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Mapping New Genetic Markers Associated with CMD Resistance in Cassava (*Manihot esculenta* Crantz) Using Simple Sequence Repeat Markers

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Abstract

Cassava mosaic disease (CMD) is the most serious disease in cassava–in India where it is grown for food, starch and sago purpose. The disease is best kept under control by exploiting the available host plant resistance, which was introgressed from *M. glaziovii* to cassava and it is known to be polygenic control. In the present study, an attempt was made to construct the genetic linkage map of cassava using SSR markers with the objective of mapping genes associated with CMD. Using single marker analysis (SMA), four CMD resistance markers were detected *viz*. SSRY28, SSRY235, SSRY44 and NS136. SSRY28 and SSRY235 were located on linkage group G and SSRY44 and NS136 on linkage group P of cassava genetic map developed by Fregene et al. (1997). Among the four markers, three (SSRY235, SSRY44 and NS136) are new markers associated with CMD resistance. The detection of markers SSRY44 and NS136 having association with CMD resistance is a new report indicating the possibility of having another genetic loci for CMD resistance in cassava in addition to the already established on linkage group G. This finding supports the polygenic control of CMD resistance.

Keywords: cassava, SSR marker, CMD, linkage map, F_1 mapping population, ICMV virus

1. Introduction

Cassava, *Manihot esculenta* Crantz., belonging to the family Euphorbiaceae, is one of the most important staple food crops in tropics and grown widely under diverse environmental conditions. This tuberous root crop has its origin in South America. It remains as the most reliable source of food for more than 700 million subsistence farmers in Africa, Asia and Latin America. The sub-Saharan Africa- currently accounts for 54% of the total world production of cassava (FAO, 2005). Cassava, in its calorie contribution in tropics, remains only fourth after rice, sugarcane and corn with a production of 202 million tonnes. India ranks first and seventh in the productivity (28 t/ha) and production (7 million tonnes) respectively in the world. Cassava is monoecious with 36 chromosomes, and is highly heterozygous due to its out-crossing nature. The crop is affected by various diseases and pests. Among them, cassava mosaic disease (CMD) is the most serious disease in Africa and India, causing yield loss ranging from 20 to 90 per cent. CMD is a viral disease caused by at least seven different geminiviruses. Transmitted of the virus by the vector whitefly, *Bemisia tabaci* is an extent of 5 per cent only. But the major spread of this disease is due to propagation of virus infected planting material.

The disease is best kept under control by exploiting the available host plant resistance, which was introgressed from *M. glaziovii* to cassava. The studies conducted by Hahn, Howland, and Terry (1980a); Hahn, Terry, and Leuschner (1980b) to establish the genetics of resistance to CMD indicated the possibility of several genes responsible for resistance. Akano, Dixon, Mba, Barrera, and Fregene (2002) reported that the resistance could be due to a major dominant gene (*CMD2*). Given the importance of *CMD2* to cassava production, and the limited knowledge on host plant resistance to geminiviruses, an effort was initiated to clone the gene. A bacterial artificial chromosome (BAC) library was constructed from the Nigerian variety, TME3, from which *CMD2* was first identified, and a fine map constructed around the region of the gene. Serial analysis of gene expression (SAGE) in

cassava revealed many differentially expressed genes of which beta-tubulin, elongation factor, importin, a transcription factor and rubridoxin were the most important (Anderson et al., 2004; Lopez et al., 2005).

The studies in the recent past on the genetics of resistance to CMD involving some of the African landraces and improved cassava clones indicated the possibility of several recessive genes responsible for CMD resistance (Lokko, Gedil, & Dixon, 2004; Lokko, Danquah, Offei, Dixon, & Gedil, 2005). Okogbenin et al. (2007) reported through marker assisted selection (MAS), the Latin American lines introgressed with dominant *CMD2* gene for cassava mosaic disease resistance. The resultant lines from introgression of the *CMD2* gene resulted in 14 genotypes combining CMD resistance and high yield are identified under African field conditions. The CMD resistance in two Nigerian cultivars *viz*., TMS 97/2205 and TMS 98/0505 was analyzed with SSR markers and in the field. Molecular data indicated, the CMD resistance in these lines was mediated by the *CMD2* gene. Okogbenin et al. (2012) reported a segregating F_1 population derived from a TMS 97/2205 x NR 8083 cross was screened using 530 SSR markers and identified (CMD3) a NS198 marker associated with CMD resistance, explaining 11% of the phenotypic variance. The combined QTL effect of CMD2 and CMD3 may account for the high level of resistance in TMS 97/2205.

Genetics of CMD resistance indicates the possibility of presence of several components responsible for CMD resistance. A better understanding on the components of resistance to CMD will be useful in breeding and in particular identifying genetic markers at DNA level to these individual components. The genetic approaches to mapping polyploid genomes with molecular markers have been reviewed by Ritter, Debener, Barone, Salamini, and Gebhardt (1991) and Wu et al. (1992). Single-dose restriction fragments (SDRF) is a class of marker useful for simplify the determination of allelism in highly heterozygous and polyploid crops (Wu et al., 1992). SDRFs are DNA markers that are present in one parent and absent in the other and segregate in a 1 : 1 ratio (absence: presence) in the progeny.

The overwhelming developments in molecular marker technology have generated various DNA marker systems. Among those systems, the SSR markers are being considered as the markers of choice. A total of 522 SSR markers were made available in cassava by Mba et al. (2001) and recently more SSR markers from new sources were available in CIAT (Fregene, Personal communication). The availability of these many markers in cassava will help to have genetic tags for various phenotypes in cassava. The objectives of the present study were to confirm the genetic locus already mapped and to identify new genomic region(s) for CMD resistance using SSR markers in a segregating population of CO2 x MNga-1.

2. Materials and Methods

2.1 Plant Materials

Two cassava varieties *viz*. MNga-1, a resistant variety and CO2, a susceptible variety, were selected based on field trials conducted at Central Tuber Crop Research Institute (CTCRI), Thiruvanthapuram and Tamil Nadu Agricultural University (TNAU), Coimbatore to develop the mapping population. CO2, a variety released from TNAU, Coimbatore, is highly susceptible to CMD, but possesses short plant type, middle branching, profuse flowering and good quality tuber with high starch content. MNga-1 (TMS30001) was developed at the IITA, Nigeria. It is a backcross derivative of cultivated cassava and wild *M. glaziovii*. It is a high yielding variety (29 t/ha) with tall plant type, top branching and good flowering.

2.2 Whitefly Vectors to Spread CMD

Whiteflies collected from the field were reared on tobacco under greenhouse conditions. The greenhouse reared whiteflies were released onto the susceptible parent CO2, grown under greenhouse conditions, to make the insects acquire the viral inoculum. The insects were allowed to feed on susceptible CO2, collected after 24 hrs and released on seedlings of CO2/MNga-1 in the greenhouse. The seedlings of susceptible CO2 infected with whiteflies were also planted along with F_1 seedlings to have the natural transmittance of virus across all the F_1 seedlings.

2.3 Screening for Resistance to CMD

The level of resistance to CMD resistance across F_1 seedlings of CO2 x MNga-1 was done six months after planting based on the scoring system adopted by Hahn et al. (1980b) and Akano et al. (2002). The level of resistance was determined based on the 1-5 scores established as per the severity of the symptoms developed, Score 1-Unaffected shoots, no symptoms in leaves; Score 2-Mild chlorosis, mild distortions at bases of most leaves, while the remaining parts of the leaves and leaflets appear green and healthy; Score 3-Pronounced mosaic pattern on most leaves, narrowing and distortion of the lower one-third of the leaflets; Score 4-Severe mosaic distortion of two thirds of most leaves and general reduction of leaf size and stunting of shoots and Score 5-Very

severe mosaic symptoms on all leaves, distortion, twisting, mis-shapen and severe leaf reductions of most leaves accompanied by severe stunting of plants. At six months stage all the seedlings were pruned and allowed to grow for three more months to record the severity of the symptom expression.

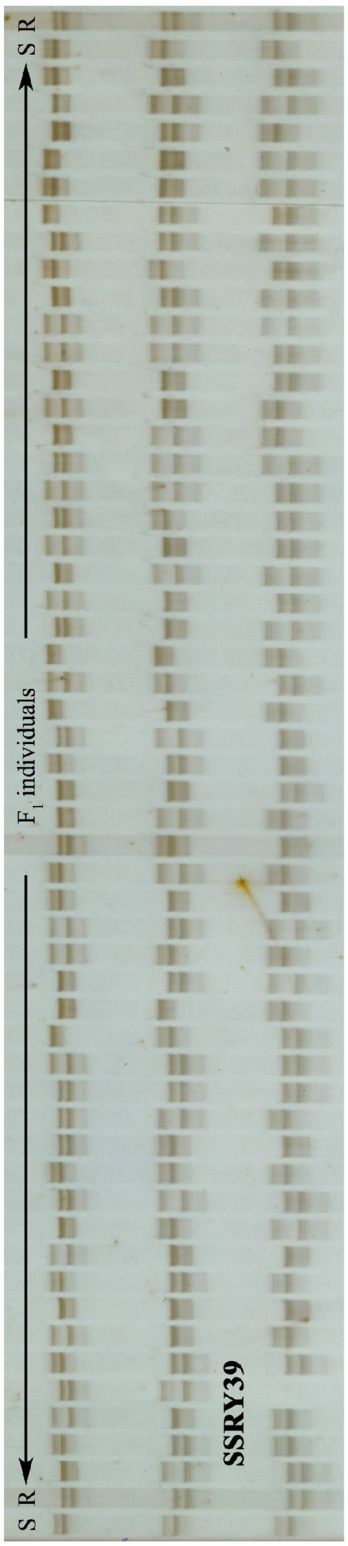
2.4 DNA Extraction

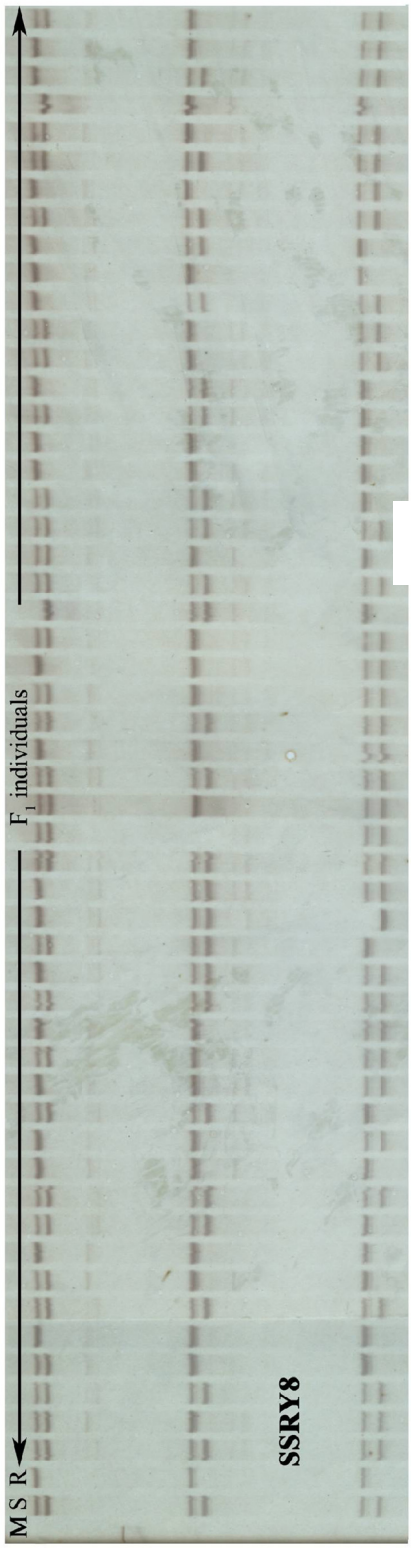
Genomic DNA of the parents and 141 F_1s were isolated from young fresh leaves adopting the procedure developed by Dellaporta, Wood, and Hicks (1983). The DNA of individual sample was quantified by using a fluorometer (DyNA Quant TM200, M/s Hoefer Pharmacia, Biotech Inc., USA) and its quality was checked on 0.8 per cent agarose gel. The final DNA concentration of all the samples was adjusted to 25 ng/µl.

2.5 Simple Sequence Repeat (SSR) Analysis

A total of 75 SSR primer pairs representing loci covering all 18 linkage groups as established by Mba et al. (2001) were synthesised from M/s Sigma Aldrich Inc. Out of 75 SSR primers, 19 primers were selected from linkage group G of Fregene et al. (1997) linkage map, where CMD resistant gene was already mapped. The primer pairs were used to identify polymorphic markers between CO2 and MNga-1. The SSR primer pairs producing polymorphic markers were surveyed on the seedlings of the 141 F_1 progenies to establish their segregation patterns because of the heterozygous nature of parents. PCR conditions were maintained as described by Mba et al. (2001). The PCR reaction was conducted in volumes of 20 µl containing 25 ng genomic DNA, 0.2 µM each of forward and reverse primers, 50 µM dNTPs, 1 X buffer (10 mM Tris-Hcl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$) and 0.3 Unit of Taq DNA polymerase (M/s Bangalore Genei Pvt. Ltd, Bangalore).

PCR amplifications were performed on a PTC100 (M/s MJ Research Inc.) Thermal Cycler with a PCR profile of 94ºC for 5 min followed by 30 cycles of 1 min at 94ºC, 2 min at 58ºC, and 2 min at 72ºC with a final extension for 5 min at 72ºC. A volume of 8 µl of loading buffer (98 per cent formamide, 10 mM EDTA, 0.005 per cent each of xylene cyanol and bromophenol blue as tracking dyes) was added to each of the amplified product and the denatured at 94ºC for 5 min, snap cooled using ice and separated on 5 per cent denaturing polyacrylamide gels (PAGE) containing 7 M urea at a constant current of 100 W. Multiplex loading of amplified products was followed based on the amplified product size range. Three sets of amplified products from the parents and F_1s were loaded at an interval of 15-30 mins when the amplified products were distinctly different for their size ranges. The patterns of amplified products across the samples were resolved by silver staining following procedure described by Panaud, Chen, and McCouch (1996) (Plate 1).





M : Marker S : CO2 R : MNga-1

Plate 1. Segregation pattern of SSRY39 and SSRY8 primers.

2.6 Data Scoring and QTL Analysis

The scoring of markers for each of the primer pairs was done as described by Wu et al. (1992). χ^2 test was carried out to establish the expected 1:1 ratio for all the SSR loci. Single marker analysis was performed by one way ANOVA to identify SSR markers associated with resistance to CMD. A significant F-test ($P < 0.01$) indicated association of marker locus with phenotype.

3. Results and Discussion

Out of 70 primer pairs, 51 primer pairs produced 89 possible patterns of segregating markers. These markers were established based on the method described by Wu et al. (1992) for SDRF markers. These 89 segregating markers were used for linkage map construction. Of the 89 markers, 9 markers (SSRY23c, SSRY23d, SSRY1, SSRY4d, SSRY20b, SSRY24a, SSRY24c, SSRY22 and SSRY303c) deviated from the regular 1:1 segregation pattern and these nine markers were not considered for linkage map construction. Forty seven markers were found to be linked into14 different groups spanning 412.9 cM and the rest of 42 markers remained unlinked. Markers were randomly distributed on the 14 linkage groups. The distance between the markers on the map also varied greatly across the different linkage groups.

Screening of 141 progenies and the parents *viz.* CO2 and MNga-1 to assess their levels of resistance to CMD indicated wide variation for the level of resistance among the progenies. CO2 and MNga-1 were identified as highly susceptible and resistant to CMD respectively. The number of plants under each category of damage score ranged from 9 (score5-highly susceptible) to 73 (score1-resistant). The number of plants falling under each category of damage score are as follows: 73 for score 1, 25 for score 2, 22 for score 3, 12 for score 4 and 9 for score 5. Based on CMD resistance screening, 73 resistant and 68 progenies susceptible to CMD and it is segregating for CMD resistance 1:1 ratio. Frequency distribution of the F_1 progenies coming under various categories of damage scores is shown in Figure 1.

Single marker analysis (SMA) was carried out using the damage scores and the marker segregation patterns 141 progenies to identify putative SSR markers linked to CMD resistance. SMA was carried out in the present study since complete genetic map could not be constructed with the available number of polymorphic markers to carry out interval analysis. The SMA between marker and phenotype resulted in the identification of four markers having association with resistance to CMD. Those four markers were SSRY28, SSRY235, SSRY44 and NS136. SSRY28 and SSRY235 were on the linkage group #5 and #11 respectively of this study (Figure 2). The other two markers showing significant association with CMD resistance were SSRY44 and NS136 on linkage group #13 and #14 respectively (Figure 3). The outcome of one-way ANOVA between markers and phenotype is given in Table 2.

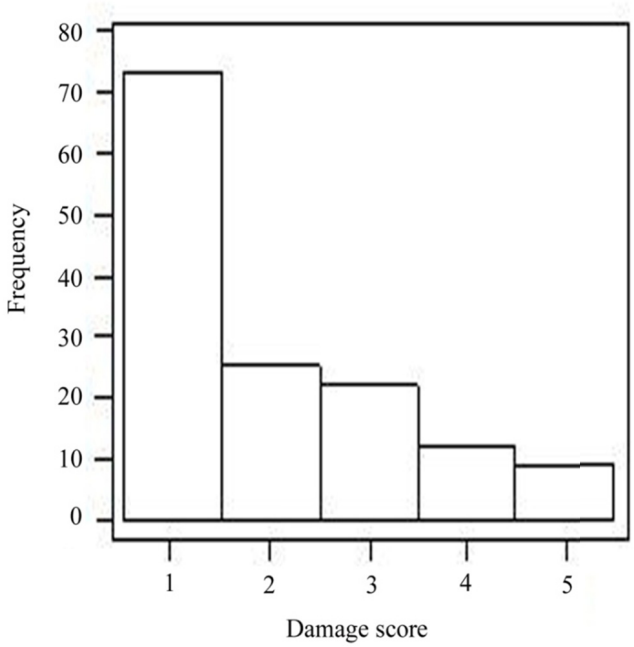


Figure 1. Frequency distribution of phenotypic values of CMD resistant F_1 mapping population

Table 2. One way ANOVA for SSR markers associated with resistance to CMD

Linked markers	R square	F (calculated)[f]	P-value	F (critical)
SSR28[a]	0.031	4.378	0.038	3.910
SSR44[b]	0.040	5.812	0.017	3.910
SSR235[d]	0.037	5.267	0.023	3.910
NS136[d]	0.032	4.669	0.023	3.910

[f] - calculated using single factor ANOVA.

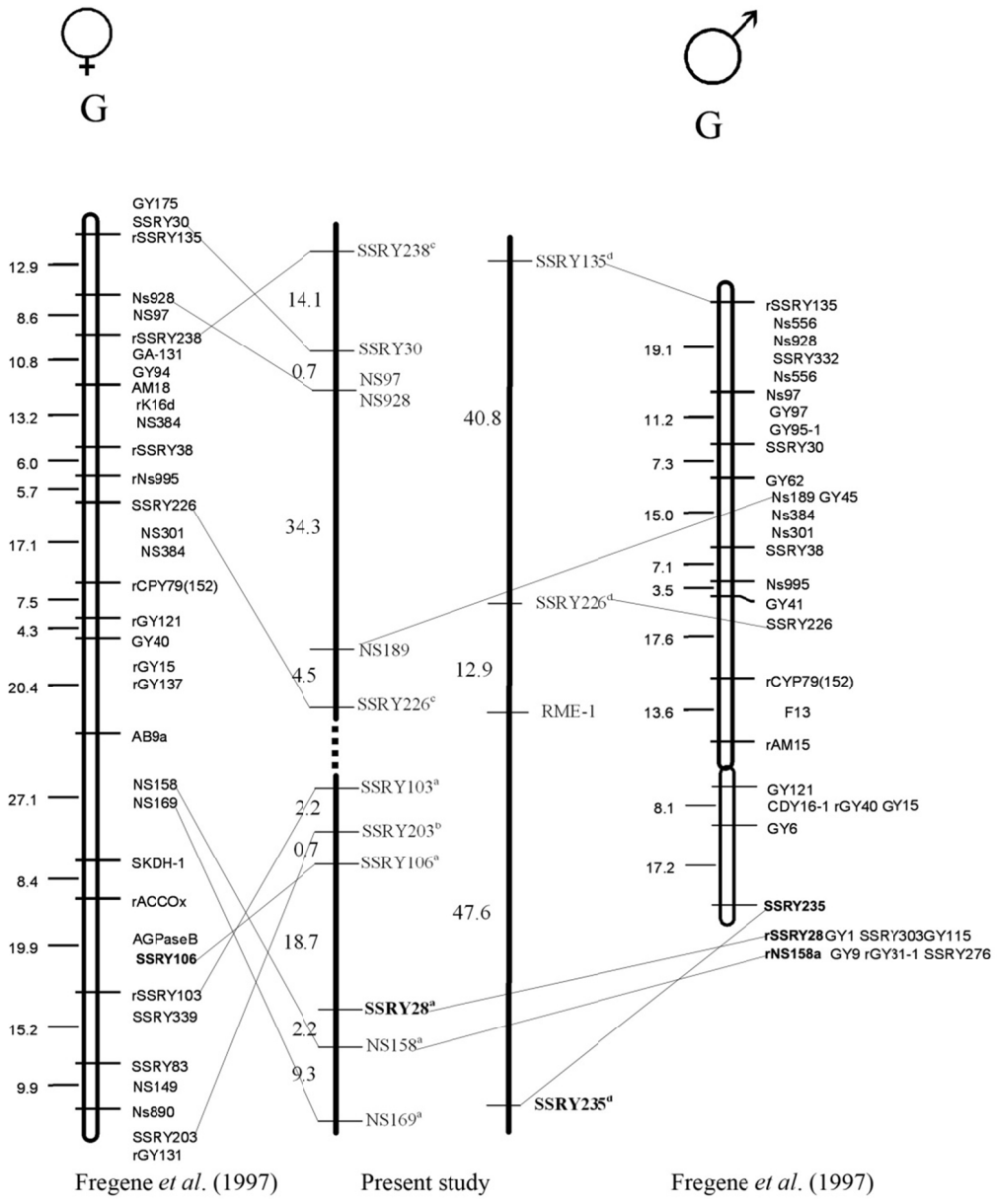


Figure 2. Combined linkage map of linkage group #5, #11 and #12 along with linkage group G of Fregene et al. (1997)

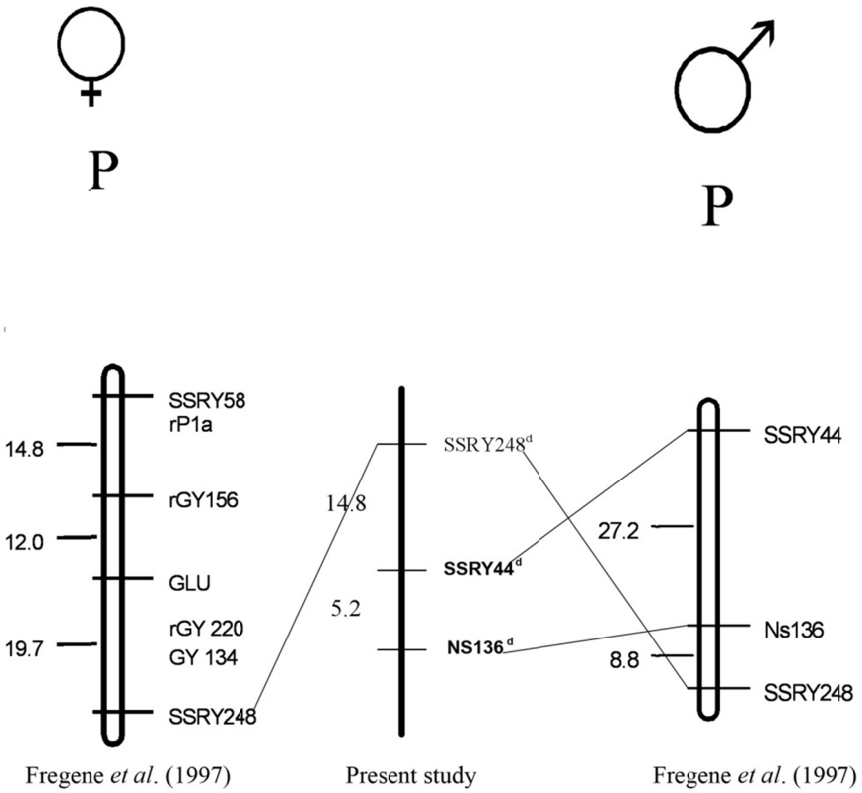


Figure 3. Linkage map of linkage group #14 along with linkage group P of Fregene et al. (1997)

In the present study, an attempt made to construct genetic maps CO2/MNga^{-1} using available 89 markers. All the 89 segregating markers were used to construct the linkage map which resulted in the grouping of 47 markers into 14 linkage groups. The other 42 markers remained unlinked. This could be because of less number markers screened and the target was to construct the linkage maps for the groups harbouring genetic loci associated with CMD resistance based on the earlier reports. The marker loci belonging to the linkage groups *viz*. #5, #11 and #12 in the present study were already mapped to a single linkage group G and similarly linkage group #14 mapped to group P (Fregene et al., 1997). The constructed genetic map containing only 47 linked SSR loci and the linkage map thus generated needs further saturation. The expected number of eighteen linkage groups for a comprehensive linkage map of cassava (2n = 36) was less than the 18 linkage groups, out of which four linkage groups had only two markers, and five linkage groups had only three markers.

The major objective of the present study was to detect the genomic regions associated with resistance to CMD caused by ICMV begomoviruses. The screening of parents and progenies for their levels of resistance to CMD revealed a continuous variation for the resistance to CMD. However, the frequency distribution of 141 progenies was skewed towards high level resistance indicating the influence of major genes. Based on CMD screening, resistance is segregating for CMD resistance 1:1 ratio. It indicates, in the resistant parent (MNga-1), CMD gene is in heterozygous condition and due to that it segregates in the F_1 progenies. This is confirmed by self pollinating the MNga-1 resistant parent and it segregate for resistance in 3:1 ratio in the S_1 progenies. The polygenic control of resistance to CMD was reported earlier by Hahn et al. (1980b); Lokko et al. (1998); Fregene, Bernal, Duque, Dixon, and Tohme (2000) and Lokko et al. (2004). However, Akano et al. (2002) reported that the resistance to CMD was under the control of single dominant gene.

The association of resistance to CMD with specific SSR markers adopting SMA by one-way ANOVA, resulted in the detection of four markers *viz*. SSRY28, SSRY235, SSRY44 and NS136. SSRY28 and SSRY235 are located on linkage group G and SSRY44 and NS136 on linkage group P of cassava linkage map (Fregene et al., 1997). Among the four markers, three (SSRY235, SSRY44 and NS136) are new markers associated with CMD resistance. Fregene et al. (1997) identified markers *viz.* SSRY235, SSRY28 and NS158 on linkage group G closer to each other. The strong association of SSRY28 and SSRY235 located on the same linkage group G with resistance to CMD established the possibility of having a major QTL for resistance to CMD in that region. In the same manner, SSRY44 and NS136 loci on linkage group P showed association with resistance to CMD indicating the control of resistance to

CMD by more than one locus. In the present study, apart from confirming the association of SSRY28 and SSRY235 with resistance to CMD, additional markers having association with CMD resistance was also established. These markers include, SSRY44 and NS136, which mapped to linkage group P of Fregene et al. (1997). The detection of markers SSRY44 and NS136 having association with CMD resistance is a new report indicating the possibility of having another genetic loci for CMD resistance in cassava in addition to the already established on linkage group G (Fregene et al., 1997). This finding supports the polygenic control of resistance to CMD as established by Hahn et al. (1980a, 1980b).

The first report of identifying a DNA marker for resistance to CMD was the detection of SSRY40 with *CMD1* gene by Fregene (2000). Similar attempts to detect the DNA markers associated with resistance to CMD led to identification of SSRY28 by two independent groups involving the Nigerian landraces *viz*. TME3 (Akano et al., 2002) and TME7 (Lokko et al., 2005). Further saturation of TME3 resistance source with more number of SSR markers and RAPD markers, two more tightly linked markers *viz*., NS158 and RME-1 were identified (Moreno Tomkins, & Fregene, 2004). However, Lokko et al. (2005) identified two more DNA markers SSRY106 and E-ACC/M-CTC (an AFLP marker) having strong association with resistance to CMD. By considering the results of the present study and the studies of Akano et al. (2002) and Lokko et al. (2005), it is very clear that the genomic regions around the marker *viz*. SSRY28 harbours one of the major gene conferring resistance to CMD irrespective of resistance sources. The SSR markers found to be associated with the CMD resistance *viz*. SSRY28 from previous studies (Akano et al., 2002; Moreno et al., 2004; Lokko et al., 2005) and NS158 and SSRY106 from the present study are on the same linkage group covering a distance of 20.9 cM. Lokko et al. (2004) identified seven different SSR markers associated with resistance to CMD in population derived from TMS30572 and TME117 and established the contribution of both the parents towards the resistance to CMD. Okogbenin et al. (2012) identified (CMD3) a NS198 marker associated with CMD resistance, explaining 11% of the phenotypic variance. The detection of several markers from different parts of the cassava genome having association with resistance to CMD unequivocally establishes the polygenic control of resistance to CMD with minor effect.

The availability of various genetic resources from TME and TMS could be used to impart CMD resistance to various elite cassava genotypes by backcross breeding. Identifying markers for the genes responsible for resistance to CMD in these genetic resources is expected to speed up the process of backcross breeding in cassava. The markers could help in future cloning of these genes.

In conclusion, in the present study, apart from the genomic region harbouring *CMD2* on the linkage group G, new genomic region having association with CMD resistance was established on the linkage group P. Established clearcut diagnostics both at phenotypic and molecular level to determine the varying levels resistance to CMD in mapping population will facilitate detection of new genetic loci for CMD resistance. Identification of more genetic loci, otherwise known as QTLs, is expected to change the strategies in breeding for CMD resistance in cassava, to have durable resistance in the progenies. Considering the availability of above genetic and genomic resources, the results from the present study involving a set of 141 progenies and limited SSR markers could be fine tuned to explore the possibilities of mapping genes associated with resistance to CMD. This could be achieved by screening the population in different environments of cassava growing areas. Moreover, the genetic map with more markers available at present and to be made available in future and the mapping population generated from CO2/MNga-1 in the present study could remain as the source for mapping genes associated with other agronomically important traits *viz.* early maturity, high dry matter, low cyanogens and high yield.

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