FIELD EVALUATION AND MOLECULAR ANALYSIS OF BEAN GENOTYPES FOR RESISTANCE TO RUST DISEASE

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Snap bean (*Phaseolus vulgaris* L.; 2n=2x=22) is a predominantly self-pollinated crop originated mainly in Latin America. From Latin America, it spread to other parts of world and now it is widely cultivated in the tropics and subtropics as well as in temperate regions of the world (Zeven, 1997; Zeven *et al.*, 1999). In Egypt, snap beans, is considered to be one of the most important vegetable crops for local market and export. The total cultivated area grown with green beans in 2012 was 57873 Feddan producing about 251279 tons with an average of 4.34 Tons/Fed (Malr, 2013). Bean rust caused by *Uromyces appendiculatus* (Pers.:Pers.) Unger var. appendiculatus is one of the most devastating fungal diseases of common bean (*Phaseolus vulgaris* L.) worldwide (Liebenberg and Pretorius, 2010; Souza *et al.*, 2008). Yield losses caused by bean rust depends on the degree of susceptibility of the dry or snap bean variety grown, the climatic conditions favoring rust infection and disease development, and earliness of the infection. An effect of this rust is the 18 to 100% reduction of grain yield in dry beans and the reduction in pod quality in snap beans (De Jesus *et al.*, 2001). The pathogen *U. appendiculatus* is not seed borne and is an obligate parasite Small holder snap bean farmers mainly rely on fungicides and insecticides to reduce production and post-harvest losses associated with diseases (Wasonga *et al.*, 2010). However, use of resistant varieties is regarded as the most effective and economically viable strategy for rust management.

One the other hand, the evaluation of genetic diversity could greatly assist and expedite selection decisions in crop breeding. The evaluation of genetic diversity could be done using several genetic markers. In contrast to morphological markers and biochemical markers, molecular markers can reveal differences among genotypes at the DNA level, providing a more direct, reliable and efficient tool for germplasm characterization, conservation and management (Virk *et al.*, 2000; Song *et al.*, 2003). Molecular markers can also facilitate quantification of existing genetic diversity and uncovering duplicate or very similar genotypes, and the identification of unique variants or genotypes for expanding the useful variation. In addition, with the aid of molecular markers, genes of interest

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were tagged, traced and used in breeding programs, and to select disease resistant germplasm (Stam, 1997). In snap bean, a number of earlier studies have investigated the genetic variation within and between bean populations as well as between cultivated and wild genotypes, using isozymes (Belletti and Lotito, 1996), seed protein analysis (Lioi *et al*., 2005) and molecular markers. Polymerase chain reaction (PCR)-based DNA markers such as random amplified polymorphic DNA (RAPDs, Williams *et al*., 1990) and Amplified fragments length polymorphism (AFLPs, Vos *et al*., 1995) were used extensively to compare the genetic structures of common bean germplasm collections (Tiwari *et al*., 2005; Durán *et al*., 2005), to assess genetic diversity within bean germplasm (Lioi *et al*., 2005; Sustar-Vozlic *et al*., 2006; Kumar *et al*., 2008), and to map resistance genes (Kelly *et al*., 2003). In particular, RAPD markers have been used in breeding programs to help in the production of rust-resistant bean cultivars (Kelly *et al*., 1994; Fábio *et al*., 2000). More recently, a number of polymorphic simple sequence repeats (SSRs) markers have been developed for beans using DNA sequence information obtained from the Gene Bank database (Yu *et al*., 2000; Blair *et al*., 2003) as well as sequencing of anonymous bean clones from genomic libraries (Gaitán-Solís *et al*., 2002; Métais *et al*., 2002). In addition, SSRs have been used by several investigators to determine the genetic diversity within and between bean populations (Métais *et al*., 2002; Durán *et al*., 2005; Diaz and Blair, 2006; Blair *et al*., 2006; 2007; Benchimol *et al*., 2007; Hanai *et al*., 2007). Accordingly, the objectives of this study were to (1) survey snap bean germplasm for new resources for resistance to leaf rust (biological and molecular evaluation of native Egyptian snap bean germplasm) (2) develop salient RAPD, ISSRs and SSRs profiles (fingerprints) of the identified valuable genotypes, and (3) assess the genetic diversity within the selected genotypes differing in their resistance and susceptibility to rust disease.

MATERIALS AND METHODS

Plant materials

Twenty two snap bean (*Phaseolus vulgaris)* genotypes, namely; Argus, Amy, Bronco, Cerdon, Coby, Concessa, Duel, Fendor, GOMI, Grenoble, Hana, Hort. gh 1-11, Hort. gh 26, Hort. 440, Hort. 407, Hort. gh 38, Hort. 9, MaGB, Paulista, Samantha, Tema and Xera were collected and evaluated for rust-resistance in this study.

Germplasm screening and field evaluation

Evaluation of collected snap bean genotypes for rust resistance was carried out at Barrage Horticulture Research Station (BHRS), Kalubia Governorate, Egypt. The 22 genotypes collections of snap beans were maintained and evaluated for rust *Uromyces appendiculatus* (Pers.) Unger reaction at BHRS during two successive fall seasons of 2012/13 and 2013/14. Seeds of the 22 snap bean genotypes were

sown on the first week of September for the two seasons in a randomized complete block design (RCBD) with three replicates. Each replicate consisted of two rows and each row was 3.5 m long and 0.7 m wide. The recommended agricultural practices were done without using fungicides. Infection types of bean rust were evaluated after the fungus had fully established under natural field conditions by using the 1-6 scale described by Stavely *et al*. (1989). Infection types 1, 2 and 3 were considered incompatible (resistant) and infection types 4, 5 and 6 were considered compatible (susceptible) as shown in Table (1). Thirty leaves/plot were taken to estimate disease severity. Plant leaves of each particular genotype were classified into six categories representing the amount of rust surface. The percentage of infection for each particular genotype was determined on lower surface using the following formula:

$$\mathrm{DI\%} = \frac{\mathrm{Sum\ of\ (n\ \times v)}}{\mathrm{6N}} \mathrm{x\ 100}$$

Where: DI = Disease index

n = Number of infected leaflets in each category.

v = Numerical value of each category.

N = Total number of leaflets in sample.

Molecular analyses

Based on the results of field evaluation, ten genotypes represent the 4 types of reactions to the rust disease; immune, highly resistant, moderate susceptible and high susceptible (Table 1) were selected for molecular analyses of the rust resistance using the three molecular systems RAPD, ISSRs and SSRs.

Genomic DNA extraction

Seeds of the selected ten genotypes were grown in a growth chamber at 27°C under a 12/12 h day/night photoperiod. Genomic DNA was isolated from the collected leaves at 10 to 15-day old seedlings (five plants per genotype) using the plant isolation kit (Jena Bioscience, Germany).

RAPD analysis

PCR reaction and condition: A total of seven random primers were used to detect the polymorphism (Table 2). The amplification performed in a 25 µl reaction volume containing about 3 µl (10 ng μL^{-1}) genomic DNA, 3 µL primer (Operon Technologies Inc.) and 12.5 µL master mix (Promega) and 6.5 µL of PCR water. The PCR temperature profile was applied through Bio-Rad C1000 thermal cycler (Germany) that was programmed with an initial step of 5 min at 94°C; the amplification reaction was carried out using 40 cycles of 60 s at 94°C, an annealing step of 1 min at 33°C and an elongation step of 1 min at 72°C; and finally a 7 min extension at 72°C. The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 µg mL^{-1}) in 1X TBE buffer at 95 volts. PCR products were visualized on UV light and photographed using a gel documentation system (Bio-Rad® Gel Doc-2000).

ISSRs analysis

Six ISSRs primers were tested using a specific and optimal annealing temperature for each one (Table 3). PCR reactions were performed in a volume of 20 µl in Bio-Rad C1000 thermal cycler (Germany). The reaction mixture contained 200 mM of each primer, 100 µM of each deoxinucleotide, 1 unit of Go Taq polymerase (Promega), 10x Taq buffer containing 2.5 mM $MgCl_2$ and 10 ng of template DNA. Amplification reaction was 94°C/5 min, followed by 30 cycles of 94°C/1 min, 45-57°C (specific for each primer)/1 min and 72°C/2 min and ending with an extension step of 72°C/7 min. PCR products were analyzed using agarose (2% w/v), electrophoretic gels stained with ethidium bromide. The gels were photographed by gel documentation system (Gel Doc 2000 Bio-Rad) and only bands with high intensity and well separated were selected.

SSRs analysis

Ten microsatellite primer pairs used for genotyping assays were identified from the Gene Bank data base. Primer names, Gene Bank accession number, sequences and corresponding annealing temperatures and allele number are listed in Table (4). PCR amplification was performed in a volume of 20 µl containing approximately 30 ng of template DNA, 1 µm of each forward and reverse primer, 10 µl 2-X Master mix of Green Go Taq DNA Polymerase (Promega) containing buffer, 200 mM of each dNTPs, 2 Mm $MgCl_2$. Reactions were conducted in Eppendorf PCR system (Germany) with initial denaturation step for 5 min at 94°C followed by 35 cycles of 94°C for 1 min, 46 to 49°C (depending on primers, Table 4) for 1 min and 72°C for 2 min; followed by a final extension at 72°C for 5 min. The PCR reaction products were evaluated for polymorphisms on 2% agarose gel. After staining with 1 µg ml^{-1} ethidium bromide for 30 to 60 min, the gels were photographed by gel documentation system (Gel Doc. 2000 Bio-Rad).

Band scoring and cluster analysis

The gels images were scanned using the Gel Doc 2000 Bio-Rad system and analyzed with Quantity One Software v. 4.0.1 (Bio-Rad Laboratories, Hercules, CA USA). For each primer, the bands were sized and then binary coded by 1 or 0 for their presence or absence, respectively in each. Percentage of polymorphism was calculated as the proportion of polymorphic bands over the total number of bands. The scores obtained using all the primers of RAPD, ISSRs and SSRs analyses were then pooled to create a single data matrix, to estimate polymorphic loci, gene diversity, genetic distance (D) and to construct a UPGMA (Unweighted Pair Group Method of Arithmetic Means) (Sneath and Sokal, 1973) dendrogram among populations using the software NTSYSpc (Numerical Taxonomy and Multivariate Analysis System) (Rohlf, 2000).

RESULTS AND DISCUSSION

Field evaluation of snap bean genotypes for natural rust resistance

Data obtained on the evaluation of snap bean genotypes reaction for rust resistance under natural infection conditions in two seasons are presented in Table (5). In each season, significant differences in disease severity percentage were found among the evaluated genotypes. Among 22 genotypes cultivated in the first season, four genotypes, *i.e.,* Concessa, Hort. 440, Coby and Hana were rated as immune to this disease. These genotypes were completely free from infection. While, other four genotypes *i.e.,* Argus, Cerdon, Duel and Samantha were recorded disease severity ranged from 1.52% to 3.17% and were categorized as highly resistant (hypersensitive). The genotype Hort. 9 was categorized as resistant with disease severity of 9.23%. The remaining 13 genotypes were susceptible having a disease severity ranged from 16.93% to 57.47%. In the second season, the same results were found except that the disease severity of the susceptible genotypes Fendor and Hort. 26 were increased from 28.27 to 36.97 and from 35.67% to 55.30% hence, their infections score were changed from 4 to 5 and 5 to 6, respectively. Ten genotypes of snap bean with various levels of resistance and susceptibility to bean rust were selected for molecular analysis of rust resistance. The selected ten genotypes were one immune cultivar (Concessa), three highly resistant (hypersensitive) cultivars (Argus, Samantha, Cerdon), two moderate susceptible cultivars (Fendor, GOMI) and four high susceptible cultivars (Bronco Tema, Paulistaand Xera).

Fingerprinting of some selected snap bean genotypes

The three molecular techniques RAPD, ISSRs and SSRs-PCR were used to get the banding patterns of ten selected genotypes of snap bean. Total of 75 bands (markers) were obtained by the three techniques among with 59 polymorphic bands represent total polymorphism of 78.7%. The highest number of bands (35) was obtained by RAPD analysis using seven primers that showed reproducible and clear banding pattern (Table 2). Six ISSRs primers and ten SSRs primers produced 22 and 15 bands, respectively. While, all the RAPD and ISSR primers produced polymorphic band pattern with total polymorphism of 76.3% and 85%, respectively, (Tables 2 and 3), only 4 SSRs primers (40%) were polymorphic with total polymorphism of 60% (Table 4). The banding patterns of some RAPD, ISSRs and SSRs primers are illustrated in Figs. (1, 2 and 3), respectively.

Genetic relationships and cluster analysis as revealed by RAPD, ISSRs and SSRs techniques

The data scored from the three techniques were compiled and analyzed using the Dice similarity coefficient to determine the genetic similarity matrix and draw the dendrogram between the selected ten snap bean genotypes (Table 6 and Fig. 4). The genetic similarities were ranged from 59.5-97.5%. While, the high-

est genetic similarity revealed by the combined data was 97.5% between the two genotypes Fendor and Xera, the lowest genetic similarity was 59.5% between the two genotypes Cerdon and Samanatha (Table 6). The UPGMA clustering dendrogram based on DICE similarity indices (Fig. 4) classified the evaluated genotypes into two major clusters. The first main cluster contains the rust-resistance genotype Cerdon only. The second main cluster is branched into two subclusters, the first contains the rust-susceptible genotypes Paulista and the second is further branched into several subclusters, where the rust-immune cultivar, Concessa and the rust-resistance cultivar, Argus are in the same subcluster and all the remaining 6 genotypes are in other different subclustars (Fig. 4).

Leaf rust *(U. appendiculatus*) is serious diseases of great negative impact on snap bean production in Egypt. Therefore, biological and molecular evaluation of any new native snap bean germplasm is vital to enrich our knowledge on the abilities of existing germplasms and enabling us to predict new cultivars performance, select parents for crossing in crop improvement programmes, and clone new natural plant resistance genes (Saker, 2005 a & b; Saker *et al*., 2005). In the present study, the biological evaluation resulted in the identification of resistance of some genotypes to leaf rust and the level of resistance varied from immune, hypersensitivity and resistant. This is considered as the most interesting and striking output. In addition, salient fingerprints of the identified valuable genotypes were successfully created using the three systems (RAPD, ISSRs and SSRs). In fact, studies aiming at evaluate the genetic relationships in legume crops using combination of two or three molecular systems have been successfully used by very few researchers (Souframanien and Gopalakrishna, 2004; Gillaspie *et al*., 2005; Dikshit *et al*., 2007). The higher percentage of polymorphism exhibited by ISSRs comparing with those of RAPD and SSRs was expected, because the technique amplifies microsatellite areas that are potentially highly polymorphic. Similar high percentage polymorphism (78%) of ISSRs techniques was obtained in earlier studies (Mcgregor *et al*., 2006). However, RAPD and ISSR generated more number of polymorphic bands but SSR have the advantage as co-dominant markers to detect homozygote and heterozygote more efficiently. The dendrogram based on the combined data of the three markers did not show clear pattern of clustering in line with the resistance or susceptibility of the genotypes for rust or according to the geographic location of the snap bean genotypes. Lack of association of diversity with geographic location may be ascribed to the substitution of snap bean seed materials by growers from agro climatic zone to neighboring zone high yielding planting materials. Sharing of breeding materials among the various institutions involved in snap bean breeding could be the other reason for low diversity between genotypes collected from distant locations. In conclusion, it can be stated that new promising resources for resistance to leaf rust were

recorded in Egyptian snap bean germplasm. The outcome of this study indicated that the molecular analysis, when combined with the biological evaluation, could proved to be a promising strategy in the selection of disease resistant germplasm, as previously reported by Haley *et al*. (1993)

SUMMARY

Bean rust, caused by *Uromyces appendiculatus* (Pers.) Unger, is an important disease in both temperate and tropical bean production regions. Twenty two genotypes of snap bean (*Phaseolus vulgaris* L.) were collected and screened for resistance to bean rust disease in two seasons. Four genotypes, *i.e.,* Concessa, Hort. 440, Coby and Hana were completely free from infection and were rated as immune to this disease. While, another four genotypes were recorded to disease severity ranged from 1.68% to 3.19% and were categorized as resistant (hypersensitive). Only one genotype was categorized as resistant with disease severity of 9.7%. The remaining 13 genotypes were susceptible having a disease severity ranged from 17.39% to 57.80%. Banding pattern of ten selected genotypes with various levels of resistance and susceptibility to bean rust and genetic diversity among them was evaluated using RAPD, ISSRs and SSRs. The three molecular systems generated total of 75 bands with polymorphism ranged from 60% to 85%. The large number of polymorphic bands allowed easy identification of the different genotypes at the DNA level. The developed dendrogram divided the common bean genotypes into two main clusters. It could be concluded that new promising resources for resistance to leaf rust were recorded in Egyptian snap bean germplasm. In addition, the developed banding patterns for these newly identified valuable Egyptian common bean accessions immune and resistant to leaf rust reported herein could support the future Egyptian snap bean germplasm collection, preservation and utilization.

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Table (1): Disease scale used for evaluation of snap bean genotypes for rust infection.

Rating Score	Description	Reaction to disease	Response
1	No visible symptoms	Incompatible	Immune
2	Necrotic spots without sporulation	"	Resistant (hypersensitive)
3	Pustules < 300 µm in diameter	"	Resistant
4	Pustules 300-500 µm in diameter	Compatible	susceptible
5	Pustules 500-800 µm in diameter	"	Moderately susceptible
6	Pustules > 800 µm in diameter	"	Highly susceptible

Table (2): The seven random primers used for RAPD-PCR analysis with their sequence, number of bands and polymorphism%.

Primer	Sequence	Total bands	Polymorphic bands	Polymorphism (%)
OPB-05	TGCGCCCTTC	7	7	100.0
OPB-08	GTCCACACGG	5	5	100.0
OPB-10	CTGCTGGGAC	7	5	71.4
OPC-02	GTGAGGCGTC	7	5	71.4
OPC-05	GATGACCGCC	4	3	75.0
OPC-06	ACCTGAACGG	3	2	66.6
OPB-08	GTGTGCCCCA	2	1	50.0
Total		35	28	Mean = 76.3

Table (3): The six primers used for ISSRs-PCR analysis with their sequence, number of bands, annealing temperature and polymorphism%.

ISSRs primers	Sequence	Total bands	Polymorphic bands	Annealing Temp	Polymorphism (%)
ISSR-12	(GA)8A	6	6	45	100
ISSR-13	(AG)8YG	5	3	55	60
ISSR-15	(GA)8YC	2	1	57	50
ISSR-17	HBH(AG)7	3	3	52	100
ISSR -18	BHB(GA)7	4	4	46	100
ISSR 19	BDB(CA)7	5	5	50	100
Total		25	22		Mean= 85

Table (4): Common bean SSRs primers (identified from the Gene Bank data base), their sequences, the annealing temperature for PCR reaction and the allele no.

SSRs name	Primer sequence (F and R)	Tm	Allele no	Polymorphic Alleles
(PV-at001)	GGGAGGGTAGGGAAGCAGT GCGAACCACGTTCATGAATG	47	(1)	0
(PV-at002)	GTTTCTTCCTTATGGTTAGGTTGTTTG TCACGTTATCACCAGCATCGTAGTA	49	(1)	0
(PV-ag004)	TTGATGACGTGGATGCATTGC AAAGGGCTAGGGAGAGTAAGTTGG	48	(1)	0
(PV-at005)	GACGTTCGCAGTATTTTGTGATATAGA CATAATACCATGCTCCTACTCTACA	48	(2)	2
(PV-at009)	ATCTTTAAATTACTAATTTTCTTGTATCGT TTCATCTTTATTACACAACCTGACTCA	46	(2)	2
(PV-atct001)	CAATTAAAACTCAACCAACCCAAATA TTTCCCGCCATAGAATATGTGAGA	49	(1)	0
(PV-at007)	ACTTCTTTCATCATCCATCCATCC TATCTTGGCTCTCTTCCTCCTCC	48	(2)	2
(PV-atcc002)	ACTTCTTTCATCATCCATCCATCC TATCTTGGCTCTCTTCCTCCTCC	48	(3)	3
(PV-gat001)	ACACCTTATCATTTAGAGGAAAAGAGA ACCCGAACTGGCTGCAACAG	47	(1)	0
(PV-atcc001)	ATGCATGTTCCAACCACCTTCTC GGAGTGGAACCCTTGCTCTCATC	49	(1)	0
Total			15	9

Table (5): Infection type and disease severity of rust disease for snap bean genotypes evaluated under natural infection conditions in two seasons.

Genotypes	Infection score		Disease severity (%)		
	First season	Second season	First season	Second season	Mean
Argus	2	2	01.52	01.83	01.68
Amy	6	6	55.88	56.90	56.39
Bronco	6	6	57.47	58.13	57.80
Cerdon	2	2	02.54	02.73	02.63
Coby	1	1	00.00	00.00	00.00
Concessa	1	1	00.00	00.00	00.00
Duel	2	2	03.17	03.20	03.19
Fendor	4	5	28.27	36.97	32.62
GOMI	4	4	30.67	31.47	31.07
Grenoble	5	5	34.27	36.9	35.59
Hana	1	1	00.00	00.00	00.00
Hort. gh 1-11	5	5	35.33	36.30	35.82
Hort. gh 26	5	6	35.67	55.30	45.49
Hort. 440	1	1	00.00	00.00	00.00
Hort. 407	6	6	56.93	58.10	57.52
Hort. gh 38	4	4	18.47	20.47	19.47
Hort. 9	3	3	09.23	10.17	09.70
MaGB	4	4	16.93	17.80	17.37
Paulista	6	6	57.37	58.10	57.74
Samantha	2	2	02.43	02.57	02.50
Tema	6	6	54.57	59.47	57.02
Xera	6	6	55.90	56.67	56.29

1= Immune, 2= Resistant (hypersensitive), 3= Resistant, 4= susceptible,
5= Moderately susceptible and 6= Highly susceptible

Table (6): Genetic similarity (GS) matrices computed according to Dice coefficient from combined data of RAPD, ISSR and SSR markers of the ten snap bean genotypes Concessa, Cerdon, Argus, Samanatha, Fendor, GOMI, Paulista, Tema, Xera and Bronco.

Genotypes	Concessa	Cerdon	Argus	Samanatha	Fendor	GOMI	Paulista	Tema	Xera	Bronco
Concessa	0.000									
Cerdon	0.682	0.000								
Argus	0.764	0.680	0.000							
Samanatha	0.682	0.595	0.950	0.000						
Fendor	0.807	0.770	0.753	0.816	0.000					
GOMI	0.759	0.715	0.770	0.813	0.864	0.000				
Paulista	0.692	0.597	0.616	0.652	0.692	0.739	0.000			
Tema	0.757	0.625	0.737	0.787	0.850	0.759	0.599	0.000		
Xera	0.837	0.755	0.761	0.846	0.975	0.827	0.722	0879	0.000	
Bronco	0.824	0.787	0.730	0.810	0.931	0.820	0.756	0.824	0.939	0.000

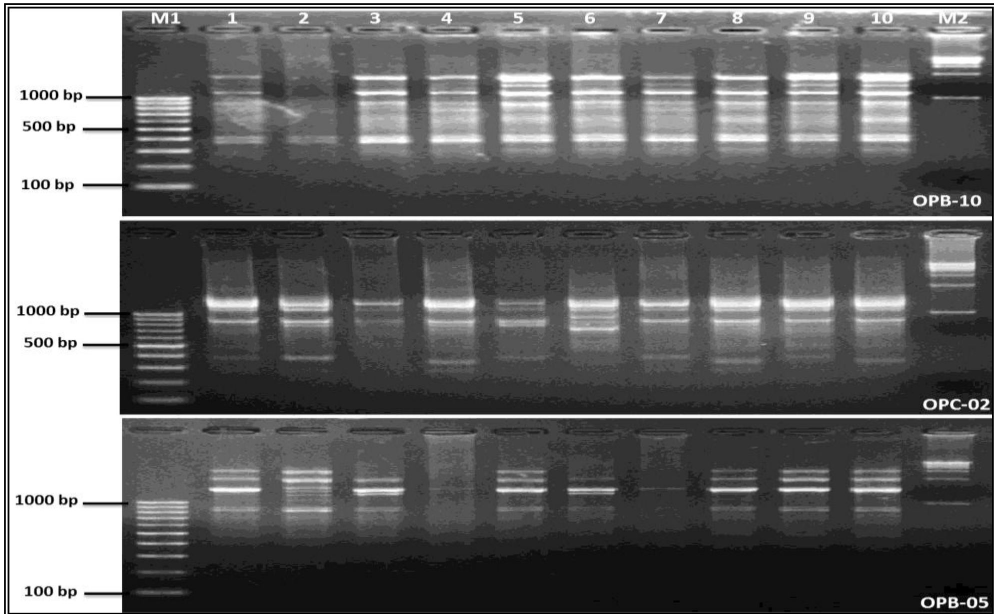

Fig. (1): RAPD profiling of ten snap bean genotypes using primers OPB-10, OPB-02 and OPC-05. Lanes 1-10 represent the snap bean genotypes: Concessa, Cerdon, Argus, Samanatha, Fendor, GOMI, Paulista, Tema, Xera and Bronco. Lane M1 is 100 bp ladder and M2 is 1 kb Ladder.

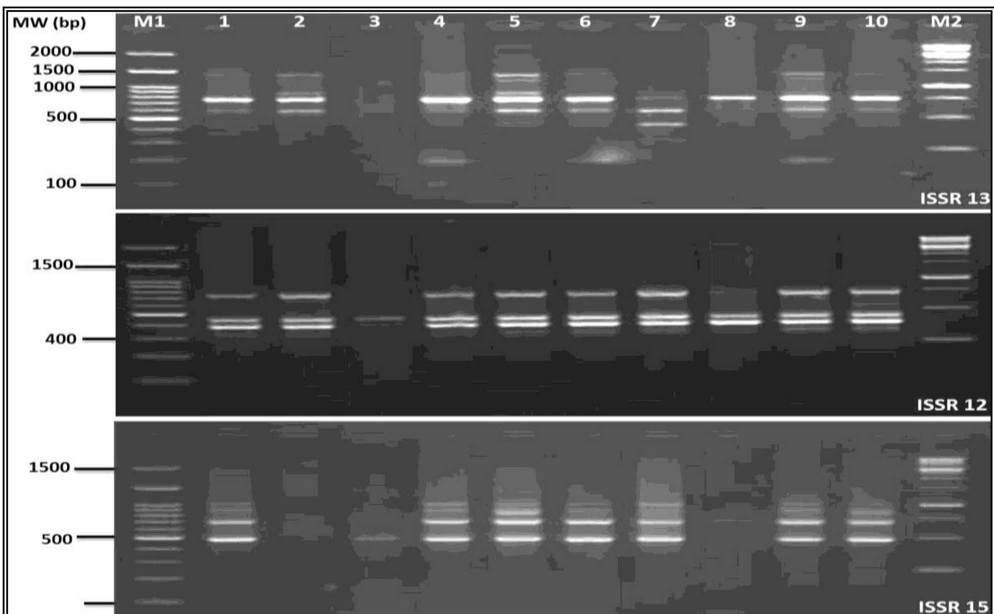

Fig. (2): ISSR profiling of ten snap bean genotypes using primers ISSRs13, ISSR12 and ISSRs 15. Lanes 1-10 represent the snap bean genotypes; Concessa, Cerdon, Argus, Samanatha, Fendor, GOMI, Paulista, Tema, Xera and Bronco, respectively. Lane M1 is 100 bp ladder and lane M2 is 1 kb Ladder.

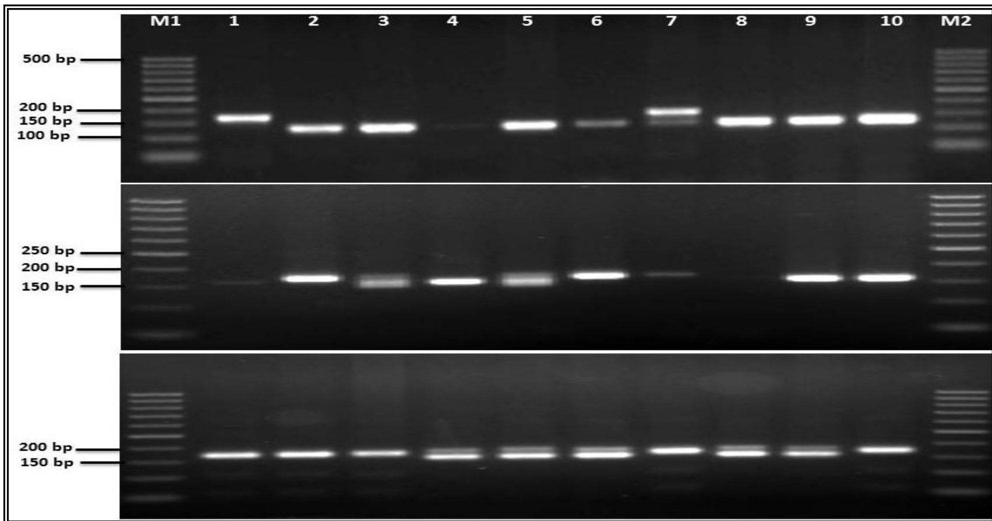

Fig. (3): SSRs profiling of ten snap bean genotypes using primers PV-at005 (upper), PV-at009 (middle) and PV-atcc002 (down). Lanes 1-10 represent the snap bean genotypes; Concessa, Cerdon, Argus, Samanatha, Fendor, GOMI, Paulista, Tema, Xera and Bronco, respectively. Lane M1 is 100 bp. ladder and lane M2 is 1 kb. Ladder.

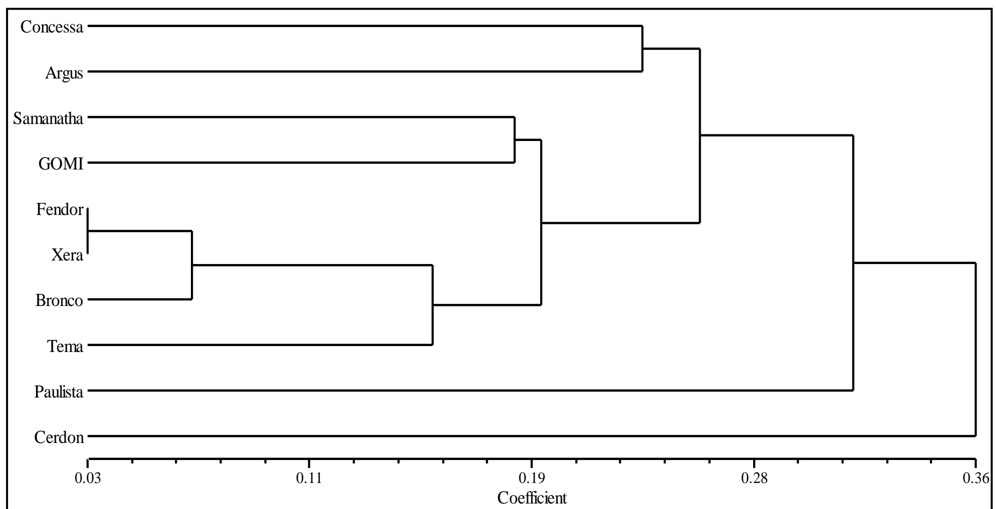

Fig. (4): Phylogenetic analysis among the ten snap bean genotypes, Concessa, Cerdon, Argus, Samanatha, Fendor, GOMI, Paulista, Tema, Xera and Bronco based on RAPD, ISSRs and SSRs data.