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Molecular Diversity Analysis of Some Chilli (*Capsicum* spp.) Genotypes Using SSR Markers

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Abstract

Chilli belongs to the genus *Capsicum* which possesses enormous wealth of genetic diversity. Extent of genetic diversity determines the success level of crop improvement programme. Simple sequence repeats (SSRs) are the most widely used marker system for molecular diversity analysis especially in cultivated species. The aim of our present study was to assess the molecular genetic diversity of 20 local chilli genotypes of Bangladesh using SSR markers. Genomic DNA was extracted from young leaves and PCR reactions were performed. Eleven SSR primers were used in PCR amplification. Total 10 alleles were detected for the five polymorphic SSR loci, with a mean of 2.00 alleles per primer. Gene diversity ranged from 0.333 to 1.00 with an average of 0.567. Polymorphic Information Content (PIC) values of the SSR primers ranged from 0.255 to 0.500 with an average value of 0.371. The similarity index matrix ranged from 0.00 to 1.000. It was highest in several germplasms *viz.* Pop-2 vs Pop-18; Pop-3 vs Pop-5 vs Pop-19 vs Pop-20 and the lowest in the germplasm Pop-8 vs Pop-18. Dendrogram based on Nei's genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) indicated the segregation of 20 chilli genotypes into two main clusters. The SSR markers showed genetic variability in the studied pepper genotypes and they are powerful tools for estimating molecular diversity of chilli. The findings of the present study have potential applications in future breeding programme for the genetic improvement of chilli.

Keywords

Capsicum, Molecular Diversity, Genotypes, SSR Markers, Polymorphism

1. Introduction

Chilli (*Capsicum* spp.) originated from tropical and humid zone of Central and

Southern America and belongs to the Solanaceae family having chromosome number 2n = 2x = 24. Among the domesticated *Capsicum* species, pungent and non-pungent forms of *Capsicum annuum* L. (pepper) are most popular and have a worldwide commercial distribution [1]. Chilli is one of the most important horticultural and spice crop in Bangladesh. It is cultivated in all parts of the country throughout the year and used as green and dry stages for their pungency and colour [2].

Chilli has numerous chemicals including steam-volatile oil, fatty oils, carotenoids, vitamins, protein, fibre and mineral elements [1] and is used for different purposes because of their nutritional value, flavour, aroma, texture, pungency and colour. It also has antifungal property against fungal species belonging to *Aspergillus* and *Fusarium* [3]. It is source of Vitamin, A, B, C and E with minerals like molybdenum, manganese, folate, potassium, thiamin, and copper. Capsaicin has significant physiological action which is used in many pharmaceutical preparations and ointments for cold, sore throat, chest congestion etc. It is also used in cosmetics like prickly heat powders and skin ointments.

The chilli landraces of different district in Bangladesh are heterogeneous and a wide variability in respect of fruit morphology, pungency, bearing habit and crop duration is found throughout country. Bangladeshi chilli varieties have been developed traditionally by selection, hybridization and back crossing with locally adapted cultivars. An important source for the introduction of new traits is the existence of a genetically diverse pool of chilli germplasm available in the country but they are mostly lying unexplored. There is a strong need to collect this germplasm and their proper characterization and classification [4]. Subjective classification based on morphological data has been reported to create confusion and difficulty in classifying the genus *capsicum* [5]. In addition, the level of polymorphism for morphological characteristics in genotypes is sometimes so limited and inadequate to allow variety/genotype discrimination [6].

Molecular markers are important tool for genotype identification and studying the organization and evaluation of plant genome [7]. With the advent of molecular biology technique, large number of highly useful DNA markers has been developed for the identification of genetic polymorphism. Molecular markers define differences in nucleotide sequences, which are unaffected by growth stage, season, location, and agronomic practice [8]. Different molecular markers such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), and simple sequence repeats (SSRs) have been developed for pepper [9] [10]. SSR markers represent highly polymorphic, reproducible, co-dominant, and multi allelic types of variation [11]. They are used in genome mapping, gene tagging, and estimation of genetic diversity, variety identification, and marker-assisted selection [12].

The main objective of this study is to capture the potential genetic diversity among chilli genotypes grown in Bangladesh and selection of suitable genotypes

for future chilli hybridization programme. Hence, the present study was carried out for following objectives.

1) Molecular diversity analysis of different chilli genotypes.
2) Polymorphism study among chilli germplasm.
3) Dendrogram establishment in some local chilli genotypes.

2. Materials and Methods

2.1. Experimental Site and Time Duration

The experiment was conducted in Biotechnology Laboratory, Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Dhaka-1207 and Regional Spices Research Centre, Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur-1701, Bangladesh.

2.2. Plant Materials

Twenty different chilli genotypes were used as plant materials for the study and were collected from different places of Bangladesh (Table 1). The genotypes were grown in poly bags placed in a shady place under the regular agronomic practices.

Table 1. List of chilli germplasm with their origin and characteristics.

Sl. No.	Genotypes	Origin	Characteristics
1	Pop-1(G_1)	Rangpur, Bangladesh	Large fruit size
2	Pop-2(G_2)	Thakurgaon, Bangladesh	Small fruit size
3	Pop-3(G_3)	Panchagarh, Bangladesh	Small fruit size
4	Pop-4(G_4)	Panchagarh, Bangladesh	Medium fruit size
5	Pop-5(G_5)	Bogra, Bangladesh	Large fruit size
6	Pop-6(G_6)	Rangpur, Bangladesh	Medium fruit size
7	Pop-7(G_7)	Bogra, Bangladesh	Large fruit size
8	Pop-8(G_8)	Bogra, Bangladesh	Large fruit size
9	Pop-9(G_9)	Dinajpur, Bangladesh	Small fruit size
10	Pop-11(G_{10})	Saidpur, Bangladesh	Medium fruit size
11	Pop-12(G_{11})	Saidpur, Bangladesh	Medium fruit size
12	Pop-13(G_{12})	Rangpur, Bangladesh	Large fruit size
13	Pop-14(G_{13})	Bogra, Bangladesh	Small fruit size
14	Pop-15(G_{14})	Thakurgaon, Bangladesh	Small fruit size
15	Pop-16(G_{15})	Panchagarh, Bangladesh	Small fruit size
16	Pop-17(G_{16})	Bogra, Bangladesh	Medium fruit size
17	Pop-18(G_{17})	Dinajpur, Bangladesh	Small fruit size
18	Pop-19(G_{18})	Saidpur, Bangladesh	Medium fruit size
19	Pop-20(G_{19})	Bogra, Bangladesh	Small fruit size
20	Pop-21(G_{20})	Bogra, Bangladesh	Medium fruit size

2.3. DNA Isolation

Genomic DNA extractions from fresh young leaf at 3 - 4 leaf stage of seedling were done in SRC lab using CTAB (cetyl trimethyl ammonium bromide) buffer. CTAB extraction buffer was prepared by Doyle & Doyle [13] method with some minor modification. Approximately 200 mg of sterilized young leaves were used for DNA extraction. Extracted DNA was visualized in 1% agarose gel. Approximate 20 - 25 ng of DNA was used as template in PCR reaction.

2.4. SSR Analysis

Eleven SSR primers *viz.* GPMS-113, CAMS-117, CAMS-142, CAMS-153, GPMS-161, GPMS-197, CAMS-327, CAMS-405, EPMS-418, CAMS-806 and CAMS-864 described previously were selected for PCR reaction on 20 local chilli germplasm for their ability to produce polymorphic band [14] [15] [16]. DNA amplification was performed in a thermal cycler (Esco Technologies Swift™ Mini Thermal cyclers, Singapore). The PCR reactions were performed in 10 µl reaction mixture containing 5.0 µl 2X Taq Master Mix, 1.50 µl primers, 1.0 µl sample DNA and 2.5 µl de-ionized water (Biolab, UK). SSRs primers were amplified under the following PCR reaction conditions: Pre-denaturation with 95˚C for 4 min; denaturation with 95˚C for 40 sec, annealing at 50˚C - 61˚C (on the basis of Tm value of primer) for 33 sec, extension at 72˚C for 40 sec, final extension at 72˚C for 5 min continuing with 31 cycles and finally stored at 4˚C.

2.5. Electrophoretic Separation of the Amplified Products

PCR products for each sample were confirmed by running it in 2% agarose gel containing 1 µl ethidium bromide in 1X TBE buffer at 90 V for 1 hour. Five microlitre (5 µl) loading dye was added to the PCR product and spinned them well. Then it was loaded in the wells. The DNA ladder (50 & 100 bp) (Promega thermal cyclers) was used in both left and right side of the gel. Under ultra-violet light on a trans-illuminator SSR bands were observed. The PCR product was saved by gel documentation system and photographed by a Gel Cam Polaroid camera.

2.6. SSR Data Analysis

The summary statistics including the number of alleles per locus, major allele frequency, gene diversity and Polymorphism Information Content (PIC) values were determined using POWER MARKER version 3.25 [17], a genetic marker data analysis software. The individual fragments were assigned as alleles of the appropriate microsatellite loci. The allele frequency data from POWER MARKER was used to export the data in binary format (presence of allele as "1" and absence of allele as "0") for analysis with NTSYS-PC (Numerical Taxonomy and Multiware Analysis System) Version 2.2 software [18]. Unweighted Pair Group Method of Arithmetic Means (UPGMA) dendrogram was constructed using a computer programme, POPGENE (Version 1.31) based on Nei's [19] genetic dis-

tance. Diversity levels of loci were evaluated by polymorphic information content (PIC value) according to the formula [20].

$$PIC = 1 - \sum_{j=i}^{n} \left(P_{ij}\right)^2$$

where, P_i is frequency of the jth pattern for marker i and the summation extends over n patterns.

3. Results

Highly polymorphic and repeatable PCR based markers Simple Sequence Repeats (SSRs) were used here to assess the polymorphism, diversity and similarity identification within those local chilli germplasm. Results obtained from the study have been presented below under the following headings.

3.1. Primer Selection and DNA Amplification through SSR Primer

Eleven SSR primer pairs were screened on twenty chilli genotypes to evaluate their suitability for amplification of DNA. Among them five primer pairs CAMS-117, CAMS-153, GPMS-161, EPMS-418 and CAMS-806 showed reproducible and distinct polymorphic amplification. A total 10 alleles were detected for the five polymorphic SSR loci, with an average number of alleles/locus of 2.00 and a range between 1 to 3 alleles (Table 2). It was observed that one SSR primer GPMS-161 (Figure 1(c)) gave maximum number of allele (3) and allelic frequency ranging from 0.675 to 0.125 followed by CAMS-117, EPMS-418 and CAMS-806 SSR primer. In CAMS 153 SSR primers showed allelic frequency

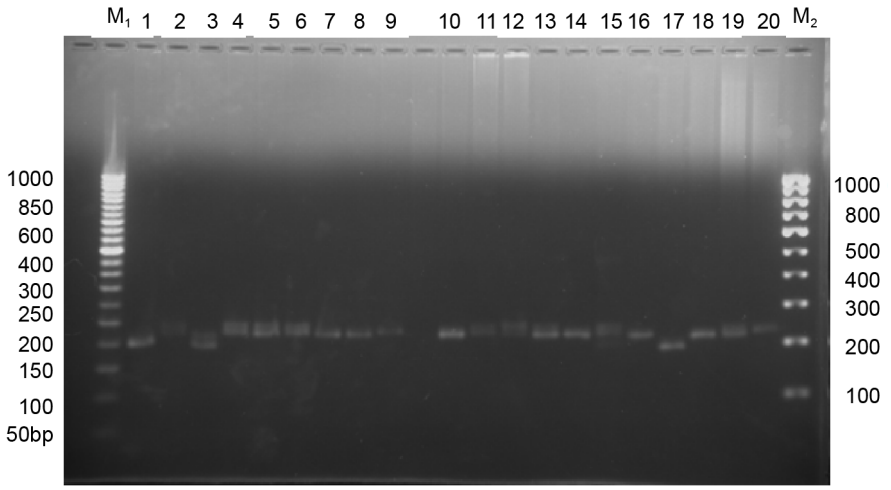
Table 2. Primer sequence, number of allele, allele size and frequency of alleles and gene diversity index and PIC value at five SSR loci across 20 chilli genotypes.

Primer code	Sequence of Primers (5'-3')	Number of Allele	DNA Fragment Size (bp)	Allele frequency	Gene Diversity	PIC Value
CAMS-117	For: TTGTGGAGGAAACAAGCAAA Rev: CCTCAGCCCAGGAGACATAA	2	220 190	0.850 0.150	0.50	0.255
CAMS-153	For: TGCACAAATATGAATCCCAAGA Rev: AGTCAGCAAACACATCTGACAA	1	200	0.737	1.00	0.388
GPMS-161	For: GAAATCCAATAAACGAGTGAAG Rev: CCTGTGTGAACAAGTTTTCAGG	3	240 200 140	0.200 0.675 0.125	0.333	0.489
EPMS-418	For: ATCTTCTTCTCATTTCTCCCTTC Rev: TGCTCAGCATTAACGACGTC	2	200 180	0.850 0.150	0.500	0.225
CAMS-806	For: TGTCACAAGTGTCAAGGTAGGAG Rev: CCCCAAAAATTTTCCCTCAT	2	210 170	0.500 0.500	0.500	0.500
Total	-	10		-	2.833	1.857
Mean	-	2.00		-	0.567	0.371

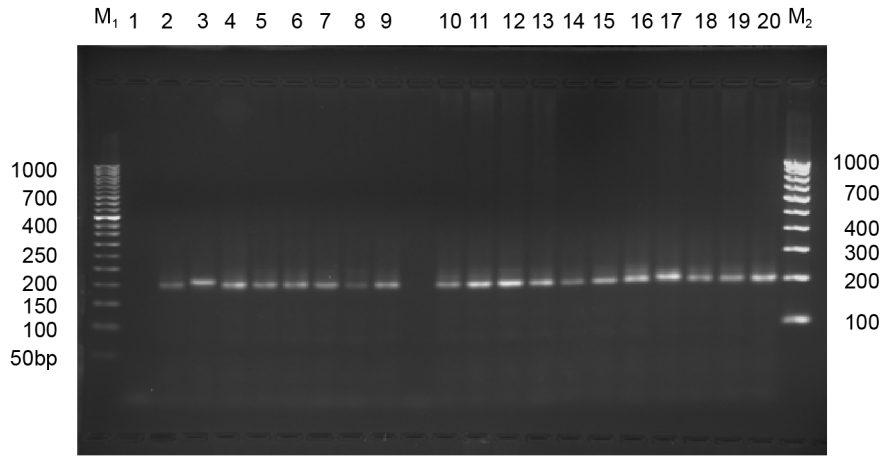
0.737 for only 1 allele (Table 2). The SSR primer CAMS-117 produced 2 DNA band ranges from 190 bp to 220 bp (Table 2) where 190 bp was amplified in the genotypes of Pop-1, Pop-3, Pop-17 (Figure 1(a)) which is polymorphic in nature while primer CAMS-153 produced 1 DNA fragment at 200 bp (Table 2) where no polymorphic band was found (Figure 1(b)). In case of GPMS-161 primer, 3 DNA fragment was observed ranges from 140 bp to 240 bp (Table 2). The genotypes Pop-2, Pop-4 and Pop-10 were amplified at 190 bp DNA fragment (Figure 1(c)) which is polymorphic. Further, the SSR primer EPMS-418 and CAMS-806 produced 2 DNA amplification which was ranges from 180 bp to 200 bp and 170 bp to 210 bp (Table 2). The primer EPMS-418 gave 180 bp fragments and it was polymorphic. It showed amplification in the genotypes Pop-1, Pop-3, Pop-18 (Figure 1(d)). The DNA band was amplified in the genotypes Pop-1, Pop-2 and Pop-3 showed polymorphism at 170 bp of DNA band in CAMS-806 (Figure 1(e)).

3.2. Gene Diversity and Polymorphism Information Content (PIC)

Gene diversity ranged from 0.333 to 1.00. Primer CAMS-153 showed highest



(a)



(b)

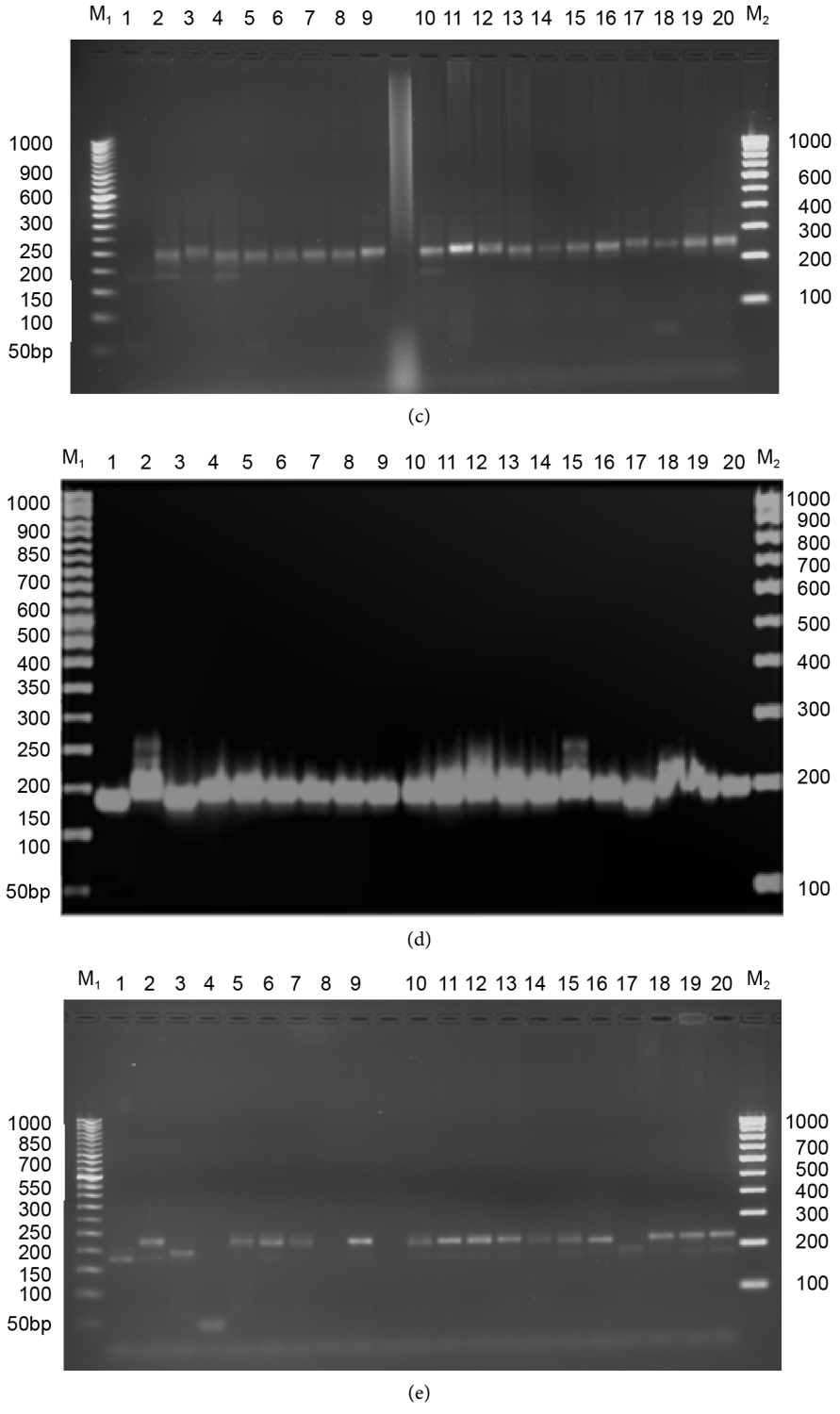


Figure 1. SSR profile of twenty chilli genotypes using primer (a) CAMS-117, (b) CAMS-153, (c) GPMS-161, (d) EPMS-418 & (e) CAMS-806. M_1: 50 bp DNA ladder and M_2: 100 bp DNA ladder. Lane 1 Pop-1; 2: Pop-2; 3: Pop-3; 4: Pop-4; 5: Pop-5; 6: Pop-6; 7: Pop-7; 8: Pop-8; 9: Pop-9; 10: Pop-11; 11: Pop-12; 12: Pop-13; 13: Pop-14; 14: Pop-15, 15: Pop-16; 16: Pop-17; 17: Pop-18; 18: Pop-19; 19: Pop-20 and 20: Pop-21.

gene diversity (1.00) followed by CAMS-117, EPMS-418, CAMS-806 which was showed same gene diversity (0.500) (Table 2). Total genetic diversity obtained 2.833 with an average 0.567. Polymorphic Information Content (PIC) value for the five markers ranged from 0.255 to 0.500 with a mean value of 0.371. The highest PIC value (0.500) was obtained for CAMS-806 followed by GPMS-161 (0.489). The total PIC was 1.857 with an average 0.370 (Table 2). PIC value revealed that CAMS-806 was considered as the best marker for 20 chilli germplasm followed by GPMS-161 and CAMS-153. Primer CAMS-117 and EPMS-418 could be considered as the least powerful marker.

3.3. Genetic Similarity Matrix

The values of similarity coefficient were computed from combined data for the five primers, ranged from 0.00 to 1.000. The highest genetic identity (1.00) was observed in Pop-2 vs Pop-18; Pop-3 vs Pop-5 vs Pop-19 vs Pop-20; Pop-4 vs Pop-18 vs Pop-19; Pop-13 vs Pop-14 vs Pop-15 vs Pop-16 vs Pop-18; whereas lowest genetic identity (0.00) was observed in Pop-8 vs Pop-18. The second largest genetic identity was 0.997 and they were present in Pop-4 vs Pop-20 and Pop-13 vs Pop-20 (Table 3). The difference between the highest and lowest

Table 3. Nei's (1972) genetic distance (below diagonal) values among 20 chilli genotypes.

Acc#	Pop-1	Pop-2	Pop-3	Pop-4	Pop-5	Pop-6	Pop-7	Pop-8	Pop-9	Pop-11	Pop-12	Pop-13	Pop-14	Pop-15	Pop-16	Pop-17	Pop-18	Pop-19	Pop-20	Pop-21
Pop-1	***																			
Pop-2	0.721	***																		
Pop-3	0.615	0.747	***																	
Pop-4	0.811	0.027	0.838	***																
Pop-5	0.204	0.161	1.000	0.195	***															
Pop-6	0.811	0.084	0.838	0.118	0.077	***														
Pop-7	0.911	0.309	0.821	0.352	0.123	0.218	***													
Pop-8	0.504	0.353	0.838	0.325	0.195	0.251	0.352	***												
Pop-9	0.298	0.453	0.620	0.506	0.503	0.506	0.452	0.506	***											
Pop-11	0.151	0.262	0.485	0.304	0.455	0.378	0.559	0.378	0.271	***										
Pop-12	0.250	0.5723	0.572	0.641	0.718	0.641	0.741	0.641	0.271	0.288	***									
Pop-13	0.197	0.520	0.520	0.588	0.665	0.588	0.688	0.588	0.218	0.235	0.053	***								
Pop-14	0.358	0.430	0.692	0.483	0.560	0.483	0.503	0.665	0.670	0.535	0.248	0.328	***							
Pop-15	0.869	0.430	0.915	0.483	0.560	0.483	0.670	0.665	0.503	0.718	0.248	0.328	0.100	***						
Pop-16	0.308	0.380	0.747	0.433	0.510	0.433	0.453	0.615	0.620	0.485	0.331	0.433	0.050	0.161	***					
Pop-17	0.358	0.597	0.915	0.665	0.742	0.665	0.670	0.888	0.871	0.718	0.381	0.483	0.100	0.211	0.161	***				
Pop-18	0.911	1.000	0.715	1.000	0.983	0.817	0.841	0.000	0.705	0.098	0.964	1.000	1.000	1.000	1.000	0.871	***			
Pop-19	0.358	0.915	1.000	1.000	0.742	0.888	0.670	0.888	0.458	0.074	0.718	0.888	0.337	0.480	0.430	0.211	0.871	***		
Pop-20	0.334	0.891	1.000	0.997	0.718	0.864	0.559	0.864	0.252	0.050	0.799	0.997	0.381	0.535	0.405	0.248	0.846	0.024	***	
Pop-21	0.728	0.145	0.062	0.341	0.195	0.118	0.219	0.341	0.219	0.171	0.834	0.782	0.608	0.608	0.809	0.407	0.219	0.240	0.296	***

Legend: 1: Pop-1; 2: Pop-2; 3: Pop-3; 4: Pop-4; 5: Pop-5; 6: Pop-6; 7: Pop-7; 8: Pop-8; 9: Pop-9; 10: Pop-11; 11: Pop-12; 12: Pop-13; 13: Pop-14; 14: Pop-15, 15: Pop-16; 16: Pop-17; 17: Pop-18; 18: Pop-19; 19: Pop-20 and 20: Pop-21.

genetic identity indicates the presence of variability among 20 germplasm of chilli. Genetic distance among 20 chilli genotypes ranged from 0.024 to 0.911. The highest genetic distance (0.911) was observed in Pop-1 vs Pop-7 vs Pop-18 genotype pairs, whereas lowest genetic distance (0.024) was estimated in Pop-19 vs Pop-20 (Table 3). The results revealed that the experimental materials showed highest genetic diversity. Genotypes pair with higher genetic distance is more dissimilar than a pair with a lower value. The lowest genetic distance indicating that they genetically much closer.

3.4. Genetic Relationship (UPGMA Dendrogram)

All the 20 chilli germplasm has been grouped into two main clusters. The first cluster further divided into two sub clusters. The sub clusters contains four genotypes (Pop-1, Pop-3, Pop-8 and Pop-18) (Figure 2). The second cluster also grouped into two sub clusters. There nine genotypes (Pop-2, Pop-4, Pop-5, Pop-6, Pop-7, Pop-9, Pop-11, Pop-12, Pop-13) in first sub cluster and seven genotypes (Pop-14, Pop-15, Pop-16, Pop-17, Pop-19, Pop-20, Pop-21) in second sub cluster of second cluster (Figure 2).

4. Discussion

SSR markers are considered more reliable because of their ability to produce highly consistent profiles [12]. Eleven SSR primers were screened on 20 chilli genotypes to evaluate their suitability for amplification of the DNA fragment.

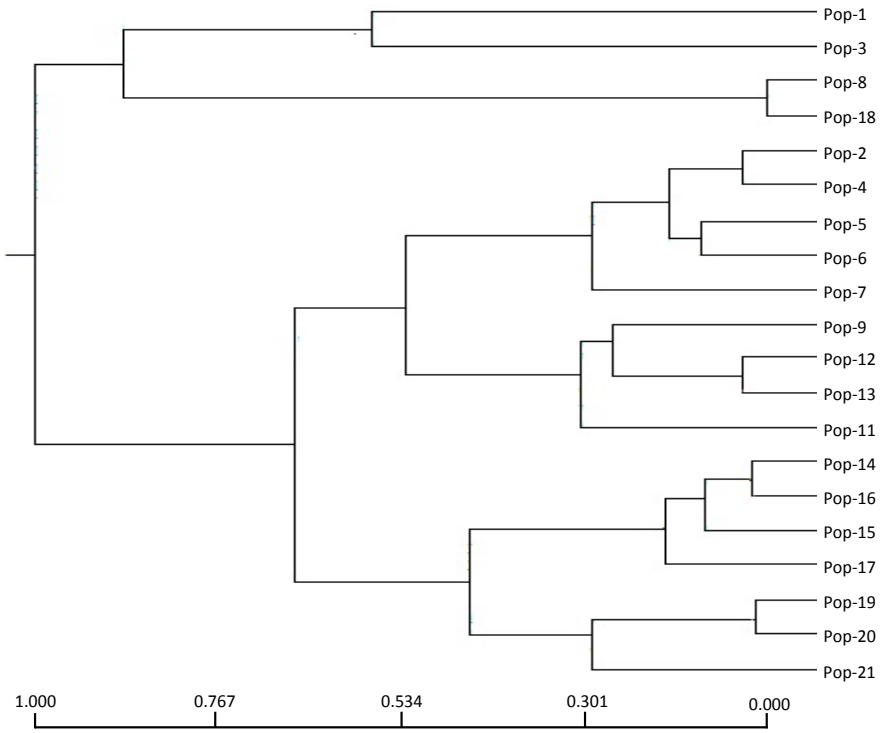


Figure 2. UPGMA dendrogram based on Nei's (1972) genetic distance, summarizing the data on differentiation between 20 chilli genotypes according to microsatellite analysis.

The average number of alleles per locus provides complementary information of polymorphism and more adequate to co-dominant markers [21]. The average numbers of alleles (2.00) per SSR primer pairs were in agreement with earlier works reported in pepper with the mean value of 2.78 alleles/locus and maximum of four alleles were amplified by the primer AVRDC PP 32 [14]. Our observation were partially supported by Tilahun *et al.* [16] where they observed 3.22 average number of alleles per primer, range from 1 to 6.

The PIC values provide an estimate of discriminating power of a marker by taking into account not only the number of alleles at a locus but also relative frequencies of these alleles. Lower PIC values might be result of closely related genotypes and vice versa. Senior *et al.* [22] opined that marker loci with an average number of alleles running at equal frequencies will have the highest PIC value. The PIC value obtained in present study varied from 0.255 to 0.500 with an average 0.371. The highest PIC value (0.500) was obtained for CAMS-806. PIC value revealed that CAMS-806 was considered as the best marker for 20 chilli germplasm followed by GPMS-161 and CAMS-153. Primer CAMS-117 and EPMS-418 could be considered as the least powerful marker. Our results were partially consistent with Yumnam *et al.* [23] (0.52) and kwon *et al.* [24] (0.53). On the contrary, 65 polymorphic markers were validated among a wide collection of 21 *Capsicum* genotypes with allele number and polymorphic information content value per marker raging from 2 to 6 and 0.05 to 0.64, respectively [25]. The varying levels of polymorphism in chilli pepper reported by various research group could be attributed to the differences in genetic structures of the populations screened and the molecular techniques used [14].

The gene diversity (GD) was 0.154, indicating a considerable amount of polymorphism within this collection. The difference between the highest and lowest genetic identity indicates the presence of variability among 20 germplasm of chilli. Genetic distance among 20 chilli genotypes ranged from 0.024 to 0.911. The highest genetic distance between them indicated that genetically they are diverged. Genotypes pair with higher genetic distance is more dissimilar than a pair with a lower value. This study indicated that the genotypes those showed the highest genetic variation can be used as parental source for breeding line to improve chilli varieties.

In this study, dendrogram revealed that the genotypes that derived of genetically similar type clustered together. Again, cluster analysis of the band patterns separated the varieties into three groups corresponding to varietal types. Morphological trait-based clustering showed some degree of similarity to dendrogram topologies based on the SSR index. This may be explained by that mostly SSR markers measures genetic variation mainly in non-coding sequences which possibly do not have a major impact on the morphology of genotype [24].

5. Conclusion

SSR markers showed genetic variability in the studied chilli genotypes and they are powerful tools for estimating genetic similarities and diversity. The genetic re-

lationships presented among the genotypes are helpful for future breeding programs through selection of genetically diverse parents. The present work was the preliminary study to characterize and detect genetic variation of chilli varieties of Bangladesh and had some limitations in term of limited number of individuals and varieties as well as number of primers used. The results indicated that the present study might be used as a guideline for developing mapping population, marker assisted selection (MAS) and crop improvement of chilli varieties and consequently enables a genetic conservation plan in Bangladesh.

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