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Tagging SSR markers associated with genomic regions controlling anthracnose resistance in chilli

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ABSTRACT

Anthracnose, caused by *Colletotrichum* spp. is a serious pre- and post-harvest disease in chilli (*Capsicum annuum* L.), which is a remunerative spice-cum-cash crop of India. An attempt was made to tag genomic regions controlling anthracnose resistance using reported microsatellite markers. Out of 60 polymorphic SSR markers screened, only four differentiated the individual constituents of resistant and susceptible bulks. Of these four, only one (HpmsE 081) was found associated with genomic regions controlling anthracnose resistance. However, the association was weak as suggested by low contribution of the marker towards the variance of response to anthracnose disease in terms of lesion size.

Key words: Anthracnose resistance, chilli, gene tagging, SSR markers.

INTRODUCTION

Chilli (*Capsicum annuum* L.) is a remunerative vegetable and spice-cum-cash crop of the Indian subcontinent. India is the largest producer accounting for 26 per cent of the global production followed by China. Andhra Pradesh and Karnataka together account for more than fifty per cent production in India. However, chilli productivity in India (1.60 t ha^{-1}) is lower than that in the developed countries such as USA and South Korea (3.4 t ha^{-1}) (Madalageri and Ukkund, 9). Among the biotic stresses that constrain the chilli production, anthracnose, caused by *Colletotrichum* spp. is a serious pre- and post-harvest disease. *C. capsici* (Syd.) Butler and Bisby, *C. gloeosporioides* (Penz.) Penz. and Sacc., *C. acutatum* (Simmonds) and *C. cocodes* (Wallr.) S.Hughes (Simmonds, 15), cause anthracnose of chilli, the former two are predominant in India. Yield losses due to anthracnose in India range from 50 (Ramachandran, 13) to 66-84 per cent (Thind and Jhooty, 17) and loss in fruit quality attributes such as oleoresin, capsaicin and phenol content due to anthracnose could be 50 per cent (Jeyalakshmi, 6) resulting in reduced market price.

Conventional breeding of chilli for anthracnose resistance is constrained owing to prevalence of multiple species/ strains, wide diversity and distribution, and wide variability in pathogenicity of *Colletotrichum*. SSR markers, as powerful surrogates for monitoring anthracnose resistance help increase pace and efficiency of breeding chilli for anthracnose resistance. Reported literature on identification of DNA markers linked to genomic regions controlling anthracnose resistance in chilli is scanty in India. Under this premise, the present study was conducted.

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MATERIALS AND METHODS

The material for the study consisted of anthracnose resistant PBC 80 (procured from World Vegetable Centre, AVRDC, Taiwan) and susceptible SB1 (provided by Dr Arvind Deshpande, of M/s JK Seeds) both belonging to *Capsicum baccatum*. The genotypes were crossed post rainy season at the experimental plots of Department of Genetics and Plant Breeding (GPB), University of Agricultural Sciences (UAS), GKVK, Bengaluru.

Disease resistance response of PBC 80 was confirmed by screening against seven *C. capsici* and four *C. gloeosporioides* isolates (data not shown). Seeds from the crossed 'PBC 80 × SB 1' fruits were sown to raise F_1 plants during second year rainy season in an insect proof nethouse. True F_1s were selfed individually to obtain F_2 seeds. F_2 seeds were sown in nursery to raise the F_2 mapping population and 40-day-old seedlings were transplanted in insect proof nethouse in November, along with their parents and F_1 by maintaining a spacing of 0.45 m between plants within a row and 0.9 m between rows. All the recommended package of practices was followed to raise a good crop.

A total of 240 F_2 plants were raised from the selfed F_1 seeds and phenotyped for reaction to anthracnose. Twenty random fruits from each F_2 plant were picked

at red ripe stage and brought to the laboratory. The fruits were surface sterilised, rinsed in sterile water and inoculated with virulent strain of *Colletotrichum capsici*, '*Cc* 38' in two replications. Thereafter, the fruits were inoculated with homogenized spore suspension containing 5×10^5 spores/ ml at two spots on the fruit (one µl/ spot) using Hamilton micro-syringe (AVRDC, 1). The inoculated fruits were incubated in plastic boxes with moist filter papers placed at the bottom and on top of the fruits to maintain relative humidity of over 90 percent and then incubated at 27±1°C for eight days. Disease reaction was recorded in terms of lesion size and was expressed as overall lesion diameter (OLD) across all inoculated points and true lesion diameter (TLD) using the following formulae;

$$OLD = \frac{\sum \text{lesion diameter}}{\text{Total No. of inoculated points}}$$

True lesion diameter (TLD): average of lesion diameter that are truly developed

$$TLD = \frac{\sum \text{lesion diameter}}{\text{No. of inoculated points that developed true lesions}}$$

Genomic DNA was extracted from young and healthy leaves of 50-day-old seedlings of parents, F_1 and F_2 plants following the extraction protocol given by Prince *et al.* (12) with a few modifications. Primers of 282 publically available microsatellite markers (Lee *et al.*, 8; Nagy *et al.*, 11; Yi *et al.*, 20) were custom synthesized from Sigma Genosys, Bengaluru. Reaction mixture for amplification consisted of template DNA (12.5 ng/ µl) 2µl, Forward primer (10 pmol/ µl) 2 µl, Reverse primer (10 pmol/ µl) 2 µl, 1 mM each dNTP 2 µl, 10 X *Taq* buffer 1 µl, 1 U *Taq* polymerase (5 u/µl) 0.2 µl. The PCR amplified products were initially visualized on 3% agarose and where clear resolution was not observed the products were denatured and separated on 6% polyacrylamide gel electrophoresis (PAGE) gel and products were visualized by silver staining.

Depending on the lesion size (mm diameter) caused upon infection with virulent '*Cc* 38' isolate, the F_2 plants were categorised as resistant and susceptible following the scale modified from Hartman and Wang (4). DNA from 10 resistant plants and ten susceptible plants were bulked. The bulks were constituted by combining equal quantity DNA (of same conc.) from selected plants, such that the final concentration of bulked DNA was made up to 12.5 ng/ µl.

Sixty SSR primers which differentiated the two parents either on 3% agarose or on 6% PAGE gel were used to genotype the two bulks. Seven primers, *viz.*, HpmsE001, HpmsE003, HpmsE070, HpmsE081, HpmsE097, HpmsE116 and HpmsE139, which differentiated the resistant and susceptible bulks were used to genotype individual constituents of the bulks along with resistant and susceptible parents for confirmation of polymorphism. A total of 125 F_2 individuals, which were randomly selected including the constituents of the resistant and susceptible bulks, were genotyped using four SSR primer combinations, which clearly differentiated the individual constituents, of the constituting bulks. The SSR marker allele segregation was recorded as binary codes. The code '1' was assigned to the F_2 individuals, which produced SSR marker amplicons size specific to the resistant parent PBC 80, '2' to those produced SSR marker amplicon specific to the susceptible parent SB 1 and '3' to those F_2 individuals that produced SSR marker amplicon specific to both parents (F_1 type), respectively.

The F_2 individuals were classified into three marker classes based on the codes. The mean lesion diameter of the individuals belonging to each of the marker classes was computed. The significance of differences among the three marker classes for mean lesion size was examined using 'F' test through one-way ANOVA approach using MS excel software. Variance explained by the SSR marker significantly associated with genomic regions controlling response to anthracnose disease infection was computed following the method suggested by Wu *et al.* (19). Broad sense heritability of the response to anthracnose infection was estimated following the method suggested by Hanson *et al*. (3). The additive and dominance genetic effects of the linked SSR marker was tested following two-sample 't' test with unequal variances (Wu *et al*., 19).

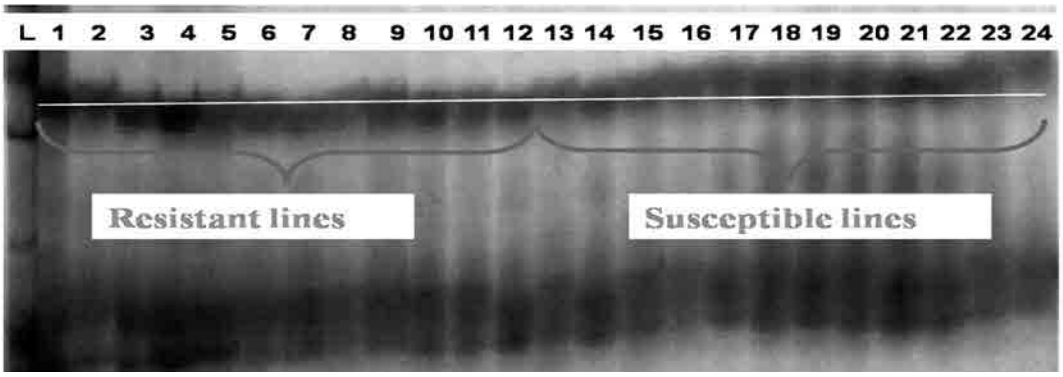
Individual plant values of each F_2 plant for anthracnose resistance were used to estimate skewness, the third degree statistics and kurtosis, the fourth degree statistics (Snedecor and Cochran, 16) to understand the nature of distribution and hence inheritance pattern using 'STATISTICA' software. Genetic expectations of skewness ($-3/2\ d^2\ h$) reveal the nature of genetic control of the traits (Fisher *et al*., 3) and Kurtosis indicates the relative number of genes controlling the traits (Robson, 14).

RESULTS AND DISCUSSION

Out of 282 SSR markers screened only 60 (Table 1) differentiated the resistant (PBC 80) and susceptible (SB 1) parents indicating low level of parental polymorphism at SSR loci (21.3%), though the parents were diverse for several morphological traits. Kwon *et al*. (7) also reported low level of polymorphism at the SSR loci among commercial chilli varieties tested. Out of seven primers, which could differentiate the resistant and susceptible bulks, only four, *viz.,* HpmsE 081 (Fig. 1), HpmsE 097, HpmsE

Table 1. List of SSR markers polymorphic to F_2 mapping population parents (PBC 80 and SB 1) in *Capsicum baccatum*.

Sl. No.	Primer	Sl. No.	Primer	Sl. No.	Primer	Sl. No.	Primer	Sl. No.	Primer	Sl. No.	Primer
1	Hpms E 001	11	Hpms E 035	21	Hpms E 070	31	Hpms E 097	41	Hpms E 146	51	AA840 689
2	Hpms E 003	12	Hpms E 036	22	Hpms E 072	32	Hpms E 100	42	Hpms E 147	52	CAN 09
3	Hpms E 005	13	Hpms E 051	23	Hpms E 074	33	Hpms E 101	43	Hpms 19	53	CM 008
4	Hpms E 012	14	Hpms E 058	24	Hpms E 075	34	Hpms E 104	44	Hpms 24	54	Gpms 3
5	Hpms E 018	15	Hpms E 059	25	Hpms E 078	35	Hpms E 116	45	Hpms 13	55	Gpms 1
6	Hpms E 019	16	Hpms E 063	26	Hpms E 081	36	Hpms E 122	46	Hpms 04	56	Gpms 4
7	Hpms E 026	17	Hpms E 064	27	Hpms E 083	37	Hpms E 125	47	Hpms 1-106	57	Gpms 93
8	Hpms E 027	18	Hpms E 065	28	Hpms E 084	38	Hpms E 139	48	Hpms 1-139	58	Gpms 159
9	Hpms E 029	19	Hpms E 066	29	Hpms E 090	39	Hpms E 141	49	Hpms 1-155	59	Gpms 147
10	Hpms E 032	20	Hpms E 067	30	Hpms E 096	40	Hpms E 145	50	Hpms 1-216	60	Gpms 140



L = 100 bp ladder, Lane 1 = Resistant parent, Lane 2 = Resistant Bulk, Lane 3-12 = Individual constituents of resistant bulk, Lane 13 = Susceptible parent, Lane 14 = Susceptible Bulk, Lane 15-24 = Individual constituents of susceptible bulk

Fig. 1. Primer HpmsE 081 differentiates the individual constituents of anthracnose resistant and susceptible bulks of F_2 mapping population in chilli.

116 and HpmsE 139 consistently differentiated the individual constituents of the two bulks. Hence, these four SSR markers were used to genotype all the 125 F_2 individuals for further confirmation of to explore their association with anthracnose resistance through single marker analysis (Fig. 3). These results suggested putative association of the four SSR markers with genomic regions controlling anthracnose resistance.

Of the four SSR markers, which consistently differentiated the resistant and susceptible bulks and their constituents, only one (HpmsE 081) was found associated with genomic regions controlling anthracnose resistance as indicated by significance of mean squares due to “between marker classes” (Table 2). Lower magnitudes of variance of response to anthracnose disease in terms of overall and true lesion size (explained by linked SSR marker), was amply reflected through low heritability (Table 3) suggesting weak association between the marker and the genetic determinants controlling anthracnose resistance. Voorrips *et al*. (18) have identified one major quantitative trait locus (QTL) with larger effects on anthracnose resistance (against *C. acutatum*) and three QTLs with smaller effects in the F_2 population (derived from *C. annuum* × *C. chinense* cross). In single marker analysis, the distance between the linked SSR marker locus and percent trait variation explained by the linked marker are confounded (Kearsey and Pooni, 6). Further as F_2 individuals are not replicable, the SSR marker-trait (anthracnose resistance) association need to be confirmed in a replicable mapping population such as recombinant inbred lines (RILs) for effective use in marker-assisted selection.

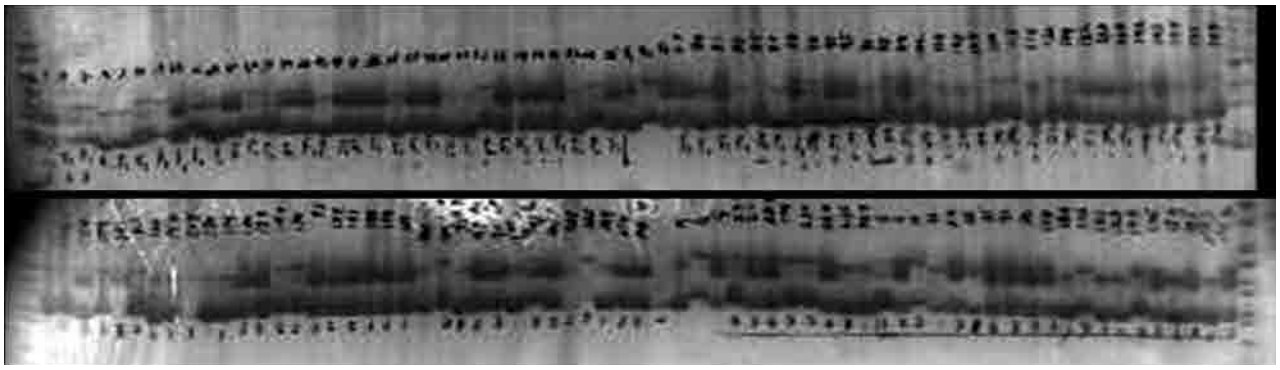


Fig. 2. Segregation of SSR marker E 116 alleles among 125 F_2 mapping individuals population in chilli.

Table 2. Analysis of variance of response to anthracnose disease between and within SSR marker (Hpms E081, Hpms E097, Hpms E116, Hpms E139) classes in an intra *Capsicum baccatum* (PBC 80 × SB 1) F_2 mapping population.

Sl. No.	Response to anthracnose disease infection	Total No. of plants	SSR marker class			'F' cal
			m_2	m_0	m_1	
Hpms E081						
1.	Average OLD for Cc (mm)	121	1.56	2.94	2.47	3.88*
2.	Average TLD for Cc (mm)	121	2.11	3.60	3.49	4.70*
Hpms E097						
1.	Average OLD for Cc (mm)	117	2.73	2.44	2.11	0.59
2.	Average TLD for Cc (mm)	117	3.36	3.04	2.86	0.33
Hpms E116						
1.	Average OLD for Cc (mm)	123	2.89	2.15	1.92	1.94
2.	Average TLD for Cc (mm)	123	3.54	2.68	2.74	1.31
Hpms E139						
1.	Average OLD for Cc (mm)	113	2.44	3.59	3.17	1.49
2.	Average TLD for Cc (mm)	112	2.44	3.59	3.17	1.49

*Significant at P = 0.05; m_2, m_0 and m_1 takes the meaning as described in the material and methods

Table 3. Estimates of variance explained by the linked SSR marker (Hpms E081), broad sense heritability and additive and dominance effect.

SSR marker	Trait	σ^2_g	Broad sense h^2	Test statistic t_1 (additive effect)	Test statistic t_2 (dominance effect)
HpmsE 081	Average Cc OLD	0.41	0.07	0.12	0.02
	Average Cc TLD	0.59	0.09	0.14	0.05

Positively skewed leptokurtic distribution of F_2 was observed for average OLD caused due to infection by '*Cc* 38' (Fig. 2) isolate while positively skewed, platy Kurtic distribution of F_2 was observed for average TLD caused due to infection by '*Cc* 38' (Fig. 5) isolate. Positively skewed distribution of individuals of F_2 for overall and true lesions produced in response to inoculation by '*Cc* 38' (Fig. 4) is on the expected lines as all *C. baccatum* lines have been reported to have some level of resistance to anthracnose. Mild selection is expected to maximize the genetic gain. However, lepto and platy kurtic distribution of F_2 individuals with respect to overall and true lesion produced upon inoculation with '*Cc* 38' (Fig. 3 & 4) indicates that fewer to large numbers of genes, respectively are involved in the

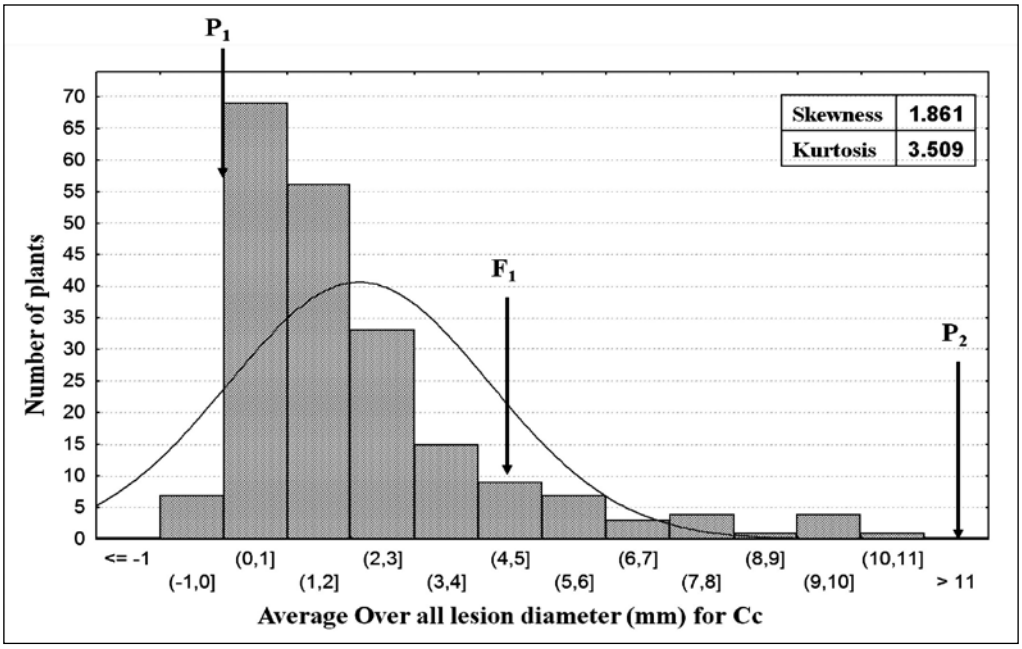


Fig. 3. Distribution of an intra *Capsicum baccatum* F_2 mapping population (PBC 80 × SB 1) individuals for average over all size of the lesion (mm) caused by *C. capsici*.

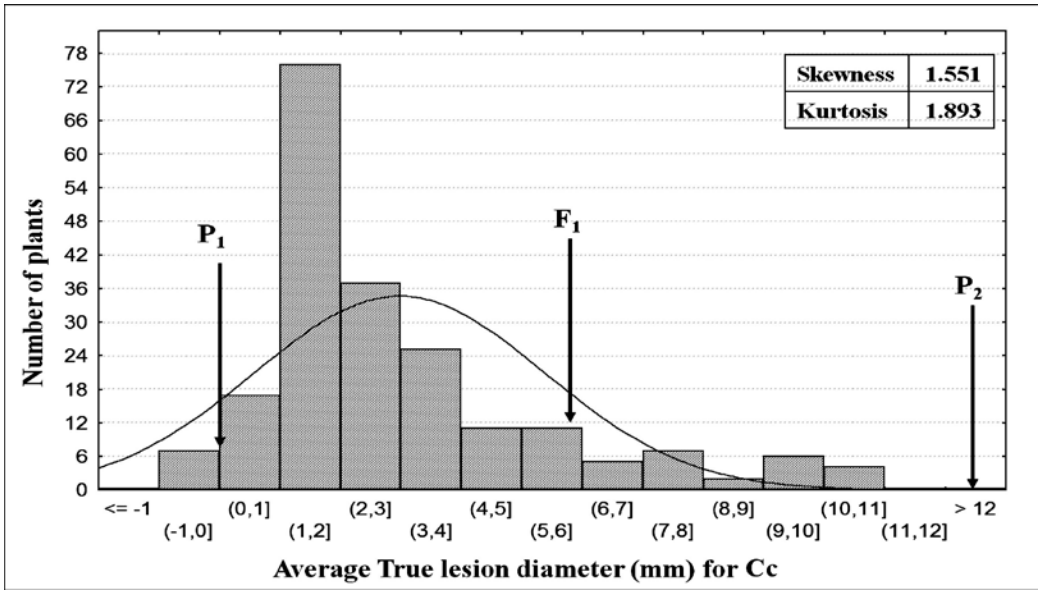


Fig. 4. Distribution of an intra-*Capsicum baccatum* F_2 mapping population (PBC 80 × SB 1) individuals for average true size of the lesion (mm) caused by *C. capsici*.

response to anthracnose disease infection. Polygenic inheritance of anthracnose resistance was also reported by Voorrips *et al*. (18). The inheritance patterns vary depending on the resistance sources and the *Colletotrichum* isolates. Several researchers have assessed the resistance to be controlled by a single recessive gene (Mahsuk *et al.*, 10).

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REFERENCES

1. AVRDC. 2004. *AVRDC Report 2003*, AVRDC Publication Number 04-599. Shanhua, Taiwan: AVRDC-The World Vegetable Center. 194 p.
2. Fisher, R.A., Immer, FR. and Tedin, O. 1932. The genetical interpretation of statistics of the third degree in the study of quantitative inheritance. *Genetics*, **17**: 107-24.
3. Hanson, C.H., Robinson, H.F. and Comstock, R.E. 1956. Biometrical studies of yield in segregating populations of Korea. *Losedezo Agron. J.* **148**: 268-72.
4. Hartman, G.L. and Wang, T.C. 1992. Characteristics of two *Colletotrichum* species and evaluation of resistance to anthracnose in pepper. **In**: *Proceedings of the Third International Conference on Plant Protection in the Tropics*. Kaula Lumpur, Malaysian Plant Protection Society. **6**: 202-05.
5. Jeyalakshmi, C., Seetharaman, K. and Ebenezer, E.G. 1994. Qualitative losses of chilli fruits due to infection by *Colletotrichum capsici* (Sydow) Butler and Bisby. *Capsicum Eggplant Newslett*. **18**: 80-82.
6. Kearsey, M.J. and Pooni, H.S., 1996. *The Genetical Analysis of Quantitative Traits*, Chapman and Hall, London, 327 p.
7. Kwon, Y.S., Lee, J.M., Yi, G.B., Yi, S.I., Kim, K.M., Soh, E.H., Bae, K.M., Park, E.K., Song, I.H. and Kim, B.D. 2005. Use of SSR markers to complement tests of distinctiveness, uniformity, and stability (DUS) of pepper (*Capsicum annuum* L.) varieties. *Mol. Cells*, **19**: 428-35.
8. Lee, J.M., Nahm, S.H., Kim, Y.M. and Kim, B.D. 2004. Characterization and molecular genetic mapping of microsatellite loci in pepper. *Theor. Appl. Genet.* **108**: 619-27.
9. Madalageri, M.B. and Ukkund, K. 2004. Management of flowering and fruiting in chilli (C*apsicum annuum* L.). *Spice India*, **17**: 20-23.
10. Mahasuk, P., Khumpeng, N., Wasee, S., Taylor, P.W.J. and Mongkolporn, O. 2009. Inheritance of resistance to anthracnose (*Colletotrichum capsici*) at seedling and fruiting stages in chili pepper (*Capsicum* spp.). *Plant Breed.* **128**: 701-06.
11. Nagy, I., Stagel, A., Sasvari, Z., Roder, M. and Ganal, M. 2007. Development, characterization and transferability to other Solanaceae of microsatellite marker in pepper (*Capsicum annuum* L.). *Genome*, **50**: 668-88.
12. Prince, J.P., Zhang, Y., Radwanski, E.R. and Kyle, M.M. 1997. A versatile and high-yielding protocol for the preparation of genomic DNA from *Capsicum* spp. (pepper). *HortSci.* **32**: 937-39.
13. Ramachandran, N., Madhavi, R.K. and Rathnamma, K. 2007. Current status of chilli anthracnose in India. **In**: *Abstracts of the First International Symposium on Chilli Anthracnose*, Oh, D.G. and Kim, K.T. (Eds.), National Horticultural Research Institute, Rural Development of Administration, Republic of Korea, pp. 25.
14. Robson, D.S. 1956. Application of K_4 statistics to genetic variance component analysis. *Biometrics*, **12**: 433-44.
15. Simmonds, J.H. 1965. A study of the species of *Colletotrichum* causing ripe fruit rots in Queensland. *Queensland J. Agri. Animal Sci.* **22**: 437-59.
16. Snedecor, G.W. and Cochran, W.G. 1994. *Statistical Methods* (5th Edn.), Iowa State University Press, Ames, Iowa, USA.
17. Thind, T.S. and Jhooty, J.S. 1985. Relative prevalence of fungal diseases of chilli fruits in Punjab. *Indian J. Mycol. Pl. Path*. **15**: 305-07.
18. Voorrips, R.E., Finkers, R., Sanjaya, L. and Groenwold, R. 2004 . TL mapping of anthracnose (*Colletotrichum* spp.) resistance in a cross between *Capsicum annuum* and *C*. *chinense*. *Theor. Appl. Genet*. **109**: 1275-82.
19. Wu, R., Ma, C.X. and Casella, G. 2007. *Statistical Genetics of Quantitative Traits: Linkage, Maps, and QTL*, New York, Springer.
20. Yi, G., Lee, J.M., Lee, S., Choi, D. and Kim, B.D. 2006. Exploitation of pepper EST-SSRs and an SSR-based linkage map. *Theor. Appl. Genet.* **114**: 113-30.

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