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Original Research article

Genetic Diversity Assessment of Chickpea Cultivars Using Dof (DNA binding with One Finger) Domain- and Gene- Specific Primers

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Abstract: Repertoires of molecular markers are being used for genetic diversity assessment among cultivars of different crops. Functional markers represent an important group of DNA markers derived from functionally characterized sequences like transcription factors. Dof (DNA- binding with one finger) is a plant specific transcription factor associated with multifarious activity involved in growth and development. Variability exists in the number of *Dof* genes and Dof domain in different crops. In the present study investigated the genetic diversity among twelve Indian cultivars of chickpea (*Cicer arietinum* L.) based on PCR amplification pattern obtained by Dof domain and gene- specific primers designed from *in silico* predicted 37 *CaDof* genes. The number of amplicons obtained with different sets of primers varied among these cultivars. The size of the amplicons ranged from 0.2 to 1.9 Kb. A total of 690 bands with 390 polymorphic and 300 monomorphic bands were recorded. The degree of polymorphism ranged from 7 to 100 percent with 27 out of 31 primers while four primers namely CaDof2, CaDof16, CaDof17 and CaDof26 showed no polymorphism. The phylogenetic tree constructed using UPGMA method revealed two major clusters comprising of four and eight cultivars, each of which was further bifurcated into sub-clusters. The genetic diversity studies attempted with selected cultivars of chickpea reveals the potential of using Dof as functional markers and could be substantially expanded by increasing the number of cultivars of chickpea along with cultivars of other legumes.

Key words: Chickpea, Dof transcription factor, functional marker, genetic diversity, polymorphism, phylogenetic tree

Introduction

Chickpea (*Cicer arietinum* L.) is an important protein rich grain legume cultivated in arid and semiarid regions of the world (Jukanti *et al*., 2012). The whole genome sequencing of chickpea (Varshney *et al*., 2013; Jain *et al*., 2013; Gupta *et al*., 2017) was an important landmark for developing appropriate strategies to improve the productivity by overcoming several constraints associated with its narrow genetic base and loss incurred by various biotic and abiotic stresses (Choudhary *et al*., 2012; Sabbavarapu *et al*., 2013; Varshney *et al*., 2014; Gaur *et al*., 2019; Roorkiwal *et al*., 2020). A variety of DNA molecular markers have been reported for assisting plant breeders to develop varieties with desired agronomic traits (Nadeem *et al*., 2018). The recent advancement in plant genomics led to the development of gene targeted and functional markers (Andersen and Lubberstedt, 2003; Poczai *et al*., 2013; Kage *et al*.,

2016). In chickpea, the development of genic (or functional) molecular markers has been reported (Gujaria *et al*., 2011; Parida *et al*., 2015).

Dof (DNA- binding with one finger) is one of the most widely studied plant specific transcription factors with highly conserved Dof domain of 50-52 amino acids including C2C2-Zn finger type motif (Yanagisawa, 2002). The Dof TFs are ubiquitously present in plants from unicellular algae to angiosperms (Moreno-Risueno *et al*., 2007). Dof factor is associated with multifarious activities in plants related to growth, development and stress tolerance (Gupta *et al*., 2015). The mining of chickpea genome revealed 37 *Dof* genes, which were extensively characterized using bioinformatics tools (Nasim *et al*., 2016). The genetic diversity among different cereals and millets were analyzed using Dof domain and gene- specific primers (Kushwaha *et al*., 2015) Similarly, the genetic diversity among barley species using 75 set of Dof domain and gene- specific primers has also been reported (Rouhian *et al*., 2017). The present study aims to reveal the potential of using *Dof* genes and domain specific primers of chickpea as functional makers for assessing genetic diversity among selected Indian cultivars of chickpea.

Materials and methods

Plant material

A total of 12 cultivars of chickpea namely Katila, DG96006, BG256, Vijay, JAKI9218, Pusa 362, DCP92-3, JG16, K850, CSG8962, Avrodhi and KWR108 were procured from ICAR-Indian Institute of Pulse Research (IIPR) Kanpur, Uttar Pradesh, India.

Genomic DNA isolation

Genomic DNA isolated from leaves of these twelve cultivars of chickpea using CTAB method (Murray and Thompson, 1980) was quantified by spectrophotometer (Dynamica, Hong Kong) and analyzed by gel electrophoresis.

PCR amplification

A total of 31 *Dof* genes and one Dof domain specific primers listed in Table 1 were designed by Primer3 from *in-silico*

Table 1. List of primers designed from the *in-silico* predicted *Dof* gene families of chickpea.

Sr. No.	Primer name	Specification	Sequence (5'-3')
1	CaDof Domain	F	AGATTCTGCCTTGTCCTCGTT
		R	GAGCTCTTACTCTTGCGCCTA
2	CaDof1	F	TTGTGGGGCTGTTTTTAGT
		R	GCAACTCCAAGAATCATCA
3	CaDof2	F	TCAGCATGGAAGGGATAAAT
		R	GGACGGTTCAGATTTTACACG
4	CaDof3	F	CCTTGTTACTTGGCTCTTTG
		R	CCATGATAGCCAACTGACA
5	CaDof4	F	ATCTCATCCCCCTGTTATCTTT
		R	GGAATTTGATCTCGGACACATA
6	CaDof6	F	CTCCACAACAAAACCTAACA
		R	GACCGGTCAACAAAAGTAGT
7	CaDof7	F	CTTCTTTGCTCACTCTCTCAA
		R	CACCCTCCATTGAAACAA
8	CaDof8	F	CCTCAACAACTTTCCCTCTC
		R	AGGTTGAGGTGAGGCTTAAT
9	CaDof9	F	TGCCTGTTTTTCTTTCTCTG
		R	CATGCACCAACCCTTTTT
10	CaDof11	F	GCAATGTTAGAGAGAAAAGC
		R	GACATTGATTCCAGTGAAAC
11	CaDof12	F	ACGCTCTCACAAACAAATCT
		R	AATCCTGCTATCAACACTGG
12	CaDof13	F	CAACCAAAACGAAAACCTTC
		R	GAAGTTTGTCCTAGCTCATGTT
13	CaDof14	F	GCAGCAAATGTCTAGCAA
		R	CCACAATAGTATCCAACCACAGT
14	CaDof15	F	ACAATCACCCTTCTTCTCTTC
		R	CTCAATTTTACCCTCCAAAG
15	CaDof16	F	CAAAACCCCACATACCTTAT
		R	CTTTCTGTTCTCCTCATTCA
16	CaDof17	F	TCTCCACTATTCTCTCCCTAAA
		R	ATCCCTAGGGTTTTCATTCT
17	CaDof19	F	GCAGCAAATGTCTAACGAA
		R	GGTTGTGCTACTGTTGTGC
18	CaDof20	F	GAGAGGGATAAAGGGTTAAA
		R	GGAGCTGTAGAAACCAATTC
19	CaDof21	F	CCCTTTCTATCCTTGTTTTG
		R	CTTCACCAATCATTCCACTC
20	CaDof22	F	CCTAAAGAGCCATCAAAACT
		R	CCGTATGAAGATTGTGGAAC
21	CaDof25	F	TTCTCCACCATCATCAAACT
		R	CTGTTCTGTTTCGTTTCTGTC
22	CaDof26	F	TGACAGAAAGATGCAAGACC
		R	CCCTTCTACTGATAACTTGAGC
23	CaDof27	F	CAACAACTTCCACATTTACG

		R	GCAGAACACAATTGTACAGG
24	CaDof28	F	GCAAGATGACGGAGTTCA
		R	TCCAACAACAAAGGCTACA
25	CaDof29	F	CCACACTTTCCACTATTTCTCA
		R	CAGGCTCTCTTTCACTTTGTT
26	CaDof30	F	TTATAAGGCCAGGATCAATG
		R	CACAACGTAATCGCAACTG
27	CaDof31	F	TCTCCAACCCATCAAACTT
		R	CTAATCTTGAACCTCCCTCA
28	CaDof33	F	TATGATTGAAAGTAAGGACCCTGC
		R	CCAACCTCAATTCTCAAACAC
29	CaDof34	F	ACCGAACAGAACAGAACAAT
		R	AGCCGTTACATTATCCAGTG
30	CaDof35	F	CCTCTTCTCCTTCTTATCACAG
		R	TGTCATTGGTGTTACTCGTT
31	CaDof36	F	ACCAACTGCAAGAAACTCAT
		R	AACCCAATTGACTCCAAAG
32	CaDof37	F	TACAATGCCTTCCTCTGACT
		R	AGCACCCTCCATTGAAAC

predicted 37 *Dof* genes of chickpea (Nasim *et al.*, 2016). The PCR amplifications were performed in 25 µl reaction volume containing ~100 ng of template DNA, 1 µM of both forward and reverse primer and 1X PCR *Taq* Mixture (HiMedia). The PCR reactions was performed using Genei thermal cycler (Bangaluru, India). The PCR conditions were an initial denaturation at 95°C of 5 min followed by 35 cycles of 1 min denaturation at 94°C, variable annealing temperature for 1 min and 2 min polymerization at 72°C and a final extension of 5 min at 72°C. Annealing temperatures were re-adjusted for each primer according to their Tm. The amplified products were analyzed on 1.0% (w/v) agarose gels stained with ethidium bromide. The size of the amplified DNA fragments was estimated with 1 Kb plus DNA Ladder (Thermo Scientific). All gels were documented using Alpha Imager 1200™ (Alpha Innotech, USA). All amplifications were performed twice for each sample and only major amplicons consistently amplified were scored for data analysis.

Data analysis

The amplicons obtained by different primers for each cultivar were scored and their presence and absence were marked as present (1) or absent (0) to finalize the binary data matrix. These data were further analyzed using the NTSYS-pc version 2.11w software to calculate the similarity values and to generate the phenogram (Rohlf 2001). Pairwise similarity matrices were obtained by Jaccard's similarity coefficient using SIMQUAL format of NTSys software (Jaccard 1908). Similarity matrices were utilized to construct the dendrogram based on unweighted pair-group method with arithmetic average (UPGMA) clustering method to reveal the genetic relationship among the cultivars of chickpea. The data recorded were

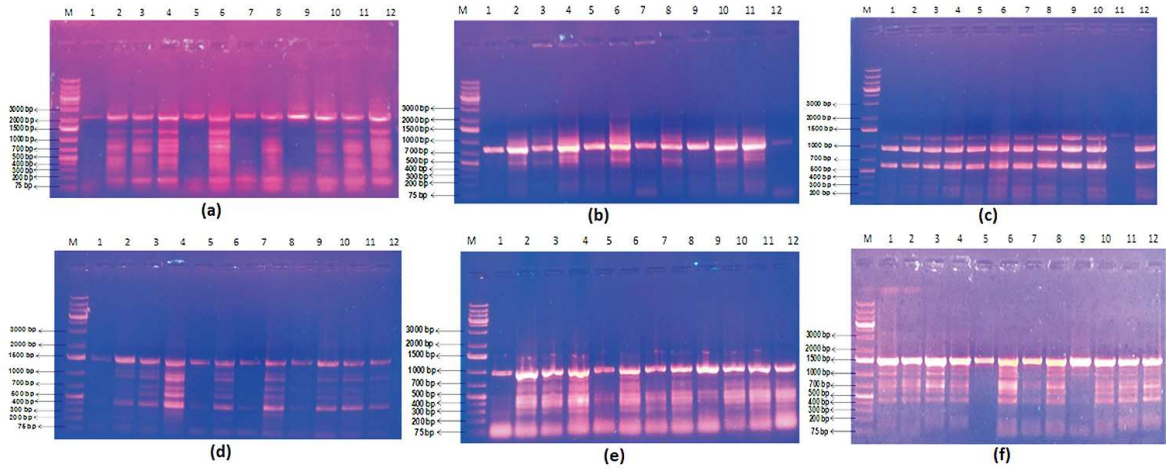

Fig. 1. Agarose gel (1%) showing PCR amplification profile of chickpea cultivars using (a)CaDof 1, (b)CaDof7, (c) CaDof8, (d) CaDof9, (e) CaDof14 and (f)CaDof 28 primers. Lane 1-12 cultivars Katila, DG96006, BG256, Vijay, JAK19218, Pusa362, DCP92-3, JG16, K850, CSG8962, Avrodhiand KWR108, respectively.Lane M-1Kb plus DNA Ladder marker (Fermentas).Numbers of monomorphic and polymorphic amplicons scored based on size of bands with different primers are mentioned in Table-2.

Table. 2. DNA banding profile obtained by PCR amplification of chickpea cultivars with Dof domain and gene specific primers.

Sl. No.	Primer code	Total number of amplicons	Size range of amplicons (Kb)	Number of Monomorphic amplicons	Number of Polymorphic amplicons	Degree of polymorphism (%)
1	CaDof1	54	0.2-2.2	12	42	77.77
2	CaDof2	12	0.9-0.9	12	00	00
3	CaDof6	25	0.45-1.1	12	13	52
4	CaDof7	19	0.5-.95	0	19	100
5	CaDof8	34	0.6-1.4	0	34	100
6	CaDof9	42	0.4-1.5	12	30	71.42
7	CaDof11	33	0.2-1.3	12	21	63.63
8	CaDof12	33	0.5-1.9	12	21	63.63
9	CaDof13	21	0.4-.85	12	09	42.85
10	CaDof14	44	0.35-1.0	12	32	72.72
11	CaDof16	12	1.3-1.3	12	00	00
12	CaDof17	12	1.5-1.5	12	00	00
13	CaDof19	20	0.35-1.4	12	08	40
14	CaDof20	26	0.3-1.3	24	02	7.69
15	CaDof21	17	0.35-1.0	12	05	29.41
16	CaDof25	46	0.3-1.4	12	34	73.91
17	CaDof26	12	0.7-0.7	12	00	00
18	CaDof28	52	0.45-1.5	12	40	76.92
19	CaDof29	48	0.3-1.1	12	36	75
20	CaDof30	14	0.55-1.1	12	02	14.28
21	CaDof31	33	0.40-1.1	12	21	63.63
22	CaDof35	26	0.4-1.0	12	14	53.84
23	CaDof36	28	0.25-1.6	24	04	14.28
24	CaDof37	27	0.55-1.1	24	03	11.11
	Total	690		300	390	

bootstrapped with 1000 replications along with Jaccard's coefficient by the computer programme WINBOOT (Yap and Nelson, 1996) while generating the dendrogram.

Results

PCR amplifications with one Dof domain and 31 *Dof* gene-specific primers (Table 1) were performed using the genomic DNA isolated from different cultivars of chickpea. The PCR amplification products were analyzed on agarose gel as shown in Fig. 1 and the variability observed were scored for further analysis. The details related with the number of bands obtained with each primer, size of the amplicons, number of monomorphic and polymorphic amplicons and degree of polymorphism is shown in Table 2.

The considerable variations in the banding pattern among selected cultivars of chickpea as visualized on agarose gels were coded by 1 or 0 based on their presence or absence respectively. Only 24 out of 32 sets of primers were used for the generation of binary matrix data. This was subjected to NTSYS software revealing phylogenetic relationship with Jaccard's similarity coefficient values ranging from 0.4556 to 0.8840 as shown in Fig.2.

The similarity matrices calculated were further utilized to construct the dendrogram using UPGMA method. It

	Katila	DG96006	BG256	Vijay	JAK19218	Pusa362	DCP92-3	JG16	K850	CSG8962	Avrodhi	KWR108
Katila	1.0000000											
DG96006	0.5588235	1.0000000										
BG256	0.6086957	0.8194444	1.0000000									
Vijay	0.5584416	0.7500000	0.8354430	1.0000000								
JAK19218	0.6200000	0.5781250	0.5362319	0.4556962	1.0000000							
Pusa362	0.5652174	0.8028169	0.8472222	0.8205128	0.5147059	1.0000000						
DCP92-3	0.6800000	0.6507937	0.6029412	0.5324675	0.7954545	0.6307692	1.0000000					
JG16	0.5757576	0.7464789	0.7671233	0.7250000	0.5714286	0.7746479	0.6721311	1.0000000				
K850	0.6938776	0.6349206	0.5652174	0.5194805	0.8139535	0.5909091	0.8000000	0.6031746	1.0000000			
CSG8962	0.6515152	0.7432432	0.8356164	0.8101266	0.5522388	0.7466667	0.6212121	0.7887324	0.6060606	1.0000000		
Avrodhi	0.5970149	0.6710526	0.8082192	0.7625000	0.5000000	0.7200000	0.5671642	0.7361111	0.5522388	0.8840580	1.0000000	
KWR108	0.6461538	0.6710526	0.7368421	0.7407407	0.5223881	0.7200000	0.6153846	0.7123288	0.5757576	0.8571429	0.7777778	1.0000000

Fig. 2. Proximity Matrix (Jaccard's similarity coefficient) for 12 cultivars of chickpea. Avrodhi and CSG8962 showed highest similarity index of 0.8840, while JAK19218 and Vijay showed lowest similarity index of 0.4556.

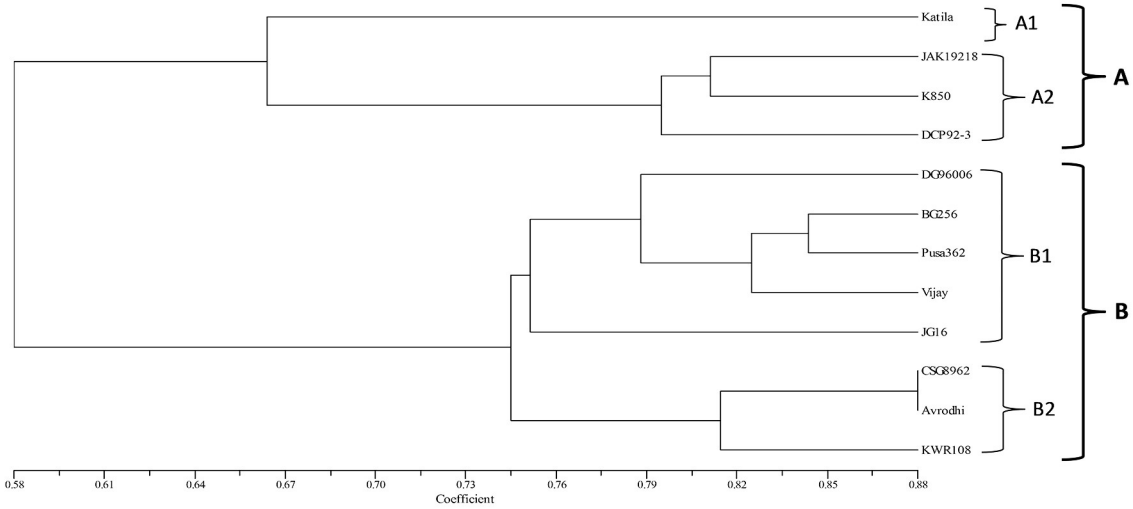

Fig. 3. Dendrogram showing relationship between different cultivars of chickpea based on DNA banding pattern obtained using different sets of *Dof*gene-specific primers. Bootstrap test with 1,000 cycles was conducted to examine the confidence of obtained tree.It revealed two major clusters designated as A and B. The major clusters A and B were further bifurcated into two sub-clusters A1-A2 and B1-B2 each.

revealed two major clusters designated as A and B. The major clusters A and B were further bifurcated into two sub-clusters A1-A2 and B1-B2 each as shown in Fig.3.

Discussion

Recent advances in functional genomics and the availability of genome sequences has led to the development of molecular markers from genes or coding regions popularly referred as genic or functional markers. These markers are better suited for marker assisted selection (MAS) and functional genetic diversity studies as compared to genomic DNA markers (Gujaria *et al.*, 2011). A repertoire of transcription factors has been revealed from the sequenced genome of chickpea. Genome-wide identification and characterization of some of the important transcription factor families studied in chickpea includes WRKY (Waqas *et al.*, 2019), NF-Y (Chu *et al.*, 2018), CCCH Zinc finger (Pradhan *et al.*, 2017), AP2/ERF (Agarwal *et al.*, 2016), Dof (Nasim *et al.*, 2016), bZIP (Wang *et al.*, 2015), MYB (Ramalingam *et al.*, 2015), and NAC (Ha *et al.*, 2014).

The variability both in terms of number of amplicons and their sizes was observed with different cultivars using Dof domain and gene-specific primers designed from predicted *CaDof* genes. A total of 690 bands comprising of 390

polymorphic and 300 monomorphic were scored. The percentage degree of polymorphism ranged from 7-100% as polymorphism could not be detected with four primers *viz*., CaDof 2, CaDof16, CaDof17 and CaDof26 primers (Table-2). The size of DNA bands ranged from 0.2 to 1.9 Kb. A very high degree of polymorphism i.e. more than 70% was obtained with primers CaDof1, CaDof7, CaDof8, CaDof9, CaDof14, CaDof25, CaDof28, and CaDof29. The genetic relatedness among different species of barley germplasm investigated using 75 sets of Dof domain and gene-specific primers has revealed high percentage of polymorphism (Rouhian *et al*., 2017). Similarly, the PCR amplification pattern as revealed by different sets of Dof domain and gene-specific primers among different cereals and millets also showed varied degree of polymorphism (Kushwaha *et al*., 2015). The highest degree of polymorphism (i.e., 100%) was observed with primers CaDof7 and CaDof8. The highest number of bands i.e., 54 was found with primer CaDof1 followed by 52 bands for CaDof28 while primer CaDof7 and CaDof8 revealed no monomorphic bands.

The phylogenetic tree constructed grouped the cultivars into two major clusters. The major cluster A included four cultivars Katila, JAK19218, K850 and DCP92-3, while cluster B has the remaining eight cultivars namely DG96006, BG256, Pusa362, Vijay, JG16, CSG8962, Avrodhi and KWR108. The sub-cluster A1 represents only one cultivar Katila while A2 sub-cluster comprised of three cultivars namely JAK19218, K850 and DCP92-3. Similarly, cultivars DG96006, BG256, Pusa362, Vijay, JG16 formed sub-cluster B1 and CSG8962, Avrodhi, KWR108 cultivars represented sub-cluster B2 (Fig.3).

Transcription factors derived microsatellite markers have been developed in chickpea and *Medicago* has immense potential for markers assisted genetic improvement (Kujur *et al*., 2013; Kujur *et al*., 2014). Mining of chickpea genome for microsatellite markers resulted in the development of databases as CicArMiSatDB exclusively for chickpea (Doddamani *et al*. 2014). In the present study, PCR amplification using the primers based on Dof gene family of chickpea were carried out to decipher the genetic diversity among selected cultivars. This could be developed as potential functional markers after extensive validation by enhancing the number of cultivars of chickpea along with cultivars of other legumes.

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Conflict of Interest: The authors declare no conûict of interests regarding the present work.

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