# Developing Genetic Mapping and Marker-Assisted Breeding Techniques in Bambara Groundnut (*Vigna subterranea* L.)

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**Abstract**

**Bambara groundnut (*Vigna subterranea* L. (Verdc.)) is an underutilised African legume crop which shows a high degree of drought tolerance. It continues to be an important leguminous crop in tropical Africa and is grown mainly by women who are small-scale farmers in Africa under traditional low input agricultural systems. It is grown mainly for its protein and carbohydrate, as a fixer of agricultural nitrogen and for its high levels of drought tolerance. Genetic studies into this species could provide important data for breeding programmes and to enhance food security in sub-Saharan Africa. The main project work was to construct two initial genetic linkage maps for Bambara groundnut and to carry out a trait QTL analysis. Genomic DNA was extracted from 73 bulked lines (effectively $F_3$) derived from a cross between the DipC and Tiga necaru landraces. A set of 94 pre-tested SSR primers designed from a 454-sequenced microsatellite-enriched library were tested for segregation in this cross. Thirty primers were polymorphic and revealed that the residual heterozygosity in this population was around 25%, consistent with an $F_3$ population. DArT markers for Bambara groundnut were previously developed and 236 polymorphic markers have also been applied in this study. The genetic map consisted of 231 markers in 25 linkage groups of two or more linked markers, totalling 498cM and covering a predicted 44% of Bambara groundnut genome. The high marker-marker linkage (at 87%) suggests a more comprehensive coverage. Nine QTL loci were identified on 7 LGs. A major QTL was identified for internode length on LG3, explaining 41% of phenotypic variation. The 'Narrow' map, in contrast with wide cross population, was constructed from two domesticated landraces and would be expected to segregate for agronomically important traits, rather than domestication traits. The development of molecular tools for mapping and QTL analysis potentially allows more rapid breeding progress to be achieved in this species.**

## INTRODUCTION

Bambara groundnut (*V. subterranea* L Verdc., $2n=2x=22$) is an underutilised African legume crop. It continues to be the third most important minor food legume crop in semi-arid Africa, after groundnut and cowpea. Bambara has undergone a prolonged period of adaptation to drought, poor soils, and pest and disease resistance through low input agriculture (Azam-Ali et al., 2001). It is a rich source of protein and, along with other local sources of protein, could help to alleviate nutritional problems in some areas (Massawe et al., 2005). Despite its importance as a part of the diet of much of sub-Saharan Africa, there are no established cultivars of Bambara groundnut and the crop is still cultivated from local landraces rather than as cultivars. Although Bambara groundnut is characterised by higher genetic diversity in the wild ancestor, many landraces also consist of multiple genotypes which potentially increases the tolerance of the landrace to

biotic and abiotic stresses. Both wild material and genotypes within landraces could represent an important potential source of beneficial traits for Bambara groundnut breeding programmes.

Genetic markers work as indicators for the presence of the desirable allele of the target gene and also reveal differences between genotypes. One of the main uses of DNA markers is the construction of linkage maps in crop species to identifying chromosomal regions that contain genes controlling simple traits and quantitative traits using QTL analysis (Mohan et al., 1997). Detecting association between the phenotype and the genotype of the markers involves dividing the population into different groups depending on a particular marker locus and testing for significant differences between groups with respect to the trait being measured (Tanksley, 1993; Young, 1996). Ideally, all the QTLs identified for marker assisted selection (MAS) should be stable across environments (Hittalmani et al., 2002; Ribaut and Betran, 1999). In addition, the reliability of these markers must be adequately tested for prediction of phenotype before they are used in MAS.

SSRs (simple sequence repeats; simple, tandemly repeated di- to tetra-nucleotide sequence motifs flanked by unique sequences) have been developed in many crop plants (Philips et al., 2001; Varshney et al., 2004). Furthermore, they are valuable as genetic markers for different applications in plant genetics and breeding, because they are co-dominant, detect high levels of allelic diversity, and are easily and economically analysed by the polymerase chain reaction (PCR; Susan et al., 1997; Powell et al., 1996). In addition they are effectual for investigating phenotypic and genotypic variation within plant species (Gupta and Varshney, 2000).

Diversity Arrays Technology (DArT) is a hybridisation-based microarray platform which is generic and cost effective as a genotyping technology. It is a suitable technique for genome-wide discovery and genotyping of genetic variation. DArT allows the simultaneous scoring of thousands of restriction site based polymorphisms between genotypes and does not require DNA sequence information or site-specific oligonucleotides (Alexander et al., 2005).

The objectives of the present study were to construct and develop a genetic linkage map of Bambara groundnut, combining microsatellite and DArT markers using the segregating population from an intra-specific cross of DipC and Tiga necaru landraces to identify marker-trait linkages, to increase the availability of marker pools and to develop this crop through marker-assisted selection, as a part of conventional breeding.

## MATERIALS AND METHODS

### Mapping Population and Genomic DNA Extraction

The segregating $F_3$ population seed derived from an intra-specific cross between the DipC (female) and Tiga necaru (male) landraces originally obtained from IITA were used as a mapping population. Total genomic DNA was extracted from all 73 lines of the segregating population using the Dellaporta protocol (Dellaporta et al., 1983) with modification. DNA collected was resuspended in approximately 500 µl of TE and stored at -20°C. DNA integrity and purity were tested using agarose gel electrophoresis with ethidium bromide staining and visualisation under UV light.

The field trail was planted out in Indonesia on 30 May 2010 for all 73 $F_3$ lines. Plants were planted in rows 40 cm apart and 40 cm between them in the row. The traits were measured according to descriptors in the book "Bambara groundnut (*Vigna subterranean*)" (IPGRI, 2000). The data were recorded for the individual plants at different growth stages and at maturation.

Anderson Darling tests (Stephens, 1974) were used to test the distribution of trait data (Table 1). The inheritance and segregation of contrasting morphological traits were studied in the segregating population of DipC and Tiga necaru landraces. Statistical software MINITAB (Release 16) was used to analyse the variance, construct residual plots and detect significant association between the traits ($p<0.05$).

**Microsatellite Assay**

A total of 94 primers derived from a number of sources, including a 454-sequenced microsatellite-enriched library were screened for polymorphism and variable loci were amplified from the segregating population. The primers were designed using the Primer3 web program version 0.4.0 (http://frodo.wi.mit.edu/primer3/; Rozen and Skaletsky, 2000).

PCR reactions were carried out in 20 µl reaction volumes using the following conditions: initial denaturation 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 50-60°C for 1 min (at the optimal annealing temperature derived from a gradient annealing temperature PCR reaction) and 72°C for 2 min, followed by a final elongation step of 72°C for 10 min. The PCR products were run on 2% agarose to check for amplification. They were then run on the Beckman CEQ 8000 and the fragment sizes analysed using CEQ$^{M}$ 8000 Fragments Analysis Software Version 8 (Beckman Coulter Inc., Fullerton, USA).

**DArT Assay**

Diversity array technology marker assays were performed by DArT Pty. Ltd. (Yarralumla, Australia; www.diversityarrays.com) as previously described (Wenzl et al., 2004; Akbari et al., 2006; Semagn et al., 2006).

**Construction of Linkage Map and QTL Mapping**

The JoinMap4 software (Van Ooijen, 2006) was used to construct the linkage map, comprising of both SSR and DArT data. Phase determination was carried out by analysing the population initially as a Cross Pollinator (CP) to determine linkage phase for the DArT markers, as parental data was not available. Phase in the linkage groups was used to convert DArT marker genotypes to different phases in an 'RIL3' population type dataset ({0,0} giving a or c, while {1,1} gave b,d) RIL loci were grouped according to 'independence LOD', groups were manually selected from LODs 3-5 and Regression mapping carried out.

The QTL analysis was carried out using MapQTL v.6 software (Van Ooijen, 2009). Nonparametric mapping using Kruskal-Wallis and also Interval Mapping were used.

**RESULTS AND DISCUSSION**

**SSR Markers**

A total of 30 SSR primer pairs out of 94 were identified as polymorphic and were mapped. Previously, a low level of genetic polymorphism in the specific gene pool of Bambara groundnut had been reported (Basu, 2005). Levels of heterozygosity in the population were found to be approximately 26% using SSR markers. This is consistent with this population being an $F_3$.

**DArT Markers**

Of the 7680 fragments detected in DArT array, 236 (3.1%) were identified as polymorphic markers in the cross and scored in this population.

A total of 266 polymorphic loci were used to assemble the genetic linkage map, including 30 SSR and 236 DArT markers. The resultant map consists of 231 linked markers, a total of 498.3 centimorgans with 25 linkage groups of two or more markers.

**Segregation Distortion**

Segregation distortion of the markers was found to be high, SSR markers showed lower distortion at 16.7% in comparison to 31.6% of the DArT markers. Previous investigations have reported 40.6% distortion in an $F_2$ intraspecific population of *Medicago tornata* (Janczewski et al., 1997). One of the highest frequencies of marker distortion of 73% was reported in an interspecific recombinant line population in tomato

(Xu et al., 1997).

**Distribution of Markers**

The 25 linkage groups spanned 498.3 cM of the Bambara groundnut genome (Table 2). The distance between two consecutive markers varied from 0-16 cM, with a mean of 2.71 cM. The longest group in cM terms, with 13 spaced markers, covered a distance of 76.4 cM. The largest number of 40 markers was in linkage group 1, spanning 58 cM, although the clustering of markers on this group perhaps suggests a repeat cluster among the DArT markers (Fig. 1).

**Linkage Map**

The final map covered approximately 498 cM which was expected to equate to about 44% of Bambara groundnut genome (roughly 11 Morgans for 11 chromosomes), although the high marker-marker linkage (231 out of 266) at 87% suggests more comprehensive coverage. Parental dissimilarity could be suppressing recombination, or potentially the developed markers could be clustered to the particular regions of Bambara genome.

**Quantitative Trait Loci (QTL) Mapping**

**1. Association of the Traits.** A Pearson's correlation analysis was conducted to determine the association between the traits of study. In the $F_3$ population seed weight/plant was found to be associated with both pod- and internode length, with correlation coefficients of +0.411 (p=0.001) and +0.392 (p=0.003), respectively (Table 3). A highly significant correlation was observed between biomass and leaflet length (r=0.947; p=0.000). Biomass (dry weight) was also correlated positively to petiole length and plant spread explaining 66.2 and 55.0% of the trait variation (p=0.000), respectively.

An association was also found between a number of traits in the $F_2$ segregating population assessed in the Tropical Crops Research Unit (TCRU) at Nottingham University. A significant positive correlation was observed between leaf no./plant and plant spread (r=0.645, p=0.000); regression analysis between the two traits explained 40.8% of the variation (Table 4). The regression analysis suggested that 100-seed weight was associated with 20.3% of the variation in the leaf no./plant. Days to emergence was also correlated with 100-seed weight (r=0.308; p=0.007).

**2. Marker and Trait Associations.**

*Leaflet Length (cm).* A QTL for leaflet length was identified by Kruskal-Wallis, mapping on LG 1. The most significant association was found at 22.3 cM, between this trait and marker bgPt-602039, scoring K*=17.78; p=0.0001 (Table 5). Two other putative QTLs located on LG 5 and 14 were associated with SSR markers PRIMER95 and mBam3co33 at significant level of p=0.05 scored; K*=7.25 and 8.32, respectively.

*Petiole Length (mm).* Analysis of QTL for petiole length indicated another QTL on LG1 for this trait at 22.3 cM position at a LOD of 2.35 which is above the significant level of threshold (2.27; by permutation, 10,000 replications). This locus explains 15.5% of phenotypic variation. Strong and positive correlation was revealed between this trait and biomass (dry weight).

*Pod Length (mm).* A single QTL for pod length was identified by IM analysis located on LG 14 at 10.1 cM, at a LOD score of 3.37 (Table 5). This genomic region explained 21.8% of the phenotypic variation of pod length in this present study. The marker mBam3co33 at 9.0 cM region is closed to this QTL (K*=14.63 at p=0.001). The locus mBam3co33 was also found to be associated with leaflet length.

*Internode Length (mm).* A major QTL on LG 3 mapped by IM at 3 cM at a very high LOD score of 7.09 was associated with the bgPabg-596988 marker and explained 40.9% of phenotypic variation and also showed a strong association in a nonparametric mapping (K*=20.93 at p=0.0001) (Table 5). Internode length was found to be associated with both seed no. and seed weight/plant (Table 3).

*Node No./Stem.* A single QTL for node no./stem was identified to be associated with the

marker bgPabg-594814 at 30.23 cM, at 2.78 LOD on the LG 2 (K*=10.75 at p=0.005) (Table 5). This QTL explained 18.4% of the phenotypic variations for the node no./stem.
*Plant Spread (cm).* A putative QTL was identified on LG 1 at 22.3 cM with a LOD score of 2.36. This genomic region explained 15.4% of the phenotypic variation for plant spread in the $F_3$ progenies of this cross. The marker locus bgPt-602039 was found to be associated with this trait (K*=9.35 at p=0.005).
*Seed No./Plant*. On LG 13 another putative QTL was identified at 15.8 cM with interval mapping, at a LOD of 2.48 which is below the significant level of threshold ratio (tested by running 10000 permutation tests). This genomic region explains 17.6% of phenotypic variations in seed no./plant and the nearest marker to this QTL was bgPt-601402 at 19.87 cM region (Table 5).
*Days to Emergence.* The Kruskal-Wallis analysis indicated a significant association between days to emergence and the markers PRIMER16 and bgPt-595387 on the LGs 19 and 6, respectively, for TCRU data (Table 5). The codominant marker SSR locus PRIMER16 was found to be the most significantly linked (K*=10.67 at p=0.005) locus to the putative QTL for this trait.
*Leaf No./Plant.* Leaf no./plant was found to have a major QTL with LOD score 3.11 at the marker locus bgPt-598428 on LG 8 (K*=8.12 at p=0.005). This QTL region explained 17.8% of the phenotypic variation observed in this cross.

**In Summary**

In order to obtain more reliable QTLs for the agronomical important traits a single QTL-model analysis of Interval Mapping, was used to tag the QTL locus for all the traits except for leaflet length and days to emergence which show non-normal distribution of the data. The Kruskal-Wallis test of Marker-QTL associations for non-normally distributed traits (van Ooijen and Maliepaard, 2001), we used a significance threshold at below $p<0.005$ to identify the QTL for traits leaflet length and days to emergence to reveal three QTLs on LGs 1, 6 and 19.

However, the population under study here was not big enough to construct a high resolution map for QTL study (Collard et al., 2005), additional work is required to evaluate the population under different environmental conditions, involving more polymorphic markers and a better understanding of agronomical complex traits to confirm these QTL markers to be used in marker assistant selection of Bambara groundnut crop.

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## Tables

Table 1. Statistical analysis and the distribution of traits in the two populations of study.

| Trait | Min. value | Max. value | Median | Mean value | Variance | Skewness | Kurtosis | Individual no. | Distribution | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Normal | Non-normal |
| Data from Indonesia | | | | | | | | | | |
| Leaflet length (cm) | 2.03 | 6.05 | 5.03 | 4.84 | 0.70 | -1.32 | 1.84 | 65 | - | non-normal |
| Petiole length (mm) | 3.17 | 15.37 | 10.62 | 10.67 | 5.68 | -0.78 | 0.81 | 64 | normal | |
| Pod length (mm) | 8.00 | 20.00 | 13.51 | 13.61 | 3.14 | 0.66 | 3.75 | 63 | normalised at 99% | - |
| Node no./stem | 4.00 | 31.00 | 14.08 | 14.59 | 15.37 | 0.93 | 4.30 | 63 | normal | - |
| Internode length (mm) | 2.80 | 22.40 | 9.00 | 10.12 | 18.31 | 0.66 | 0.27 | 62 | normal | - |
| Plant spread (cm) | 2.60 | 41.18 | 27.75 | 27.06 | 76.60 | -0.68 | 0.57 | 65 | normal | - |
| Seed no./plant | 1.00 | 20.40 | 6.83 | 6.88 | 17.51 | 0.72 | 0.46 | 59 | normal | - |
| Data from TCRU at Nottingham University | | | | | | | | | | |
| Days to emergence | 1.456 | 790.046 | 0.662 | 1.456 | 16.707 | 0.818776 | 0.629145 | 76 | - | non-normal |
| Leaf no./plant | 1.456 | 790.046 | 0.662 | 1.456 | 790.0456 | 0.469311 | -0.16038 | 76 | normal | - |

Table 2. Distribution of the markers, length of linkage groups and marker density in the genetic map constructed with $F_3$ population of DipC × Tiga necaru cross.

| Linkage groups | Length (cM) | No. of markers mapped in the groups | | | Mean distances (cM/marker) |
|---|---|---|---|---|---|
| | | Total marker | SSR marker | DArT marker | |
| 1 (Map 2) | 58.2 | 40 | 3 | 37 | 1.46 |
| 2 | 38.1 | 15 | 0 | 15 | 2.54 |
| 3 | 33.5 | 13 | 3 | 10 | 2.58 |
| 4 | 36.6 | 11 | 0 | 11 | 3.33 |
| 5(Map 3) | 15.7 | 10 | 3 | 7 | 1.57 |
| 6(Map 2) | 23.3 | 9 | 2 | 7 | 2.59 |
| 7 | 2.3 | 9 | 0 | 9 | 0.26 |
| 8(Map 2) | 20.9 | 13 | 0 | 13 | 1.61 |
| 9 | 13.3 | 12 | 2 | 10 | 1.11 |
| 10 | 26.7 | 7 | 0 | 7 | 3.81 |
| 11 | 16.8 | 5 | 2 | 3 | 3.36 |
| 12 | 1.6 | 3 | 0 | 3 | 0.53 |
| 13 | 76.4 | 23 | 4 | 19 | 3.32 |
| 14 (Map 3) | 11.8 | 15 | 1 | 14 | 0.79 |
| 15 | 3.1 | 9 | 1 | 8 | 0.34 |
| 16 | 9.4 | 8 | 0 | 8 | 1.18 |
| 17 | 19.1 | 6 | 0 | 6 | 3.18 |
| 18 | 8 | 5 | 1 | 4 | 1.60 |
| 19 | 23.5 | 4 | 2 | 2 | 5.88 |
| 20 | 18.9 | 4 | 1 | 3 | 4.73 |
| 21 | 2 | 2 | 0 | 2 | 1.00 |
| 22 | 5.1 | 2 | 1 | 1 | 2.55 |
| 23 | 0 | 2 | 0 | 2 | 0.00 |
| 24 | 32.6 | 2 | 0 | 2 | 16.30 |
| 25 | 1.4 | 2 | 0 | 2 | 0.70 |
| Total | 498.3 | 231 | 26 | 205 | |
| Range | 0-76.4 | 2-40 | 0-4 | 1-37 | 0.26-5.88 |

Table 3. Pearson correlation coefficient between the traits data from the Indonesia planting of the cross between DipC × Tiga necaru in 2010.

| Traits | Plant spread (cm) | Leaflet length (cm) | Pod length (mm) | Node no./ stem | Biomass dry weight (g/plant) | Internode length (mm) | Seeds weight (g/plant) | Seed no./ plant |
|---|---|---|---|---|---|---|---|---|
| Leaflet length (cm) | **0.770 p=0.000 $R^2$=58.6%** | | | | | | | |
| Pod length (mm) | 0.237 p=0.64 | 0.242 p=0.58 | | | | | | |
| Node no./stem | 0.148 P=0.250 | 0.267 P=0.036 | 0.218 P=0.089 | | | | | |
| Biomass dry weight (g/plant) | **0.746 p=0.000 $R^2$=55.0%** | **0.947 p=0.000 $R^2$=89.6%** | **0.358 p=0.004 $R^2$=11.4%** | 0.116 p=0.196 | | | | |
| Internode length (mm) | 0.171 P=0.188 | 0.265 P=0.039 | -0.046 P=0.726 | **0.519 P=0.000 $R^2$=25.7%** | 0.138 P=0.289 | | | |
| Seeds weight (g/plant) | 0.187 p=0.159 | 0.171 p=0.199 | **0.411 p=0.001 $R^2$=15.4%** | 0.116 p=0.389 | 0.077 p=0.560 | **0.392 p=0.003 $R^2$=13.8%** | | |
| Seed no./plant | 0.116 p=0.384 | 0.076 p=0.56 | 0.264 p=0.046 | 0.112 p=0.401 | -0.030 p=0.824 | **0.418 p=0.001 $R^2$=16.0%** | 0.947 p=0.947 | |
| Petiole length (mm) | **0.766 p=0.000 $R^2$=58.1%** | **0.847 p=0.000 $R^2$=77.3%** | **0.427 p=0.001 $R^2$=16.8** | 0.221 p=0.087 | **0.817 P=0.000 $R^2$=66.2%** | 0.107 p=0.415 | 0.154 p=0.249 | 0.075 p=0.511 |

Table 4. Correlation coefficients between the traits data from Tropical Crops Research Unit growth of the cross between DipC × Tiga necaru in 2004.

| Traits | Days to emergence | Leaf no./plant | Plant spread | Pod no./plant |
|---|---|---|---|---|
| Leaf no/plant | -0.100<br>p=0.390 | | | |
| plant spread (cm) | 0.061<br>p=0.599 | **0.645**<br>**p=0.000**<br>**$R^2$=40.8%** | | |
| Pod no/plant | 0.084<br>p=0.472 | **0.537**<br>**p=0.000**<br>**$R^2$=27.9%** | **0.731**<br>**p=0.000**<br>**$R^2$=53.5** | |
| 100-seed weight (g) | **0.308**<br>**p=0.007**<br>**$R^2$=8.3%** | **0.463**<br>**p=0.000**<br>**$R^2$=20.3%** | 0.397<br>p=0.000 | 0.177<br>p=0.126 |

Table 5. A QTL mapping in Bambara groundnut using non-parametric (Kruskal Wallis analysis) and interval mapping.

| Traits | Kruskal-Wallis analysis (non-parametric mapping) | | | | | | Interval mapping | | | | | Permutation test |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Linkage group | Map Location (cM) | Locus | KW K* | df | Significance level | Interval mapping (cM) | LOD | Variance | % explained | Additive | |
| Leaflet length | 1 | 22.31 | bgPt-602039 | 17.78 | 1 | ******* | | | | | | |
| | 14 | 8.95 | mBam3co33 | 8.32 | 2 | ** | | | | | | |
| | 5 | 11.64 | PRIMER95 | 7.25 | 2 | ** | | | | | | |
| Petiole length | 1 | 22.31 | bgPt-602039 | 11.01 | 1 | ***** | 22.308 | 2.35 | 4.726 | 15.5 | 1.256 | 2.27 |
| Pod length | 14 | | | | | | 10.08 | 3.37 | 2.41 | 21.80 | 0.97 | 2.53 |
| | 14 | 8.95 | mBam3co33 | 14.63 | 2 | ***** | 8.95 | 3.04 | 2.47 | 19.90 | 0.89 | |
| Internode length | 3 | 2.98 | bgPabg-596988 | 20.93 | 1 | ******* | 2.98 | 7.09 | 10.64 | 40.90 | 3.17 | 2.9 |
| Node no./stem | 2 | 30.23 | bgPabg-594814 | 10.75 | 1 | **** | 30.20 | 2.79 | 12.33 | 18.40 | 2.18 | 2.6 |
| Plant spread | 1 | 22.31 | bgPt-602039 | 9.35 | 1 | **** | 22.31 | 2.36 | 63.83 | 15.40 | 4.57 | 2.8 |
| Seed no./plant | 13 | | | | | | 15.79 | 2.48 | 14.19 | 17.60 | 2.27 | 2.8 |
| | 13 | 19.87 | bgPt-601402 | 3.71 | 1 | * | 19.87 | 2.28 | 14.41 | 16.3 | 2.13 | |
| Days to emergence | 19 | 23.47 | PRIMER16 | 10.67 | 2 | **** | | | | | | |
| | 6 | 14.06 | bgPt-595387 | 9.95 | 1 | **** | | | | | | |
| Leaf no./plant | 8 | 8.96 | bgPt-598428 | 8.12 | 1 | **** | 8.96 | 3.11 | 658.98 | 17.80 | -15.19 | 2.65 |

Significant level of K * values: *: 0.10, **: 0.05, ***: 0.01, ****: 0.005, *****: 0.001, ******: 0.0005, *******: 0.0001.

## Figures

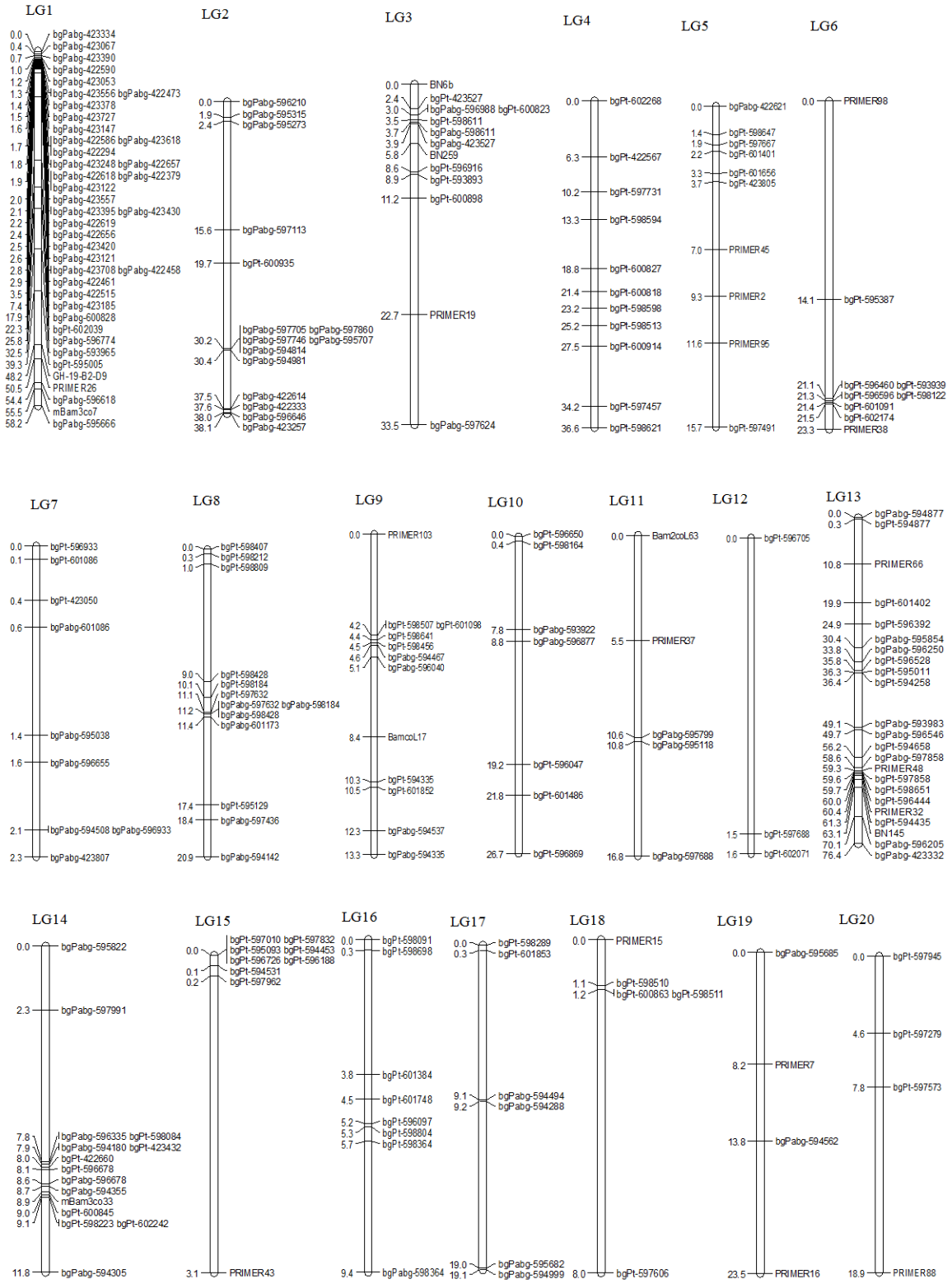


Fig. 1. Genetic linkage map of $F_3$ segregation population of 73 lines derived from crossing of DipC × Tiga necaru.