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Development of chloroplast microsatellite markers in *Capsicum:* Insight into evolution of Bhut Jolokia - a clad of ghost chilli landraces

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Abstract

In the present study, a total of 27 chloroplast specific SSRs (CpSSR) have been identified in the chloroplast genome of *Capsicum annum* L. The frequency of the SSRs was about one in 5.7 kb of the chloroplast genome. Out of 27 SSRs, 26 were mono-nucleotide repeats of A/T and one was a tri-nucleotide repeat (TTA). Further a set of seven markers were validated by genotyping 48 capsicum accessions comprising of cultivars from five different species and landraces of unknown identity. The seven SSR markers generated a total of 27 alleles among 48 samples used in this study. The size of the amplicons varied from 161 bp (CaCpM22 & 26) to 339 bp (CaCpM06). The polymorphic information content (PIC) value for the set of the primers used ranged from 0.11 to 0.48 with an average of 0.33. The number of alleles for markers ranged from three to six with an average of 3.28 alleles per marker. The phylogenetic analysis of the chilly accessions showed that the Bhut jolokia land race is clustered along with the *C. frutescence* indicating the it's probable parentage. The chloroplast genome based SSR markers identified in the present study can be further used for the marker-assisted genomic studies.

Key words: Bhut Jolokia, Capsicum, Chilly, Chloroplast, SSR

Introduction

The genus *Capsicum* belongs to the Solanaceae family and it comprises of around 30 species. Among them, five are mostly grown all over the world for human consumption as food and spice (Bhutia et al. 2019). All capsicum species are found to be diploid in nature with 24 chromosomes (2n=2x=24), however species containing 32 (Wang and Bosland 2006) 48 (Dafadar et al. 2012) chromosomes have also been reported. These species are believed to be originated in South and Central Americas where these were domesticated around 7,000 BC. Christopher Columbus introduced them into Europe during end of 15th century, from where these were spread to rest of the world. It is probably introduced to India by Portuguese explorers (Basu and De 2003). The North east (NE) India has a rich natural heterogeneity due to its distinctive geographical location which has been identified as one of the twelve "Genetic Epicentres" for the evolution of world flora. The NE India exhibits natural diversity for many vegetable crops belonging to Cucurbitaceae, Papilionaceae and Solanaceae families. Chillies grown in this region exhibit a wide range of morphological, biochemical, and genetic variation (Islam et al. 2015; Islam et al. 2016; Yumnam et al. 2012). Several capsicum landraces are traditionally grown in NE india, which are differentiated based on their pungency level and morphological characters. Among these landraces, Bhut Jolokia (BJ) is being famous for their high pungency (Islam et al. 2015; Islam et al. 2016). Despite of uniqueness and importance a less efforts have been made to solve the systemic position of these landraces including Bhut Jolokia. In this direction the molecular markers will act as better tools to analyse the genetic relatedness of these landraces from the well characterized cultivars of the capsicum.

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Genetic variations existed at any specific locus has great importance for functional annotations as well as to be used as a molecular marker (Lai et al. 2010; Ram et al. 2007). Several molecular markers are available for ascertaining genetic relatedness among the members of plant species (Katara et al. 2010; Ram et al. 2019; Yadav et al. 2019). Among them the microsatellites are very popular for their co-dominant nature, high polymorphism and genome wide distribution (Singh et al. 2010). Microsatellite also called simple sequence repeats (SSRs) is referring to the DNA sequence having a variable number of repeats motifs of 1-6 bp length. SSRs can be found abundantly in plant genomic DNA. The SSRs are also identified in the organelle genome such as chloroplast (Powell et al. 1995) and mitochondria (Soranzo et al. 1999). However, SSR in organelle genome has not been well characterized in plants even though their genomes in several plants species have been sequenced (Sonah et al. 2011). The SSRs from organelle genome have the similar characteristics as that of nuclear genome. Over the last two decades, organelle genome (chloroplast and mitochondria genome) has been extensively used for the phylogenetic and molecular evolutionary studies in both plants and animals due to uniparental inheritance, effectively haploid and non-recombinant nature. However in plants, chloroplast genome showed several advantages over mitochondria such as high copy number, availability of vast number of primer, relatively lack of homoplasmy and recombination which make them popular for extensive use in phylogenetic studies (Provan et al. 2001). The conservative nucleotide substitution rate in chloroplast genome makes it important for evolutionary and ecological studies (Provan et al. 2001; Tsunewaki 1993). Very few studies of chloroplast genome specific marker validation have been performed in capsicum (Cheng et al. 2016). The availability of the genome of the capsicum provided the opportunity to analyse the distribution of SSRs in chloroplast genome and to utilize them for genetic analysis

The present study was conducted with two prime goals: firstly, identify a large number of polymorphic cpSSR markers in the genus *Capsicum* and secondly, study the phylogenetic relationship of "Bhut Jolokia" landrace with other cultivars from different *Capsicum* species.

Materials and methods

Plant material

A set of 48 diverse accessions of *Capsicum* were used in the present study. Out of these, 37 accessions were collected from NE states of India, *viz*., Arunachal Pradesh, Assam, Manipur and Sikkim and remaining accessions represented five cultivated species namely *C. annuum*, *C. frutescens*, *C. chinense*, *C. baccatum* and *C. pubscense*. The accessions from NE India were collected from remote hilly regions including farmer's fields, backyard gardens and local markets. The details of samples, accession IDs, vernacular names, species and place of collection are provided as Table 1. For each accession, 8-10 fully matured (red ripe) fruits were collected and seeds were extracted after drying in oven at 40^{o}C. Seeds were germinated in hycotrays and transplanted in the field with a spacing of 60 cm x 45 cm.

Chloroplast microsatellite markers identification and primers designing

To identify the chloroplast based microsatellite markers, two newly annotated peppers genome (Kim et al. 2014; Qin et al. 2014) were searched with the following repeat threshold criteria, 10 repeats for mono-nucleotides, 6 for di-nucleotides, 5 for tri-nucleotides repeats, 4 for tetra nucleotides repeats and 3 for penta-hexa nucleotides repeats using MISA perl script (Thiel et al. 2003). The sequences having microsatellite repeats were used to design primer pairs from their flanking regions using program Batch Primer 3 (You et al. 2008). PCR primers were generated with a number of set parameters such as annealing temperature 50-60^{o}C, GC content of 30-70 %, and a product size of 100-350 bp.

DNA isolation and genotyping with microsatellite markers

Young leaf samples were pooled from three randomly selected plants from each accession and considered as the representative leaf sample for that accession. Total genomic DNA was isolated from leaf sample using a method describes by Doyle (1990). Genotyping was carried out using indirect florescent labelling methods described by Schuelke (2000). Each forward primer was tagged with M13 sequence (5'-TGTAAAACGACGGCCAGT-3') which was labelled with both the IRDye-700 and IRDye-800 fluorescent dye. Reaction was carried out in a 15 µl reaction containing 50 ng DNA, 1x reaction buffer, 1.5 mM $Mgcl_2$, 0.2 mM of each dNTP, 1 U of *Taq* polymerase, 8 pmol of extended Forward primers and Reverse primers and 2 pmol of M13 IRDye labelled primer. PCR amplification was set as follows: 94 for 5 min, followed by 25 cycles at 94^{o}C for 30 s, 56^{o}C for 30 s, and 72^{o}C

Table 1. Details of the accessions of *Capsicum* species used in the present study

Acc ID	Vernacular	Pungency	Place of collection	Acc ID	Vernacular	Pungency	Place of collection
CC001	BJ	Pungent	Kamrup, Assam	CC139	*C. chinense*	Pungent	AVRDC, Tiwan
CC004	BJ	Pungent	Kamrup, Assam	CC156	Unknown	Pungent	Sikkim
CC015	BJ	Pungent	Goalpara, Assam	CC157	BJ	Pungent	Karbi Anglong, Assam
CC017	BJ	Pungent	Dibrugarh, Assam	CC158	BJ	Pungent	Karbi Anglong, Assam
CC018	BJ	Pungent	Dibrugarh, Assam	CC159	Mem jolokia	Pungent	Karbi Anglong, Assam
CC022	BJ	Pungent	Dibrugarh, Assam	CC160	Beka jolokia	Pungent	Karbi Anglong, Assam
CC028	PBC-904	Pungent	AVRDC, Taiwan	CC162	BJ	Pungent	Karbi Anglong, Assam
CC032	Unknown	Pungent	Nagaon, Assam	CC172	BJ	Pungent	Karbi Anglong, Assam
CC034	Unknown	Pungent	Nagaon, Assam	CC174	Khorika jolokia	Pungent	Karbi Anglong, Assam
CC051	BJ	Pungent	Dhemaji, Assam	CC175	Krisna jolokia	Pungent	Karbi Anglong, Assam
CC055	BJ	Pungent	Dhemaji, Assam	CC176	Long mem jolokia	Pungent	Karbi Anglong, Assam
CC056	BJ	Pungent	Dibrugarh, Assam	CC179	BJ	Pungent	Karbi Anglong, Assam
CC057	BJ	Pungent	Namsai, Arunachal Pradesh	CC180	BJ	Pungent	Karbi Anglong, Assam
CC058	BJ	Pungent	Kamrup, Assam	CC195	BJ	Pungent	Karbi Anglong, Assam
CC060	BJ	Pungent	Kamrup, Assam	CC198	BJ	Pungent	Karbi Anglong, Assam
CC061	CW	Non-pungent	Nirmal Seeds Pvt Ltd, Maharashtra	CC202	BJ	Pungent	Karbi Anglong, Assam
CC062	KTPL-19	Non-pungent	Nirmal Seeds Pvt Ltd, Maharashtra	CC203	BJ	Pungent	Karbi Anglong, Assam
CC063	IVPBC-535	Non-pungent	AVRDC, Tiwan	CC206	BJ	Pungent	Karbi Anglong, Assam
CC101	BJ	Pungent	Jorhat, Assam	CC217	BJ	Pungent	Karbi Anglong, Assam
CC103	BJ	Pungent	Tinsukia, Assam	CC221	*C. frutescence*	Pungent	AVRDC, Tiwan
CC107	Lota Bhut	Pungent	Tinsukia, Assam	CC222	*C. frutescence*	Pungent	AVRDC, Tiwan
CC114	BJ	Pungent	Manipur	CC226	*C. chinense*	Pungent	AVRDC, Tiwan
CC119	BJ	Pungent	Tinsukia, Assam	CC227	*C. frutescence*	Pungent	AVRDC, Tiwan
CC138	*C. pubscence*	Pungent	AVRDC, Tiwan	CC228	*C. baccatum*	Pungent	AVRDC, Tiwan

for 1 min, followed by 15 cycles at 94°C for 30 s, 52°C for 30 s and 72°C for 1 min with a final extension at 72°C for 15 min. For each primer amplicon product were separated using 6.5% denaturing PAGE (polyacrylamide gel electrophoresis) on LI-COR 4300 DNA analyser (LI-COR BioSciences Lincoln). Microsatellites fragment were analysed using GENEIMAGIR 4.2 software. Only clear and unambiguous fragment were scored with their fragment size.

Data analysis

The entire summery statistic such as number of alleles, allele frequency, average heterozygocity, polymorphic information content (PIC), were calculated using Power Marker v.30 (Liu and Muse 2005). Furthermore, pairwise genetic similarity was calculated based on simple matching methods. And a phylogenetic dendrogram was constructed based on the neighbor joining method (NJ) using Darwin v5.00 (Perrier et al. 2003).

Results

Distribution of cpSSR markers in Capsicum chloroplast genome

A total of 27 chloroplast specific SSRs (CpSSR) have been identified in the chloroplast genome of *Capsicum annuum* (Table 2). The frequency of the SSRs was

Table 2. Details of the simple sequence repeat markers developed from the *Capsicum* chloroplast genome

ID	SSR	Forward Primer (5'-3')	Tm (°C)	Reverse Primer (5'-3')	Tm (°C)	Product size (bp)	Start (bp)	End (bp)
CaCpM1	(A)10	GCAACCCGAAGAAAATATCG	59.5	GCCAATCCAACACAAGTCCT	60.0	293	4405	4697
CaCpM2	(T)10	TTCTCTTTTCGACATCACATCA	58.4	TACGAACACCTCCCCATTCT	59.4	285	6649	6933
CaCpM3	(T)11	AGAGCTTCGTTGCTTGGTGT	60.1	GGATTACGTCCGGGATCATT	60.9	196	8339	8534
CaCpM4	(A)10	AATGATCCCGGACGTAATCC	60.9	TTTCAATTGGGAGAGATGGC	60.0	254	8515	8768
CaCpM5	(T)11	TCGGTGGAAACAAACTCCTT	59.6	TTTCATTCGGCTCCCTTATG	60.0	242	9788	10029
CaCpM6*	(T)12-(A)15	AGCCTTCCAAGCTAACGATG	59.5	TGCGTCCAATAGGATTTGAA	59.1	293	10098	10390
CaCpM7	(T)12	ATTCATTTGGCTCTCACGCT	59.8	AAAAACGGGGATTTTACCCC	61.1	274	12583	12856
CaCpM8	(A)11	TCATTTCTCCCCACAACCTC	59.9	TTCACGACATACCCGAACAA	60.0	271	16841	17111
CaCpM9	(T)10	ACTTCTTGAATCACCCACCG	60.0	TCTTGTGCAGTCCAAAACAAA	59.4	211	33186	33396
CaCpM10	(T)11	GCAGCAGGATTTGAAAAAGG	59.8	GAAAGAAAGGGGAATGGCTC	60.0	264	36563	36826
CaCpM11	(A)10	TACGGCTCCTCCCTTATGTG	60.1	AACATCCATCTCGTTCGTCC	59.9	298	45202	45499
CaCpM12	(A)10	CCCTATGCTGCCTATTAGCG	59.9	TTACAGAGATGGTGCGATTTG	58.8	292	45599	45890
CaCpM13	(T)10	TGCTGTACCTCACAAGCCAC	59.9	AATTAACCGATCGACGTGCT	59.6	295	56580	56874
CaCpM14	(A)10	TACCACTGTCAAGGGGGAAG	60.0	CCATGGTATTTGATTTGCCA	59.2	147	57300	57446
CaCpM15	(A)10	CCAAATTCTTGTTGGCGATT	59.9	GATGCTGGGAATCCCTTGTA	59.9	130	66002	66131
CaCpM16*	(T)14	TGGCTACTCTAACCTTCCCG	59.3	AGAGCCAAAGCGTGTGAACT	60.1	288	72096	72383
CaCpM17	(T)11	TGTGATTCAGCAATCCCAAA	60.0	CTATCGGGAGTTCGGTTCAG	59.7	152	73575	73726
CaCpM18	(T)11	CCGCGATGGTATTTTCTTGT	60.0	TCCAGGCTCCGTTTAGAAAA	59.8	295	74504	74798
CaCpM19	(T)10	TCCTGGAAGGCAATTCTGAT	59.6	CACGGTTCCTTTATCCCCTT	60.2	116	81208	81323
CaCpM20	(A)10	ACACATCAATTCTCGAGCCC	60.1	GCAGCATCCAAAATGCCTAT	60.1	223	84075	84297
CaCpM21*	(T)19	GCCCGGGACCAAGTTACTAT	60.2	GGGTTATCCTGCACTTGGAA	59.9	268	87166	87433
CaCpM22*	(A)20	GAAAAATAGCTCGACGCCAG	60.0	GTGGGAGAGAAGGGATGTCA	60.0	139	110526	110664
CaCpM23	(T)10	CGAAAGCTATTTCTACTGGGGA	59.8	ATAGGTCGGTGGGGAAAATC	60.0	288	115579	115866
CaCpM24	(A)11	CCAATGGAATTCTGTCTGCT	57.7	ATTGCAAAAGCAAAAAGGGG	61.3	300	122748	123047
CaCpM25*	(TTA)5	TGGTCGATTCCACCGATTAT	60.2	TCGAGTGAATGGAAAGGACA	59.2	259	130699	130957
CaCpM26*	(T)19	GTGGGAGAGAAGGGATGTCA	60.0	GAAAAATAGCTCGACGCCAG	60.0	138	133160	133297
CaCpM27*	(A)19	GGGTTATCCTGCACTTGGAA	59.9	TGATTGGCTACAAAGGGATTTT	59.8	199	156390	156588

*List of Selected CpSSR markers used for genotyping the *Capsicum* accessions

one in ~5.7 kb of the chloroplast DNA. All the observed SSRs are of A/T repeats. Out of 27 SSRs, 26 were mono-nucleotide repeats and one was a tri-nucleotide repeat (TTA).

Efficiency of CpSSR markers

Out of 27 CpSSRs identified, seven were employed for genotyping the *Capsicum* accessions (Table 3). The seven SSRs generated a total of 27 alleles among 48 samples used in this study. All the markers showed a sharp and clear amplification across all the species (Fig. 1). The size of the amplicons varied from 161 bp (CaCpM22 & 26) to 339 bp (CaCpM06). All the samples amplified a single allele for the primer sets used, except one of the genotype (CC034) which showed two alleles each for two primer sets (CaCpM02 & 25). The polymorphic information content (PIC) value for the set of the primers used ranged from 0.11 to 0.48 with an average of 0.33. The number of alleles for markers ranged from three to six with an average of 3.28 alleles per marker (Table 3). The markers, CaCpM-16, CaCpM-22, and CaCpM-27 amplified allele specifically from *C. annuum*, *C. frutescens*, and *C. baccatum*, respectively.

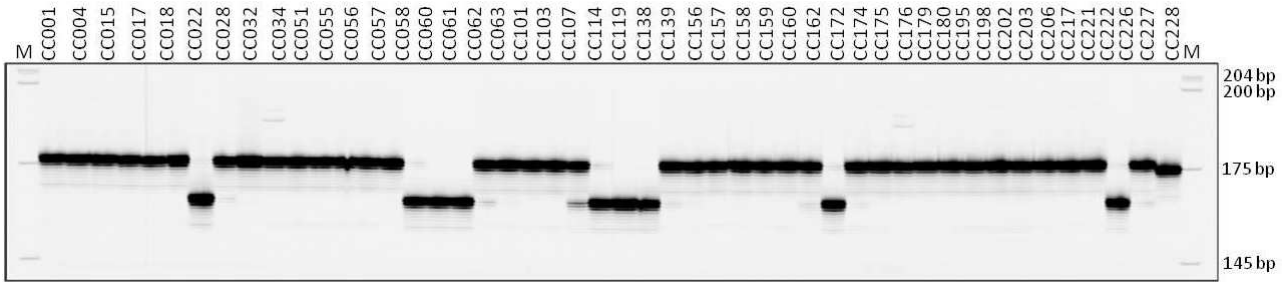

Fig. 1. Banding pattern of amplification product of CaCpM_26 microsatellite markers showing allelic variation among 48 *Capsicum* accessions. Base pairs (bp), marker (M)

Table 3. Characteristic features of seven CpSSR markers defining polymorphic information content (PIC), and number of alleles and hterozygocity observed with genotyping of a set of 48 capsicum genotypes

Marker	Major allele frequency	Alleles	Hetero-zygosity	PIC
CaCpM-06	0.8229	5.0000	0.0208	0.3031
CaCpM-16	0.7708	4.0000	0.0000	0.3402
CaCpM-21	0.7500	3.0000	0.0000	0.3249
CaCpM-22	0.6875	4.0000	0.0000	0.4362
CaCpM-25	0.8021	6.0000	0.0208	0.3315
CaCpM-26	0.8125	2.0000	0.0000	0.2583
CaCpM-27	0.9375	3.0000	0.0000	0.1151

Genetic relationship of Bhut Jolokia with other cultivars of capsicum

Genetic relationship of BJ accessions along with taxonomically known capsicum species were analysed using CpSSR markers. NJ cluster analysed revealed unambiguous relationship between BJ genotypes and known species. Most BJ genotypes were grouped in one cluster along with *C. frutescen* (Fig. 2). The cluster clearly separated the major taxonomic species i.e. *C. annuum*, *C. frutescens* and *C. chinense*.

Discussion

The markers based on the genomic DNA are widely used for genetic analysis in crop plants. Recently the markers based on the organaller genome are adopted for such studies. Among the available markers microsatellites received more importance because of their innate advantages over other markers. In the present study, we used the chloroplast genome based SSRs (CpSSR) to ascertain the genetic relatedness of the Buth jolokia, a popular landrace from north east India.

Resent advances in sequencing technology has made it possible to perform whole genome sequencing, transcriptome sequencing, and organelles genome sequencing at affordable cost. Due to such technological advances, genomic resources have been increased many folds (Bhat et al. 2018; Shivaraj et al. 2019). As demonstrated in the present study and many previous reports, genomic resources can be used effectively for the marker development using simple bioinformatics tools along with many other obvious applications like gene family characterization,

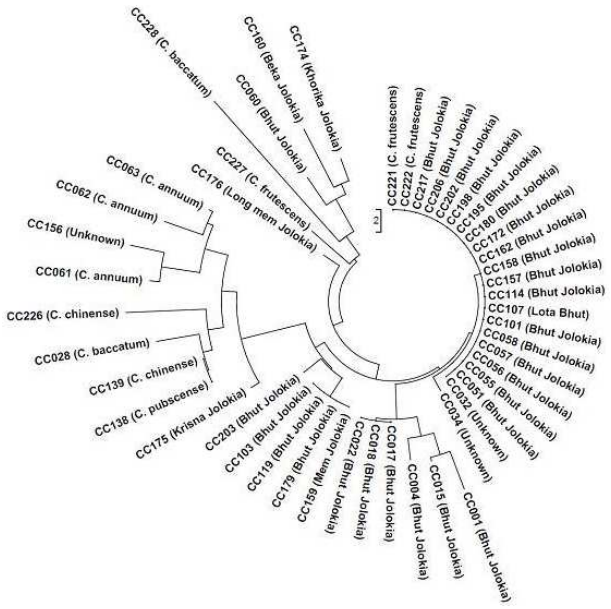

Fig. 2. Dendrogram depicting genetic diversity among 48 capsicum genotypes characterized by using CpSSRs. The landrace Bhut jolokia was found to cluster along with the *C. frutescence*

genome-wide studies describing different structural and functional features (Mir et al. 2020; Shivaraj et al. 2014; Shivaraj et al. 2017; Tyagi et al. 2018).

In this study a total of 27 CpSSRs have been identified from *C. annuum* which is in the range of CpSSRs previously reported in plant species like wheat (25 CpSSRs) (Tomar et al. 2014) and sorghum (31 CpSSR) (Li et al. 2010). Among the SSRs identified in *C. annum*, seven SSRs were tested on DNA isolated from 48 capsicum accessions. For all the primers tested, 100% amplification was observed in all the genotypes indicating that these markers can be successfully used for analysing the genetic polymorphism and various organeller studies in capsicum germplasm. Similarly, CpSSRs are successfully used in *Capsicum* (Cheng et al. 2016) and other crops such as citrus (Cheng et al. 2003), sorghum (Li et al. 2010), and wheat (Tomar et al. 2014). All the seven markers used showed polymoprphism among the accessions used. The high level of the polymorphism observed in this study might be due to use of LI-CORE for detection, which is having high resolution power compared to the agarose gel. Several studies reported very low level of polymorphism i.e 50% (Yumnam et al. 2012) and 26% (Hanáèek et al. 2010) for the genomic SSRs used to analyse *Capsicum*. The PIC value found to be 0.33 which is lower than the value of 0.46, described by (Minamiyama et al. 2006) and 0.76 reported previously by (Lee et al. 2004) in chilly using SSR marker. The average alleles per locus observed was 3.8, higher than 3.5 described by (Hanáèek et al. 2010). Compared to the nuclear genome SSRs, CpSSRs showed low PIC value although few cpSSR detected large number of alleles. In certain cases the markers showing high polymorphism are not always preferred under such conditions CpSSRs can be effective (Hedrick 1999).

Several studies have used genic SSR markers for the mutant screening. Such studies mostly observed high level of polymorphism in mutant lines generated through EMS mutagenesis. For instance, Manzila et al. (2020) reported an average 5.2 alleles per genic SSR marker used to screen a mutant population of *C. annuum.* In such cases it is always difficult to verify the parentage (source of variation) or any possibility of seed mixing or out-crossing (Bansal et al. 2019; Chaudhary et al. 2019). However, no report published up to now showing such polymorphism for CpSSR in mutants. Use of CpSSRs or mitochondrial SSR for the genetic purity testing and also for mutation breeding looks promising .

It has been observed that in chilly it is difficult to differentiate even the individual species of *C. frutescens*, *C. chinense* and *C. annuum* based on morphological characteristics (Pickersgill et al. 1979). Hence under such circumstances the molecular markers will help ascertain the genetic relatedness among different species. Three CpSSRs (CaCpM-16, CaCpM-22, and CaCpM-2) used in this study showed amplification specifically from three different *Capsicum* species, hence these markers can be further validated on large collection of accessions and can be used as diagnostic markers. The phylogenetic analysis of the *Capsicum* accessions showed that the Bhut jolokia land race is clustered along with the *C. frutescens* indicating the it's probable parentage. The seeds used for growing the Bhut Jalokia are collected from different areas of the north east region and subsequently selfed for several generations to make them genetically pure. However possibility of outcrossing before procurement of the seed cannot be ruled out.

In conclusion, the chloroplast genome based SSR markers identified in chilly can be used for the marker-assisted genomic studies. The set of well-characterized markers showing species specific amplification will serve as a good tool to identify the accessions from those species. The further validation of all the markers is required to analyse the chilly

accessions in a more robust way.

Authors contribution

Conceptualization of research (AI, SMS,HS); Designing of the experiments (HS, SBT, RD); Contribution of experimental materials (AI,SBT); Execution of field/lab experiments and data collection (AI); Analysis of data and interpretation (AI,SMS,VK, DP,RD); Preparation of the manuscript (AI, SMS, SBT).

Declaration

The authors declare no conflict of interest.

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