JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2016. Vol. **10**(2), p. 1559-1563

Genetic Analysis of Pigeonpea (*Cajanus cajan* L. Millsp.) Genotypes for *Fusarium* wilt using Microsatellite Markers

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(Received: 05 February 2016; accepted: 08 March 2016)

Genetic analysis of seventeen pigeonpea genotypes for *fusarium* wilt using twenty eight microsatellite markers produced 88 alleles. Difference in allele size was observed among resistant and susceptible genotypes. The average number of alleles per locus was found to be 3.14 the average PIC values was 0.49 using these microsatellite markers. The Jaccard's similarity coefficients based on microsatellite markers data analysis was 0.49. A dendrogram constructed based on the UPGMA clustering method revealed two major clusters. Cluster-I comprised of nine cultivars and the cluster-II included remaining eight cultivars. Genotypes that are susceptible to *fusarium* wilt of pigeonpea *viz*., GT-1, GT-100, GT-101, GT-102, BANAS, AVPP-1, AGT-2, T-15-15 and LRG-41 formed one cluster. The dendogram showed that genotypes that are resistant to *fusarium* wilt of pigeonpea *viz*., C-11, BDN-2, ICPL-87, ICPL-87119, ICPL-84060, ICP-8863, BSMR-853 and WRGE-119 were closely related and they formed another cluster. The study revealed that microsatellite markers can be efficiently used for discriminating resistant and susceptible pigeonpea genotypes for *fusarium* wilt.

Keywords: Pigeonpea, *Fusarium* wilt, and Microsatellite.

Pigeonpea [*Cajanus cajan* (L.) Millsp.] (2n = 22) is an important food legume crop of the semi-arid tropical regions of Africa and Asia. It belongs to the family *Leguminosae* and sub family *Papilionaceae*. The chromosome number of all *Cajanus* species is n=11, with a genome size of 808 Mbp (Greilhuber and Obermayer, 1998). India is the largest pigeonpea growing country, cultivating it on 36.3 lakh hectares with an annual production of around 27.6 lakh tonnes and productivity of 760.33 kg/ha. The productivity of this crop remained low at around 700–800 kg/ha over the last five decades. This is mainly due to limited exploitation of available natural variability of genus *Cajanus* in breeding lines. It is drought tolerant but highly susceptible to some abiotic (water-logging) and biotic (*Fusarium* wilt and sterility mosaic disease, *etc*.) stresses.

Fusarium wilt of pigeonpea is a soil borne disease caused by fungus *Fusarium udum*. Therefore, to minimize yield losses due to *fusarium* wilt, it is necessary to tackle these problems at molecular level by developing cultivars which resist/ tolerate these biotic stresses and have greater recovery from damage. Genomic tools especially molecular markers have facilitated breeding in many cereal crops leading to development of several improved cultivars/ varieties with enhanced resistance / tolerance to biotic or abiotic stresses (Varshney *et al*., 2006). Molecular markers, such as RAPD, SCAR, SSR, RFLP, AFLP *etc.*, have been used to assess genetic variations at DNA level.

Microsatellites (Tautz and Rentz 1984), also known as Simple Sequence Repeat (SSR) markers, are DNA-based molecular markers that

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offer several advantages because they are reproducible, polymorphic, polymerase chain reaction (PCR)-based and readily portable within a species (Edwards *et al*. 1996). In case of pigeonpea, although a few SSR markers have been reported recently (Burns *et al*. 2001, Odeny *et al*. 2007), not a single genetic map is available so far. This can be attributed to mainly two factors: (i) availability of meager number of molecular markers and (ii) a very low level of polymorphism in cultivated pigeopea germplasm (Odeny *et al*. 2007). In present investigation studied twenty eight microsatellite markers for genetic analysis of genetic analysis of seventeen pigeonpea genotypes for *fusarium* wilt.

MATERIALS AND METHODS

Plant material

Total seventeen pigeonpea genotypes namely, GT-1, GT-100, GT-101, GT-102, BANAS, AVPP-1, AGT-2, T-15-15, LRG-41, C-11, BDN-2, ICPL-87, ICPL-87119, ICPL- 84060, ICP-8863, BSMR-853 and WRGE-119 were collected from Agricultural Research Station, Dahod and Pulse Research station, Vadodara, Anand Agricultural University, Anand and used in present study. Plants were grown in pots and young leaf samples of all plants of each cultivar were collected for genomic DNA isolation.

Genomic DNA isolation

Extraction of DNA from young leaves was done using modified Cetyl-Trimethyl Ammonium Bromide (CTAB) method (Murray and Thompson, 1980) with some minor modifications.

PCR amplification using microsatellite markers

Twenty eight microsatellite markers were selected from available literatures (Table 1) which were synthesized from MWG biotech, Germany. PCR was carried out in 25ìl reaction volumes containing 2.5 ìl of 10 x Taqassay buffer (Tris with15mM MgCl2), 10 mM of each dATP, dCTP, dGTP and dTTP, 5U Taq polymerase (BioLabs, UK), 10 picomole of forward – reverse primer and 50 ng of template DNA. Amplification were carried out in a thermo-cycler (Applied Biosystem Veriti, CA, USA) programmed for 35 cycles with an initial denaturation at 94°C for 5 min followed by cycling conditions of denaturation at 94°C for 45 second, annealing at 56°C for 45 second and extension at 72°C for 1 min. After 35 cycles, there was a final extension step of 7 min at 72°C. The amplicons were analyzed on 2.5 % agarose gels and detected by staining with ethidium bromide. UV trans-illuminated gels were photographed with gel documentation system (SYNGENE, USA).

Data collection and analysis

The clear and distinct bands amplified by microsatellite markers were scored for the presence and absence of the corresponding band among the genotypes. The scores 1 and 0 indicates the presence or absence of bands respectively. The software used for the analysis of the scored data was NTSYSpc version 2.02 (Rholf 1994). The molecular weight of the PCR products was estimated by Alpha EaseFC4.0.0 software (Alpha Innotech Corporation, USA) for each primer to analyze allele range and cluster analysis was performed by agglomerative technique using the UPGMA (Un-weighted Pair Group Method with Arithmetic Mean) method by SAHN clustering function of NTSYSpc.

RESULTS AND DISCUSSION

The microsatellite markers which are more effective comparable to RAPD markers due to their co-dominant nature, also as it could distinguish the resistant and susceptible genotypes. The data collected from 28 microsatellite markers produced 88 alleles. The average number of alleles per locus was found to be 3.14. The effective number of alleles was 2.39. The maximum number of alleles was six which were recorded for markers CCttc005 and CCttc008 followed by Ccat006, CCB2, CCB4, CCB6, CCB9, CCtta006, and CCttc007 which produced four alleles. Ccac010, CCac026, CCB7, CCB10, CCttc002, CCttc006, CCttc012, CCttc033, ICPM1A08, ICPM1G01, ICPM1G04 and ICPMTC20 produced three alleles. Whereas CCB1, CCB8, CCtc013 and ICPM2BM08 produced two alleles each and CCgtt002, ICPM1E10 produced single allele which was the lowest in the present investigation. The highest allele frequency found by CCB1 marker was 0.813 which was similar to Songok *et al*., (2010). The highest PIC value was recorded for CCB10 (0.68), CCttc005 (0.68) which was similar to Saxena *et al*., (2010) and the lowest for CCtc013 (0.11). The average PIC value and number of alleles were 0.60 and 4.8 for the markers developed by Odeny *et al*., (2007) while 0.49 and

3.14 for markers used in present study. The molecular weight of the amplified PCR products ranged from 110bp (ICPM1G04) to 295bp (CCtc013) (Table 1).

Based on the microsatellite markers data, cluster analysis was performed using genetic similarity values and a dendrogram was generated showing the grouping of genotypes according to their resistance and susceptibility to *fusarium* wilt. The highest similarity index value of 0.78 was found between GT-1 and GT-100 in susceptible genotypes, whereas in resistant genotypes the highest similarity index value, 0.77 was found between ICPL-87 and ICPl-87119 while the least similarity index value of 0.05 was found between GT-100 and ICPL-87119. The average similarity coefficient among genotypes was 0.49 (Table 2).

The microsatellite markers cluster pattern is presented Figure 1. It showed two major clusters namely A and B formed at a similarity coefficient of 0.13 (Fig. 1). Cluster A was divided into two sub clusters A1 and A2. Grouping of nine genotypes that are susceptible to *fusarium* wilt were in one major cluster 'A'. Sub-cluster A1 included pigeonpea genotypes viz., GT-1, GT-100 and GT-101. Sub-cluster A2 consists of genotypes GT-102, BANAS, AVPP-1, AGT-2, T-15-15 and LRG-41. Cluster B was divided into two sub-clusters B1 and B2. Grouping of eight genotypes that are resistant to *fusarium* wilt were in one major cluster

Table 1. Results of microsatellite markers analysis

S. No	Locus name	Repeat Motif	No. of bands amplified	Molecular Size rang (bp)	Total no. Alleles	PIC
1	Ccac010	$(CA)_7aca(TA)_3$	17	175bp-192bp	3	0.62
2	CCac026	(AC)7	15	245bp-260bp	3	0.62
3	CCac036	(CATA)3ta(TG)6	11	212bp-221bp	2	0.42
4	Ccat006	(TA)7(CA)6	15	220bp-290bp	4	0.64
5	CCB1	(CA)10	16	197bp-205bp	2	0.21
6	CCB2	(CA)21	12	135bp-168bp	4	0.29
7	CCB4	(CA)31	16	235bp-265bp	4	0.61
8	CCB6	(CA)6	17	178bp-195bp	4	0.58
9	CCB7	(CT)16	16	152bp-170bp	3	0.59
10	CCB8	(CT)30	15	128bp-135bp	2	0.29
11	CCB9	(CT)22	16	152bp-180bp	4	0.65
12	CCB10	(CA)15	17	225bp-240bp	3	0.68
13	CCgtt002	(TGT)4	17	212bp	1	0
14	CCtc013	(TC)6	17	285bp-295bp	2	0.11
15	CCtta006	(ATT)21	16	220bp-246bp	4	0.53
16	CCttc002	(GAA)5g(GAA)5	13	165bp-180bp	3	0.50
17	CCttc005	(CA)8	17	265bp-290bp	6	0.68
18	CCttc006	(GAA)11gag(GAA)5 gaggaagag(GAA)17	14	160bp-172bp	3	0.45
19	CCttc007	(GA)4ca(GA)4 cagagt(GA)8	16	235bp-265bp	4	0.57
20	CCttc008	(AC)7	16	225bp-290bp	6	0.73
21	CCttc012	(TTC)7	13	165bp-175bp	3	0.65
22	CCttc033	(CTT)8	17	210bp-232bp	3	0.58
23	ICPM1A08	(CA)6	15	250bp-265bp	3	0.63
24	ICPM1E10	(CA)7	17	165bp	1	0
25	ICPM1G01	(CA)8	17	270bp-286bp	3	0.49
26	ICPM1G04	(T)21	17	110bp-120bp	3	0.66
27	ICPM2BM08	(TG)5n(TG)5	15	130bp-140bp	2	0.48
28	ICPMCT20	(GA)14(AAGA)5	17	120bp-140bp	3	0.65
Total			437	-	88	13.91
Avg.			15.60	197bp-218bp	3.14	0.49

Table 2. Genetic similarity matrix of microsatellite markers data based on Jacard's similarity coefficient

	GT-1	GT-100	GT-101	GT-102	BANAS	AVPP-1	AGT-2	T-15-15	LRG-41	C-11	BDN-2	ICPL-87	ICPL-87119	ICPL-84060	ICP-8863	BSMR-853	WRGE 119
GT-1	1.00																
GT-100	0.78	1.00															
GT-101	0.48	0.51	1.00														
GT-102	0.40	0.43	0.58	1.00													
BANAS	0.45	0.44	0.41	0.75	1.00												
AVPP-1	0.34	0.37	0.45	0.63	0.65	1.00											
AGT-2	0.35	0.35	0.38	0.47	0.57	0.71	1.00										
T-15-15	0.28	0.25	0.25	0.39	0.52	0.52	0.54	1.00									
LRG-41	0.21	0.22	0.21	0.32	0.33	0.48	0.38	0.58	1.00								
C-11	0.08	0.07	0.06	0.10	0.12	0.16	0.17	0.30	0.34	1.00							
BDN-2	0.08	0.08	0.11	0.10	0.10	0.12	0.17	0.20	0.25	0.61	1.00						
ICPL-87	0.06	0.05	0.08	0.12	0.13	0.10	0.10	0.25	0.28	0.61	0.68	1.00					
ICPL-87119	0.06	0.05	0.08	0.12	0.12	0.09	0.12	0.22	0.25	0.55	0.61	0.77	1.00				
ICPL-84060	0.09	0.08	0.11	0.13	0.13	0.10	0.15	0.21	0.24	0.40	0.54	0.59	0.62	1.00			
ICP-8863	0.11	0.10	0.09	0.10	0.11	0.10	0.13	0.18	0.20	0.47	0.48	0.57	0.60	0.58	1.00		
BSMR-853	0.11	0.10	0.09	0.10	0.11	0.10	0.13	0.20	0.23	0.43	0.52	0.52	0.65	0.63	0.66	1.00	
WRGE-119	0.11	0.10	0.08	0.06	0.08	0.05	0.14	0.17	0.17	0.41	0.50	0.38	0.44	0.54	0.52	0.62	1.00

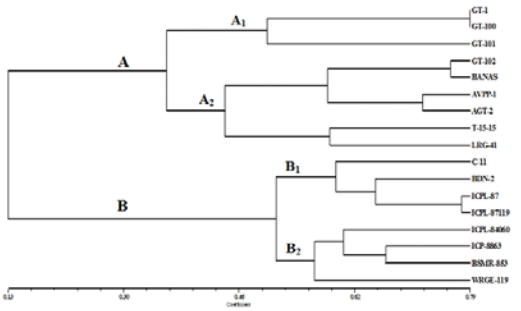


Fig. 1. Dendrogram constructed using UPGMA cluster analysis for microsatellite markers.

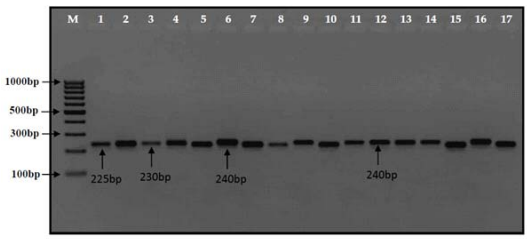
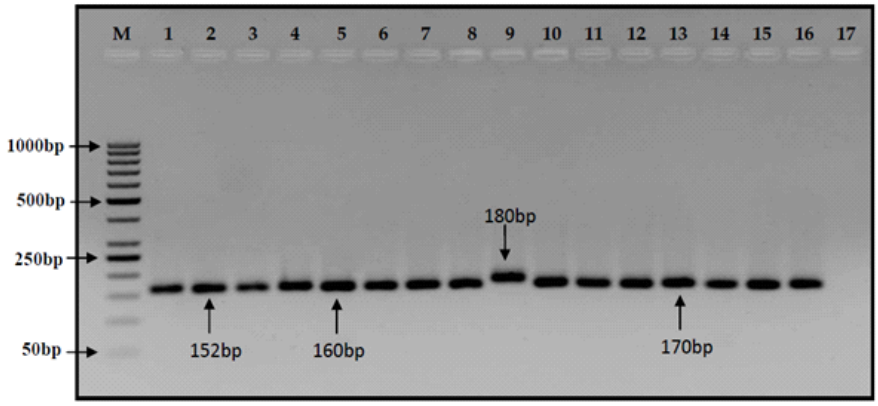


Fig. 2. Amplification of microsatellite markers CCB10



M = 100 bp DNA Ladder ; (1) GT-1 ; (2) GT-100 ; (3) GT-101 ; (4) GT-102 ; (5) BANAS ; (6) AVPP-1 ; (7) AGT-2 ; (8) T-15-15 ; (9) LRG-41 ; (10) C-11 ; (11) BDN-2 ; (12) ICPL-87 ; (13) ICPL-87119 ; (14) ICPL-84060 ; (15) ICP-8863 ; (16) BSMR-853 ; (17) WRGE-119

Fig. 3. Amplification of microsatellite markers CCB9

'B'. Sub-cluster B1 consists of genotypes viz., C-11, BDN-2, ICPL-87 and ICPL-87119; whereas sub-cluster B2 consists of genotypes viz., ICPL-84060, ICP-8863, BSMR-853 and WRGE-119.

The dendrogram constructed using UPGMA the pooled SSR loci data shows that *fusarium* wilt susceptible pigeonpea genotypes viz., GT-1, GT-100, GT-101, GT-102, BANAS, AVPP-1, AGT-2, T-15-15 and LRG-41 are closely related. It also revealed that *fusarium* wilt resistant genotypes of pigeonpea viz., C-11, BDN-2, ICPL-87, ICPL-87119, ICPL-84060, ICP-8863, BSMR-853 and WRGE-119 are closely related.

CONCLUSION

Microsatellite markers attained great significance in characterization of plant germplasm resources. They were used to distinguish the 17 pigeonpea genotypes according to their resistance and susceptibility to *fusarium* wilt. Dendogram based on microsatellite markers data showed the clustering of genotypes according to their reaction to *fusarium* wilt. The genetic similarity among the susceptible and the resistant genotypes was expressed more clearly using these markers. The study also revealed that from the tendency of resistant and susceptible genotypes to cluster together, it can be inferred that these genotypes share a common phylogenetic pathway and the resistancy and susceptibility to *fusarium* wilt may be due to mono or oligogenes which can in turn to be targeted using more efficient marker (molecular) systems.

ACKNOWLEDGEMENTS

We thank Retd. Head and Professor Dr. J. C. Jadeja, Department of Genetics and Plant Breeding, B. A. College of Agriculture for providing the research facility as well as encouragement during these study.

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