AFLP-based Genetic Linkage and Crossover Analysis in F_{10} Inbred Lines of Cowpea (*Vigna unguiculata* (L.) Walp.)

I. K. Asante[1]* and C. Fatokun[2]
[1.] *Department of Botany, P. O. Box LG55, University of Ghana, Legon-Accra, Ghana*
[2.] *International Institute of Tropical Agriculture, University of Ibadan, Ibadan, Nigeria*
** Corresponding author; E-mail: ikasante@ug.edu.gh*

Abstract

Genetic linkage map was constructed within the cultivated gene pool of cowpea from F_{10} recombinant inbred population of 94 individuals. The recombinant population was derived from a cross between the breeding lines Kanannado and 88DM-345 which were developed in Nigeria. Kanannado is day sensitive while 88DM-345 is day neutral. Twenty-three AFLP primer combinations were tested on the inbred lines and the parentals generating 141 marker loci. A total of 96 AFLP marker loci identified 11 linkage groups spanning 2551.6 cM with an average distance of 231.9 cM. Average marker density was 26.58 per marker loci. Linkage groups ranged from 17.2 to 1137.8 cM and included 3–35 marker loci, respectively. A total of 4330 crossing-over with an average of 2.0 per cM was observed. The average number of crossing-over per individual over the genome was 4.5 per chromosome per individual.

Introduction

The amplified fragment length polymorphism (AFLP) technique is a method of DNA fingerprinting (Vos *et al.*, 1995). This technique involves the combination of the strategies of restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR), and is carried out in four distinct steps (Miyashita *et al*., 1999): restriction enzyme digestion of DNA, ligation of adaptors to the restricted sites, PCR amplification of restricted fragments with primers that bind to the adaptor sequence and the adjacent selective nucleotides, and acrylamide gel electrophoresis. Large numbers of fragments are generated by using restriction enzymes with two different specificities such as *EcoRI* and *MseI*. Reliable and reproducible restricted fragments are achieved by PCR amplification with the specific primers. Acrylamide gel electrophoresis is employed to distinguish fragments differing in length by only one base pair. Therefore, AFLP can be a powerful technique to detect a large number of bands with high reproducibility and sensitivity.

AFLP analysis has been applied to construct genetic maps of many crop plants (barley: Becker *et al.,* 1995; melon: Wang *et al.,* 1997; potato: Van Der Voort *et al.,* 1997; *Arabidopsis thaliana*: Alonso-Blanco *et al.,* 1998), and to study phylogenetic relationship and genetic diversity among cultivars (barley: Russell *et al.,* 1997; Schut *et al*., 1997; cassava: Roa *et al*., 1997; Eucalyptus: Gaiotto *et al*., 1997; hop: Hartl & Seefelder 1998; maize: Marsan *et al*., 1998; wheat: Donini *et al*., 1997). AFLP analysis has been used to construct a fine genetic map (Alonso-Blanco *et al.,* 1998).

Knowledge of genetic maps of chromosomes is extremely useful in breeding programmes. Genetic maps enable the

breeder to accurately predict the inheritance of characters whose genes ere mapped and make it easier for him/her to select the right pairs of parents for crossing. This paper reports the results of genetic linkage map constructed within cultivated gene pool of cowpea from F_{10} recombinant inbred population of 94 individuals.

Materials and methods

Plant material

The mapping population of the cowpea used in the investigation was derived from a cross between two pure lines, Kanannado and 88DM-345. Kanannado is a day sensitive type, whereas 88DM-345 is a day neutral type. The recombinant inbred population was developed at the International Institute of Tropical Agriculture, Ibadan, Nigeria.

DNA extraction and AFLP markers

Seedlings were raised in the greenhouse and genomic DNA was extracted from the leaves of each F_{10} plant using the protocol as described by Dellaporta (1983).

Amplified fragment length polymorphism

The AFLP procedure was performed following the Life Technologies AFLP Analysis System 1 (GicoBRL, Gathersburg, MD) instructions and using the products of the kit. Genomic DNA of 250 ng from the two parental lines and the mapping population was restricted with 2.5 U each of *EcoRI* and *MseI* in a 25-µl reaction mixture for 2 h at 37 °C. Twenty-five microlitres of a mixture containing *EcoRI* and *MseI* adapter, 1 U T_4 DNA ligase, 0.4 m*M* ATP in 10 m*M* Tris-HCl (*p*H 7.5), 10 m*M* magnesium acetate, and 50 m*M* potassium acetate were added. Ligation was incubated at 20 °C for 2 h. Five microlitres of a 10-fold-diluted ligation were amplified for a preselective amplification using a pair of primers based on the sequence of *EcoRI* and *MseI* adapters, including one additional selective nucleotide at the 3' end. Selective amplification reaction was performed with the *EcoRI* and *MseI* primers including three additional selective nucleotides at the 3' end. The *EcoRI* primer was labeled at the 5' end with [γ-^{33}P] ATP using a T_4 polynucleotide kinase.

The reaction was performed in a 10-µl reaction mix containing 5 µl of 50-fold-diluted preamplified DNA, *EcoRI* and *MseI* primers, dNTPs, 10x PCR buffer 100 m*M* Tris-HCl (*p*H 8.3), 15 m*M* $MgCl_2$, 500 m*M* KCl and 0.5 U Taq DNA polymerase (GibcoBRL). Amplifications were performed using MJ Research (Watertown, Mass.) PTC 100 or PTC 200 thermocyclers. One cycle was performed at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 60 s, followed by 0.7 °C each cycle, and finally 23 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s. The amplification products were denatured at 90 °C for 3 min after addition of 5 µl of loading buffer (98% v/v formamide, 0.5 m*M* EDTA, 0.25% w/w bromophenol blue, 0.25 w/w xylene cyanol). Later 7 µl of each sample were loaded onto a 6% w/v denaturing polyacrylamide gel (7.0 *M* urea, 10x TBE buffer and 40% bisacrylamide) and electrophoresed in a 1x TBE buffer at 60 W for 2.5 h, or until xylene cyanol was two-thirds down the length of the gel.

Linkage analysis

Each polymorphic AFLP marker was identified by primer pair combination with a

band number. Polymorphic bands were numbered serially in a descending order of molecular size. Clear and unambiguous bands were scored for the presence or absence of the corresponding band in the parents and among the segregating recombinant inbred population. All markers were tested for the expected 1:1 segregation by the χ^2 test at the $P = 0.01$ level of significance with the SAS software. Linkage analysis was performed using *Mapl*. Markers were assigned to linkage groups using the "group" and "order" commands with a LOD score of 5.0. Map distances were calculated using Kosambi mapping function (Kosambi, 1994).

Results and discussion

Molecular markers

Twenty-three selective AFLP primer pairs were tested on the cowpea inbred population. These primer combinations were highly polymorphic.

Marker segregation

Cowpea is a diploid with disomic inheritance. From a total of 141 loci scored for mapping, 21% segregated in normal Mendelian segregation, with approximately 73% segregating 1:1, while the remaining 17% showed a 3:1 segregation ratio (data not shown).

Map construction

A total of 96 AFLP marker loci were distributed in 11 LGs. Markers in the 11 LGs covered a length of 2,551.6 cM with an average of 9.0 loci per LG. LG 2 was the longest group with 35 loci spanning 1,137.8 cM and LG 5 was the shortest with three loci spanning 17.2 cM. The average size of the LGs was 231.9 cM. The average marker density was 26.58 cM per marker. Generally, the marker loci were not evenly distributed throughout the genome; large gaps of over 54.9 cM were present in all LGs except in LGs 8, 9, 11 and 5.

The total crossing-over over the total map length of 2,551.6 cM was 4,330 with an average of 2.0 per cM. Average number of crossing-over per plant over the genome was 4.50 per chromosome per plant. LG2 recorded the highest number of crossing-over with a mean of 20.21 per plant and a variance of 175.18 (Table 1). LGs 5 and 9 recorded the lowest number of crossing-over of 32 per plant each with an average of 0.34 each and variance of 1.26 and 0.57, respectively. In LGs 5 and 9, crossing-over occurred in about 7.0% and 14% of plants. In LG2 crossing-over occurred in about 83% of plants.

The vast majority of the markers showed 1:1 Mendelian segregation ratio; this is indication that most of the loci were in a heterozygous state in one parent and in a homozygous state in the other parent. Only 17% of the markers showed a 3:1 segregation pattern, indicating a heterozygous state in both parents. Similar segregation pattern was observed by Saha *et al.* (2004) in the study of SSR- and AFLP-based genetic linkage map of tall fescue (*Festuca arundinacea* Schreb.) Using RAPDs markers Menedez *et al.* (1997) observed that the vast majority of loci showed 3:1 Mendelian segregation ratio but about 14% showed 1:2:1 segregation pattern in domesticated inbred lines of cowpea. A similar pattern was observed in AFLP markers linked to resistance of cowpea to parasitism by *Striga gesnerioides* (Ouedraogo *et al.*, 2001).

TABLE1
Statistics of crossover for the linkage groups

Linkage group	*Total map length (cM)*	*Total number of crossover*	*Mean number of crossover per plant*	*Variance*	*Standard deviation*
1	427.1	735	7.82	26.52	5.15
2	1137.8	1900	20.21	175.18	13.24
3	366.8	598	6.36	24.17	4.92
4	109.9	180	1.91	2.57	1.60
5	17.2	32	0.34	1.26	1.12
6	259.8	467	4.97	12.10	3.48
7	81.0	140	1.49	2.06	1.43
8	29.7	50	0.53	0.79	0.89
9	17.7	32	0.34	0.57	0.76
10	84.3	148	1.57	2.31	1.52
11	20.6	38	4.0	0.65	0.81

Markers present in both parents and showing a 3:1 segregation ratio proved useful for identifying homologous groups between maps in aligning male and female linkage maps of apple (*Malus pumila* Mill.) using multi-allelic markers (Maliepaard *et al.*, 1998). In the present work the proportion of Kanannado alleles was slightly higher (0.519) than that of 88DM-345 alleles (0.481).

On LG9, six markers showed excess of Kanannado alleles whereas three showed excess of 88DM-345. Two markers at one end showed segregation distortion (22.2%). Distorted segregation was also observed in cowpea in genetic linkage map of domesticated inbred lines (Menedez *et al.*, 1997). Similar proportions of distorted segregation were found in a cross between wild and cultivated cowpea cross (22%) (Menancio-Hautea *et al.*, 1993) and in potato (25%) (Gebhardt *et al.*, 1989). High levels of segregation distortion were observed in a cross between two distantly related heterozygous tall fescue genotypes (Saha *et al.*, 2004) and in rye (Warnke *et al.*, 2004). Segregation distortion can be caused by various processes, among which are gametic selection and/or faulty chromosome pairing, an association between heterozygosity and plant vigour, the selection of one parent type, and genetic self-incompatibility (Xu *et al.*, 1995; Saha *et al.*, 2004). However, the involvement of genetic self-incompatibility genes in segretaion distortion in the present work is doubtful since cowpea is a predominantly self-pollinating plant.

The present cowpea map spans 2,551.6 cM as against 972 cM and 684 cM in previous work based on RAPDs and RFLPs, respectively. The differences might be due to the higher level of polymorphism revealed by AFLPs. The average length of 231.9 cM per LG corresponds to approximately 4.5 chiasmata per chromosome. This is the first report on an attempt to analyse chiamata frequency in the cowpea. In the tall fescue genotypes an average length of 102.2 cM corresponds to approximately 2.0 chiasmata per chromosome (Saha *et al.*, 2004). In 50 genotypes of a Norwegian cultivar of

meadow fescue, Simosen (1975) showed that the mean number of chiasmata per chromosome ranged from 1.5 to 1.96. Alma *et al.* (2003) also reported an average map length of 94.1 cM per chromosome that corresponds to approximately 1.9 chiasmata per chromosome. The average marker density was 26.58 cM per marker. Markers were highly clustered in some regions, but there were gaps of 8.6 cM–50 cM in some LGs. This indicates that either recombination events or mapped loci were not evenly distributed throughout the genome.

References

Alm V., Fang C., Devos K. M., Vollan K., Grieg Z and **Rognli O. A.** (2003). A linkage map of meadew fescue (*Festuca pretense* Huds.) and comparative mapping with other *Poaceae* species. *Theor. appl. Genet.* **108**: 25–40.

Alonso-Blanco C., Peeters A. J., Koornneef M., Lister C. and **Dean C.** (1998). Development of an AFLP-based linkage map of Ler, Col and Cvi *Arabidopsis thaliana* ecotypes and construction of Ler/Cvi recombinant inbred line population. *Pl. J.* **14**: 259–271.

Becker J., Vos P., Kuiper M., Salamini F. and **Heun M**. (1995). Combined mapping of AFLP and RFLP markers in barley. *Mol. Gen. Genet.* **249**: 65–73.

Dellaporta S. L., Wood J. and **Hicks J. B.** (1983). A plant DNA mini preparation: version II. *Pl. Mol. Biol. Rep*. **1**: 19-21.

Donini P., Elias L. M., Bougourd S. M. and **Koebner M. D.** (1997). AFLP fingerprint reveals pattern differences between template DNA extracted from different plants. *Genome* **40**: 521–526.

Fatokun C. A., Danesh D., Menancio-Hautea D. and **Young N. D.** (1993). A linkage map for cowpea (*Vigna unguiculata* (L.) Walp) based on DNA markers. In *A compilation of linkage and restriction maps of genetically studied organisms, genetic maps 1992* (J. S. O'Brien, ed.), pp. 6256–6258. Cold Spring Harbor Laboratory Press, New York.

Gaiotto F., Bramucci M. and **Grattapaglia** (1997). Estimation of outcrossing rate in a breeding population of *Eucalyptus urophylla* with dominant RAPD and AFLP markers. *Theor. appl. Genet.* **95**: 842–849.

Gebhardt C., Ritter E., Debbener T., Schachtschabe V., Walkemeier B., Ubrg H. and **Salamini F.** (1989) RFLP analysis and linkage mapping in *Solanum tuberosum*. *Theor. appl. Genet.* **78**: 65–75.

Hartl L., and **Seefelder S.** (1998). Diversity of selected hop cultivars detected by fluorescent AFLPs. *Theor. appl. Genet.* **96**: 112–116.

Kosambi D. D. (1944). The estimation of map distances from recombination values. *Ann. Eugenics* **12**: 172–175

Marsan P. A., Castiglioni P., Fusari K., Kuiper M. and **Motto M.** (1998) Genetic diversity and its relationship to hybrid performance in maize as revealed by RFLP and AFLP markers. *Theor. appl. Genet*. **96**: 219–227.

Mayiishata N. T., Innan H. and **Terauchi R.** (1996). Intra- and interspecific variation in the alcohol dehydrogenase locus region of wild plants *Arabis gemmifera* and *Arabidopsis thaliana*. *Mol. Biol. Evol*. **13**: 433–436.

Maliepaard C., Alston F. H., van Arkel G.., Brown L. M., Chereau E., Dunemann F., Evans K. M., Gardiner S., Guilford P., van Heusden A. W., Janse J., Laurens F., Lynn J. R., Manganaris A. G., den Nijs A. P. M., Periam N., Rikkrink E., Roche P., Ryder C., Sansavani S., Schmidt H., Tartarini S., Verhaegh J. J., Vrielink-van G. M. and **King G. J.** (1998). Aligning male and female linkage maps of apple (*Malus pumila* Mill) using multiallelic markers. *Theor. appl. Genet.* **97**: 60–73.

Menancio-Hautea D., Fatokun C. A., Kumarl, Danesh D. and **Young N. D.** (1993). Comparative genome analysis of mung bean (*Vigna radiata* L., Wilezek) and cowpea (*Vigna unguiculata* L. Walpers) using RFLP mapping data. *Theor. appl. Genet.* **86**: 797–810.

Menendez C. M., Hall A. E. and **Gepts P.** (1997). A genetic linkage map of cowpea (*Vigna unguiculata* developed from a cross between two inbred, domesticated lines. *Theor. appl. Genet*. **95**: 1210–1217.

Ouedraogo J. T., Maheshwari V., Berner D. K., St-Pierre C. A., Belzile F. and **Timko M. P.**

(2001). Identification of AFLP markers linked to resistance of cowpea (*Vigna unguiculata* L.) to parasitism by *Striga gesnerioides*. *Theor. appl. Genet.* **102**: 1029–1036.

Roa A. C., Maya M. M., Duque M. C., Tohme J. and **Allem A. C.** (1997). AFLP analysis of relationships among cassava and other *Manihot* species. *Theor. appl. Genet.* **95**: 741–750.

Russel J. R., Fuller J. D., Macaulay M., Hatz B. G. and **Jahooir** (1997). Direct comparison of levels of genetic variation among barley accessions detected by RFLPs, AFLPs, AARs and RAPDs. *Theor. appl. Genet.* **95**: 714–722.

Saha M. C., Mian M. A. R., Eujayl I., Zwonitzer J. C., Wang L. and **May G. D.** (2004). Tall fescue EST-SSR markers with transferability across several grass species. *Theor. appl. Genet.* **109**: 783–791.

Schut J. T., Qi X. and **Stam P.** (1997). Association between relationship measures based on AFLP markers, pedigree data and morphological traits in barley. *Theor. appl. Genet.* **95**:1161–1168.

Simonsen Ø. (1975). Cytogenetic investigations in diploid and autotetraploid population of *Festuca pratensis* Huds. *Heriditas* **79**: 73–108.

Vos P., Hogers R., Bleaker M., Reijans M., van de Lee T., Harnes M., Fritjers A., Pot J., Peleman J., Kuiper M. and **Zabeau M.** (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* **23**: 4407–4414.

Van der voort J. R., Wolters P., Folkertsma R., Hutten R. and **Zandvoort P.** (Year?). Mapping of the cyst nematode resistance locus Gpa2 in potato using a strategy based on comigrating AFLP markers. *Theor. appl. Genet.* **95**: 874–880.

Wang Y. H., Thomas C. E. and **Dean R. A.** (1997). A genetic map of melon (*Cucumis melo* L.) based on amplified length polymorphism (AFLP) markers. *Theor. appl. Genet.* **95**: 791–798.

Warnke S. E., Barker R. E., Jung G., Sim S. C., Mian Mar, Saha M. C., Brilman L. A., Dupal M. P. and **Forster J. W.** (2004). Genetic linkage mapping of an annual x perennial ryegrass population. *Theor. appl. Genet.* **109**: 294–304.

Xu W. W., Sleper D. A. and **Chao S.** (1995). Genome mapping of tall fescue (*Festuca arundinacea* Shreb.) with RFLP markers. *Theor. appl. Genet.* **91**: 947–955.

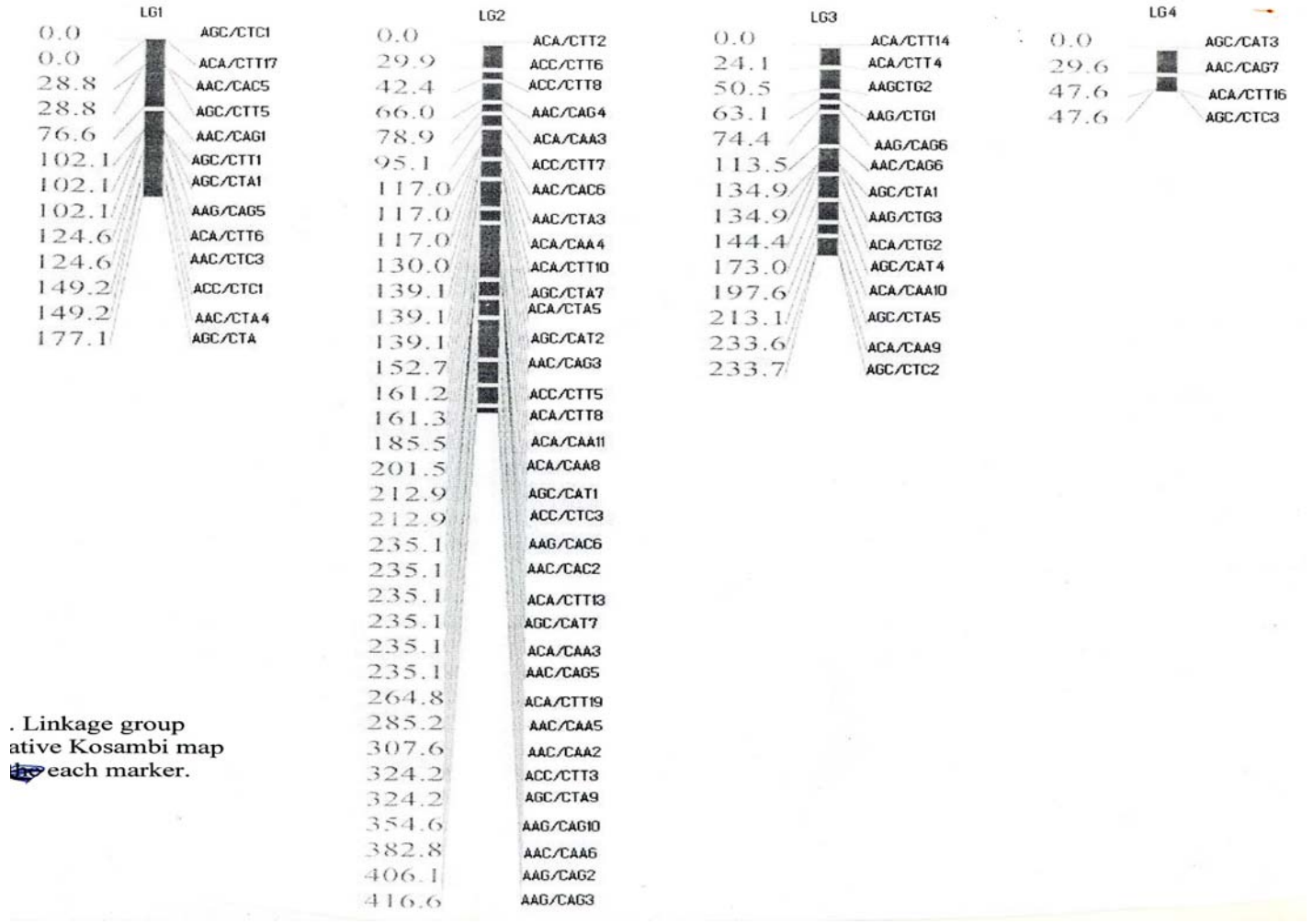

Fig.1. Linkage map of *Vigna unguiculata*. Linkage group numbers are indicated on the top. Cumulative Kosami map distances are indicated at the left side of each marker.

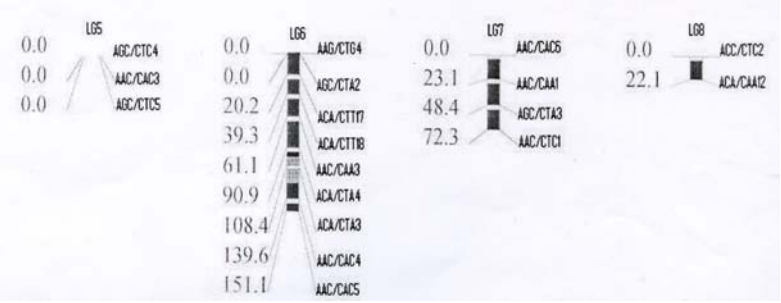

Fig.1 cont'd. Linkage map of *Vigna unguiculata.* Linkage group numbers are indicated on the top. Cumulative Kosami map distances are indicated at the left side of each marker.