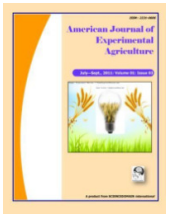

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Analysis of AFLP Markers for Screening Resistance to Common Bean Roots Rot (*Pythium* spp.)

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Authors' contributions

This work was carried out in collaboration between all authors. Author VAP and NKM designed the study, wrote the protocol and wrote the first draft of the manuscript. Author KAS reviewed the experimental design and managed laboratory analysis. Author NKM conducted the laboratory experiments. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To investigate AFLPs for suitability as potential markers for identification of sources of *Pythium* resistance in bean genotypes preferred by poor small holder farmers.
Place and Duration of Study: Department of biological sciences Masinde Muliro University of Science and Technology, Kenya and Bangor University, North Wales, United Kingdom, between September 2010 and December 2012.
Methodology: 45 common bean accessions comprising 35 seed samples collected from farmers, market centers, as well as seed stockists and ten common bean lines generated from a conventional breeding program with differential resistance to the pathogen *Pythium* were subjected to AFLP analysis. Genetic characterization using cluster and principal component analysis were conducted to determine segregating patterns of bean accessions in relation to tolerant and

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susceptible bean lines. Average genetic distances were calculated and similarity coefficients subjected to unweighted pair group method of arithmetic averages to generate dendograms.
Results: Two informative AFLP primer combinations yielded 194 polymorphic loci. Genetic distance of bean samples from KARI Kakamega had 56 to 414 base pairs with a variability index of 0.63 to 0.90. Combined analysis of bean accessions from KARI breeding program and market class common bean samples revealed a variability index range of 0.62 to 0.90 with 56 to 420 base pairs. PCA contributed about 51.58% on the genetic variation. Cluster analysis of the 10 KARI-Kakamega bean lines revealed that resistant bean varieties were genetically different from the susceptible bean varieties. The dendogram generated revealed four sub-groups and with the exception of *Alulu,* a mildly resistant cultivar*,* that segregated alongside resistant cultivars, resistant varieties clustered differently from susceptible cultivars. However, screening with farmers' germplasm produced a dendogram that revealed a mixture of distinct and relatively non-distinct categorization with regard to resistance. Only resistant cultivars *AN1062, R2075, R719 and R1946* and susceptible cultivars *GLP2* and *GLP585* clustered together as expected. The others segregated randomly alongside the farmers' germplasm. Resistant varieties *AND1062*, *R2075*, *R719, R1946 and SCAM80* were more genetically related to marketable *class of beans. R1946* R719, *R2075* and *AND1062* are closely related genetically compared to *Mw001, KK15, Alulu* and *GLP2*.
Conclusion: AFLP is a relatively informative technique that has a great potential of delineating susceptible and resistant *Pythium* root rot dry bean varieties, and can be used as a preliminary guide to carry out further analysis. It is notable that the AFLP markers used were not able to clearly distinguish all the cultivars comprehensively and should not be used alone in determining resistance levels. The information generated in this study will contribute to the propagation of acceptable market class bean lines with resistance to *Pythium* root rot for improved livelihood and increased food security.

Keywords: Pythium root rot; resistance; susceptibility; common beans; AFLP markers.

1. INTRODUCTION

Bean production is an important and widespread farming activity in Kenya. In tropical regions, common bean is characterized by low and unstable grain yields due to various ecological and agronomic parameters. The common bean suffers from several biotic and abiotic production constraints. Among these parameters, bean root rot and a decline in soil fertility are among the major causes leading to bean yield losses [1,2]. Biotic constraints include diseases such as angular leaf spot, anthracnose, root rots, rusts, halo blight, and bean common mosaic virus [3-6]. These diseases and pests are classified as high to moderate in importance in Kenya highlands [7].

There has been an increase in the importance of *Pythium* bean root rots in several countries of Eastern and Central Africa, such as Burundi, the Democratic Republic of Congo, Kenya and Uganda and Rwanda, due to severe break out of root rots. *Pythium* is caused by a complex of soil borne pathogens that occur singly or in a complex of two or more organisms, with the most important ones being *Fusarium solani* f. sp. *phaseoli* and *Pythium ultimum* var. *ultimum* that results in considerable losses of about 221,000 million tonnes/year [4,7-9]. Based on spatial distribution damage and effect on yield, *Pythium* species are more frequently associated with severe outbreaks resulting in 70-100% bean loss depending on severity [3,10]. *Pythium* is known to survive in the soil for several years as oospores that germinate to produce zoospores which infect the root and lower stem [8]. The pathogen is very persistent in the soil and when susceptible varieties are used, complete yield losses usually occur when the environmental conditions are favorable for pathogen development [11-13]. Unfortunately, almost all commercial varieties released in Kenya are susceptible to this fungus [14]. This is partially due to lack of widespread utility of proficient procedures for delineating resistant and susceptible cultivars in crop improvement programmes. This therefore necessitates a need to urgently develop and deploy efficient protocols that can tag the disease.

Generally resistance to *Pythium* bean root rot disease is quite complicated and not well elucidated [13,15-18]. Resistance is reportedly genetic although it seems to vary depending on the stage of development and cultivar. Polygenic resistance in bean seed decay and pre-emergence damping off has previously been

noted [15,16]. Complex resistance in a bean line *A300* has been reported [18]. However upon crossing a cultivar *A300* with several *Navy* beans to develop root rot resistance, quantitative mechanism of genetic resistance was achieved. However [19] have indicated that resistance is qualitative while tolerance could be quantitative. However, substantial research has been undertaken in East Africa to understand genetics of inheritance to root rot resistance [13,19]. Resistance to the disease is thought to be inherited as a dominant character following experiments conducted using resistant and susceptible varieties to *Pythium* [13,19]. These experiments concluded that resistance to *Pythium* spp. is inherited as a single and dominant character. Molecular markers have also been deployed characterize bean root rot resistance and to ascertain the allelic relationship between the resistant genes present in different genotypes that had been conventionally developed. This indicates that inheritance of resistance to bean root rot maybe both qualitative and quantitative with modifying effects.

Marker assisted selection (MAS) is a protocol that permits the use of DNA markers that are tightly linked to target loci as a substitute for or to assist in phenotypic screening. A marker can either be located within the gene of interest or be linked to a gene determining a trait of interest. MAS can assist in the selection for a trait based on genotype using associated markers rather than the phenotype of the trait [20,21]. Reliance on phenotypic selection to improve plant varieties has been used by plant breeders to achieving breeding progress through the assessment of phenotypic and genotypic characteristics including disease resistances traits. This has resulted in the increased utility of easily detectable DNA markers in crop improvement. To examine changes in diversity over time in an objective manner, AFLPs or microsatellite markers appear most useful [22] amongst other molecular markers as they are appropriate tools for distinguishing plant varieties or lines [23]. The use of DNA markers for screening and selection of plants for disease resistance in a breeding program offers several advantages over conventional methods. DNA marker based genotypes can be obtained from almost any plant tissue, plants can be screened already at the seedling stage or even as seeds, thus allowing early selection for traits which may be expressed in adult plants only such as, grain or fruit quality, male sterility, photoperiod sensitivity. DNA markers can easily target alleles that are difficult, expensive and/or time consuming to score phenotypically [24]. In addition, selection for desirable traits can be made on the basis of a single plant whereas this would not be possible by phenotypic selection. Poor heritability does not pose a problem if selection is based on marker information. For traits with complex inheritance every individual genetic component contributing to the trait can be selected separately. Also, multiple characters that would normally be epistatic can be maintained and ultimately fixed [25]. Molecular markers are essential in breeding as recessive genes can be maintained without the need for progeny tests in each generation, as homozygous and heterozygous plants can be distinguished with the aid of co-dominant markers [26-28]. The paramount importance of marker assisted selection is achievement of breeding goals in a shorter time than is achieved through conventional breeding, by a precise assemblage of target traits with less unpremeditated crop loss [29].

MAS can never replace phenotypic selection especially for disease resistance because a final testing of breeding lines is always obligatory, regardless of how tight a marker is linked to a gene or QTL [30]. It has been applied in the main agricultural crops, mainly wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare*), potato (*Solanum tuberosum*), maize (*Zea mays*), fruits and vegetables, particularly tomato (*Solanum lycopersicum*), complemented with some examples from rice (*Oryza sativa*) and soybean (*Glycine max*). Molecular markers have been used at CIMMYT (International Maize and Wheat Improvement Center) in applied wheat breeding to shows 45,000 MAS data points per year are generated between the two wheat growing seasons [31]. In their work, MAS is considered as a technology which will increasingly be adopted in breeding programs in the private and public sectors. According to CIMMYT, the success of utilization of markers in wheat breeding depends to a high degree on the understanding of biotechnologists and breeders working together. MAS in Barley have progressed further than in wheat, which is probably due to the simpler, diploid genome. In contrast to wheat, barley varieties have been released that are based on MAS [32]. Breeding for barley yellow mosaic virus resistance and rust resistance are a main focus of marker selection in Barley. The most important use of MAS in maize is backcrossing of transgenes into elite

inbred lines [33], use of microsatellite markers for the conversion of normal maize lines into Quality Protein Maize (QPM), containing more lysine and tryptophan than the native lines [34], or the introgression of favourable QTL for earliness and grain yield between maize elite lines [35]. Marker- assisted resistance breeding in potato and marker-assisted introgression has been successful [36]. Although various markers have been developed [36], practical applications in breeding are still rare. Most marker applications are carried out in experimental populations of diploid potatoes [37], which impede the application of experimental results in practical breeding work. Bean germplasm lines have been improved through MAS too [38-44]. One white bean (*P. vulgaris* L.) variety that is resistant to bean golden yellow mosaic virus has been released successfully through MAS and carries QTLs for common bacterial blight resistance [45].

In order to increase productivity and yield stability on farms, scientists need faster and proficient protocols to tag root rot resistance. Such procedures have the potential to facilitate development of common bean cultivars that are acceptable, well adapted, marketable and disease resistant. So far, the level of *Pythium* root rot resistance in the local farmers' bean germplasm remains unknown. The bean breeding program at Kenya Agricultural Research Institute (KARI) Kakamega station has over time developed several root rot resistant bean lines with albeit low farmers acceptability using classical crop improvement methods. This programme could be made more efficient by the development of MAS markers that tag root rot resistance. Since farmers germplasm may possess sources of resistance to this disease, there is need to develop faster seed trait identification processes and screen them for he traits in order to harness germplasm potential. Because the KARI Kakamega bean breeding program relies on conventional techniques of selection characterized by introgression of desirable traits from exotic bean cultivars, incorporation of marker assisted techniques into the program will reduce duration taken in developing and releasing desirable bean lines. This will ultimately lead to the development of suitable *Pythium* root rot resistant germplasm for marketing purposes and for improved bean productivity in the region [13,14]. This study therefore investigated the potential of AFLP markers to differentiate resistant and susceptible *Pythium* root rot phenotypes and explored the potential of AFLP markers in enhancing the identification and selection of *Pythium* resistant germplasm for probable utilization in bean improvement programs in the region. Knowledge about identified resistance sources of beans combined with information of the polymorphic levels that exists between the improved resistant bean lines and commercial varieties will reveal the level of diversity and contribute towards development of appropriate protocols with potential to decrease adoption rates and duration taken to release improved bean varieties in Eastern and Central Africa.

2. MATERIALS AND METHODS

2.1 Plant Materials

This study was based on a sample of 45 bean seeds obtained from Kenya Agricultural research institute (KARI Kakamega Station) and from a survey conducted in 2010 (Tables 1&2). The KARI common bean lines that had been developed in a conventional breeding program were used in developing a marker assisted protocol. Pathogenicity status of the KARI samples has been previously determined [14,20] as described in Table 1. The germplasm included 6 resistant one moderately resistant and 4 susceptible lines (Table 1). Thirty five marketable common bean samples obtained from a survey conducted in 2010 were used for screening purposes by determining the comparative genetic diversity with the susceptible/resistant bean lines using AFLP markers (Table 2).

2.2 Leaf Sample Preparation, Isolation of Genomic DNA and Primer Selection

Plant materials selected for the study were grown in the greenhouse sterilized soil filled in 20 cm plastic pots for three weeks. For each bean accession, young fresh leaves about 5 - 6 leaf stage were harvested for DNA extraction using the Qiagen DNA extraction kit, following the instructions of the manufacturer with minor modifications. 300gms of fresh tissue were used. 50 ng/ul of DNA was used in subsequent AFLP analysis. A total of 5 primers (Table 3) previously used for screening dry bean samples and two universal primers were initially used to analyze the breeding lines collected from KARI [14,46]. Of these, three AFLPs primer combinations were selected due to reproducibility of polymorphic bands and information content.

Table 1. *Pythium* resistance status, growth habit and seed size characteristics of bean lines used in the study

Serial no.	Variety	Reaction to *Pythium spp.*	Rate of disease development	Growth habit	Seed size
1	RWR- 719	Resistant (RR)	Very low	Determinate	Small
2	AND 1062	Resistant (RR)	Very low	Determinate	Large
3	RWR 1946	Resistant (RR)	Very low	Determinate	Medium
4	RWR 2075	Resistant (RR)	Very low	Determinate	Medium
5	KK 15	Resistant (RR)	Very low	Indeterminate	Medium
6	Scam 80	Resistant (RR)	Very low	Determinate	Medium
7	GLP 585	Susceptible (SS)	Fast	Indeterminate	Small
8	GLP 2	Susceptible (SS)	Fast	Determinate	Medium
9	Mwitemania	Susceptible (SS)	Fast	Determinate	Medium
10	Alulu	Moderately resistant (MR)	Moderate	Determinate	Large

Adapted from [14], [20]

Table 2. Characterization of 35 bean accessions used in the genetic diversity study

Accession code	Local name	Colour and seed size	Longitude	Latitude
Mw01	Mwitemania	Pinto and medium	00.26589°	037.75072°
Ro02	Rose coco	Calima and medium	00.26836°	037.66205°
GA03	Gasele	White and medium to large	00.31809°	037.66199°
Mw04	Mwitemania	Pinto bean and medium	00.33229°	037.64431°
Gas05	Gasele	White and medium to large	00.04799°	037.65575°
Can06	Canadian wonder	Kidney red and medium to large	00.12636°	037.56236°
Wai07	Wairimu	Red haricot small	00.56743°	037.49013°
Can08	Canadian wonder	Kidney red and medium to large	00.60266°	037.51808°
Rmw09	Red Mwitemania	Pinto and medium	00.49228°	037.46354°
Roc10	Rose coco	Rose coco; Variegated, purple on cream, medium sized, globular.	00.40642°	037.62504°
Mw11	Mwitemania	Pinto and medium.	00.39233°	037.60790°
Rmw12	Red Mwitemania	Pinto and medium.	00.38413°	037.58708°
Ny13	Nyayo	Calima and medium	01.56349°	037.25222°
Kb14	KATB1	Yellow and medium oblong.	01.56406°	037.25496°
Kik15	Kikuyu	Rose coco; Variegated, red on cream, medium sized, globular.	01.53483°	037.26243°
Roc16	Rose coco	Grey calima and medium	00.12736°	034.73612°
KKf17	KK15	Black and medium oblong	00.05426°	034.74751°
Rh18	Red Haricot	Red haricot small	00.06007°	034.72225°
Sugo19	Sugar 1	Surgar and Medium size oblong.	00.12375°	034.78494°
KKt20	KK 20	Rose coco; variegated with large red flecks on cream, medium to large sized, oblong.	00.11862°	034.80309°
Rosc021	Rose coco	Rose coco; Variegated, purple on cream, medium sized, globular.	00.28283°	034.72213˚
Rosc022	Rose coco	Rose coco; Variegated, purple on cream, medium sized, globular.	00.28405°	034.70447°
Sugf23	Sugar 5		00.28305°	034.72014°
Wai24	Wairimu	Red haricot: small to medium sized, oblong.	00.29542°	034.54762°
Okuo25	Okuodho	Canadian wonder; Purple- black, medium to large sized, oblong	00.29649°	034.54858°
Kik26	Kikuyu	Rose coco; Variegated, red on cream, medium sized, globular	00.2964°	034.54858°
Rai27	Raila	Rose coco; variegated with large	00.56354°	034.57097°

Accession code	Local name	Colour and seed size	Longitude	Latitude
		red flecks on cream, medium sized and oblong.		
Katv28	Kat. Variety	Canadian wonder; Purple- black, medium to large sized, oblong	00.25069°	034.75063°
Gi3029	Gasele	Canadian wonder; white to light grey, medium to large sized, oblong	00.24770°	034.75199°
Okuo30	Okuodho	Canadian wonder; Purple- black, medium to large sized, oblong	00.33535°	034.47865°
Mwitemania	Mwitemania	Mwitemania; resembles Pinto bean, variegated, green – grey on cream, small to medium sized globular.	Nil	Nil
Mwezi moja	APC	Mwezi moja; many fine purple spots, medium to large sized, oblong.	Nil	Nil
Mwitemania	Mwitemania	Mwitemania; resembles Pinto bean, variegated, green – grey on cream, small to medium sized globular.	Nil	Nil
Raco4C	Rose coco	Rose coco; Variegated, purple on cream, medium sized, globular.		
Gac03C	White haricot	Canadian wonder; white to light grey, medium to large sized, oblong	Nil	Nil

Table 3. Sequence of AFLP primers used for evaluation of improved bean types from KARI- Kakamega

No.	Code	Primer sequence (5`→3`)	Source	Reference
1.	E24	GACTGCGTACCAATTCTC	Applied biosystems, CA, USA	[47]
2.	E33	GACTGCGTACCAATTCAAG	Applied biosystems, CA, USA	[47]
3.	E35	GACTGCGTACCAATTCACA	Otsyula et al. 2010	[14]
4.	M50	GATGAGTCCTGAGTAACAT	Sustar-Vozlic et al., 2006	[46]
5.	M62	GATGAGTCCTGAGTAACTT	Sustar-Vozlic et al., 2006	[46]
6.	**E00	GTAGACTGCGTACCATTC	Applied biosystems, CA, USA	[47]
7.	**M00	GACGATGAGTCCTGAGTAA	Applied biosystems, CA, USA	[47]

*NB: **E00 **M00 are universal primers*

2.3 AFLP Analysis

The AFLP assay was conducted using a modified version of [48] and adapted for use with the CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc). Total genomic DNA of each bean accession was digested with restriction endonucleases (EcoR I and Mse I). The restriction digestion involved 4 µl of NEB_4 buffer, 0.1 µl of 5 units EcoR I (50 U/µl), 0.5 µl of 5 units Mse I/Tru 91 adaptors (10 U/µl) and 4 µl of PCR water added to a volume containing 500 ng DNA and made up to 40 µl with PCR grade water. A total of 40 µl of restriction mixture and DNA was incubated for 1 hour at 37°C. The ligation step involved mixing 1 µl of 5 pMol EcoR I adaptor (Adapter 1: 5'-CTCGTAGACTGCGTACC-3'; Adapter 2: 5'-ATTGGTACGCAGTCTAC-3'), 1 µl of 50 pMol MseI/Tru 91adaptors (adapter 1: 5'-GACGATGAGTCCTGAG-3'; adapter 2: 5'-TACTCAGGACTCAT-3'), 1.0 µl of T_4 DNA ligase buffer (10x), 0.3 µl T_4 DNA ligase, (1 unit) and 6.7 µl PCR grade water, to a total volume of 10 µl of the ligation component. The 10 µl was pipetted to microfuge tubes containing the results of each restriction digest mixture and incubated at 37°C for 3 hours. PCR was conducted using primers E00 universal primer for EcoR I and M00 universal primer for Mse I were used for the preamplification step. Selective amplification stage involved the use of E33, E35, M50 and

M62 primers (Table 3). The preamplification primer mix for one sample comprised of universal primer 50 ng/µl (E00 for EcoR I) 0.6 µl, universal primer 50 ng/µl (M00 for Mse I) 0.6 µl and 3.8 µl of PCR grade water. 5 µl of the preamplification mix, 10 µl Bioline Biomix^{TM} PCR master mix containing 2.0 mM $MgCl_2$and 5 µl DNA from the restriction /ligation reaction were put in PCR tubes and placed in a thermal cycler with 30 amplification cycles (Denaturation at 94°C; annealing at 56°C for 1 min; polymerization at 72°C for 1 min and further held at 72°C for 10 min.).

The preamplification product was then diluted in PCR grade water in 1: 10, 1:20 and 1: 30 ratios. A PCR mix for selective primers was prepared and it comprised of 5 µl of diluted preamplification PCR product, 5 µl of selective primer mix [0.25 µl of labeled primer (50 ng/µl), 0.3 µl of unlabelled primer (50 ng/µl) and 4.45 µl of PCR grade water] and 10 µl of Bioline Biomix ^{TM} containing 2.0 mM $MgCl_2$. Each PCR tube contained a final volume of 20 µl. The thermo cycler was programmed to amplify the product in two sets of temperatures. The first set had 13 cycles of amplification while the second had 23 (denaturation at 94°C for 30 sec; annealing at 65°C; polymerization at 72°C for 1 min; and held at 10°C for 12 hrs) before fragment analysis.

2.4 Detection and Scoring of AFLP Fragments

The dilution from the selective PCR was optimized for fragment analysis. The PCR product was run on Beckman Coulter CEQ 8000 to analyze the fragments generated from the AFLP analysis. A master mix of 40 µl of the SLS (deionized formamide) and 0.5 µl the DNA size standard was loaded in each sample well. 0.5 µl of the final PCR product was added to the sample well carefully and covered by a drop of mineral oil to avoid evaporation. The buffer plate was immersed in loading buffer and loaded into the sequencer. The software package for CEQ8000 genetic analysis system (Beckman Coulter, Inc.) was used to input the data detailing well contents, prior to loading the plate into the sequencer and running the analysis. A DNA size standard (PA400) was used to size the fragments. The laser detected fragments present in the spectrum of each fluorophore, producing an electronic profile of relative fluorescence units (RFUs) versus fragment size [49] to detect insertions or a deletion of nucleotides. The traces of samples were compared and visually scored. Peaks were scored if they were between 1000 and 130000 RFUs (vertical axis).

2.5 Data Analysis

2.5.1 Ring and cluster analysis for the genetic structure between *Pythium*- developed and farmers' dry bean samples

Data analysis was performed using NTSYS-pc version 2.1. Each accession was scored (1) for present and (0) for absent of each polymorphic loci. The genetic distance was calculated basing on simple matching coefficient method. The similarity matrix was subjected to cluster analysis by unweighted pair- group method with arithmetic averages. The generated binary data transformed into spreadsheets and subjected to population diversity analysis. Nei's genetic distances were determined. Cluster analysis was performed using simple matching coefficients. The similarity coefficients was subjected to unweighted pair group method of arithmetic averages (UPGMA) and a dendogram generated using GenAlEx software and NTSYS- PC software version 2.1, Execter software, New York [50]. Principal component analysis was performed on the dataset. Since the first three components of the multidimensional data set explains the variation in the observed relationships among the population under a study [50], the first two Eigen values were plotted to show the degree of similarity among the entries.

3. RESULTS

3.1 Polymorphism

AFLP profiles of the *Pythium* related samples were generated using two AFLP primer combinations (E-TC/M-CAT, E-ACA/M-CTT) and produced a total of 194 polymorphic loci. The size of the AFLP generated fragments by KARI Kakamega dry bean samples ranged between 56 and 414 base pairs and 56- 420bp for the 35 dry bean market samples respectively. Dry bean samples from KARI kakamega revealed a genetic distance of 0.62 to 0.90 (Fig. 1). Genetic distance of dry bean samples from farmers and Kari Kakamega samples analyzed together revealed a genetic distance of 0.63 – 0.90 (Fig. 1). *KK15* had a distance matrix of 0.71, close to the distance generated from *SCAM80* and *Alulu* of 0.77. There is close genetic relationship between *KK15* and the sub cluster of *SCAM80* and *Alulu* compared to the susceptible sub cluster of *GLP585* and *GLP2* with the

distance 0.79. A sub cluster comprising of four resistant varieties (*AND1062, R2075, R719* and *R1946*) had a distance matrix 0.86. The genetic similarity within these samples is closer compared to the other samples used in the group. *Mw001* was most dissimilar to the rest of the samples used in this analysis with a distance matrix of 0.62, indicating a wide genetic dissimilarity with the rest of the bean samples analyzed.

3.2 Cluster Analysis

The AFLP marker used to delineate the KARI-Kakamega bean lines was able to distinguish the cultivars clearly. A neighbor-joining dendogram based on Nei's genetic distances [51] clustered the KARI-Kakamega bean lines into four sub groups in which all except *Mw001* that was susceptible segregated together (Fig. 1). The major cluster segregated into three subgroups in which susceptible and tolerant accessions grouped together with the exception of *Alulu* a mildly resistant linethat clustered alongside resistant lines in Cluster 1. Cluster 2 formed two subgroups, Cluster 2a and 2b, all comprising of resistant accessions. Cluster 3 comprised of *GLP585 and GLP2* which were all susceptible. Cluster 3 and 4 were located closely and thereby formed the susceptible group while cluster 1 and 2 formed the resistant grouping.

Since the first three components of the multidimensional data set explains the variation in the observed relationships among the population under a study [50], the first two Eigen values were plotted to show the degree of similarity among the entries (Table 4). Principal coordinates analysis clearly a major split between the KARI-Kakamega populations into distinct clusters. Principal component analysis based on allele frequencies among the groups across mapped AFLP markers clustered the bean lines into four groups (Fig. 2). As observed, 27.25% of the variation could be attributed to the first two principal components, 13.02% to the second and 11.31% to the third. Group 1 was comprised of accessions belonging exclusively to resistant group, group 2a and 2b to also resistant cultivars and group 3 and 4 to susceptible cultivars. The first three Eigen values of the ten dry bean lines from KARI Kakamega using NTSYS-PC version, revealed 51.59% similarity (Table 4). As observed in the dendogram (Fig. 1), the lines clustered out into 4 groups that clearly delineated susceptible and tolerant lines. *R1946, R719*, *R2075* and *AND1062* are closely related genetically compared to *Mw001*, *KK15*, *Alulu* and *GLP2*. The clustering revealed how diversely related market class Kenyan dry beans are in relation to the bean samples developed from the breeding program at KARI- Kakamega. Principal component analysis of the *Pythium* samples (Fig. 2) revealed a closer clustering of the resistant varieties *AND1062, R1946* and *R719* and susceptible varieties *GLP2, GLP585* clustered together.

Table 4. Description of genetic variation percentage in the observed relationship among 10 improved bean lines used in the study

Eigen Value	Percent (%)	Cumulative
2.72	27.25	27.25
1.30	13.02	40.27
1.13	11.31	51.59
1.09	10.98	62.58
0.89	8.99	71.57
0.74	7.40	79.04

On screening the entire bean population using the AFLP markers, the dendogram produced did not clearly depict the differences as observed in the KARI-Kakamega bean lines. A similarity analysis made on all the bean samples used in this study including the *Pythium* related germplasm was performed to validate the genetic relatedness that existed within the KARI – Kakamega bean samples that had already been evaluated and approved in terms of their reaction to *Pythium* species together with bean samples present in the market and with farmers (Fig. 3). The degree of similarity within the samples products from a conventional breeding exercise at KARI- Kakamega breeding station generated a dendogram with 8 clusters (Fig. 3). *Mw001*, a susceptible variety to *Pythium* again segregated differently from the rest of the bean population as earlier observed (Fig. 1). Susceptible lines *GLP2* and *GLP585* grouped together in cluster 4 alongside *GA03*. There was distinct clustering between the resistant varieties and susceptible varieties in cluster 1 and cluster 4. Cluster 1, 2 and 3 can be said to comprise of *Pythium* root rot tolerant accessions. Cluster 4 comprised of only susceptible accessions. Cluster 5 comprised accessions that segregated alongside *Alulu,* a mildly resistant line and thus may be portrayed to also be mildly resistant. Cluster 7 comprised of accessions that clustered together with *KK15* which is a tolerant line. Thus, clustering results for the 45 dry bean samples (35 bean samples from farmers and 10 bean samples from KARI kakamega) revealed 8 clusters, with the resistant

varieties clustering together with some local seed samples from farmers in cluster 1. *AND1062*, *R2075, R719* and *R1946* which are resistant varieties clustered with bean samples from Eastern province (Meru and Embu population). Several resistant varieties clustered in cluster 5 and 7, with susceptible varieties being found in cluster 4 and 8 (Fig. 3).

4. DISCUSSION

The AFLP markers used in this study were able to clearly delineate the KARI-Kakamega bean lines into susceptible and tolerant groups with a relatively high degree of precision. *Mw001* a susceptible cultivar is definitely very different from all the assessed accessions. Because *AN1062, R2075, R719* and *R1946* were tightly linked together, they could be the best placed tolerant accessions for use in screening other accessions for tolerance in comparison to *KK15* and *SCAM80.* However it should be noted that *Alulu,* a mildly resistant bean line segregated together with the resistant lines *SCAM80* and *KK15*. This therefore suggests that it may be closely associated with the resistant lines or the AFLP marker used was not tightly linked to the allele for resistance. That the association may have had to do with unrelated gene loci. From these results it is clear that the AFLP markers used were able to segregate the KARI-Kakamega bean lines with regard to root rot resistance. But as a result of the inability to clearly distinguish *Alulu* from the other tolerant lines, there is need to use another protocol to test the reproducibility of the results before it can clearly be stated that the markers were proficient.

Principal components analysis is one of the most important methods of ordination analysis. It constructs a new set of orthogonal coordinate axes such that the projections of points onto them have maximum variance. While defined in terms of variances and co variances, PCA is usually applied to standardized data since the results are sensitive to the often arbitrary choices of units of measurement used in a study. Cluster analysis involves performing various types of agglomerative cluster analysis of some type of similarity or dissimilarity matrix. From the bean samples analyzed, a data matrix was computed using the distance coefficients among the columns of the standardized data matrix. The distance matrix is clustered using the single-link clustering method to form of a phenogram. The samples developed from a conventional breeding exercise were subjected to analysis to yield a distance matrix ranging from 0.62 to 0.89.

On screening the whole bean population using the AFLP markers earlier used on the KARI-Kakamega lines, it was evident that although some accessions were clearly segregating alongside either tolerant or susceptible lines as could therefore be identified as so. Thus the AFLP markers used clearly delineated those accessions that clustered out in Clusters 1, 2 and 3 and can be identified as being tolerant. However, some accessions especially those in cluster 7 could not be identified clearly as being tolerant or susceptible. This necessitates a need to explore other AFLP markers or to screen the population with a tried and tested protocol before it can be conclusively be employed as credible marker. Thus the AFLP markers used should not be used alone but in combination with other markers.

AFLP markers have the potential to resolve genetic differences at the level of 'DNA fingerprints' for individual identification and parentage analysis. In the ideal case, a few primer combinations will suffice to generate an adequate number of polymorphic markers. The key feature of AFLP–PCR is its capacity for the simultaneous screening of many different DNA regions that are distributed randomly throughout the genome. To achieve high reliability of the screen, genomic DNA is prepared in an ingenious, but technically straightforward. The large number of percentage (over 50%) on the component analysis indicates this was a successful analysis [52]. We observed considerable genetic diversity within the marketable common beans of Kenya and resistant and susceptible bean samples conventionally developed from KARI Kakamega. Bean root rot caused by several *Pythium* species has been a quandary on beans *(Phaseolus vulgaris L.*) increasing in importance recently in East and Central Africa as total crop loss when susceptible dry bean varieties are used is evident [3]. Previous studies to monitor *Pythium* root rot disease development have been conducted in a field and screen house setting [53]. Authors uprooted seedlings and scored lesion development using the CIAT scale of 1-9 [54]. This formed the basis of conventionally producing the ten common bean samples used in this study. In their experiments, RWR719, MLB 4989A and AND1062 were controls and received disease interaction phenotype score of either 1, 2 or 3 denoting resistance to *Pythium ultimum* while GLP 2 was susceptible (8-9 score) under same conditions [53].

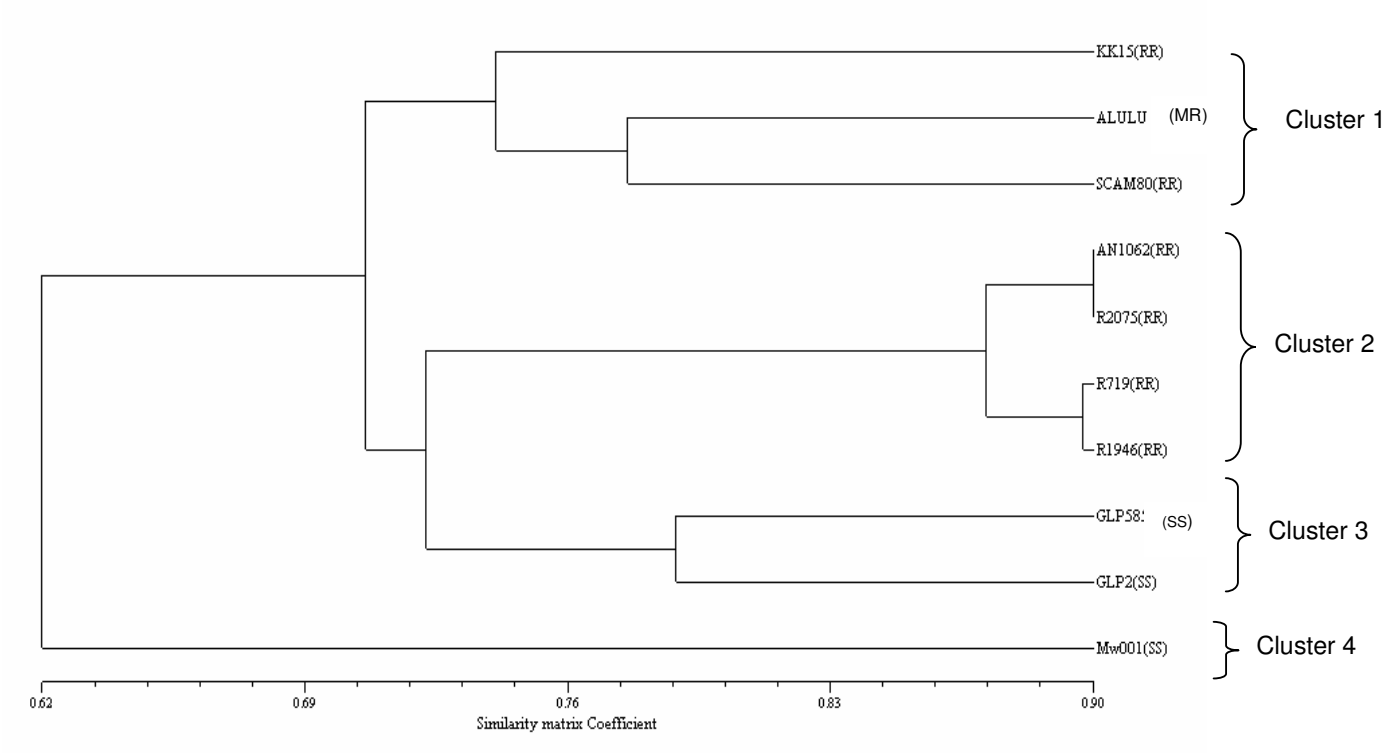

Fig. 1. A Neighbor-joining dendogram of 10 KARI-Kakamega bean lines based on genomic DNA fingerprinting with 194 polymorphic bands generated from two primer combinations of AFLP. The dendograms were drawn based on Nei's (1978) genetic similarity distances using the GenAlEx and NYSYS-PC version 2 software

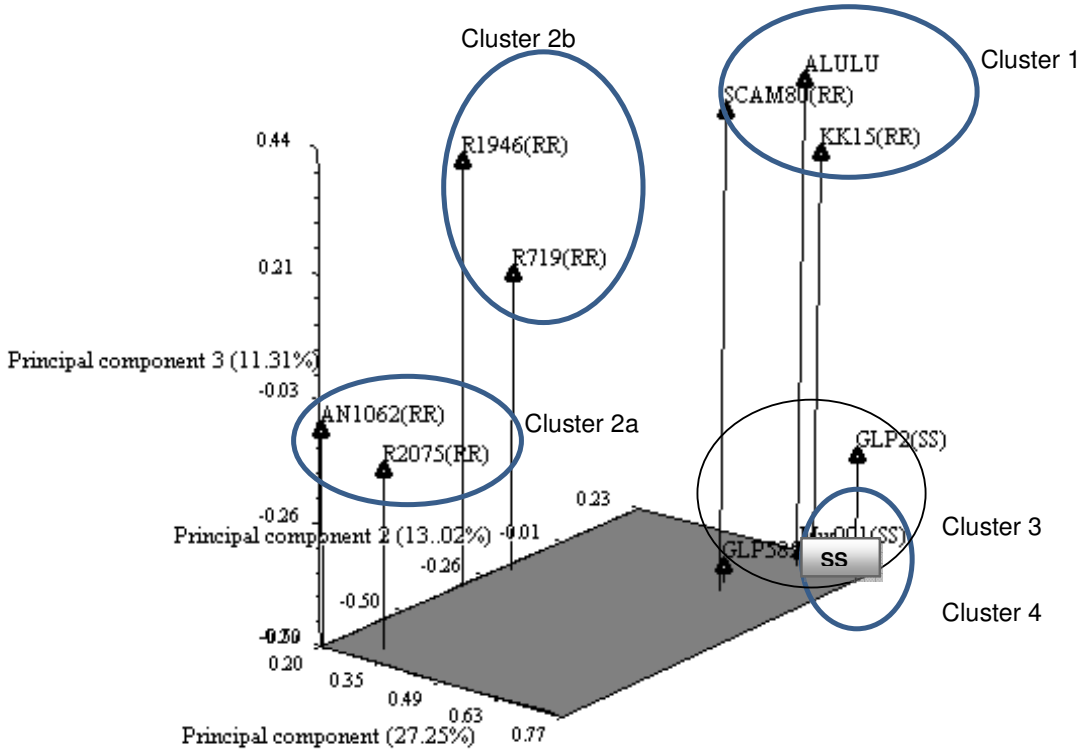

Fig. 2. Principal component analysis of 10 breeding bean lines developed in a conventional breeding program based on Nei's genetic distance. The first, second and third principal coordinates are indicated 10 common dry bean lines developed by the KARI Kakamega conventional breeding program on *Pythium* root rot resistance. The PCA describes 51.58% of the variation in the observed relationships existing in the bean samples

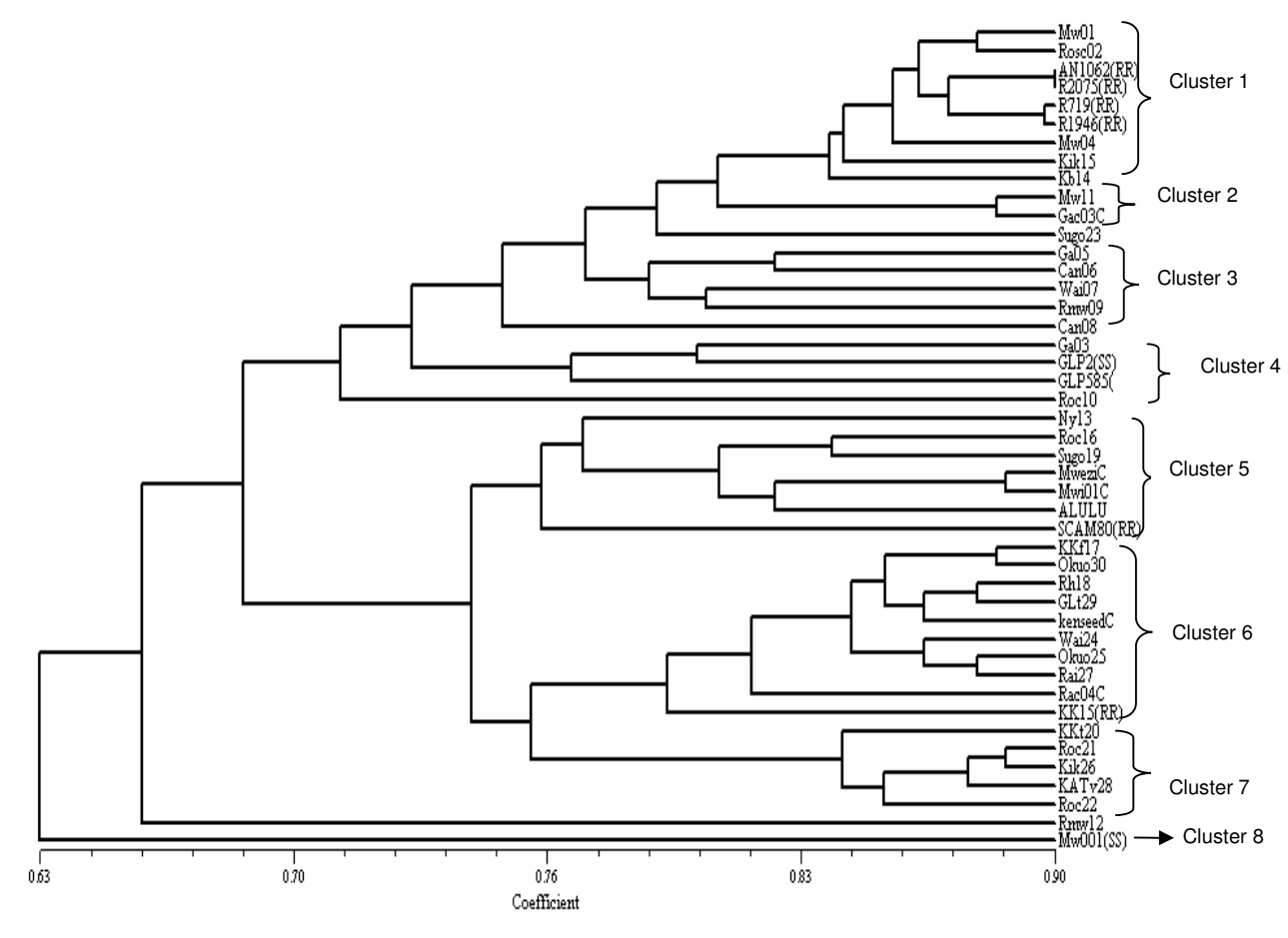

Fig. 3. AFLP clustering dendogram generated from matrices of genetic distances obtained by the complement of the similarity coefficients of 45 bean samples of *P. vulgaris* including *Pythium* resistant/susceptible accessions from KARI – Kakamega

Molecular characterization of 35 different Pythium strains affecting the Ugandan common bean was done using RFLP marker, to demonstrate the wide diversity that exists among different bean plants affected by *Pythium* root rot [55]. Difficulties in interpreting the relatedness among the isolates were experienced because of wide intra specific variation that exists within the *Pythium* species. To significantly reduce the effects of this disease on the bean crop, identification of sources of resistance to bean genotypes preferred by resource poor small holder farmers is imperative. Resistant varieties have been identified in Kenya but they were not of preferred market classes [14]. Farmers are reluctant to absorb the new release of resistant bean varieties due to presence of undesirable characteristics. For this reason, farmers prefer to hold on to their preferred from KARI institutions. The farmers save the seed of bean varieties that possess preferred traits such as high yielding, adaptation to local growing conditions, vigorous, early maturing and posing good culinary qualities. Farmers indicate that saving their own seed is economical and there is assurance of seed growth once replanted.

Subsequent work of characterization has been done of susceptible common bean varieties from Africa [56] in different seed sizes, such as the Kenyan varieties GLP 2 and GLP 585 which are respectively large seeds (Andean gene pool) and small seeds (Mesoamerican gene pool), the Ugandan variety CAL 96 (Calima) with large seeds (Andean gene pool) and the Rwandan variety Urugezi with an intermediate seed size (Mesoamerican gene pool). All the resistant common bean varieties have been characterized into different sizes and are advanced lines from an international breeding nursery run by CIAT (Cali, Colombia). This include the small-seeded variety RWR 719 from the Mesoamerican gene pool, the intermediate-seeded varieties *MLB49-89A* and *SCAM 80-CM/15*, both from the Mesoamerican gene pool, and the large-seeded varieties AND 1055 and AND1062, both from the Andean gene pool [3].

Pythium root rot resistance is controlled by a single dominant gene [57]. Experiments in earlier studies involved investigating the genetic relationship between *Fusarium and Pythium* resistance inheritances in a resistant bean line *RWR 719*. There is an association of $PYAA19_{800}$ with *Pythium ultimum* resistance in *RWR 719, MLB 49-89A* and *AND1062*, located 1.5cM from the resistance gene [58]. These common bean varieties are possible sources of resistance to *Pythium* root rot disease to market acceptable common beans in Kenya. The most effective way of managing bean root rot for small scale farmers is exploiting the host plant resistance [59,60]. *KK15* had a distance matrix of 0.71, close to the distance generated from *SCAM80* and *Alulu* of 0.77. There is close genetic relationship between KK15 and the sub cluster of *SCAM80* and *Alulu* compared to the susceptible sub cluster of GLP585 and GLP2 with the distance 0.79. A sub cluster comprising of four resistant varieties (*AND1062, R2075, R719* and *R1946)* had a distance matrix 0.86. The genetic similarity within these samples is closer compared to the other common bean samples used in the group. Major crosses can be conducted to transfer resistance from resistant varieties to the market acceptable varieties.

Resistance to *Fusarium solani* is complex and is conditioned by two or more genes [61-63]. *Pythium ultimum* resistance is controlled by a single dominant gene, marked by a dominant SCAR marker-$PYAA19_{800}$ [3,14,63]. The presence of joint resistance to both *Fusarium* and *Pythium* species in root rots have been observed in several resistance sources [4,9,64]. Some market class samples are closely related genetically to the *Pythium* related samples developed in a conventional breeding exercise at KARI- Kakamega. In a further breeding exercise, these bean samples can be used as susceptible parents in efforts to obtain crosses for transfer of the resistant genes in improvement programs.

Eight major clusters were obtained with the cluster analysis majority of the resistant bean samples from KARI clustering with bean varieties from Eastern province (Meru and Embu population) and Western province (Kakamega region) (Fig. 2). In the first cluster four resistant varieties clustered among market classes (AND1062, R2075, R719 and R1946). The fifth and seventh cluster had two resistant varieties SCAM 80 and KK15. KK15 is a recent bean line that is resistant to *Pythium* root rot, is high yielding and fast maturing. Most farmers find the colour of the seed coat unacceptable, therefore as much as it is a wonder seed that is being taken up by small scale farmers to counteract poverty and improve food availability, further crossing should be done to break the linkage between the gene responsible for resistance and seed coat colour, to increase the acceptance rate of this new improved variety among the bean farmers in Kenya. However, since the clustering produced mismatching, it is possible that there may be linkage to the pathogenicity of the bean

germplasm. There is need to determine the pathogenicity status of the 35 bean samples and comparisons made with the molecular markers' findings of this study.

Efforts to integrate *Pythium* root rot resistant gene into Rwandan susceptible varieties have been successful after carrying out crosses between three local susceptible varieties and two known sources of resistance to Pythium root rot, R719 and AND1062 [65]. The Rwandan bean profile of resistance to *Pythium* found in the progenies from back-crosses between (RWR 1668, R617-97A and Urugenzi, as recipient parents) and the selected sources of resistance (RWR 719 and AND 1062, as donor parents) is achievable. At each back cross generation A SCAR marker PYAA 19_{800} has been used and found to be linked tightly to the gene responsible for resistance in R719 and AND1062. This is a feasible exercise which can be experimented in Kenyan bean breeding institutions to help improve resistance of market acceptable beans to attacks by root rots. However, needs further work with SCAR marker $PYAA_{800}$ and propagation to enhance the transference of the resistant gene to acceptable bean samples of market class in Kenya.

Knowledge about identified resistance sources of beans combined with information of the polymorphic levels that exists between the improved resistant bean lines and commercial varieties will reveal the level of diversity and contribute towards development of appropriate protocols with potential to decrease adoption rates and duration taken to release improved bean varieties in Eastern and Central Africa. Effective bean root rot management calls for the use of integrated approaches with a strong component of resistant varieties. To design resistant gene development in bean varieties ideal by resource poor small holder farmers, long lasting strategies are dependent on the identification of useful genes and a good understanding of the host – pathogen interaction. The use of resistant cultivars is the most viable option for controlling bean root rot, particularly for small-scale growers to solve this quagmire in the agricultural sector of bean breeding. To increase the productivity and yield stability of the dry bean of Kenya, there is need to come up with varieties that are well adapted to the changing climate, fit consumers' trait preferences and are resistant to root rots. Knowledge about identified resistance sources and mode of inheritance of resistant genes is needed to develop more stable resistant bean cultivars to be adapted to regions of Eastern and Central Africa where bean root rot is an increasing problem.

In view of the lack of public access to primers like SCAR marker $PYAA_{800}$ with proven capability to tag resistant and susceptible varieties, there is a need to redesign the primers used in this study with an aim to increasingly target the resistant gene. However, since there are primers already designed in similar studies in Rwanda and Colombia, it is desirable that the same population be subjected to screening using such markers and comparisons with this study made.

5. CONCLUSION

This study has revealed the genetic relationships between and within bean population from the study areas by use of AFLP marker. It is a preliminary work to be advanced towards developing a protocol for a breeding exercise for marketable *Pythium* resistant common bean. It has yielded useful information on the level of polymorphism and genetic relatedness between common bean varieties developed from KARI Kakamega through a convectional breeding program and bean varieties of market classes of Kenya. However, it did not conclusively segregate resistant and susceptible bean lines to distinction and should be used cautiously. Therefore AFLP is potential markers which can be used to help breed for *Pythium* resistances in market class of beans thus relieve the small scale farmer of food insecurity and increasing poverty.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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