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Characterization of cultivated and wild species of *Capsicum* using microsatellite markers

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ABSTRACT

Diversity of twenty four genotypes of hot pepper representing cultivated species *Capsicum annuum, C. frutescens, C. baccatum and C. chinense* as well as a wild species *C. chacoense* was analyzed in the present study using 99 microsatellite loci distributed uniformly throughout the genome. The 85 polymorphic loci, out of 99 simple sequence repeat (SSR) loci used, amplified a total of 192 alleles among the 24 genotypes with one to five allele per loci. The average number of alleles per loci was found to be 2.25. The highest polymorphism information content (PIC value) was observed to be 0.729 for the marker located on linkage group 6. Principal component analysis provided useful information regarding genetic relationship among genotypes as it distributed all the genotypes studied into three major groups each including different species. All the *C. annuum* genotypes were grouped together while other cultivated species formed a separate group. The *C. chacoenese* was the only wild species studied which, although, fell within the first group but was placed separately from *C. annuum*. Besides, all the Chilli leaf curl resistant genotypes were grouped together.

Keywords: Chilli, genetic diversity, hot pepper, DNA markers, simple sequence repeats

INTRODUCTION

Chilli or Hot pepper is one of the most economically important vegetable crops that belongs to the genus *Capsicum*, family-Solanaceae. Globally it is cultivated on an area of approximately 1.5 million hectares with a total production of about 7 million tons (Geetha and Selvarani, 4). The genus *Capsicum* had originated from tropical and humid zone of Central and Southern America. China and India account for about half of the World production of fresh pepper and chillies. Moscone *et al.* (10) had reported the existence of 31 Capsicum species, five of which are domesticated*: Capsicum annuum, Capsicum frutescens, Capsicum chinense, Capsicum baccatum* and *Capsicum pubescens*. In addition, considerable variation has also been observed within each Capsicum species with respect to several traits, including colour, shape and size of seeds, flowers and fruits, resistance to biotic and abiotic stresses as well as level of pungency. A thorough understanding about the extent of genetic diversity available in the germplasm collection of a particular crop is very important for strategic germplasm collection, maintenance, conservation and utilization. Several taxonomic studies have been conducted in the past on characterization of genetic resources of capsicum using morphological, biochemical and hybridization techniques (Barboza and Bianchetti, 2) however, the application of molecular markers provided useful insights on discrimination of the species within the existing complexes (Nicolai *et al.,* 12). The genetic relationships existing in a collection of 24 pepper genotypes collected from various sources was assessed to find reliable molecular markers for breeding programs. SSR markers were chosen for this purpose keeping in mind their advantageous features such as co-dominance, abundance, multiple allelic and hyper variable nature (Powell *et al.*, 15). Moreover, in order to give additional information on the genotypes under study for transfer of useful alleles from different backgrounds; genotypes with useful attributes such as resistance to chilli leaf curl disease, bacterial leaf spot resistance as well as different levels of pungency and different fruiting habits were included in the study.

MATERIALS AND METHODS

Plant Material

The germplasm used in this study consisted of 24 genotypes representing 19 genotypes from *Capsicum annuum*, three from other cultivated spp viz. *C. frutescens, C. baccatum, C. chinense* and one genotypes from wild relatives *C. chacoense.* The details regarding different genotypes are mentioned in Table 1. Young, healthy and uninfected leaves from each genotype were collected and brought to the laboratory in liquid nitrogen (-196^{0}C) where they were kept in deep freezers at -80^{0}C till further use.

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Table 1. Characteristics features of different *capsicum* genotypes used in the study.

Name of the genotype	Characteristic features
WBC-sel-5 (C. annuum)	Resistance to Leaf curl, fruiting -cluster erect
DLS-sel-10 (C. annuum)	Resistance to Leaf curl, fruiting -cluster erect
DKC-8 (C. annuum)	Resistance to Leaf curl, fruiting -cluster erect
CJL-S-1 (C. annuum)	Resistance to Leaf curl, fruiting -cluster erect
Tiwari (C. annuum)	Tolerance to Leaf curl, fruiting -single erect
DCL-2 (C. annuum)	Resistance to Leaf curl, fruiting -cluster erect
Phule Mukta (C. annuum)	Susceptible to Leaf curl, fruiting –single pendent, 8-10 cm fruit
LCA-334 (C. annuum)	Susceptible to Leaf curl, fruiting –single pendent, national check
Kashi Anmol (C. annuum)	Susceptible to Leaf curl, fruiting –single pendent, national check
GVC-111 (C. annuum)	Susceptible to Leaf curl, fruiting –single pendent, 10-12 cm fruit
Anugraha (C. annuum)	Susceptible to Leaf curl, Bacterial wilt resistant, fruiting –single semi erect, early fruiting (one month)
Vellayani Attulya (C. annuum)	Susceptible to Leaf curl, fruiting –single pendent, high fruit weight
LCA-333 (C. annuum)	Susceptible to Leaf curl, fruiting –single pendent
Punjab Guchhedar (C. annuum)	Susceptible to Leaf curl, fruiting –Cluster erect, High capsaicin
DSL-352 (C. annuum)	Susceptible to Leaf curl, fruiting –single pendent, flood tolerant
DSL-524 (C. annuum)	Susceptible to Leaf curl, fruiting –single pendent, drought tolerant
Uttakal Yellow (C. annuum)	Susceptible to Leaf curl, fruiting –single erect, High capsanthin
Phule jyoti (C. annuum)	Susceptible to Leaf curl, fruiting –cluster pendent, tolerant
PC-2062 (C. annuum)	Susceptible to Leaf curl, fruiting –single pendent, bushy
Vellayani Sambridhi (C. frutescens)	Susceptible to Leaf curl, fruiting –single erect, high pungency
EC783777 (*C. frutescens)*	Susceptible to Leaf curl, fruiting –single pendent
C. baccatum (PBC-80)	Susceptible to Leaf curl, fruiting –single pendent, anthracnose resistance
C. chinense	Susceptible to Leaf curl, fruiting –single pendent, TMV,CMV resistance
C. chacoense	Susceptible to Leaf curl, fruiting –single pendent, Bacterial leaf spot resistance

DNA extraction

Genomic DNA was extracted from young leaf tissue following the C-TAB procedure of Murray and Thompson, 11). DNA quality and quantity were assessed on a 1% (w/v) agarose gel stained with ethidium bromide and also by using a NanoDrop ND-1000 spectrophotometer

Selection of the primer

99 SSR markers were selected from already published sequences of Yi *et al.* (20) and were custom synthesized (SBS Genetech Co.Ltd., Beijing, China). The markers were selected in such a way so that all the chromosomes were represented. The details of the primers are mentioned in Table 2.

Polymerase Chain Reaction

All the SSR markers were amplified by PCR in 15 µL volumes with 50 ng genomic DNA, 1.0 U *Taq* DNA polymerase (Hi media Laboratories, Mumbai, India), 1.0 µM of each primer, 0.6 uL of 10 mM dNTP mix (Hi media Laboratories, Mumbai, India) and 1.5 uL of 10 × PCR buffer having 17.5 mM $Mg\ Cl_2$ (Hi media Laboratories, Mumbai, India). All the primers were amplified using touchdown PCR in an Eppendorf Mastercycler. Amplification conditions used were, one cycle of 94°C for 3 min; 10 cycles of 94°C for 0.5 min, 65–55°C decreasing by 1°C per cycle for 1 min, and 72°C for 1 min; 30 cycles of 94°C for 0.5 min, 55°C for 1 min, and 72°C for 1 min; and a final cycle of 72°C for 5 min.

Amplified products were resolved on 3.0% agarose gels with Tris/Acetate /EDTA (TAE) stained with ethidium bromide, at a constant voltage of 60 V for 3 h using a horizontal gel electrophoresis system (BioRad, USA) and visualized and photographed under UV light in a gel documentation unit (Alpha imager, Cell bioscinces, Santa Clara, CA).

Table 2. Allelic variations in 85 Microsatellite loci used for characterization of 24 hot pepper genotypes.

S. No.	Marker name	LG	Polymorphism status	Expected Product size (bp)	No of alleles in total 24 genotypes	No of alleles in 20 *C. annuum* genotypes	Observed Product size	PIC in total 24 genotypes	PIC in *C.annuum* genotypes
1.	HpmsE034	1	P	202	2	2	200, 210	0.413	0.188
2.	HpmsE035	1	PWOC	226	2	1	225, 235	0.219	0
3.	HpmsE036	1	P	261	4	2	260, 270, 290, 300	0.608	0.499
4.	HpmsE104	1	M	212	1	1	210	0	0
5.	HpmsE137	1	PWOC	189	3	1	160, 180, 200	0.244	0
6.	HpmsE019	1	P	232	5	3	180, 190, 210, 220, 250	0.671	0.508
7.	HpmsE021	1	PWOC	250	3	1	250, 260, 270	0.156	0
8.	HpmsE022	1	M	206	1	1	200	0	0
9.	HpmsE027	1	P	230	3	3	230, 250, 270	0.611	0.588
10.	HpmsE121	1	M	198	1	1	200	0	0
11.	HpmsE047	2	P	260	2	2	240, 260	0.148	0.18
12.	HpmsE118	2	PWOC	193	2	1	190, 200	0.278	0
13.	HpmsE135	2	PWOC	209	2	1	200,210	0.153	0
14.	HpmsE144	2	PWOC	236	2	1	320, 340	0.0798	0
15.	HpmsE148	2	P	205	2	2	200, 210	0.33	0.188
16.	HpmsE001	2	P	207	3	3	200, 210, 220	0.538	0.349
17.	HpmsE008	3	P	230	3	2	230, 240, 250	0.497	0.488
18.	HpmsE010	3	P	198	2	2	180, 200	0.486	0.499
19.	HpmsE050	3	PWOC	247	2	1	250, 270	0.153	0
20.	HpmsE073	3	P	220	4	2	220, 240, 260, 270	0.625	0.487
21.	HpmsE126	3	PWOC	192	3	1	190, 210, 230	0.571	0
22.	HpmsE060	3	PWOC	206	4	1	200, 220, 230, 240	0.358	0
23.	HpmsE006	4	P	243	2	2	200, 240	0.486	0.487
24.	HpmsE055	4	PWOC	275	3	1	250, 275, 300	0.226	0
25.	HpmsE071	4	P	188	3	2	220, 230, 250	0.631	0.475
26.	HpmsE081	4	P	185	2	2	180, 200	0.413	0.388
27.	HpmsE099	4	M	163	1	1	165	0	0
28.	HpmsE140	4	M	225	1	1	300	0	0
29.	HpmsE015	5	M	146	1	1	140	0	0
30.	HpmsE116	5	P	189	4	4	175, 180, 190, 200	0.663	0.681
31.	HpmsE129	5	PWOC	233	2	1	230, 250	0.278	0
32.	HpmsE011	6	M	151	1	1	150	0	0
33.	HpmsE014	6	P	106	5	3	80,100, 110, 120, 130	0.729	0.660
34.	HpmsE072	6	P	199	4	3	160, 180, 190, 210	0.726	0.660
35.	HpmsE078	6	PWOC	203	3	1	150, 170, 200	0.363	0
36.	HpmsE088	6	P	199	3	3	200, 210, 230	0.628	0.642
37.	HpmsE076	6	M	239	1	1	240	0	0
38.	HpmsE052	7	M	221	1	1	220	0	0
39.	HpmsE068	7	PWOC	232	2	1	230, 240	0.083	0
40.	HpmsE103	7	P	177	2	2	150, 170	0.340	0.332

S. No.	Marker name	LG	Polymor-phism status	Expected Product size (bp)	No of alleles in total 24 genotypes	No of alleles in 20 *C. annuum* genotypes	Observed Product size	PIC in total 24 genotypes	PIC in *C.annuum* genotypes
41.	HpmsE114	7	P	190	3	2	185, 190, 200	0.559	0.487
42.	HpmsE020	7	P	200	2	2	190, 200	0.444	0.332
43.	HpmsE082	9	P	232	2	2	220, 230	0.278	0.099
44.	HpmsE084	9	PWOC	220	2	1	210, 220	0.0798	0
45.	HpmsE102	9	P	163	3	2	160, 170, 180	0.531	0.499
46.	HpmsE013	10	P	256	2	2	240, 250	0.365	0.18
47.	HpmsE031	10	P	167	2	2	170, 180	0.486	0.499
48.	HpmsE065	10	M	199	1	1	200	0	0
49.	HpmsE096	10	M	237	1	1	240	0	0
50.	HpmsE059	10	P	235	2	2	220, 240	0.423	0.432
51.	HpmsE012	11	P	208	2	2	200, 215	0.413	0.388
52.	HpmsE046	11	P	277	4	3	240, 250, 260, 270	0.649	0.633
53.	HpmsE124	11	P	227	4	3	200, 220, 230, 240	0.674	0.549
54.	HpmsE132	11	P	197	2	2	185, 195	0.469	0.432
55.	HpmsE023	11	P	206	3	3	200, 210, 220	0.626	0.609
56.	HpmsE054	12	M	219	1	1	220	0	0
57.	HpmsE064	12	P	221	2	2	170, 190	0.499	0.498
58.	HpmsE108	12	M	200	1	1	200	0	0
59.	HpmsE075	12	P	205	3	3	200, 210, 230	0.628	0.632
60.	HpmsE110	A	M	191	1	1	190	0	0
61.	HpmsE040	B	M	245	1	1	240	0	0
62.	HpmsE086	B	M	221	1	1	220	0	0
63.	HpmsE067	C	M	212	1	1	210	0	0
64.	HpmsE087	C	M	247	1	1	245	0	0
65.	HpmsE002	U	P	177	3	2	170, 180, 210	0.510	0.401
66.	HpmsE028	U	PWOC	231	3	1	220, 230, 240	0.366	0
67.	HpmsE017	U	P	199	3	3	190, 200, 250	0.390	0.380
68.	HpmsE018	U	P	267	2	2	240, 250	0.255	0.255
69.	HpmsE032	U	PWOC	231	2	1	200, 230,	0.087	0
70.	HpmsE058	U	M	202	1	1	200	0	0
71.	HpmsE091	A	M	194	1	1	194	0	0
72.	HpmsE093	C	M	207	1	1	200	0	0
73.	HpmsE097	U	M	250	1	1	250	0	0
74.	HpmsE130	U	P	221	4	4	170, 180, 200, 210	0.678	0.568
75.	HpmsE133	U	P	205	3	3	200, 210, 220	0.608	0.519
76.	HpmsE145	U	P	222	4	3	170, 190, 220, 240	0.507	0.434
77.	HpmsE147	U	PWOC	178	2	1	180, 190	0.0798	0
78.	HpmsE063	1	P	209	2	2	180, 200	0.332	0.391
79.	HpmsE077	1	P	235	2	2	200, 220	0.498	0.5
80.	HpmsE083	1	P	209	4	3	190, 210, 220, 230	0.684	0.614

S. No.	Marker name	LG	Polymorphism status	Expected Product size (bp)	No of alleles in total 24 genotypes	No of alleles in 20 *C. annuum* genotypes	Observed Product size	PIC in total 24 genotypes	PIC in *C.annuum* genotypes
81.	HpmsE100	1	P	220	4	2	200, 210, 220, 230	0.370	0.099
82.	HpmsE112	1	M	206	1	1	200	0	0
83.	HpmsE115	1	M	216	1	1	210	0	0
84.	HpmsE131	1	PWOC	246	2	1	250, 260	0.236	0
85.	HpmsE003	2	P	164	2	2	150, 160	0.287	0.277
					192	146			
								0.302541	0.22368

Data Analysis

The amplified products were scored for each accession based on presence and absence of band using binary code 1 and 0 for the presence and absence of band, respectively. Molecular size (bp) of amplified DNA fragment was determined by comparison with 50 bp ladder (BR biochem, bioscience, life sciences) using image acquisition analysis software of alpha imager gel documentation system. The binary matrix was used to estimate Jaccard's genetic similarity coefficients for SSRs. Principal Coordinate Analysis was performed using NTSYS-pc 2.02 analytical package after calculating eigen values. (Rohlf, 17).

For single-locus evaluations of the SSR data, all DNA fragments were scored as allele sizes at each locus. The polymorphic information content (PIC) of each marker locus, which combines the number of alleles and their frequency distribution within a population and serves as a measure of allele diversity at a locus, was evaluated by applying the following equation, as suggested by Anderson (1):

$$PIC = 1-\sum_{i=1} P_i^2$$

Where Pi is the frequency of the i-th allele among a total of n alleles (Liu, 9).

RESULTS AND DISCUSSION

Knowledge of population diversity in a crop is the first step towards effective utilization of the genetic variability available to breeders. Furthermore, it is essential to have an unequivocal identification method to verify the material obtained. The traditional methods are now being complemented by molecular techniques, enabling breeders to make better decisions when choosing the germplasm used in breeding programs (Cubero, 3). Molecular markers can be regarded as efficient and accurate tools for identification and assessment of genetic variation in a rapid and thorough manner. In fact they provide us advanced and, possibly, the most effective means for understanding the basis of genetic diversity.

The information on the nature and degree of genetic divergence is essential for the breeder to choose the right type of parents for purposeful hybridization in heterosis breeding (Patel *et al.*, 14; Farhad *et al.*, 5). In order to benefit transgressive segregation, the knowledge of genetic distance between parents is necessary. Present study was aimed at understanding genetic diversity and clustering pattern of chilli genotypes grown in India using SSR markers so as to get an idea about the suitability of genotypes for future chilli hybridization programme.

A total of 99 SSR markers uniformly distributed throughout pepper genome were used for diversity analysis of the 24 chilli genotypes. These microsatellite loci were selected in such a way that at least four markers were selected from each linkage group. Out of 99 microsatellite markers, 14 did not amplify in any of the genotype studied and 24 markers were found to be monomorphic (denoted by letter M in Table 2) across all the genotypes selected and hence were unable to differentiate between these genotypes. Out of the remaining 61 polymorphic markers, 18 markers did not show polymorphism in the *C. annuum* genotypes but were polymorphic in other cultivated genotypes (denoted by PWOC in Table 2) *viz., C. frutescens, C. baccatum* and *C. chinense* as well as a wild species *C. chacoense* (Fig. 1a). Forty three out of total microsatellite loci (denoted by letter P in Table 2) studied were found to be highly polymorphic across all the genotypes (Fig. 1 b,c).

The 85 polymorphic SSRs loci amplified a total of 192 alleles across the twenty four genotypes studied (Table 2) and the number of alleles per loci ranged from one to five with an average allele frequency of 2.25 per loci. Maximum five alleles were observed for the marker HpmsE014 located on linkage group 6. Furthermore, when just the *C. annuum* genotypes

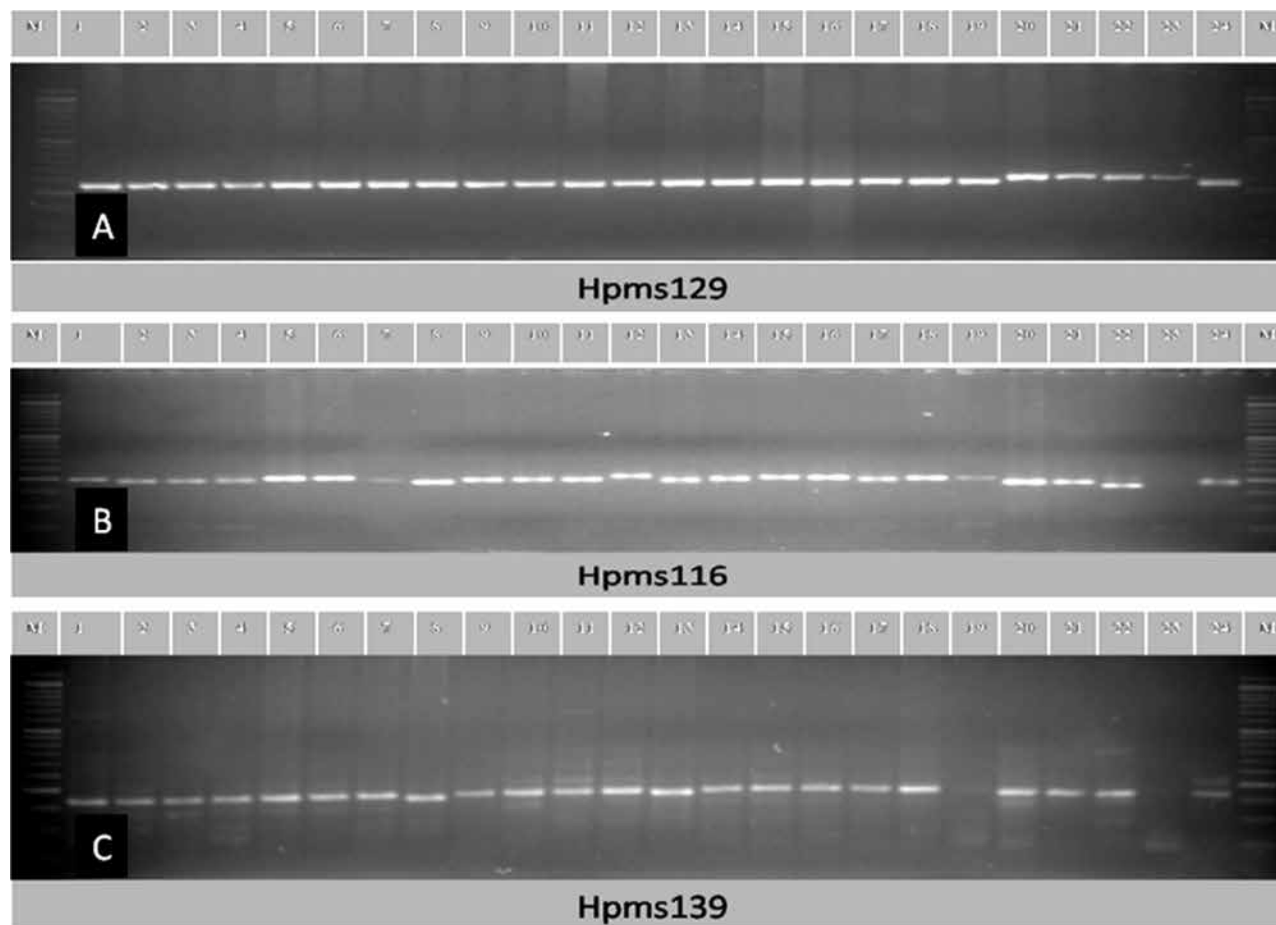

Fig. 1. Polymorphism observed in 24 *Capsicum* genotypes using different SSR markers of Hpms series. A: Polymorphism observed only in other cultivated and wild spp. with primer Hpms E129 ; B&C: Polymorphism observed in all the genotypes.

were considered, it was found that allele number ranged from one to four. Maximum of four alleles were observed in the *C. annuum* genotypes for markers HpmsE116 (located on linkage group 5) and HpmsE130 (location unassigned). A total of 146 alleles were amplified in the nineteen *C. annuum* genotypes (Table 2) with an average allele frequency of 1.72. In earlier works 3.5 alleles per locus has been reported in pepper (Hanáček *et al.*, 7), however this value is bound to change with the diversity in germplasm and number of loci studied.

The highest value of PIC was observed to be 0.729 across all the 24 genotypes and 0.660 among the 19 *C. annuum* genotypes for marker HpmsE014 located on linkage group 6 (Table 2). Marker HpmsE072 located on the same linkage group had the same PIC value (0.660) among the *C.annuum* genotypes but when the whole set of 24 genotypes was considered, it had a slightly lower (0.726) PIC value.

Fig. 2 represents the 3D Principal coordinate analysis plot of the 24 genotype of chilli based on 85 SSR markers using NTSYS pc 2.02 software package (Rohlf, 17). In this plot, twenty four genotypes under study have been separated in such a way that all the *C annuum* genotypes are clustered together in one major group (group I) while the genotypes belonging to *C. frutescens, C. baccatum, C. chinense* formed a separate group (group II). The wild genotype *C. chacoense* which is known to be bacterial leaf spot resistant was found to fall in group I alongwith *C. annuum* genotypes, however, it was an outlier within group I. This result is also in line with the results obtained by Ince *et al.* (8) and Rai *et al.* (16). Several studies have reported that genetic diversity between commonly grown improved *C. annuum* genotypes is less than the diversity between semi-wild and landrace genotypes (Oyama *et al.* 13). This is expected, as during and after domestication nearly all domesticated crop species have gone through a

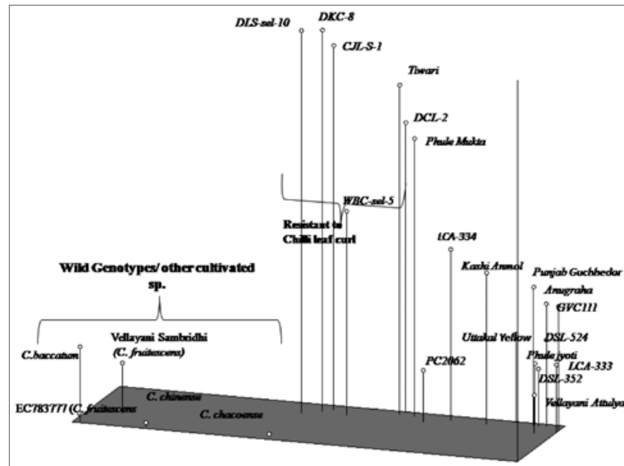

Fig. 2. 3D PCA plot of the 24 genotype of chilli based on 85 SSR markers.

decline in genetic diversity (Gepts, 6). The frequent use of selected elite breeding lines in commercial breeding worldwide has further narrowed genetic diversity in many crop plant species. The magnitude of the observed genetic bottleneck, however, depends on the type of marker (molecular or phenotypic) used to measure genetic diversity (Rai *et al.*, 16). The 3D PCA plot generated using similarity coefficient of twenty four capsicum genotypes provided useful information regarding genetic relationship among the genotypes as all the genotypes viz. WBC-Sel-5, DLS Sel-10, DKC-8, CJL-S-1, Tiwari, DCL-2, which have been shown resistant to chilli leaf curl disease in our earlier studies (Srivastava *et al.*, 18, 19) were grouped together within group I. Similarly DSL-352 and DSL 524 which are two different selections from the cross between same parents were found to lie together. The two *C. frutescens* genotypes viz. Vellayani Sambridhi and EC 783777 also clustered together under group II. However the PCA plot did not show any specific pattern of scattering or clustering among the genotypes on the basis of fruiting habits, as different genotypes with different fruiting habits (cluster erect, cluster pendent, single pendent, single semi erect and single erect) were found to be distributed randomly across all the groups. This appears to be in agreement with the earlier reports of Rai *et al.* (16). The present study has provided useful insight on mapping of gene for resistance to chilli leaf curl disease, as all the resistant genotypes clustered together in same cluster within group I which emphasizes the utility of markers used in the present study for this purpose.

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