the genotypes CHFPUM-6 (Pasighat, Arunachal Pradesh), CHFPUM-18 from (Gangtok, Sikkim) and CHFPUM-25 from (Agartala, Tripura) showed maximum numbers (26) of protein bands while the minimum numbers (13) of bands were present in genotypes CHFPUM-13 from (Aizwal, Mizoram).

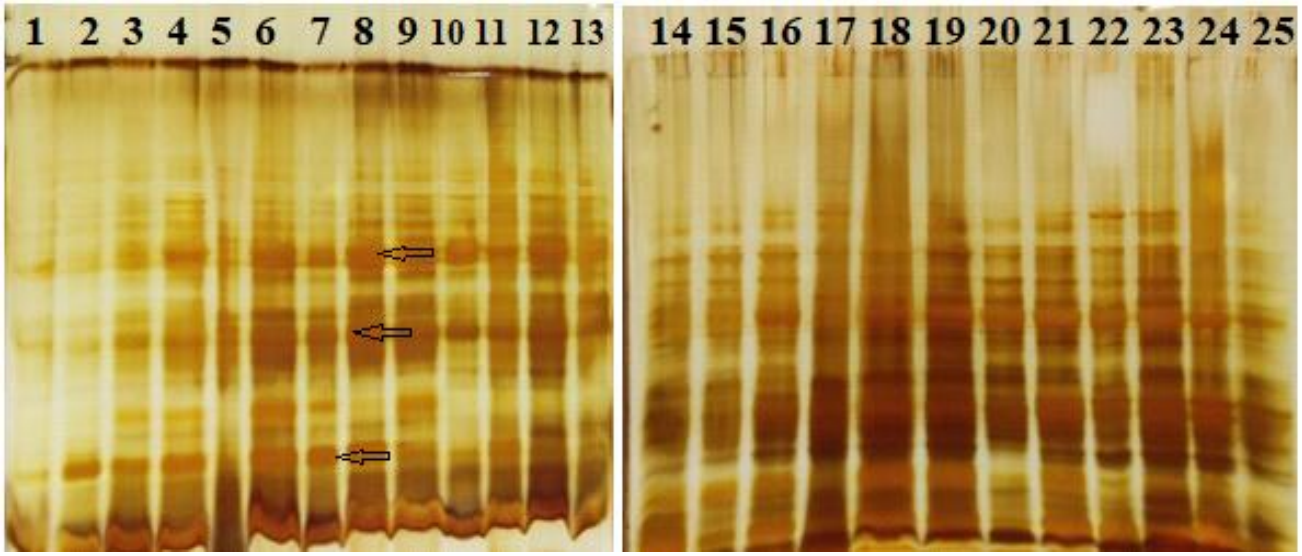

Figure 1 Seed protein banding pattern of 25 genotypes of pumpkin, arrow indicates bands present in maximum number of genotype

Table 2 Major cluster produced by SDS-PAGE analysis in 25 genotypes of pumpkin

Cluster	Genotypes
I	CHFPUM-5, CHFPUM-12, CHFPUM-9, CHFPUM-6 CHFPUM-2, CHFPUM-7 CHFPUM-14, CHFPUM-10 CHFPUM-4, CHFPUM-3 CHFPUM-13,
II	CHFPUM-24 CHFPUM-25, CHFPUM-8 CHFPUM-19, CHFPUM-21
III	CHFPUM-15
IV	CHFPUM-20 CHFPUM-23
V	CHFPUM-16 and CHFPUM-18
VI	CHFPUM-11
VII	CHFPUM-17
VIII	CHFPUM-22
IX	CHFPUM-1

Band number 2 (Rm=0.11) and 5 (Rm=0.14) was recorded in genotypes CHFPUM-5 only. Band number 7 (Rm=0.16) and 8 (Rm=0.17) only was present in genotype CHFPUM-3 and CHFPUM-6, respectively. Band 72 was present in CHFPUM-4 only. Band number 1 (Rm=0.10) was present in genotype CHFPUM-3 and CHFPUM-6. Similarly, band number 6 (Rm=0.15) was to present in CHFPUM-2 and CHFPUM-6. 60 (Rm=0.69) present in CHFPUM-2 and CHFPUM-12 and band number 82 (Rm=0.91) was found to be present only in genotype CHFPUM-11 and CHFPUM-19. Band number 15 (Rm=0.24) was present in CHFPUM-2, CHFPUM-4 and CHFPUM-5 only. Band number 23 (Rm=0.32), band number 39 (Rm=0.48) and band number 81(Rm=0.91) was found to be present in maximum (13) number of genotypes.

Clustering Analysis

Based on the dendrogram, all the genotypes were divided into 9 clusters at 50 percent genetic distance from each other on the basis of cluster analysis. The germplasm CHFPUM-15, CHFPUM-11, CHFPUM-17, CHFPUM-22 and CHFPUM-1 did not show similar with any other germplasm. Cluster 1 consists of 11 genotypes out of which CHFPUM-2, CHFPUM-3, CHFPUM-4, CHFPUM-5 and CHFPUM-6 were from east siang, Arunachal Pradesh where as CHFPUM-12, CHFPUM-13 and CHFPUM-5, CHFPUM-14 were from Aizwal Mizoram. Cluster 2 includes 5 genotypes out of which CHFPUM-24 and CHFPUM-25 was from Agartala, Tripura and remaining genotypes were from different locations. Cluster 4 (CHFPUM-20, CHFPUM-23,) and Cluster 5 (CHFPUM-16, CHFPUM-18,) consists of 2 genotypes each.

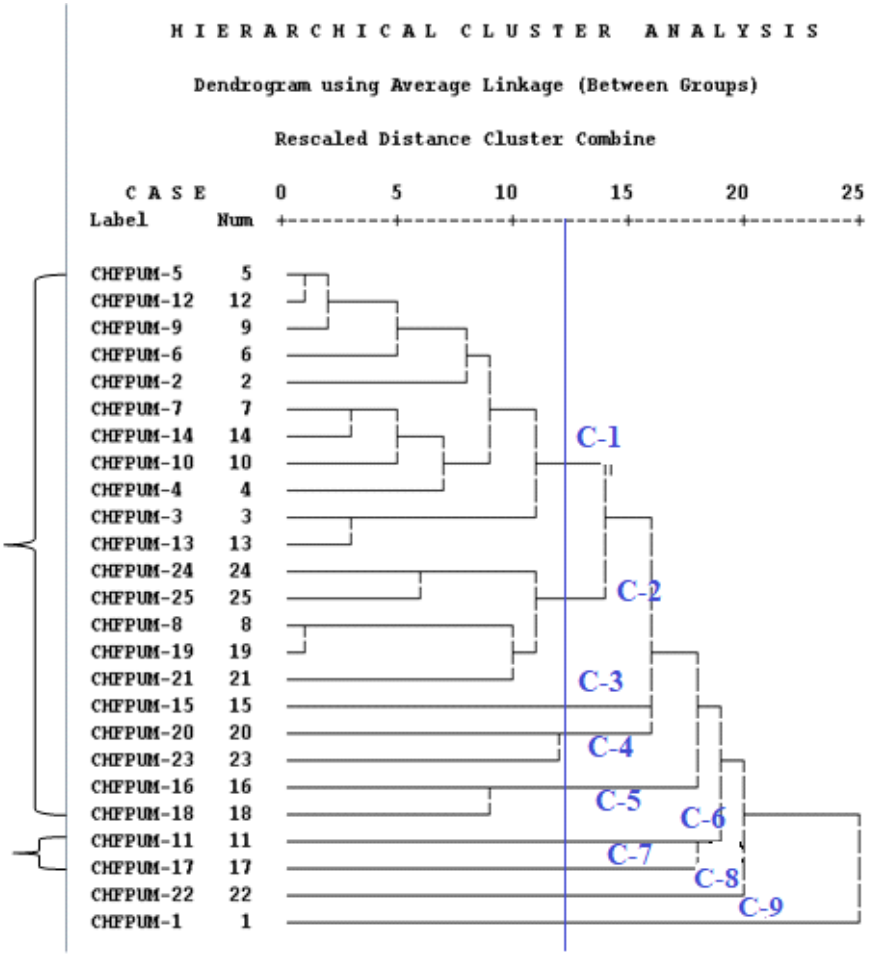

Figure 2 UPGMA of 25 pumpkin genotypes based on total seed protein profiles obtained by SDS-PAGE

A dendrogram drawn based on the similarity index coefficient matrix utilizing SDS-PAGE analysis for the 25 genotypes of pumpkin under study has been presented in (**Table 3)**. Percentage similarity index generated by SDS-PAGE analysis in the germplasm ranged from (0 to 62.86%). Values of percentage similarity index coefficient matrix suggested least genetic distance of genotypes was observed between CHFPUM-3 with CHFPUM-13 and CHFPUM-20 (0.00%) while maximum similarity distance was observed between the genotypes CHFUM-1collected from East Siang, Arunachal Pradesh with genotypes CHFUM-11 was collected from Aizwal, Mizoram as evident by coefficient value of (62.86%).

The seed storage profiling showed distinct polymorphism in electrophoretic banding patterns and led to the detection protein revealed altogether 89 scorable polypeptide bands (Figure1). The polymorphism was marked in all storage proteins fraction of the pumpkin genotypes on the basis of their molecular weight [14].

Application of different methods of storage protein electrophoretic patterns has been used for the identification and the characterization of crop. Some investigators proposed that seed protein profiles may be useful as an indicator of taxonomic relationships within some species [15] but ones said that this method was insufficient for the discrimination at the cultivar level [16]. In present our finding indicated that, a total of 89 protein bands as per Rm values were recognized by silver staining. The genotypes exhibited considerable variation in protein band number ranging from 13-26, indicated that SDS PAGE of seed protein supplied additional banding pattern for the discrimination of the pumpkin genotypes. The results were in agreement with the findings of [17]. Several studies suggested that the application of numerical analysis, coupled with the utilization of a standardized identification system instead of simple quantitative comparison of protein patterns provides an effective approach to the investigation of taxonomic relationships among crop species [18]. Here in our experiment investigation, SPSS for windows package (version 21) was used to data analyse because of the difficulties in the visual interpretation of SDS-PAGE of seed protein profiles. The electrophoretic seed protein profiles have been outlined in the form of electrophorograms based on UPGMA resulting in distinct clusters (Figure 1) and were studied and summarized in (Table 2). Among the genotypes CHFPUM-6 (Pasighat, Arunachal Pradesh), CHFPUM-18 (Gnagtok, Sikkim) and CHFPUM-25 (Agartala, Tripura) showed maximum numbers (26) of protein bands while the minimum numbers (13) of bands were present in genotypes CHFPUM-13 (Aizwal, Mizorum). Similar findings were also reported by Kumar and Tata (2010) [19], Berber and Yasaf (2011) [20].

Table 3 Percentage similarity index estimate among 25 genotype of pumpkin using SDS-PAGE analysis

Genotypes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
CHF PUM-1	0.00																								
CHF PUM-2	15.78	0.00																							
CHF PUM-3	21.62	9.30	0.00																						
CHF PUM-4	15.10	39.1	17.7	0.00																					
CHF PUM-5	30.00	4.34	35.5	25.0	0.00																				
CHF PUM-6	14.28	12.5	25.5	28.0	20.0	0.00																			
CHF PUM-7	10.00	21.7	13.3	29.1	25.0	36.0	0.00																		
CHF PUM-8	15.38	22.2	27.2	29.7	34.0	28.5	34.0	0.00																	
CHF PUM-9	25.00	17.3	31.1	25.0	12.5	24.0	20.8	25.5	0.00																
CHF PUM-10	16.12	27.9	9.52	26.6	22.2	34.0	31.1	27.2	26.6	0.00															
CHF PUM-11	62.86	14.6	20.0	37.2	27.9	26.6	27.9	28.5	18.6	30.0	0.00														
CHF PUM-12	18.18	5.12	26.3	19.5	19.5	13.9	29.2	20.0	14.6	15.7	27.7	0.00													
CHF PUM-13	13.17	17.1	0.00	17.0	12.7	10.2	21.2	38.8	32.4	23.5	12.5	20.0	0.00												
CHF PUM-14	6.00	15.3	10.0	19.5	14.6	18.6	4.87	30.0	9.75	10.5	33.3	17.6	26.6	0.00											
CHF PUM-15	14.29	29.7	17.3	12.2	24.4	31.3	20.4	41.6	20.4	17.3	9.90	14.2	26.3	33.3	0.00										
CHF PUM-16	21.00	18.1	32.5	21.7	17.3	25.0	17.3	35.5	13.0	37.2	43.0	25.7	17.1	30.7	38.2	0.00									
CHF PUM-17	25.00	30.4	13.3	29.1	25.0	24.0	37.5	38.2	25.0	40.0	27.9	14.6	32.4	29.2	44.6	52.1	0.00								
CHF PUM-18	9.52	0.83	25.5	24.0	28.0	34.6	20.0	36.6	24.0	29.7	22.2	27.9	15.3	41.8	58.8	20.8	44.0	0.00							
CHF PUM-19	10.00	17.3	26.6	29.1	20.8	44.0	20.8	17.0	20.8	22.2	27.9	24.3	21.6	29.2	44.8	34.7	41.6	48.0	0.00						
CHF PUM-20	9.41	0.41	0.00	44.0	16.0	26.9	36.0	40.8	32.0	34.0	26.6	23.2	5.64	27.9	31.3	37.5	44.0	38.6	36.0	0.00					
CHF PUM-21	0.00	35.8	10.5	39.2	9.75	23.2	34.1	25.0	19.5	15.7	27.7	17.6	13.3	35.2	18.6	15.3	24.3	46.3	14.6	51.1	0.00				
CHF PUM-22	10.52	13.9	31.8	34.0	29.7	20.4	25.5	39.1	34.0	40.8	52.0	35.7	27.9	35.3	33.8	48.0	55.9	48.0	34.6	32.0	25.5	0.00			
CHF PUM-23	15.10	35.1	13.8	42.0	17.3	24.0	29.7	34.0	34.0	22.2	14.6	15.3	16.3	15.3	41.8	35.5	38.2	28.5	25.5	44.0	40.0	26.0	0.00		
CHF PUM-24	5.00	31.5	22.7	25.5	12.7	28.5	29.7	26.0	12.7	31.8	28.5	20.0	11.1	40.0	29.1	44.0	42.8	28.5	25.5	36.5	35.7	34.0	43.0	0.00	
CHF PUM-25	14.28	37.5	34.0	28.5	28.5	42.8	28.5	32.0	40.8	25.7	26.6	18.6	20.8	23.2	47.0	25.7	24.3	46.3	40.0	36.5	27.9	32.0	40.8	8.16	0.00

Cluster analysis of banding pattern of twenty five pumpkin genotypes based on similarity index is shown in Figure 2. The numerical analysis of SDS-PAGE of seed protein profiles showed that each cluster had slight discriminative protein banding profile. In our finding, all the genotypes were divided into 9 major clusters at 50 percent genetic distance from each other on the basis of cluster analysis. The distribution of different genotypes in different cluster of the dendrogram has been presented in Figure 2. In our study, Cluster 1 consists of 11 genotypes, Cluster 2 includes 5 genotypes. Cluster 4 genotypes and Cluster 5 consists of 2 genotypes each (Table 2). However, the genotypes CHFPUM-15, CHFPUM-11, CHFPUM-17, CHFPUM-22 and CHFPUM-1 did not show similar with any other germplasm in clustering, the conformity of present work with [17].

Based on the similarity index coefficient matrix utilizing SDS-PAGE analysis for the 25 genotypes of pumpkin under study has been presented in (Table 2). Additionally, our finding showed that the genotype CHFPUM-1 was most distantly related to CHFUM-11. Similar report with present findings was also reported by (Akbar *et al*. 2010 [21] and Yatung*et al*. 2014) [4] in their study on phylogeny and genetic diversity studies in *Capsicum* germplasm using seed storage proteins. Hence, it was recommended that these two genotypes could be utilized for crossing programme to create more segregates of desired characteristics through pumpkin breeding programmes, with conclusion that genotypes from different regions were observed to be closely related to each other and genotypes from the same region had different genetic back ground. Inter regional diversity could be as a valuable source as intra regional diversity for pumpkin improvement.

Conclusion

The genotype CHFPUM-1 (East Siang, Arunachal Pradesh) was most distantly related to CHFPUM-11, hence, it can be recommended that these genotypes could be utilized for crossing programme to create more genetic diversity or segregants of desired characteristics through pumpkin breeding programmes.

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