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Genetic diversity assessment of *Fusarium oxysporum* f. sp *ciceris* isolates of Indian chickpea fields as revealed by the SRAP marker system

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Abstract

An experiment was conducted to study the precise geographical distribution and racial complexity of *Fusarium oxysporum f.sp ciceris* (*Foc*) isolates representing 12 states of 4 agro-climatic zones of India at morphological, pathogenic and molecular level. The DNA based sequence related amplified polymorphism (SRAP) markers was employed to differentiate Foc isolates at genome level. The genotypic data output of the isolates was examined for diversity parameter as marker's Polymorphic percentage (PM %), Polymorphic Information Content (PIC), Marker Index (MI) and Gene Diversity Index (DI). As a result, 15 primers used in this study could generated total of 154 reproducible alleles ranging from 100-2100 bp (average allele per marker 10.26) in size, of that 149 (97%) were found to be polymorphic. The neighbor-joining analysis effectively classified the isolates of North East Plain Zone (NEPZ), Central Zone (CZ), North West Plain Zone (NWPZ) and South Zone (SZ) into four clusters. In summary, DNA based marker analysis could differentiate as per isolates geographical location, however pathogenic interaction of isolates from same geographical location could not match the genetic differentiation. Accordingly, considering the present complexity in racial profile, precise classification based on homologs virulence genes specific to races would give a more meaningful in correlating isolates with their native geographical distribution and helps in future resistance breeding programs for sustainable management of vascular wilt disease.

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Introduction

Fusarium oxysporum f.sp. *ciceris* (Padwick) Matuo and K. Sato (*Foc*), causal organism of vascular wilt in chickpea, is a major reason for its low productivity worldwide. As per reports, the average annual yield loss in chickpea at the national level, attributable to fresh weight ranged from 10 to 100% depending on infection stage, type of variety and environmental conditions, however it may leads to complete loss especially when disease comes in vegetative and or reproductive stages (Soregaon and Ravikumar, 2012). The *Foc* races prevail over the different legumes cultivating region of the country. Globally, eight races have been reported (0, 1A, 1B/C, 2, 3, 4, 5 and 6) in chickpea (Gasco and Diaz 2003) having distinct geographic distribution. In India predominantly, race 1, 2, 3, and 4 have been reported, whereas, race 0, 1A/C, 5 and 6 have been reported from the Mediterranean region and United States of America (USA). However, remaining three races have also been reported from India recently with the same chickpea differential set used (Dubey *et al.,* 2012). Due to this widespread nature of racial distribution, chickpea breeders are unable to select suitable

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donor lines use for transferring resistant gene in the breeding programme to develop durable resistant crops. In this context it becomes essential to identify specific race specific DNA based diagnostic marker and its population distribution across different geographical region.

Molecular markers are an important tool for evaluating genetic variability, diagnostic marker development and population distributions study in fungal species. Invariably, different marker systems *viz*. as RAPD (Kumar *et al.,* 2014); ITS and EF-1 alpha (Soren *et al.,* 2015); RFLP (Sharma *et al.,* 2009), AFLP (Sivaramakrishnan *et al.,* 2002), DArT (Sharma *et al.,* 2014), ISSR and SSR (Dubey and Singh, 2008), has been reported in genetic variability study in *Fusarium* sp.

Sequence-related amplified polymorphism (SRAP) is a PCR-based dominant marker system (Li and Quiros, 2001), which simplifies the AFLP detection procedure and also to increase throughput for multiple loci and improve reproducibility. In principle, this marker system work based on the difference in GC content between gene encoding region and neighbouring spanning sequences in the genome. Accordingly, two sets of primer pairs with 16-22 long nucleotide: forward primer (containing GGCC box near to 3' end preferentially annealing to the GC-rich regions) and reverse primer (containing AATT preferentially bind to the introns and intergenic spacer) are designed and employed (Li *et al.,* 2013). The PCR conditions were used as: 94ºC for 1 min, 35ºC for 1 min and 72ºC for 1 min for the first 5 cycles and followed by 30 cycles at the raised annealing temperature of 50ºC. Since, PCR starts at 35ºC annealing temperature allows the primers to bind at multiple loci in the target DNA and as a consequence, binding targets of the primers at 50ºC for significantly improves the efficiency and reproducibility of the alleles.

Recently, SRAP marker technology has been extensively used in different fungal species like Ganoderma *lucidum* (Sun *et al.,* 2006), Chinese *Auricularia auricula* (Tang *et al.,* 2010) and *Lentinula edodes* (Fu *et al.,* 2010) to successfully dissect the diversity and phylogeny analysis. In addition to the fungi, the technique was successfully employed in many crops, such as corn, cotton and some vegetables, including mustard, potato, hot pepper, cucurbits, pea, eggplant and safflower for diversity and phylogeny analysis. With this background, a comprehensive study was carried out for genetic diversity and race profiling of Indian isolates to establish correlation between geographic distribution and racial profiling, so as to breed crops for specific race for durable resistance.

Materials and Methods

A total of 34 *Foc* isolates representing 4 agro-ecological zones, namely Central zone (CZ), South zone (SZ), North-Eastern Plains zone (NEPZ), North-West Plains zone (NWPZ) signifying 12 states of India were used in the study (Table 1). The pathogenic fungi were primarily isolated from the infected tissues of target crops (size of 5-8 mm) and subsequently tissue were surface sterilized in 1% sodium hypochlorite solution and incubated on 2% potato dextrose agar (PDA) plates for 7 days at 25±1°C for inducing sporulation. The pure cultures of isolate were obtained using spore dilution plate method for morpho-genomic and pathogenicity test (Singh and Singh, 1970).

Mycelial growth estimation and pathogenicity test : To study the cultural and morphological variation of isolates, active cultures of isolates were inoculated in fresh PDA plates for 6-7 days at 25±1°C to observe mycelial growth, color, type of septum, pathogenicity and conidial size.

The pathogenicity of isolates was studied using a soil inoculation method (Pande *et al*., 2007) in different set of chickpea genotypes including genotype "JG-62" as susceptible check. To carry out the pilot experiments, a mixture of sand-chickpea (8:2 w/w) medium were used to inoculate and incubated for 15 days at 25±1°C. The multiplied inoculums with appropriate load (1 x 10^7 conidia/ml) were mixed with sterilized garden soil at 5% (w/w) concentration and sterilized seeds of differential chickpea genotypes (*Viz;* JG62, C104, JG74, JG315, BG212, JG11, IPC04-52, Chaffa, DCP92-3 and Vijay) were placed to observe the host- pathogen reaction. Thus, the pathogenicity and racial identity of each isolate could be recorded based on the symptoms of vascular wilt.

Extraction and quantification of genomic DNA : DNA from 12-15 days old mycelia was extracted as per Lee and Taylor (1990) with minor modifications. DNA was dissolved in $T_{10}E_1$ buffer and stored at -80ºC. The quality and quantity of DNA were estimated by spectrophotometer (Eppendorf). Finally, DNA was diluted to working concentration of 30 ng μl^{-1} for PCR analysis.

PCR based Identification of *Foc* : The authenticity of isolates was confirmed through PCR assay using forma specialis *Fusarium sp. ciceris* specific primer (*Foc*0-12f/r) of Gasco and Diaz (2003). The PCR reaction was performed in total volume of 10µl following the PCR set: initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 1 min, annealing at 58°C for 1 min, elongation at 72°C for 1 min, with a final elongation at 72°C for 10 min. Amplified products were resolved in 2% agarose gel in 0.5X TBE buffer.

SRAP primers and data analysis : Initially, all possible primer combinations were screened in a set of samples, and accordingly 15 primers combination (Table 2 and 3) were found to produce scorable and reproducible bands. These primer pairs were further screened on defined isolates with applied PCR conditions; initial denaturation at 94°C for 5

Table 1 : Name of *Fusarium oxysporum* f.sp *ciceris* with its place of origin, morphological characteristics and pathogenicity test

Isolates	States	Pulses growing agro ecological zone	Pathogenicity test			
			Pathogenicity	Conidia size	Mycelial growth rate	Pathological reaction
KFoc 29	Uttar Pradesh	NEPZ	Highly	Medium	Fast	Race-0
KFoc 33	Uttar Pradesh	NEPZ	Weakly	Small	Medium	Race-2
KFoc 40	Uttar Pradesh	NEPZ	Highly	Large	Fast	Race-5
KFoc 32	Uttar Pradesh	NEPZ	Highly	Small	Slow	Race-3
KFoc 42	Uttar Pradesh	NEPZ	Moderately	Medium	Fast	Race-1
KFoc 81	Uttar Pradesh	NEPZ	Highly	Large	Slow	Race-0
JFoc 58	Chattisgarh	CZ	Highly	Large	Fast	Race-1
JFoc 71	Madhya Pradesh	CZ	Weakly	Small	Fast	Race-1
JFoc 37	Madhya Pradesh	CZ	Weakly	Large	Slow	Race-3
JFoc 20	Madhya Pradesh	CZ	Weakly	Medium	Medium	Race-4
JFoc 22	Madhya Pradesh	CZ	Weakly	Medium	Slow	Race-3
JFoc 21	Madhya Pradesh	CZ	Weakly	Small	Medium	Race-0
KFoc 63	Andhra Pradesh	SZ	Moderately	Small	Medium	Race-4
JFoc 35	Madhya Pradesh	CZ	Moderately	Large	Fast	Race-3
JFoc 32	Madhya Pradesh	CZ	Moderately	Small	Medium	Race-1
JFoc 53	Madhya Pradesh	CZ	Moderately	Large	Slow	Race-0
KFoc 72	Karnataka	SZ	Highly	Large	Medium	Race-1
JFoc 8	Madhya Pradesh	CZ	Weakly	Large	Slow	Race-1
JFoc 5	Madhya Pradesh	CZ	Weakly	Medium	Fast	Race-1
KFoc 65	Andhra Pradesh	SZ	Highly	Medium	Medium	Race-6
JFoc 77	Chattisgarh	CZ	Highly	Medium	Slow	Race-0
NDFoc 72	Rajasthan	NWPZ	Weakly	Large	Slow	Race-1
NDFoc 83	Rajasthan	NWPZ	Weakly	Small	Fast	Race-1
NDFoc 50	Rajasthan	NWPZ	Moderately	Large	Fast	Race-4
NDFoc 7	Rajasthan	NWPZ	Moderately	Medium	Fast	Race-0
NDFoc 36	Rajasthan	NWPZ	Moderately	Small	Medium	Race-4
NDFoc 6	Rajasthan	NWPZ	Highly	Large	Slow	Race-2
JFoc 60	Gujarat	CZ	Highly	Small	Slow	Race-0
JFoc 88	Maharashtra	CZ	Moderately	Medium	Medium	Race-4
NDFoc 66	Haryana	NWPZ	Highly	Small	Medium	Race-4
NDFoc 100	Jharkhand	NEPZ	Moderately	Large	Fast	Race-1
NDFoc 90	Punjab	NWPZ	Moderately	Small	Medium	Race-2
NDFoc 64	Haryana	NWPZ	Moderately	Small	Slow	Race-1
NDFoc 108	New Delhi	NWPZ	Weakly	Large	Fast	Race-1

NWPZ= North West Plain Zone; NEPZ= North East Plain Zone; CZ= Central Zone, SZ= South Zone; Weakly (0-30%), Moderate (31-50%), Highly (>51%); Small (12.5 - 30.0 x 2.5 - 4.5 mm), Medium (15 – 37.5 x 2.5 – 5 mm), Large (20 – 45x 2.5 – 5 mm); Slow (<70 mm), medium (70-79 mm), fast (>79 mm)

min followed by 5 cycles of 94°C for 1 min, annealing at 35°C for 1 min 20 second, elongation at 72°C for 1 min and 35 cycles of 94°C for 1 min, 50°C for 1 min 20 second, 72°C for 1 min with final elongation at 72°C for 10 min. Amplified products were resolved in 2% agarose gel in 0.5X TBE buffer.

To test phylogenetic correlation and molecular evolutionary analyses, genotypic data were analysed using DARwin5 software (URL: http://darwin.cirad.fr/Home.php) (Perrier and Jacquemoud-Collet 2006). The utility of each marker system was evaluated by markers discriminatory power *viz*; polymorphic information content (PIC), marker index (MI) and Nei's (1973) gene diversity index (DI) using Popgene software, version 1.31 (www.ualberta.ca/~fyeh/popgene.pdf). Effective multiplex ratio (EMR) of primer was calculated as the "product of, fraction of polymorphic bands and number of polymorphic bands for an individual marker system" as described by Milbourne *et al.* (1997). The product of two functions *i.e.*, DI and EMR was used to calculate MI, as described by Prevost and Wilkinson (1999).

Results and Discussion

The cultural and morphological variation is the key factor for conventional taxonomic classification of the fungus. However, grouping of isolates based on key indicator as colony pattern, mycelial growth rate is quite difficult.

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Table 2 : List of SRAP primer pairs showing amplification along with percentage of PM and MM bands

Primers combinations	Number of allele	Amplicon size (bp)	PM	PM%	PIC value	DI	MI
ME2/EM3	12	180-2100	11	92	0.87	0.38	3.84
ME3/EM7	15	120-1510	15	100	0.89	0.40	0.60
ME5/EM3	14	110-1800	14	100	0.88	0.35	0.49
ME5/EM7	10	120-1990	10	100	0.84	0.20	0.20
ME2/EM7	13	100-1570	12	92	0.88	0.32	3.53
ME1/EM6	8	120-1900	7	92	0.77	0.30	1.93
ME5/EM1	12	150-1510	12	100	0.86	0.22	0.26
ME2/EM2	9	110-1400	9	100	0.80	0.20	0.18
ME3/EM3	10	110-1000	9	92	0.84	0.27	2.24
ME1/EM4	9	300-2000	9	100	0.83	0.25	0.23
ME5/EM2	5	260-900	5	100	0.73	0.30	0.15
ME2/EM1	7	170-1100	6	92	0.77	0.29	1.60
ME3/EM6	7	110-1100	7	100	0.76	0.28	0.20
ME5/EM10	16	100-2010	16	100	0.87	0.26	0.41
ME5/EM6	7	110-1400	7	100	0.74	0.28	0.20
Average	10.26		9.93	97	0.82	0.29	1.07

PM: Polymorphic. PM%: Percentage of polymorphism. PIC: Polymorphic information content.DI: diversity index per polymorphic band. MI: marker index

Table 3 : Sequence of SRAP primers used for genetic diversity studies

Primer	Sequence of primer 5´———3´	Primer Reference	Primer	Sequence of primer 5´———3´	Primer Reference
ME 1	TGAGTCCAAACCGGATA	Li and Quiros, 2001	EM 1	GACTGCGTACGAATTAAT	Li and Quiros, 2001
ME 2	TGAGTCCAAACCGGAGC	Li and Quiros, 2001	EM 2	GACTGCGTACGAATTTGC	Li and Quiros, 2001
ME 3	TGAGTCCAAACCGGAAT	Li and Quiros, 2001	EM 3	GACTGCGTACGAATTGAC	Li and Quiros, 2001
ME 5	TGAGTCCAAACCGGAAG	Li and Quiros, 2001	EM 4	GACTGCGTACGAATTTGA	Li and Quiros, 2001
			EM 6	GACTGCGTACGAATTAAC	Li and Quiros, 2001
			EM 7	GACTGCGTACGAATTCAA	J.J. Lu *et al.*, 2012
			EM 10	GACTGCGTACGAATTGCA	J.J. Lu *et al.*, 2012

Therefore, allele based classification is an authentic and important option, which is primarily based on the genetic makeup of the isolates and has now become a choice of modern genetic classification. In the present study, the level of pathogenic variability of isolates in differential chickpea genotypes was set to measure the virulence in term of disease severity index (DSI). The disease symptoms appeared below the hypocotyl and above epicotyl region during the crop growth were rated for disease in susceptible chickpea variety "JG 62" to classify the isolates as highly pathogenic (>50%), moderate (>20-50%), and weakly pathogenic (0-20%) (Table.1). Accordingly, based on differential behavior of isolates in host-pathogen interaction study, 34 *Foc* isolates were classified into seven different races *viz*. 0, 1, 2, 3, 4, 5 and 6. Accordingly, seven isolates from NEPZ showed mixed type of racial picture (race-0, 1, 2, 3 & 5), while isolates from SZ showed race-1, 4 and 6. Similarly, there was an occurrence of race-0, 1, 3, 4 and 6 in four districts of CZ, whereas race-0, 1, 2 and 4 were present in 4 districts of NWPZ.

F.oxysporum f.sp. *ciceris* was subjected to PCR analysis with forma specialis *Foc* specific primer (Foc0-12f/r) and amplified common band of 1500 bp (Fig. 1) for ascertaining *Foc* studied *Fusarium* strains. 15 SRAP marker combinations were employed in 34 isolates to capture multiple loci to dissect the proper classifications. The SRAP fingerprinting of all the 15 primer gave an average of 154 reproducible alleles ranging from 100-2100 bp (average band of 1100bp), of which 149 (97%) were found to be polymorphic and further data was used it for statistical analysis (Fig. 2).

The PIC values of primer pairs ranged from 0.73 (ME5/EM2) to 0.89 (ME3/EM7) with an average of 0.82. Furthermore, the usefulness of each marker was evaluated through DI and MI values as shown in Table 2. The highest level of gene diversity (0.40) was detected for ME3/EM7. The ME3/EM3 resulted in highest marker indices (2.24) followed by ME3/EM7 and ME2/EM1.

Establishing correlation between geographical distribution of isolates and racial distribution of *Foc* was the hypothesis. In fact, several limitations are associated with classical taxonomic methods in precise classification.

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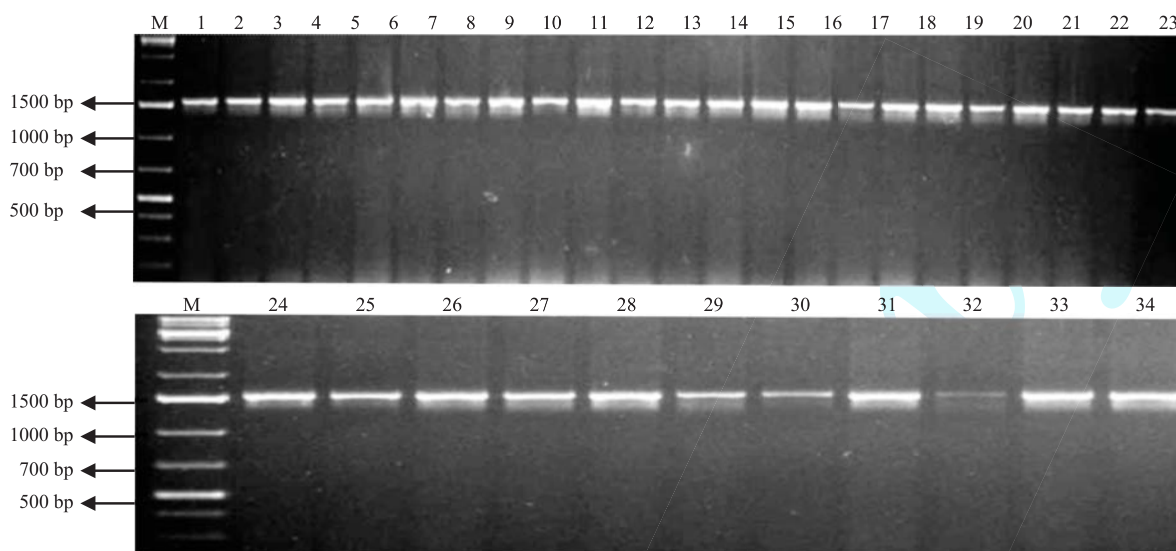

Fig. 1 : Gel photograph showing the profile for *Fusarium oxysporum* f.sp.*ciceris*isolates as shown by forma specialis*F. sp. ciceris* primer (Foc0-12f/r). (Where M = 1 Kb plus DNA ladder).

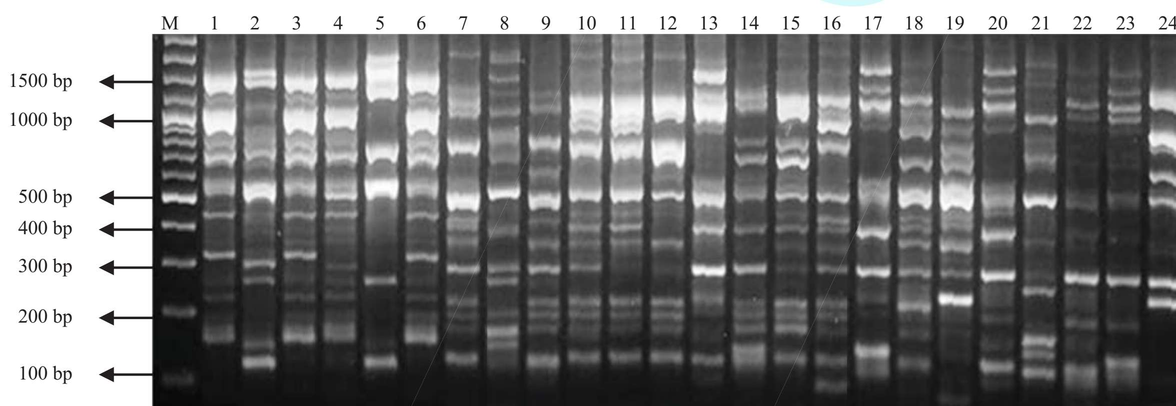

Fig. 2 : SRAP profile of 34 *Fusarium oxysporum* f.sp.*ciceris isolates* as shown by primer pair ME3/EM7. (Where M = 100 bp plus DNA ladder, Lane 1-24 represent the *Fusarium oxysporum f.sp. ciceri* isolates as mentioned in table1).

Thereby, DNA based marker system always offers an appropriate tool for overcoming the traditional classification methodology (Gurjar *et al*., 2009). Accordingly, information generated by marker system was used by employing binary database using dissimilarity matrix through neighbor-joining methods which grouped isolates into four major clusters (Fig. 3). The Cluster I comprised 14 isolates from four states of CZ (Madhya Pradesh, Chhattisgarh, Maharashtra and Gujarat) representing race-0, 1, 3 and 4. Likewise, Cluster II included 10 isolates from four states of NWPZ (Rajasthan, Haryana, Punjab and Delhi) representing race-0, 1, 2 and 4. The cluster III grouped 3 isolates from two states of SZ (Andhra Pradesh and Karnataka) representing race-1, 4 and 6, respectively. Additionally, Cluster IV consisted of 7 isolates from two different states of NEPZ (Jharkhand and Uttar Pradesh) represented race-0, 1, 2, 3 and 5. As a result of correlation analysis, isolates fell together having same geographical origin but did not match with their same racial status. Most of the diversity existed within the population and thereby, alleles generated with the primers combinations did not share the same haplotype. Hence, none of the amplified sequence of collected isolates from the same climatic region was found identical. Altogether, an attempt was made analyze the correlation between the pathogenic reactions and its geographical distributions, but it was not possible to establish the same as of our hypothesis.

The study highlights the existence of genetic variation in pathogen at species level establishing the evidence about the gradual genetic evolution of novel *spp*. alongside the

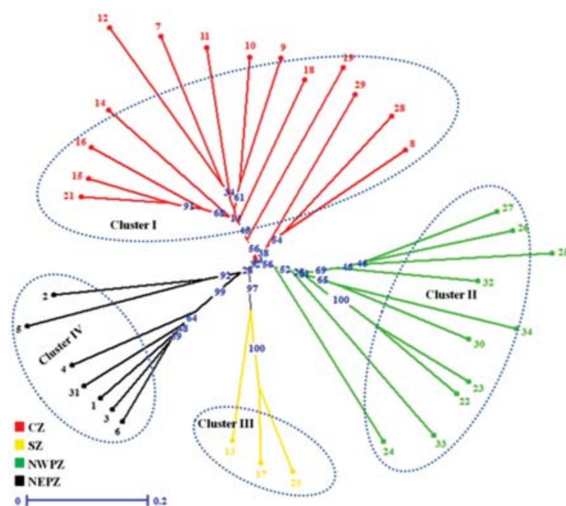

Fig. 3 : A Neighbor-joining tree showing a genetic relationship among 34 isolates of *Fusarium oxysporum* f. sp. Ciceris

existing one. High rate of this genetic change occurred in the isolates might be mediated by single gene mutation, insertion of transposable elements, or by loss of chromosomal segments and cropping system might favor evolution of large population of fungus (Peterson, 2005; Kistler and Miao, 1992). The reason could also be due to man-made dispersal of different genotypes during farmers to farmers seeds exchange program. However, isolates from major cluster I representing the CZ showed more occurrence of race-1. Similarly, isolates from cluster II representing the NWPZ also showed occurrence of race-1 without excluding the possibility of other races. Therefore, the result indicated major occurrence of race-1 in CZ and NWPZ. Hence, the marker system studied might be suitable to study the genetic diversity, but not for racial classification. Additionally, for classifying the isolates on the basis of homologue virulence gene(s)/markers of SIX1, SIX4, SIX8 and SIX9 as reported in *F. oxysporum* f. sp. *Lycopersici* (Thatcher *et al*., 2011), would be more meaningful in correlating the isolates with its geographical distribution and racial status (Gasco *et al.,* 2001, 2004b; Abd-Elsalam *et al.,* 2004; Sharma *et al.,* 2009). The current findings support the previous observation made by other researchers using markers that no correlation exist between molecular as well as pathogenicity of the isolates (Cramer *et al.,* 2003; Dubey *et al.,* 2012). This article highlights the present demand for pyramiding multiple gene governing different QTLs into agronomical superior cultivar against various races. This observation would help in identifying racial status and their geographical distributions in the chickpea growing region for sustainable chickpea production in resistance breeding programme.

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