# An SSR-Based Molecular Genetic Map of Cassava

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**Short title:** $F_2$ linkage map of Cassava

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## Abstract

Microsatellites or simple sequence repeats (SSR) are the markers of choice for molecular genetic mapping and marker-assisted selection in many crop species. A microsatellite-based linkage map of cassava was drawn using SSR markers and a $F_2$ population consisting of 268 individuals. The $F_2$ population was derived from selfing the genotype K150, an early yielding genotype from an $F_1$ progeny from a cross between two non-inbred elite cassava varieties, TMS 30572 and CM 2177-2 from IITA and CIAT respectively. A set of 472 SSR markers, previously developed from cassava genomic and cDNA libraries, were screened for polymorphism in K150 and its parents TMS 30572 and CM 2177-2. One hundred and twenty two polymorphic SSR markers were identified and utilized for linkage analysis. The map has 100 markers spanning 1236.7 cM, distributed on 22 linkage groups with an average marker distance of 17.92 cM. Marker density across the genome was uniform. This is the first SSR based linkage map of cassava and represents an important step towards quantitative trait loci mapping and genetic analysis of complex traits in *M. esculenta* species in national research program and other institutes with minimal laboratory facilities. SSR markers reduce the time and cost of mapping quantitative loci (QTL) controlling traits of agronomic interest and determination of gene actions and for marker-assisted selection (MAS).



**Key words** Cassava - SSR Markers - Linkage map

## Introduction

Cassava (*Manihot esculenta* subsp. *esculenta* Crantz) is the principal or second most important source of calories for more than 500 million people (Cock 1985; Best and Henry 1992). As a staple food, it is the sixth most important crop worldwide (Mann, 1997). Cassava is an alloploid with 36 chromosomes having a DNA content of 1.67pg per cell nucleus (Awoleye et al., 1994). This value corresponds to 772 mega base pairs in the haploid genome and puts cassava's genome size at the lower end of the range of higher plants (Bennet et al., 1992).

The first genetic linkage map for cassava was constructed with predominantly RFLP markers and a full-sib intra-specific cross (Fregene et al. 1997). The map has so far provided initial tools for genetic analysis of important traits of cassava (Jorge et al. 2000, 2001; Akano et al., 2002; Okogbenin and Fregene 2002; Okogbenin and Fregene 2003), as a first step towards such a rational use of molecular markers in cassava breeding.

However an $F_1$ progeny is not the ideal population for genetic analysis of complex quantitative traits. It cannot be used to detect recessive or epistatic interactions, important gene actions in traits of agronomic interest. The use of full-sib crosses from heterozygous parents alters QTL mapping by redefining mating type at a locus level rather than all loci in parents and also detection of QTL alleles is based on separate maps for each parent. The marker genotype in the $F_1$ progeny populations results from the independent meioses and crossovers in the maternal and paternal parents thus individual maps are often constructed for each parent (Grattapaglia et al. 1994; Groover et al., 1994; Van Eck et al. 1994).

Furthermore, RFLPs cannot be transferred readily to national programs of the developing world because it is expensive and the laborious, furthermore facilities for the radioactive procedures are not available in most laboratories. Microsatellite or simple sequence repeat (SSR) markers are preferable, because they are easy to implement in most laboratories and amenable for high throughput marker genotyping, furthermore, PCR-based marker systems such as SSR produce results within a day. SSRs are small tandem repeats of DNA, usually 2-5 bp in length, that occur in most eukaryotic genomes. They are widely applied in plant genome mapping and genetic analysis because of their co-dominant inheritance, high degree of polymorphism and ease of analysis (Akkaya et al. 1992, 1995; Senior and Heun 1993; Jarret and Bowen 1994; Plaschke et al., 1995; Roder et al., 1995; Rongwen et al., 1995, Hamwieh et al., 2005).

To overcome the problems associated with the use of labor intensive RFLP markers and problems with genetic analysis in an $F_1$ cross of non-inbred parents, we used simple sequence repeat (SSR) markers to conduct genetic mapping in an $F_2$ population. A genetic map derived using an $F_2$ population should be much more informative than an $F_1$ population. In addition, an SSR-based $F_2$ – derived map of cassava will allow for a higher level of map saturation unlike parent-specific mapping in the $F_1$. The $F_2$ – derived map will be of value to studies designed to identify markers associated with traits of interest and for comparative analysis with other related species. In addition, SSR markers associated with traits of interest will facilitate marker-assisted selection (MAS) in a modest cassava breeding program. We report here the construction of the first SSR marker-based genetic map of cassava.

## Materials and Methods

*Plant material and DNA isolation*

The $F_1$ cassava mapping population described in Fregene et al. (1997) was analyzed for early yield and related traits in 1998 and 1999. Based on results obtained and profuse flowering abilities, three $F_1$ individuals (K68, K145 and K150) were pre-selected and selfed to produce $F_2$ populations. These $F_1$ individuals were derived from the cross between 'TMS30572' (female parent), an elite cassava cultivar from the breeding program at the International Institute of Tropical Agriculture (IITA), Nigeria, and CM2177-2' (the male parent), a successful cassava resulting from breeding activities at the Centro Internacional Agricultura Tropical (CIAT) in Colombia. The highest germination rate was recorded in K150, with 372 seedlings as compared with 316 and 245 seedlings for K68 and K145 respectively, K150 also showed the highest heterozygosity with SSR markers . The progeny of K150 were therefore selected for genetic mapping studies.

The progeny used for map construction consisted of 268 individuals produced from selfing K 150. From each $F_2$ genotype, approximately 3g of young leaf tissue from greenhouse-grown plants was collected in a mortar and immediately frozen in liquid nitrogen. Genomic DNA was extracted from the frozen leaf samples of each individual of the $F_2$ population and from the grand parents (TMS 30572 and CM 2177-2) and K150 as described by Dellarporta et al. (1983). DNA concentrations were quantified using a DNA fluorometer. DNA quality and integrity were assessed by electrophoresis on agarose gels.

*Molecular Marker analysis*

One hundred and eighty six SSR markers from a genomic library (Mba et al., 2001), 132 SSR markers from a cassava root and leaf cDNA library (Mba et al., 2001 unpublished data), and 154 SSR markers from a genomic library (Fregene et al., 2002 unpublished data) were used, a total of 472 markers. The SSR primer sequences used in this study are available upon request from the International Center for Tropical Agriculture (CIAT, the Spanish acronym) Colombia.

All of the primer pair combinations were first screened with the grandparents (TMS 30572 and CM 2177-2) and K150 ($F_1$ parent of the $F_2$ population) to find the polymorphic SSR marker. The selected polymorphic markers were then used for evaluating the whole population. Some of the polymorphic Markers with non-specific amplifications and/or too faint products were discarded from the final population assay.

PCR was performed in 96-well plates in PTC200 thermocyclers (MJ Research, Watertown, Mass). Amplifications were carried out in 12.5-µl reactions containing 25 ng of DNA, 5pmoles of each primer, 10 X of *Taq* polymerase buffer (500 mM KCl, 100mM Tris-HCI (pH 8.5), and 1 mg/ml gelatin), 1.0 mM of $MgCl_2$, 0.5mM of dNTPs and 0.25 U of *Taq* polymerase. The final volume was adjusted with sterile distilled $H_20$. The PCR profile was: $94^OC$ for 10 min, followed $95\ ^OC$ for 4 min, 25 cycles at $95\ ^OC$ for 1 min, 55 $^OC$ for 2 min and finally 10 min at $72\ ^OC$ for the final extension. The PCR products were separated by running on 6% polyacrilamide denaturing gels (PAGE) gels and electrophoresed in 1X TBE at 100W for 2h using a Bio-Rad sequencing gel rig (BIORAD, California). The amplified products were visualized by silver staining. Two sequential

loadings, after an interval of about 20 min, of PCR amplification product of the progeny was done to increase the efficiency of the mapping process.

*Genetic linkage analysis*

SSR alleles segregating in the mapping population were scored according to the expected classes for an $F_2$ population. Alleles derived from female grandparent were scored as "A" alleles whereas alleles from the male grandparent were designated "B" alleles. Individuals homozygous for maternal grandparental alleles were scored "AA", heterozygous "AB" and homozygous for paternal grandparental "BB". Marker classes at each locus were summarized for all individuals into the three different genotypic classes expected for a $F_2$ population and a chi square tests for segregation distortion were carried out to compare the observed ratio with the expected, 1:2:1. Chi square analysis was performed at the threshold of P=0.05 to test for significant deviations from expected ratios (segregation distortion).

The linkage analysis was with MAPMAKER/EXP, version 3.0 (Lander et al., 1987). Linked markers were identified using the group command and a recombination value of 0.30 and LOD of 3.0. For each group of markers, three point analysis was performed. Markers within groups were then ordered using the order command with LOD > 2.0. The resulting marker order was examined using the "ripple" command to ascertain the order was at least 100 times better than the second best order. The marker order was considered as the framework for each linkage group**.**

Recombination frequencies were converted to map distances (cM) using the Kosambi mapping function (Kosambi, 1944). The remaining markers were then placed

with the try command. The "error detection" command was used to check for unexpected mistakes in data entering.

## Results

*SSR polymorphism and segregation of markers.*

Of the 472 SSR markers, 163 were found to be heterozygous in K150. Eight markers, which were polymorphic in K150, TMS 30572, and CM 2177-2, did not segregate in the $F_2$ progeny, revealing that these markers may be duplicated loci. Seventy three percent (122) of the markers evaluated segregated in 1:2:1 ratio. Thirty three markers (27%) showed distorted segregation ($P \leq 0.05$, chi-square test). Results of linkage analysis revealed that markers with distorted segregation were distributed throughout the genome. Deviation from the expected segregation ratios was observed for markers on thirteen LGs. The number of markers showing segregation distortion varied from 1 to 4 per LG. The most extreme examples of segregation distortions in the $F_2$ was found with marker SSRY 100 on LG 16 where only 16 of 260 plants were "AA" homozygotes, and NS 33 on the same linkage group where only 17 of 235 plants scored were also "AA" homozygotes.

*An SSR Linkage map*

One hundred and twenty two markers were employed in the linkage analysis and 100 of these markers could be assigned to 22 linkage groups (LG1 – LG22), which had 2 -8 markers, and a linkage group length varying from of 9.7 cM (LG19) to 129.9 cM (LG3) (Table 1). The linkage map of the $F_2$ population spans a total of genetic distance of 1236.7 cM (Kosambi cM), with 22 markers remaining unlinked. Markers were randomly distributed on the 22 linkage groups.

The distance between the markers on the map also varies greatly across the different linkage groups. The average marker distance was 17.92 cM, with intervals between loci ranging from 5.6 to 39.8 cM (Fig. 1). The size of the LG does not necessarily reflect the number of linked markers. For instance, LG 2, with a total linkage distance of 84.3 cM had 8 mapped loci, whereas in LG 12, with a distance of 105 cM was covered by only 5 markers. However the correlation between linkage distance and number of markers was r = 0.75 indicating that the SSR markers were fairly distributed randomly across the genome. Table 1 provides a summary of SSR marker distribution on different linkage groups showing the size, number of markers and the average marker interval of each LG. The number of LG in this map (22) exceeds the haploid number of chromosomes for cassava (n=18), indicating that the map is not saturated.

*Comparison with $F_1$ map*

The length of the cassava genome based on genetic mapping in an $F_1$ cross is estimated to be about 1610 cM (Fregene et al 1997). The $F_1$ map (Fregene et al., 1997) of this species differed from the $F_2$ map with respect to marker type and number, genome coverage (span) and marker density. The $F_1$ female parent-derived map spans 931.6 cM with 168 markers compared to the F2 map with 1236.7 cM and 100 markers.

Mapping of SSR markers in the $F_1$ map have also been conducted (Zarate et al. 2002 unpublished results; Libreros et al., 2002 unpublished results), so far, about 200SSR markers have been placed on the $F_1$ map and sixty-seven of the SSR markers are common to both the $F_2$ population and the $F_1$ map. A majority (44) of the common SSRs showed colinearity between $F_1$ and $F_2$ maps (Zarate et al., unpublished data) indicating the

reliability of both maps. The 44 markers are shown in Table 2. However some differences was detected for some markers. A few differences in order were evident for some markers in LG5 (SSRY 35, SSRY 13), LG 7 (NS40, NS 9) and LG 12 (NS 74) in the $F_2$ map. These differences were probably due to statistical inaccuracy associated to the limited number of individuals studied in the $F_1$ (150 individuals). The mean and variation in the lengths of the linkage groups in the $F_2$ is also similar with that found in the $F_1$.

Some marker intervals were found to be consistent between the $F_1$ and $F_2$ maps (for example, in LG2 the interval NS 260-NS 217 was 10.5 cM in the $F_1$ and 10.7 cM in the $F_2$; for SSRY 83 –NS 870 the interval was 9.9 cM in the $F_1$ and 9.5 cM in the $F_2$) (Table 2). However, there were also recombination differences between the two maps in some genomic regions (for example in LG10, the interval between SSRY172-SSRY101 was 75 cM in the $F_1$ and 36.7 in the $F_2$; for SSRY101-SSRY229 it was 11.3 cM in the $F_1$ and 23.3 cM in the $F_2$). The average marker intervals based on 22 marker pairs (Table 2) were 19.57 cM in the $F_1$ and 18.42 in the $F_2$ (Table 2), indicating that the average recombination frequencies between both maps were similar.

**Discussion**

SSR markers are advantageous to applied plant breeding because they are co-dominant, easily assayed and detect high levels of polymorphism (Morgante and Olivieri, 1993) and for these reasons SSR markers have become more valuable markers to breeders for the purposes of genome and QTL mapping. SSR markers have, thus, become the marker class of choice for the molecular mapping of many crop species (Roa et al., 2000).

The high degree of microsatellite polymorphism, 50%, that we observed in cassava is not surprising and is comparable to the results of other crop species (Udupa et al., 1999; Winter et al., 1999). The polymorphism detected with RFLPs in cassava is lower, an average of 40%. A few markers revealed monomorphic double bands indicating the possibility of duplicated loci for such genomic regions.

Segregation distortions have been reported in several studies and a high frequency of markers showing distorted segregation is common in outcrossing species (Kubisiak et al., 1995; Hanley et al 2002; Dettori et al.; 2001; Liebhard et al., 2002). The level of segregation distortion observed in this study (27%) is within the range reported in plant molecular studies (Schon et al. 1993; Lin et al., 1996; Wang et al. 1998). Deviations of Mendelian segregation ratios may be due to various processes amongst which may be the presence of gametophytic selection for sub lethal genes i.e. genes controlling the viability of pollen, zygote or seedlings, putatively located on one or more of the these linkage groups (Yan et al., 2005). Cassava is an outcrossing species with high genetic load and suffers from severe inbreeding depression. Segregation distortion in cassava may therefore not be unrelated to the association between heterozygosity and plant vigor found in cassava.

We have constructed the first PCR marker-based genetic linkage map of cassava that contains only SSR loci but the map requires further saturation. The expected number of eighteen linkage groups for a comprehensive linkage map of cassava (2n=36) was exceeded by seven linkage groups, out of which three linkage groups had only two markers, and three linkage groups had only three markers. Since most linkage groups are small, it is safe to conclude that the apparent excess of linkage groups might be due to

incomplete coverage of the genome with the marker loci. We anticipate that the smaller groups will be brought together as new markers are identified.

Marker distribution along the linkage groups (LG) was not uniform, as evident by the mixture of tightly linked loci and regions with low density as observed in the constructed map. This suggests that either recombination events or mapped loci were not evenly distributed throughout the genome. The low density of markers in some of the linkage groups might also correspond to regions highly homozygous and subject to higher recombination frequencies events (Castiglioni et al., 1999).

Differences in map length between the $F_1$ and $F_2$ can result from a variation in the number of recombination events in the two maps as well as variations in the numbers and locations of mapped loci. For most of the linkage groups, the order of the markers in both maps is consistent apart from minor differences on some linkage groups. The presence of common markers in both maps favor, not only the identification of homologous linkage groups but also the integration of the $F_1$ and $F_2$ maps. Multi-parental genetic mapping recommended by Murranty (1996) is a potential field of application. Through such common markers, QTLs identified using the $F_1$ map for important agronomic traits, can be revalidated in the $F_2$. This is useful from the point of view of breeding and stability in different genetic backgrounds, prerequisites for using molecular markers for marker-assisted selection, can be found.

This $F_2$ population holds great potential for the detection of QTL of agronomic interest in view of marker-assisted selection. This SSR map will complement genetic analysis in cassava and should provide us the additional opportunity to estimate genetic effects of QTLs. Development of an $F_2$ map provides a different generation to study the

QTLs and their genetic effects. In an $F_2$ population, one can determine the effect of different gene action on phenotype because all three possible gene dosages at a locus are represented. This can not be done exhaustively in an $F_1$ population. Thus an $F_2$ population can be used to map recessive and epistatic genes from either parent (Patterson et al., 1991) unlike the $F_1$.

In marker assisted breeding, co-dominant markers such as SSRs are effective in identifying desirable genotypes at early stages of selection. Therefore an $F_2$ SSR based map is an important pre-requisite for molecular marker-assisted selection (MAS) to increase the efficiency of cassava breeding. Efforts are on-going develop more SSR markers and to construct a saturated $F_2$ map for use in tagging genes controlling traits of agronomic importance and for marker assisted selection.

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Table 1: Linkage group size, number of markers, and the average marker interval per linkage group of the $F_2$ linkage map

| Linkage group | Size (cM) | No. of markers | Average marker interval (cM) |
|---|---|---|---|
| 1 | 40.4 | 5 | 10.1 |
| 2 | 84.3 | 8 | 12 |
| 3 | 129.9 | 8 | 16.2 |
| 4 | 94.6 | 7 | 15.7 |
| 5 | 117.8 | 6 | 23.5 |
| 6 | 58.4 | 5 | 14.6 |
| 7 | 72.6 | 7 | 12.1 |
| 8 | 65.5 | 7 | 10.9 |
| 9 | 51.1 | 5 | 12.8 |
| 10 | 88.6 | 6 | 17.7 |
| 11 | 38.7 | 7 | 6.45 |
| 12 | 105.3 | 5 | 26.3 |
| 13 | 49.8 | 3 | 24.9 |
| 14 | 11.2 | 3 | 5.6 |
| 15 | 63.8 | 3 | 31.9 |
| 16 | 22.6 | 3 | 11.3 |
| 17 | 14.8 | 2 | 14.8 |
| 18 | 10 | 2 | 10 |
| 19 | 9.7 | 2 | 9.7 |
| 20 | 30.3 | 2 | 30.3 |
| 21 | 37.5 | 2 | 37.5 |
| 22 | 39.8 | 2 | 39.8 |
| ∑/mean | 1236.7 | 100 | 17.9 |

Table 2:Marker pairs intervals in the $F_1$ and $F_2$ Maps

| Linkage Group | Marker (SSR) | $F_1$ (cM) | $F_2$ (cM) |
|---|---|---|---|
| 20 | NS82-SSRY314 | 26.1 | 30.3 |
| 14 | SSRY296-SSRY21 | 20.7 | 11.2 |
| 4 | NS980-SSRY40 | 7.8 | 12.9 |
| | SSRY3-SSRY23 | 8.2 | 15.6 |
| | SSRY251-NS717 | 19.8 | 23.9 |
| 12 | NS260-NS217 | 10.5 | 10.7 |
| 2 | SSRY83-NS890 | 9.9 | 9.5 |
| | NS928-SSRY226 | 44.3 | 46 |
| | NS189-NS995 | 1.2 | 33.4 |
| 9 | SSRY12-NS340 | 7.9 | 1.6 |
| | SSRY52-NS340 | 7.3 | 3.1 |
| 5 | SSRY35-SSRY284 | 14.6 | 28.1 |
| | SSRY13-SSRY284 | 48.7 | 44.6 |
| 10 | SSRY172-SSRY101 | 75 | 36.7 |
| | SSRY101-SSRY229 | 11.4 | 23.3 |
| 16 | NS33-SSRY100 | 71.5 | 16.3 |
| 18 | NS308-SSRY20 | 14 | 10 |
| 13 | SSRY10-NS185 | 10.9 | 19.1 |
| 11 | NS210-NS347 | 5.8 | 7.8 |
| | NS347-NS10 | 4.6 | 8.9 |
| | NS10-SSRY90 | 5.2 | 6.2 |
| | SSRY90-SSRY19 | 5.2 | 6.1 |
| Average | | 19.57 | 18.42 |

**Captions for Tables and Figures**

Table 1: Linkage group size, number of markers, and the average marker interval per linkage group of the $F_2$ linkage map

Figure 1. A genetic linkage map of cassava (*Manihot esculenta* Crantz) based upon a $F_2$ cross and SSR marker

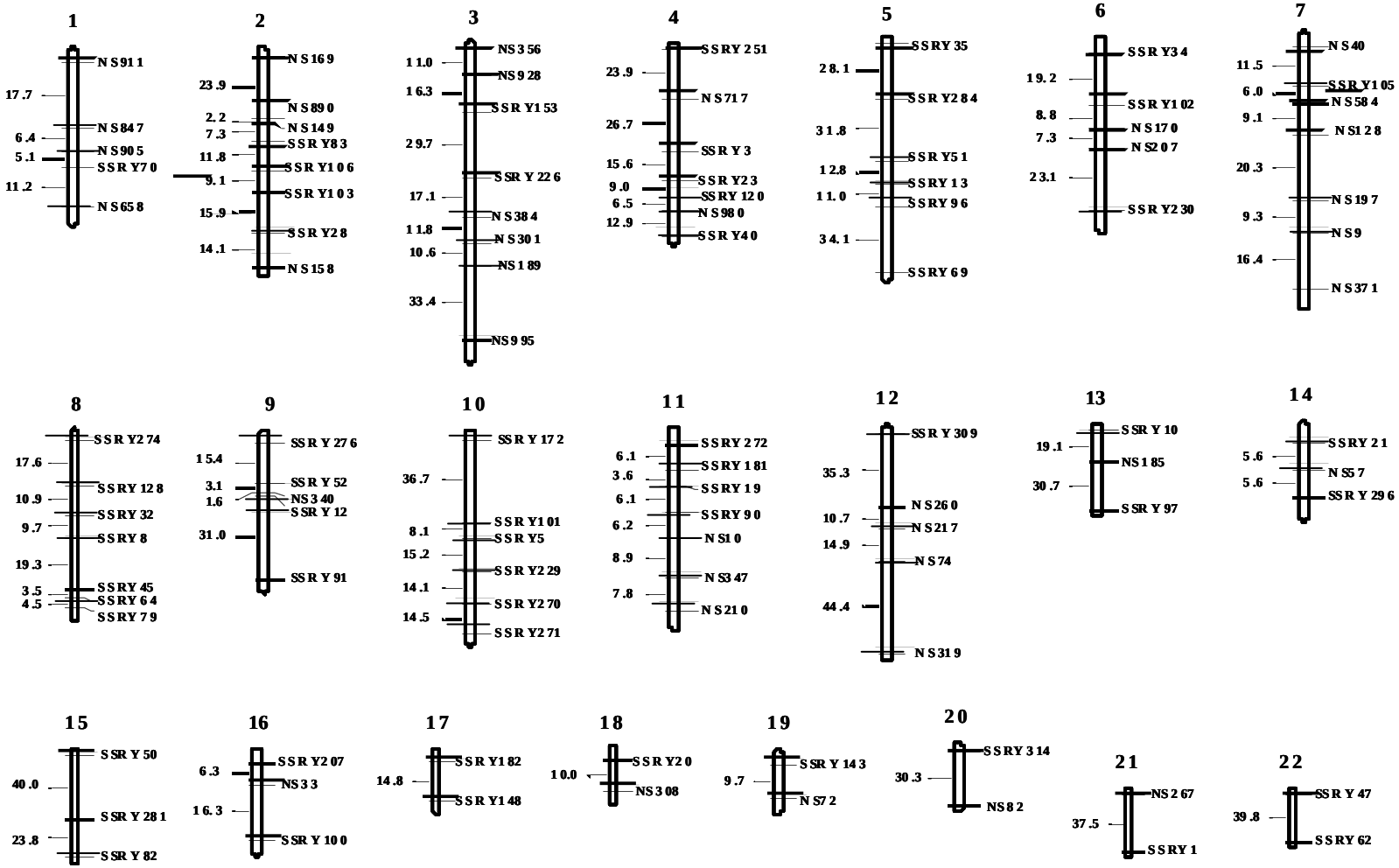


Figure 1. A genetic linkage map of cassava (*Manihot esculenta* Crantz) based upon a $F_2$ cross and SSR markers