Omonrice 17: 29-40 (2010)

INVESTIGATION OF GENETIC RELATIONSHIP AMONG YELLOW MOSAIC VIRUS RESISTANT COWPEA LINES USING MICROSATELLITE MARKERS

Tran Dinh Gioi[1], K.S. Boora[2] and Kamla Chaudhary[3]

[1]Cuulong Delta Rice Research Institute (CLRRI)
[2,3]Haryana Agricultural University, Hisar 125 004, Haryana, India

ABSTRACT

Microsatellite markers were used to investigate the genetic basis of cowpea yellow mosaic virus (CYMV) resistance in 40 cowpea lines. A total of 60 SSR primers were used to screen polymorphism between stable resistance (GC-3) and susceptible (Chrodi) genotypes of cowpea. Among these, only 4 primers were polymorphic and these 4 SSR primer pairs were used to detect CYMV resistance genes among 40 cowpea genotypes. The polymorphism information content (PIC) of these SSR markers ranged from 0.30 to 0.72. A dendrogram of these genotypes based on microsatellite polymorphisms generally agreed with the CYMV resistant phenotype of these lines. All the genotypes could be divided into two major groups, separated at 45% similarity. The resistant group comprised of 18 cowpea lines with 77% to 100% similarity, in which 10 genotypes shared 100% similarity. Also, the two resistant lines were classified in a separate group with one susceptible line and joined with resistant group at 47% similarity. The susceptible group consisted of two subgroups with 71% and 77% similarity within each subgroup.

Key words*:* cowpea, CYMV resistance, genetic similarity, microsatellite, polymorphism, SSR markers.

INTRODUCTION

Cowpea, *Vigna unguiculata* (L.) Walp, is an important grain legume crop in a number of countries of the tropics and subtropics, especially in sub-Saharan Africa, Asia, Central and South America (Singh *et al*., 1997). Its value lies in its high protein content (23-29%) and its ability to fix atmospheric nitrogen, which allows it, not only to grow but improve poor soils as well (Steele, 1972). Cowpea is considered to be one of the most widely well adapted and versatile crops which can tolerate high temperature and drought compared to other crop species. It can thrive in dry environments and produce the dry grain yield of about 1000 kg/ha in a Sahelian environment with only 181 mm of rainfall and high evaporation rate (Hall and Patel, 1985). It is estimated that cowpea is now cultivated on more than 12.5 million hectares, with an annual production of over 3 million tons worldwide (Singh *et al.* 1997). Cowpea productivity is greatly affected by a number of biotic factors such as insect pests, fungi, bacteria and viruses. Among these, viral diseases are considered to be a major limiting factor for low productivity of cowpea in the tropical and sub-tropical countries (Mali and Thottappilly 1986). More than 20 different viruses have been reported from various cowpea-growing areas of the world (Thottappilly and Rossell 1985). Cowpea yellow mosaic disease caused by cowpea yellow mosaic virus (CYMV) is one of the most serious diseases of cowpea. In fact, CYMV causes the maximum damage to different cowpea cultivars and it may cause yield reduction up to 80-100 % (Chant, 1960; Gilmer et al., 1974; and Williams, 1977). However, there are some cowpea cultivars available which show resistance to CYMV also.

Microsatellites or Simple Sequence Repeats (SSRs) are co-dominant markers that are routinely used to study genetic diversity in different crop species. These markers occur at high frequency and appear to be distributed throughout the genome of higher plants. These are DNA

sequences that consist of two to five nucleotide core units such as $(AT)_n$, $(CTT)_n$ and $(ATGT)_n$, which are tandem repeats. The regions flanking the microsatellites are generally conserved among genotypes of the same species, allowing the selection of PCR primers that will amplify the intervening SSR in all genotypes. Variation in the number of tandem repeats, *n*, results in different PCR product lengths. Due to mutations these repeats are highly polymorphic even among closely related cultivars, causing variations in the number of repeating units. They can detect a large number of alleles; level of heterozygosity is high and follows Mendelian inheritance (Wu and Tanksley 1993). Microsatellites have become the molecular markers of choice for a wide range of applications in genetic mapping and genome analysis (Li *et al*. 2000), genotype identification and variety protection (Senior *et al*. 1998), seed purity evaluation and germplasm conservation (Brown *et al.* 1996), diversity studies (Xiao *et al.* 1996), paternity determination and pedigree analysis (Ayres *et al*. 1997), gene and quantitative trait locus analysis (Blair and McCouch 1997) and marker-assisted breeding (Weising *et al.* 1998). For identification of molecular markers linked to agronomically important genes, SSR is also one of the best choices as compared to RAPD and AFLP in a more polymorphic information or more cost effective manner, respectively (Young, 1999). Therefore, this study was undertaken to investigate genetic relationship within and between yellow mosaic virus resistant and susceptible cowpea lines by using microsatellite markers.

MATERIALS AND METHODS

Plant materials

The present investigation was conducted using pure line population of cowpea created in the Forage section, Plant Breeding Department, Chaudhary Charan Singh, Agricutural University (CCS HAU), Hisar, India. Initially ninety cowpea lines were used to screen CYMV resistance under the field condition of Forage section, Plant breeding Department in July 2005. After screening of 90 cowpea genotypes, 20 clearly resistant lines and 20 susceptible lines were selected for further analysis using SSR markers. Genomic DNA was isolated from the young leaves of 3 to 4 week old seedlings of cowpea lines using CTAB (Cetyl trimethyl ammonium bromide) extraction method of Murray and Thompson (1980) modified by Saghai-Maroof *et al*. (1984) and Xu *et al.* (1994).

Molecular markers

Sixty SSR primers were used to study the genetic basic of 40 cowpea lines against cowpea yellow mosaic virus. The SSR primers specific for cowpea (*Vigna unguiculata*) and moth bean (*Vigna aconitifolia*) were obtained from Life Technologies Pvt. Ltd., N-Delhi India. The sequences of primers used in this study are given in Table 1 and Table 2

Table 1. Summary of cowpea SSR primer pairs specific for cowpea.

Primer		Primer sequence (5'-3')	SSR sequence	Predicted size (bp)*
VM1	F	CACCCGTGATTGCTTGTTG	$(TC)_{20}$	135
	R	GTCCCCTCCCTCCCACTG		
VM2	F	GTAAGGTTTGGAAGAGCAAAGAG	$(AG)_{32}$	162
	R	GGCTATATCCATCCCTCACT		
VM3	F	GAGCCGGGTTCAATAGGTA	$(AG)_{27}$	171
	R	GAGCCAGGGCAGAGGTAGT		
VM4	F	AGTAAATCACCCGCACGATCG	$(CT)_{20}$	248
	R	AGGGGAAATGGAGAGGAT		
VM5	F	AGCGACGGCAACAACGAT	$(AG)_{32}$	188
	R	TTCCCTGCAACAAAAATACA		
VM6	F	GAGGAGCCATATGAAGTGAAAAT	$(AG)_{26}$	248
	R	TCGGCCAGCAACAGATGC		
VM7	F	CGCTGGGGGTGGCTTAT	$(AG)_{13}$	158

Primer		Primer sequence (5'-3')	SSR sequence	Predicted size (bp)*
	R	AATTCGACTTTCTGTTTACTTG		
VM8	F	TGGGATGCTGCAAAGACAC	$(AG)_{16}$	295
	R	GAAAACCGATGCCAAATAG		
VM9	F	ACCGCACCCGATTTATTTCAT	$(CT)_{21}$	271
	R	ATCAGCAGACAGGCAAGACCA		
VM10	F	TCCCACTCACTAAAATAACCAACC	$(AC)_3(CT)_{10}(AC)_3$	278
	R	GGATGCTGGCGGCGGAAGG		
VM11	F	CGGGAATTAACGGAGTCACC	$(AT)_{4..}(AC)_{12}$	195
	R	CCCAGAGGCCGCTATTACAC		
VM12	F	TTGTCAGCGAAATAAGCAGAG	$(AG)_{27}$	157
	R	CAACAGCAGACGCCCAACT		
VM13	F	CACCCGTGATTGCTTGTTG	$(CT)_{21}$	135
	R	GTCCCCTCCCTCCCACTG		
VM14	F	AATTCGTGGCATAGTCACAAGAGA	$(AG)_{24}$	144
	R	ATAAAGGAGGGCATAGGGAGGTAT		
VM15	F	CGGCTGCAGCAAACAAGAG	$(AG)_{4..}(GT)_{10}$	162
	R	AAACCCGTGCAAGAAACCAA		
VM16	F	TCCTCGTCCATCTTCACCTCA	$(CT)_7\ldots(CT)_7$	203
	R	CAAGCACCGCATTAAAGTCAAG		
VM17	F	GGCCTATAAATTAACCCAGTCT	$(CT)_{12}$	152
	R	TGTGTCTTTGAGTTTTTGTTCTAC		
VM18	F	AGCCGTGCACGAATGAT	$(GA)_{13}$	257
	R	TGGCCTCTACAACAACACTCT		
VM19	F	TATTCATGCGTGACACTA	$(AC)_7\ldots(AC)_5$	241
	R	TCGTGGCACCCCCTATC		
VM20	F	GGGGACCAATCGTTTCGTTC	$(GT)_{17}$	246
	R	ATCCAAGATTCGGACACTATTCAA		
VM21	F	TAGCAACTGTCTAAGCCTCA	$(AT)_{17}$	179
	R	CCAACTTAACCATCACTCAC		
VM22	F	GCGGGTAGTGTATACAATTTG	$(AG)_{12}$	217
	R	GTACTGTTCCATGGAAGATCT		
VM23	F	AGACATGTGGGCGCATCTG	$(CT)_{16}$	174
	R	AGACGCGTGGTACCCATGTT		
VM24	F	TCAACAACACCTAGGAGCCAA	$(AG)_{15}$	144
	R	ATCGTGACCTAGTGCCCACC		
VM25	F	CCACAATCACCGATGTCCAA	$(TC)_{18}$	240
	R	CAATTCCACTGCGGGACATAA		
VM26	F	GGCATCAGACACATATCACTG	$(TC)_{14}$	294
	R	TGTGGCATTGAGGGTAGC		
VM27	F	GTCCAAAGCAAATGAGTCAA	$(AAT)_{5..}(TC)_{14}(AC)_3$	207
	R	TGAATGACAATGAGGGTGC		
VM28	F	GAATGAGAGAAGTTACGGTG	$(TC)_{20}$	250
	R	GAGCACGATAATATTTGGAG		
VM29	F	CGTGACACTAATAGTAGTCC	$(TC)_{11}$	295
	R	CGAGTCTCGGACTCGCTT		
VM30	F	CTCTTTCGCGTTCCACACTT	$(TC)_{10}$	140
	R	GCAATGGGTTGTGGTCTGTG		
VM31	F	CGCTCTTCGTTGATGGTTATG	$(CT)_{16}$	200
	R	GAAAAAGGGAGGAACAAGCACAAC		
VM32	F	GCACGAGATCTGGTGCTCCTT	$(AG)_{10}$	177
	R	AGCGAAAACACGGAACTGAAATC		
VM33	F	GCACGAGATCTGGTGCTCCTT	$(AG)_{18}(AC)_8$	270
	R	CAGCGAGCGCGAACC		

Primer		Primer sequence (5'-3')	SSR sequence	Predicted size (bp)*
VM34	F	AGCTCCCCTAACCTGAAT	$(CT)_{14}$	216
	R	TAACCCAATAATAAGACACAT		
VM35	F	GGTCAATAGAATGGAAAGTGT	$(AG)_{11}(T)_9$	127
	R	ATGGCTGAAATAGGTGTCTGA		
VM36	F	ACTTTCTGTTTTACTCGACAACTC	$(CT)_{13}$	160
	R	GTCGCTGGGGGTGGCTTATT		
VM37	F	TGTCCGCGTTCTATAAATCAGC	$(AG)_5(CCT)_3\ (CT)_{13}$	289
	R	CGAGGATGAAGTAACAGATGATC		
VM38	F	AATGGGAAAAGAAAGGGAAGC	$(AG)_{10}(AC)_5$	135
	R	TCGTGGCATGCAGTGTCAG		
VM39	F	GATGGTTGTAATGGGAGAGTC	$(AC)_{13}(AT)_5(TACA)_4$	212
	R	AAAAGGATGAAATTAGGAGAGCA		
VM40	F	TATTACGAGAGGCTATTTATTGCA	$(AC)_{18}$	200
	R	CTCTAACACCTCAAGTTAGTGATC		

** The predicted size was determined from the sequencing results of the isolated clones. F, R: Forward and reverse sequences*

Table 2. Summary of SSR primer pairs specific for moth bean.

Primer	Accession	Primer sequence (5'-3')	SSR motif	Predicted size (bp)*
AGB1	AG1	CATGCAGAGGAAGCAGAGTG	$(GA)_8GGTA(GA)_5GGGGACG(AG)_4$	132
	AF48383	GAGCGTCGTCGTTTCGAT		
AGB2	GATS11	CACATTGGTGCTAGTGTCGG	$(CT)_8CA(CT)_2GTTT\ (CT)_4$	306
	AF48384	GAACCTGCAAAGCAAAGAGC		
AGB3	GATS11B	CCCACACATTGGTGCTAGTG	$(CT)_8$	160
	AF48384	AGCGCAATGCTACTCGAAAT		
AGB4	GATS54	GAACCTGCAAAGCAAAGAGC	$(GA)_5AACAGAGT\ (GA)_8$	114
	AF48384	TCACTCTCCAACCAGATCGAA		
AGB5	GATS91	GAGTGCGGAAGCGAGTAGAG	$(GA)_{17}$	229
	AF48384	TCCGTGTTCCTCTGTCTGTG		
AGB6	BM3	CAGAAGTGCTTATCCCCGAG	$(GAA)_3GATGAA\ (GCA)_2(GAA)_4$	193
	AF48384	TGAAATCTTCCCCTCCTTCA		
AGB7	BM6	AGGGTTTACACACGACAGGC	$(GAAAA)_3$	153
	AF483844	GGTTGATATGCCCTCATGGT		
AGB8	BM16	CACCGGGAGTGGCTGACA	$(CA)_{21}TA(CA)_5$	149
	AF483845	GTTTGGGGCGGAGTTCGA		
AGB9	BM20	ATCCGTAGAGAGGTGAACGG	$(CAGA)_3GACA\ (CAGA)_{12}$	146
	AF483846	ATGAGTGCAGTTTGGTGCAG		
AGB10	BM25	CGCCTCCAACGGTCTTCT	$(CA)_{17}CG(CA)_2$	227
	AF483847	CAAGCAGGTGCGAATCCA		
AGB11	BM48	GCCGTTGAGCTGGAGAGCA	$(GA)_5$	232
	AF483848	CCTTCTTCTTGAGCCCGCTG		
AGB12	BM53	AACTAACCTCATACGACATGAAA	$(CT)_{21}(CA)_{19}(TA)_9$	287
	AF483849	AATGCTTGCACTAGGGAGTT		

Primer	Accession	Primer sequence (5'-3')	SSR motif	Predicted size (bp)*
AGB13	BM67	CCAATGCTGCCACACAGATA	$(CA)_{31}(CG)_5(CA)_{10}$	289
	AF483850	CGCCCTTATGATCCAGTCCT		
AGB14	BM68	TTCGTTCACAACCTCTTGCATT	$(CA)_6TA(CA)_4(TA)_4(CA)_5$	170
	AF483851	TGCTTGTTATCTTGCCCAGTG		
AGB15	BM79B	CATGGAGGTAGAGGATAATAAGGAG	$(GA)_{28}$	125
	AF483852	CATTAGAGCCGCCACTTG		
AGB16	BM98	GCATCACAAAGGACTGAGAGC	$(CA)_8(CT)_3$	247
	AF483853	CCCAAGCAAAGAGTCGATTT		
AGB17	BM114	AGCCTGGTGAAATGCTCATAG	$(TA)_8(GT)_{10}$	234
	AF483854	CATGCTTGTTGCCTAACTCTCT		
AGB18	BM137	CGCTTACTCACTGTACGCACG	$(CT)_{33}$	155
	AF483855	CCGTATCCGAGCACCGTAAC		
AGB19	BM138	TGTCCCTAAGAACGAATATGGAATC	$(GT)_{13}$	203
	AF483856	GAATCAAGCAACCTTGGATCATAAC		
AGB20	BM139	TTAGCAATACCGCCATGAGAG	$(CT)_{25}$	115
	AF483857	ACTGTAGCTCAAACAGGGCAC		

**The predicted size was determined from the sequencing results of the isolated clones.*

Microsatellite marker analysis

Sixty SSR primer pairs were used to detect polymorphism between standard resistant (GC-3) and susceptible (Chirodi) cowpea genotypes. The polymorphic markers were then used to carry out PCR for all individuals of 40 cowpea lines to detect the resistance genes. PCR for the amplification of template DNA was performed in PTC 100TM thermo-cycler (MJ Research Inc., Watertown, MA, USA). Total volume of PCR reaction mixture was made to 20 µl, which contained 1x PCR buffer, 200 µM dNTPs, 0.5 µM of primer (both), 2 mM $MgCl_2$, 2.0 units of *Taq* polymerase and 50 ng of template DNA. PCR conditions for the microsatellite analysis included an initial pre-denaturation step of three minutes at 94^0C and following 30 cycles of 92^0C for 1 min (denaturing), 55^0C for 1.5 min (annealing) and 72^0C for 1.5 min (extension), with the final step of extension was carried out at 72^0C for 10 minutes.

The PCR products were separated on 3% agarose gel by electrophoresis. The polymorphism information content (PIC) of each microsatellite was calculated based on allele pattern of all the genotypes as described by Weir (1996). PIC = 1-ΣP_i^2, where P_i is the frequency of the i^{th} allele in the test lines. NTSYSpc (version 2.0) was used to calculate the genetic similarity (Jaccard's coefficient), principal coordinate, and cluster analyses (Unweighted Paired Group Method Using Arithmetic Averages).

RESULTS

Polymorphism of microsatellites in 40 cowpea lines

To detect polymorphism between resistant and susceptible cowpea genotypes, genomic DNA of standard resistant variety (GC-3) and susceptible variety (Chirodi) were first used as template for PCR amplification using SSR markers. Among 60 SSR markers used, 40 cowpea specific SSR primer pairs showed amplification, 18 primer pairs out of 20 moth bean SSR primers failed to give amplification and only two primer pairs (AGB1 and AGB16) showed amplification. These 42 SSR primer pairs produced 110 amplified fragments, in which only 9 polymorphic bands were obtained. The number of alleles ranged from one (monomorphic primer pair) to seven (data not shown).

Out of 42 SSR primer pairs which showed amplification, four SSR markers gave clearly polymorphic bands on the 3 per cent agarose gel (Fig. 1). These 4 primer pairs with their polymorphic alleles, monomorphic alleles and polymorphism information content (PIC) listed in the Table 3, were used to analyze 40 cowpea genotypes. The number of polymorphic alleles ranged from 2 to 3 with the average of 2.25. The polymorphism information content varied from 0.30 to 0.72 with the average of 0.54.

Table 3. Polymorphism in cowpea genotypes as revealed by SSR primers

SSR primers	Total no. of alleles	Polymorphic alleles	Monomorphic alleles	Percent polymorphism (%)	PIC value
VM1	3	2	1	66.7	0.72
VM3	2	2	0	100.0	0.30
VM31	2	2	0	100.0	0.45
AGB1	4	3	1	75.0	0.67
Average	2.75	2.25	0.5	85.43	0.54

Genetic diversity of the cowpea lines

As reported earlier, SSR markers showed high levels of polymorphism in cowpea (Li *et al*., 2001). In the present study it was confirmed that SSR markers could distinguish CYMV resistant lines in cowpea. In fact, four polymorphic microsatellites were able to distinguish 13 to 17 resistant lines out of the 20 resistant genotypes (data not shown). Among these 4 SSR markers, moth bean designed SSR marker AGB1 (AG1/AF48383), amplified 100 bp allele in 17 of 20 susceptible genotypes. Three genotypes HC98-08, HC2-62 and FS-68 (S2, S6 and S10) did not show 100 bp fragment amplification (Figure 2). In the other group of 20 resistant genotypes, 15 genotypes did not show amplification of 100 bp allele. However, in three genotypes HC98-50, HC98-63 and HC1-10 (R7, R10 & R16, respectively) amplification of 100 bp allele was detected.

A dendrogram of the 40 cowpea lines was constructed by the Unweighted Paired Group Method Using Arithmetic Averages on the basis of the genetic similarity (Jaccard's coefficient) (Fig. 3). In the population of 20 CYMV resistant lines and 20 CYMV susceptible line of cowpea, it was expected that the lines shared the common genetic similarity within resistant and susceptible genotypes. In fact, groupings of the 40 cowpea lines based on microsatellite polymorphisms generally agreed with the resistant phenotype of these lines. Two major groups separated at 45% similarity. Among 20 resistant cowpea lines anlysed, 18 lines shared 77% to 100% similarity, and 10 cowpea lines showed 100% similarity. These 10 genotypes were HC98-30, CS88, HC98-45, HC98-58, HC98-64, HC1-3, HC2-9, HC2-11, CPD26-0 and HC1-14 (R1, R3, R4, R9, R11, R12, R13, R14, R15 and R18, respectively). The other two resistant lines were classified in a separate group with one susceptible line and joined with resistant group at 47% similarity. The susceptible group consisted of two subgroups with 71% similarity for 7 cowpea lines (HC3-25, HC3-39, HC3-22, HC3-30, HC3-2, HC3-31, HC3-29), and 77% similarity among 12 cowpea lines (HC97-39, HC9B-28, HC2-59, HC2-72, HC2-85, HC98-08, HC2-62, HC2-61, HC2-69, HC3-40, HC2-87, HC3-21). The same pattern of similarity between resistant and susceptible groups was observed in two-dimensional (Fig. 4) and three-dimensional principle coordinate analysis (Fig. 5). There were also two separate groups of resistant and susceptible genotypes on the dendrogram with resistant genotypes HC1-19, HC98-33, HC1-10, HC1-11, HC1-14 and HC1-15 (R20, R2, R16, R17, R18 and R19, respectively) lying closer to each other in one group, and the susceptible genotypes HC2-62, HC2-72, HC2-85, HC2-87, HC3-22, HC3-25, HC3-30, HC3-31, HC3-39 and HC3-40 (S6, S8, S9, S11, S14, S15, S17, S18, S19 and S20, respectively) could be placed in another group. Cowpea genotypes

HC98-48, HC98-51, HC98-63, FS-68 and HC3-29 (R6, R8, R10, S10 and S16) were separate from these two groups.

DISCUSSION

Microsatellite markers have been used to detect polymorphism in many important crop species. They have reported to show high levels of polymorphism in different genotypes of crops such as rice (*Oryza sativa* L., Chen *et al*., 1997), wheat (*Triticum aestivum* L., Devos *et al*., 1995), barley (*Hordeum vulgare* L., Liu *et al*., 1996), oat (*Avena sativa* L., Li *et al*., 2000), maize (*Zea mays* L., Senior *et al*., 1998), sorghum [*Sorghum bicolor* (L.) Moench, Brown *et al*., 1996], soybean [*Glycine max* (L.) Merr., Akkaya *et al*., 1992], beans (*Phaseolus* and *Vigna*, Yu *et al*., 1999), *Brassica* species (Szewc-McFadden *et al*., 1996), alfalfa (*Medicago* spp., Diwan *et al*., 1997), sun-flower (*Helianthus annuus* L., Brunel, 1994), tomato (*Lycopersicon esculentum* Mill., Smulders *et al*., 1997), and cowpea (*Vigna unguiculata* L. Walp, Li *et al*., 2001).

The present study showed that microsatellite markers could be used to distinguish CYMV resistant lines in cowpea.. All the microsatellite primer pairs of cowpea could successfully amplify DNA from 40 cowpea lines. In fact, four polymorphic microsatellites markers were also able to distinguish 13 to 17 resistant lines out of the 20 resistant genotypes Furthermore, two microsatellite primer sets designed from the sequences of moth bean (AG1/AF48383 and BM98/AF483853; AGB1 and AGB16) were able to amplify DNA of cowpea in which AG1/AF48383 (AGB1) could distinguish 15 resistant lines among 20 resistant genotypes investigated. Therefore, microsatellite markers of cowpea could be used to detect CYMV resistance genes and map these genes to cowpea linkage map. In addition, these microsatellite primers could be used for comparative genome analysis between the different *Vigna* species.

To differentiate the genetic basis of a number of cultivars in the same species, microsatellite markers could also be used. Application of SSR markers have been used to estimate the genetic diversity of a large number of cultivars in rice (Yang *et al*., 1994), soybean (Rongwen *et al*., 1995), wheat (Plaschke *et al*., 1995), maize (Senior *et al*., 1998), and cowpea (Li *et al*., 2001). The number of alleles amplified per primer pair was from 3 to 25 for rice, 11 to 26 for soybean, 3 to 16 for wheat, 2 to 23 for maize and 2 to 7 for cowpea. In the present study, similar results were obtained as reported earlier (Li *et al*., 2001). It was observed that only one to seven alleles per primer pair were amplified from the 40 cowpea lines (In the present study, microsatellite bands were detected on 3% agarose gel by electrophoresis). This showed that the level of microsatellite polymorphism in cowpea is much lower than other crops. The materials used in the present study were all from the pure line of cowpea created and maintained in Haryana Agricultural University (HAU) and thus had a relatively narrow genetic base. In a study on genetic diversity in soybean, 11 to 26 alleles per microsatellite primer pair were amplified from 96 soybean genotypes while this number was reduced to five to 10 alleles per primer pair in 26 cultivars from North American breeding programs (Rongwen *et al*., 1995).

The other possible reason for the low level of microsatellite polymorphism is that the cultivated cowpea is relatively low in genetic diversity compared with other crops. It has been suggested that cowpea was only domesticated one (Pasquet. 1999), unlike *P. vulgaris* (Singh *et al*.. 1991) or rice (Second. 1981). The low genetic diversity in cultivated cowpea may be a result of this narrow genetic base.

The low level of genetic diversity among cowpea breeding lines at the DNA level and cultivars could be increased by using its wild relatives to broaden the genetic base. Li *et al*. (2001) demonstrated that microsatellite markers were conserved among *Vigna* species. Hence microsatellite markers could provide a simple approach to assaying the introduction of such genetic material.

Polymorphic information content (PlC) provides an estimate of the discriminatory power of a locus by taking into account, not only the number of alleles that are expressed, but also the relative frequencies of those alleles (Smith *et al*., 1997). PlC values range from 0 (monomorphic) and 1 (very high discriminative, with many alleles in equal frequencies). Senior *et al*. (1998) reported that PlC is synonymous with the term “gene

diversity" as described by Weir (1996). The PIC value of SSR markers in the present study was not very high and ranged from 0.30 to 0.72 but only four out of 42 SSR primer pairs gave polymorphism. The PIC values of SSR markers can be compared to results reported by Li *et al*. (2001) with PIC ranged from 0.02 to 0.73. Presently, even poly-acrylamide gels were used to detect the DNA alleles, the polymorphic information could not be compared with other crop species.

Groupings of the 40 cowpea lines based on microsatellite polymorphisms generally agreed with the CYMV resistant phenotype of these lines. Two major groups separated at 45% similarity (Fig. 4). The resistant group including 18 resistant lines with 77% to 100% similarity. The other two resistant lines were classified in a separated group with one susceptible line placed with resistant group at 47% similarity. The susceptible group consisted of two subgroups with 71% and 77% similarity within each subgroup.

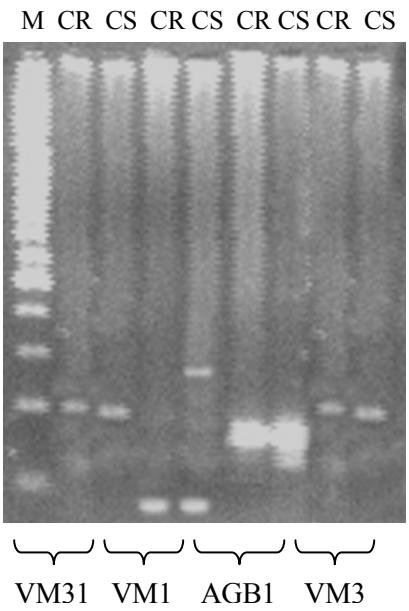

Figure 1. Polymorphic pattern of VM31, VM1, AGB1 and VM3 PCR products between standard resistant and susceptible varieties.
Lane M: 100bp DNA ladder;
CR: check (standard) resistant variety (GC-3)
CS: check (standard) susceptible variety (Chirodi)

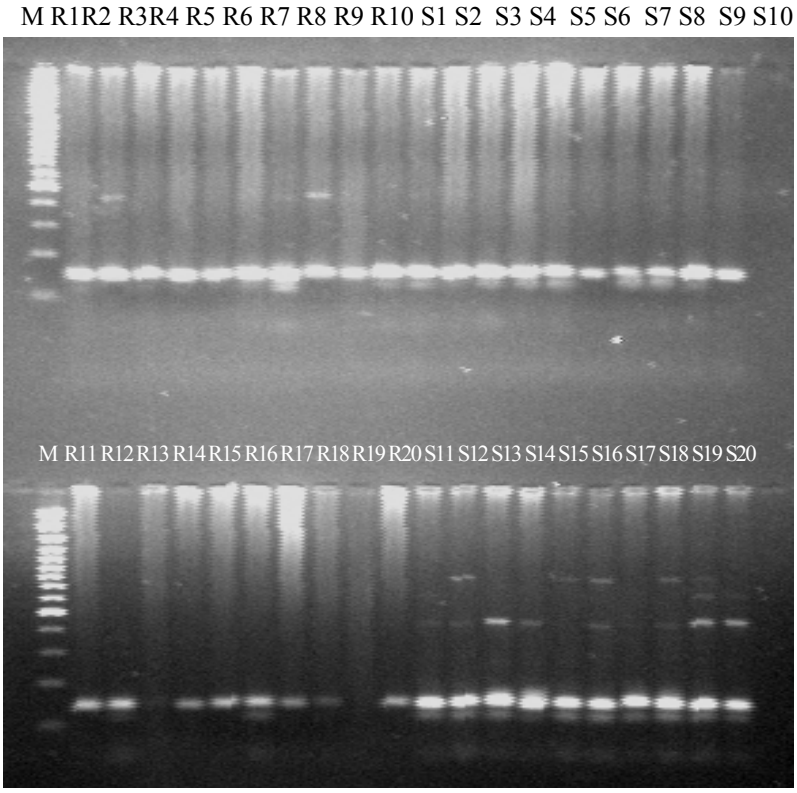

Figure 2. Electrophoresis pattern of PCR amplified fragments of 20 susceptible and 20 resistant genotypes with SSR marker AGB1

Lane M: 100bp DNA ladder;
Upper lanes R1-10: resistant genotypes, S1-10: susceptible genotypes
Lower lanes R11-20: resistant genotypes; S11-20: susceptible genotypes

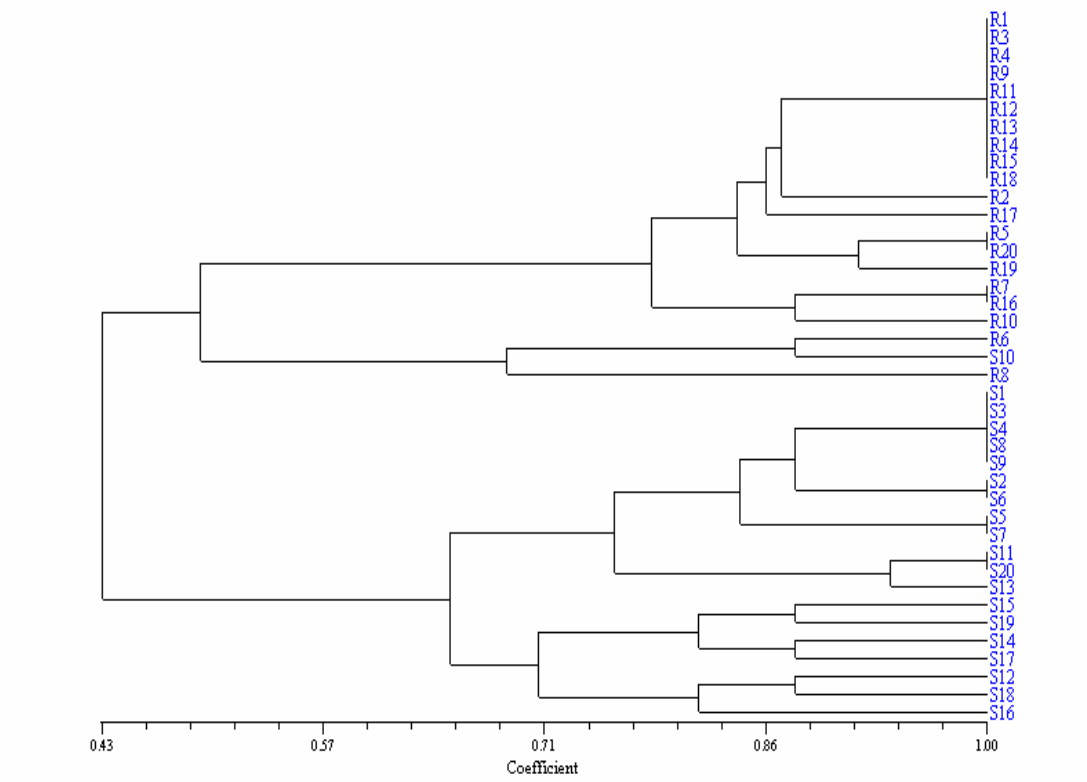

Figure 3. Phylogenetic relationship of the 40 cowpea lines constructed using four polymorphic microsatellites.

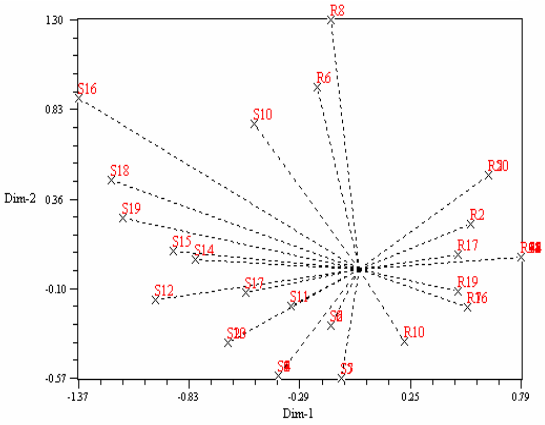

Figure 4. Two-dimension principle oordinate analysis of the 40 cowpea lines

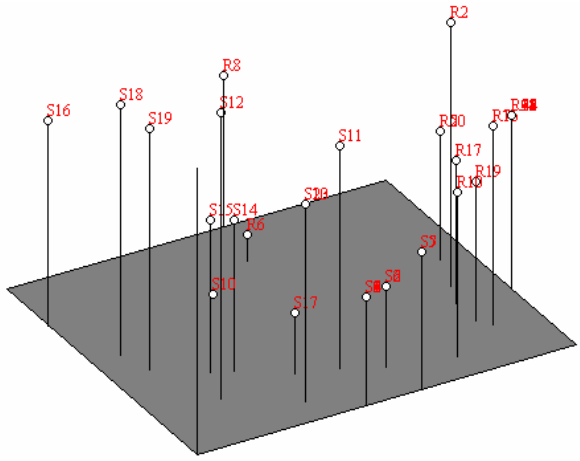

Figure 5. Genetic similarity among 40 cowpea genotypes revealed by three-dimensional view of dendrogram

Comparison of the dendrogram produced by the present study with that constructed using 90 cowpea breeding lines and one wild relative as reported by Li et al. (2001), the genetic similarity concentrated into two groups of cowpea lines resistance to CYMV disease and susceptible ones, but not disperses discrepancies and incongruities as the earlier dendrogram. This agreement showed that microsatellite marker can be used to estimate the genetic basis of CYMV resistance in cowpea.

In conclusion, microsatellite markers are polymorphic in cowpea. They can be used to distinguish CYMV resistant lines of cowpea from susceptible lines. A dendrogram constructed based on microsatellite polymorphism generally agreed with the reaction of cowpea lines with CYMV disease. The degree of the polymorphism is relatively low in cowpea compared with other crops.

Acknowledgement

This research was supported by a grant from 'PhD. scholarship between Vietnam-India Culture Exchange Program'. The first author TDGioi is grateful to Indian Council for Cultural Relations, Azad Bhawan, New Delhi for providing financial support during PhD. Degree programme.

REFERENCES

Akkaya MS, AA Bhagwat, and PBCregan. 1992. Length polymorphisms of simple sequence repeat DNA in soybean. *Genetics*. **132**: 1131-1139.

Ayres NM, AM McClung, PD Larkin, HFJ Bligh, CA Jones, and WD Park. 1997. Microsatellite and a single nucleotide polymorphism differentiate apparent amylose classes in an extended pedigree of US rice germplasm. *Theor. Appl. Genet.* **94**:773–781.

Blair M, and SR McCouch. 1997. Microsatellite and sequence tagged site markers diagnostic for the bacterial blight resistance gene, *xa-5*. *Theor. Appl. Genet*. **95**:174–184.

Brown SM, MS Hopkins, SE Mitchell, ML Senior, TY Wang, R Powell, R Duncan, F Gonzalez-Candelas, and S Kresovich. 1996. Multiple methods for the identification of polymorphic simple sequence repeats (SSRs) in sorghum [*Sorghum bicolor* (L.) Moench]. *Theor. Appl. Genet*. **93**:190–198.

Brunel D. 1994. A microsatellite marker in *Helianthus annuus* L. *Plant Mol. Biol*. **24**: 397–400.

Chant SR. 1960. The effect of infection with tobacco mosaic and cowpea yellow mosaic viruses on the growth rate and yield of cowpea in Nigeria. *Emp. J. Exp. Agric*. **28**: 114-120

Chen X, S Temnykh, Y Xu, YG Cho, and SR McCouch. 1997. Development of a microsatellite framework map providing genome-wide coverage in rice (*Oryza sativa* L.). *Theor. Appl. Genet*. **95**:553–567.

Devos KM, GJ Bryan, AJ Collins, P Stephenson, and MD Gale. 1995. Application of two microsatellite sequences in wheat storage proteins as molecular markers. *Theor. Appl. Genet*. **90**:247–252.

Diwan N, AA Bhagwat, GB Bauchan, and PB Cregan. 1997. Simple sequence repeat DNA markers in alfalfa and perennial and annual Medicago species. *Genome*. **40**:887–895.

Gilmer RM, WK Whitney, and RJ Williams. 1974. Epidemiology and control of cowpea mosaic in Western Nigeria. In pro. 1st IITA Grain legume improvement workshop. International Institute of Tropical Agriculture, Ibadan, Nigeria. Pp: 325.

Hall AE and PN Patel. 1985. Breeding for resistance to drought and heat. In: S.R. Singh and K.O. Rachie (Eds.). Cowpea research, production and utilization. Wiley, New York. pp: 137-151

Kelly JD, and PN Miklas. 1998. The role of RAPD markers in breeding for disease resistance in common bean. Molecular breeding: new strategies in plant improvement **4**: 1-11

Lee M. 1995. DNA markers and plant breeding programs. *Adv. Agron*. **55**:265-344

Li CD, CA Fatokun, U Benjamin, BB Singh, and GJ Scoles. 2001. Determining Genetic Similarities and Relationships among Cowpea

Breeding Lines and Cultivars by Microsatellite Markers. *Crop Sci*. **41**:189-97

Li CD, BG Rossnagel, and GJ Scoles. 2000. The development of oat microsatellite markers and their use in identifying Avena species and oat cultivars. *Theor. Appl. Genet*. **101**:1259-1268.

Liu ZW, RM Biyashev, and MA Saghai Maroof. 1996. Development of simple sequence repeat markers and their integration into a barley linkage map. *Theor. Appl. Genet.* **93**:869-876.

Mali VR and G Thottappilly. 1986. Virus disease of cowpeas in the tropics. pp. 361-403. In: Review of Tropical Plant Diseases. Raychandhuri, S.P and Varman, J.P. (Eds). Volume. 3.

Murray, H.G., and W.F. Thompson. 1980. Rapid isolation of higher weight DNA. *Nucleic Acids Res*. **8**: 4321-4325.

Pasquet, R.S. 1999. Genetic relationships among subspecies of *Vigna unguiculata* (L.) Walp. Based on allozyme variation. *Theor. Appl. Genet*. **98**:1104-1119.

Plaschke J, MW Ganal, and MS. Roder. 1995. Detection of genetic diversity in closely related bread wheat using microsatellite markers. *Theor. Appl. Genet*. **91**:1001-1007.

Roder MS, V Korzum, K Wendehake, J Plaschke, MH Tixier, P Leroy, and MW Ganal,.1998. A microsatellite map of wheat. *Genetics*. **149**: 2007-2023.

Rongwen J, MS Akkaya, AA Bhagwat, U Lavi, and PB Cregan. 1995. The use of microsatellite DNA markers for soybean genotype identification. *Theor Appl. Genet.* **90**:43–48.

Saghai-Maroof MA, KM Soliman, RA Jorgensen, and RW Allerd. 1984. Ribosomal spacer length polymorphism in barley Mendelian inheritance, chromosomal location and population dynamics. *Proc. Natl. Acad. Sci*. USA. **81**: 8014-8019.

Second G. 1981. Evolutionary relationships in the *Sativa* group of *Oryza* based on isozyme data. *Genet. Sd. Evol*. **17**:89-114.

Senior ML, JP Murphy, MM Goodman, and CW Stuber. 1998. Utility of SSRs for determining genetic similarities and relationships in maize using an agarose gel system. *Crop Sci*. **38**:1088–1098.

Singh BB, OL Chambliss, and B Shamar. 1997. Recent advances in cowpea breeding. In Singh BB, Mohan Raj DR, Dashiell KE, Jackai Len (ed.). Advances in cowpea research. IITA-JIRCAS, Ibadan, Nigeria.

Singh SP, R Nodari, and P Gepts. 1991. Genetic diversity in cultivated common bean. 1. Allozymes. *Crop Sci*. **31**:19-23.

Smith JSC, EC Chin, H Shu, OS Smith, SJ Wall, ML Senior, SE Mitchell, S Kresovich and J Ziegle. 1997. An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays* L.) comparisons with data from RFLPS and pedigree. *Theor.Appl. Genet*. **95**: 163-173.

Smulders MJM, G Bredemeijer, W Rus-Kortekaas, P Arens, and B Vosman. 1997. Use of short microsatellites from database sequences to generate polymorphisms among *Lycopersicon esculentum* cultivars and accessions of other *Lycopersicon* species. *Theor. Appl. Genet*. **97**:264-272.

Steele WM. 1972. Cowpea in Africa. Doctoral thesis. University of Reading, United Kingdom.

Szewc-McFadden AK, S Kresovich, SM Bliek, SE Mitchell, and JR McFerson. 1996. Identification of polymorphic, conserved simple sequence repeats (SSRs) in cultivated Brassica species. *Theor. Appl. Genet*. **93**:534–538.

Thottappilly G and HW Rossel. 1985. Worldwide occurrence and distribution of virus Dis., pp: 155-171. In: Cowpea Research, Production and Utilization. Singh, S.R. and Rachie, K.O (Eds.). Chichester: John Wiley and Sons, England.

Weir BS. 1996. Genetic data analysis II. 2nd ed. Sinaeur Associates Inc., Sunderland, MA.

Weising K, P Winter, B Huttel, and G Kahl. 1998. Microsatellite markers for molecular breeding. *J. Crop Prod*. **1**:113-143.

Williams RJ. 1977. Identification of multiple disease resistance in cowpea. *Trop. Agric*. **54** (1): 53-59.

Wu KS and SD Tanksley. 1993. Abundance, polymorphism and genetic mapping of microsatellites in rice. Mol. Gene. Genet. **241**: 225-235.

Xiao J, J Li, L Yuan, SR McCouch, and SD Tanksley. 1996. Genetic diversity and its relationship to hybrid performance and heterosis in rice as revealed by PCR-based markers. *Theor. Appl. Genet*. **92**:637–643.

Xu GW, CW Magill, KF Schertz, and GE Hart. 1994. A RFLP linkage map of *Sorghum bicolor* (L.) Moench. *Theoretical and Applied Genetics*. **89**(2-3):139 – 145.

Yang GP, MA Saghai Maroof, CG Xu, QF Zhang, and RM Biyashev. 1994. Comparative analysis of microsatellite DNA polymorphism in landraces and cultivars of rice. *Mol. Gen. Genet*. **245**:187-194.

Young ND. 1999. A cautiously optimistic vision for marker-assisted breeding. *Mol. Breed*. **5**: 505-510

Yu K, SJ Park, and V Poysa. 1999. Abundance and variation of microsatellite DNA sequences in beans (*Phaseolus* and *Vigna*). *Genome*. **42**:27-34.

Nghiên cứu tương quan về di truyền giữa các dòng cowpea kháng bệnh khảm virus bằng microsatellites

Microsatellite markers được sử dụng để nghiên cứu di truyền tính kháng bểnh khảm do virus trên 40 dòng đậu cowpea (CYMV). Với 60 SSR primers, người ta xem xét tính đa hình giữa dòng kháng (GC-3) và dòng nhiễm (ChrodiChỉ có 4 primers biểu thị đa hình và 4 SSR primer này được sử dụng để tìm kiếm dòng kháng CYMV trong 40 giống cowpea thí nghiệm. Thông tin đa hình (PIC) của những SSR markers này biến thiên từ 0,30 đến 0,72. Giản đồ phân nhóm kiểu gen trên cơ sở microsatellite thống nhất với kiểu hình kháng CYMV. Kết quả cho thấy có 2 nhóm di truyền ở mức độ tương đồngt 45%. Nhóm giống kháng bao gồm 18 dòng cowpea ở mức tương đồng 77% đến 100%, trong đó 10 kiểu gen đạt 100% tương đồng. Hai dòng kháng bệnh virus cũng được xếp nhóm khác với nhóm nhiễm ở mức độ tương đồng 47%. Nhóm nhiễm được chia thành 2 nhóm phụ ở mức tương đồng là 71% và 77%.