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Full Length Research Paper

Genetic diversity analysis of the medicinal herb *Plantago ovata* (Forsk.)

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***Plantago ovata* (Forsk.) (2n = 8) used as laxative, emollient and demulcent, has great commercial and medicinal importance. With India being the largest producer in the world there is still a lack of defined varieties of the species and no coordinated breeding efforts are being made. In the present study, we report the phylogenetic analysis of the crop for its utilization in future breeding programs for defining varieties of the crop. A total of 302 clear and reproducible bands were obtained with random amplified polymorphic DNA (RAPD) techniques involving 35 random primers in 18 selected lines, out of which 198 (65.5%) were polymorphic with an average 8.6 bands per primer. Amplified DNA fragments ranged from 300 to 3400 bp. Dissimilarity indices based on Nei and Li equation ranged from 0.07 to 0.29 indicating moderate level of genetic polymorphism. Hierarchical cluster analysis using SPSS method showed genetic variation amongst genotypes dividing them into three major clusters comprising 10, seven and one genotypes, respectively. The result of present study indicates that RAPD analysis has determined the genetic relationships and estimated the genetic diversity among the genotypes of *P. ovata*.**

Key words: *Plantago ovata* (Forsk.), random amplified polymorphic DNA (RAPD) markers, Nei and Li equation, genetic diversity.

INTRODUCTION

Plantago ovata (Forsk.) commonly known as Isabgol and commercially as Blond psyllium (Dalal and Sriram, 1995) is grown in India for its use in ayurvedic medicines (Bist et al., 2001). It belongs to the family Plantaginaceae. It is a diploid (2n = 8), and predominantly a cross-pollinated annual herb. The genus *Plantago* comprises of 200 species of which 10 species occur in India. Out of these 10 species, only two species, *P. ovata* Forks. and *P. psyllium* are economically important but *P. ovata* is the main source of Isabgol due to its high quality and yield of husk.

In this crop, seeds have medicinal value, and the husk which is the rosy-white membranous covering of the seed (epicarp of seed) constitutes the drug. Seeds are believed to have cooling, demulcent effect and useful in inflammatory (Beara et al., 2010) and biliary disorders of digestive organs. They are also applied as a poultice for rheumatic and gouty swelling and thought to be beneficial in treatment of dysentery and irritation of the intestinal tract. The seeds are used in ayurvedic, Unani and allopathic medicines also (Dalal and Sriram, 1995). The husk, which is about 25 to 30% of the seed, has the

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Figure 1. Plants of eighteen cultivated genotypes of Isabgol

property of absorbing and retaining water and hence, it works as an antidiarrheal drug. It is also used in calico printing, dyeing, agar-agar media preparation, gum and jelly making, as binder in tablets, as thickner and a fixative in ice-cream, confectionary and in cosmetics industries (Upadhayay et al., 1978).

India currently ranks first in the production and trade of *P. ovata* in the world market and earn a sizeable foreign exchange. The genotypes of Isabgol do not have much variability morphologically. So to improve the Isabgol, genetic variability in various genotypes is a prerequisite. Thus, there is an urgent need to characterize the cultivated genotypes for their genetic variability. Molecular markers can be helpful in order to find out polymorphism among the Isabgol genotypes. Keeping in view the medicinal and economic importance of Isabgol, the present study was conducted on 18 Indian grown Isabgol genotypes for the assessment of genetic diversity by using the random amplified polymorphic DNA (RAPD) markers and its utilization in future breeding programs, as a prelude to crop improvement.

MATERIALS AND METHODS

Plant

Eighteen Indian grown Isabgol (*P. ovata* Forsk.) genotypes comprising fifteen indigenous and three exotic accessions of diverse origin were procured from Medicinal and Aromatic Plant Section, Department of plant Breeding, CCS HAU, Hisar. These genotypes were selected on the basis of their origin and morphological characteristics. The seedlings were raised in pots under natural environmental conditions in the screen house (Figure 1).

DNA extraction, purification and polymerase chain reaction (PCR) amplification

Total genomic DNA from 18 genotypes of Isabgol was isolated initially following standard cetyl trimethylammonium bromide (CTAB) method given by Murray and Thompson (1980), modified by Saghai-Maroof et al. (1984) which was further modified and standardized for Isabgol genotypes. PCR was carried out in 20 µl of reaction mix containing 100 ng genomic DNA, 1.5 unit of Taq DNA polymerase, 2 µl of 10× Taq DNA polymerase buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1 Triton X-100), 1.5 mM $MgCl_2$, 100 mM of each dNTP's and 0.2 mM of decanucleotide primers.

Amplification was carried out in PTC-100 programmable thermal cycler (MJ Research and Biometra Personal). An annealing temperature of 40°C was found to be optimum for generation of clear and reproducible bands. Amplified DNA fragments were resolved by submerged horizontal electrophoresis in 1.6% (w/v) agarose gel and visualized by staining with ethidium bromide. PCR amplification products were viewed under long wavelength (350 nm) ultraviolet (UV) trans-illuminator and photographed using VDS Image Master of Pharmacia, Biotech. 38 primers (Table 3) were selected from 10-base random oligonucleotides primers and were used to find out polymorphism among the Isabgol genotypes.

Statistical data analysis

The banding patterns from RAPD analysis for each primer were recorded by visual observation. Based on presence (1) or absence (0) data, genetic similarity was calculated to estimate all pairwise differences in the amplification products for all genotypes. The dissimilarity matrix was obtained after multivarient analysis using

Table 1. Effect of DNA extraction methods on DNA yield and quality from leaves of Isabgol genotypes.

Parameter	DNA extraction method			Mean
	CTAB	SDS potassium acetate	SDS miniprep	
	Yield of DNA (µg DNA/g of leaves)			
Palampur-3	318.0	246.0	70.0	634.0
Ahmedabad	290.5	210.0	64.6	565.1
MIB-125	255.0	198.0	61.6	514.6
HI-96	82.0	70.0	55.1	207.1
Mean	945.0	734.0	251.3	
Absorbance ratio ($A_{26}o/A_28o$)	1.83	1.65	1.6	

Nei and Li (1979) equation. Based on this data, hierarchical cluster analysis was done using SPSS method to obtain a dendrogram.

RESULTS AND DISCUSSION

Random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and microsatellites are undoubtedly valuable tools for addressing population genetics and plant breeding (Ibrahim et al., 2010). Morphologically, the cultivated genotypes of Isabgol are very similar to each other. So phenotypically, it is difficult to identify and differentiate them. Polymerase chain reaction (PCR) based markers like RAPD allow the rapid detection of DNA polymorphism from various medicinal and aromatic plants, and were shown to be potentially useful for studying genetic variation in them. To avoid erratic amplification, good quality DNA free of contaminants and optimization of PCR reaction conditions is a prerequisite. Accordingly, the present investigation was undertaken to standardize the DNA extraction methods and to optimize the PCR reaction conditions and generate RAPDs to study genetic variability in Indian grown Isabgol genotypes.

DNA extraction and purification

Total genomic DNA was extracted from freshly emerged leaves of 2 to 3 weeks old seedlings of 18 genotypes of Isabgol with three different DNA extraction methods: Cetyl trimethyl ammonium bromide (CTAB) method, sodium dodecyl sulphate (SDS)-potassium acetate method and sodium dodecyl sulphate (SDS) miniprep method. The highest amount of DNA per gram of fine ground leaf tissue was extracted using CTAB method (3180 µg/g of leaves) followed by SDS potassium acetate method (246.0 µg/g of leaves). The quantity of DNA extracted by following SDS miniprep method was lowest (70 µg/g of leaves). Among the different genotypes, the significantly highest amount of DNA was extracted from Palampur-3 by using all methods. Following CTAB method, Palampur-3 genotype yielded DNA to the tune of 3180 µg/g of leaves followed by SDS potassium acetate method (246.0 µg/g of leaves). The yield of DNA from Palampur-3 by using SDS miniprep method was lowest (70 µg/g of leaves). The genotype HI-96 yielded lowest amount (82.0 µg/g) of DNA with CTAB method. The absorbance ratio (A260/A280), which reflects the quality of DNA extracted was high for DNA extracted by CTAB method (1.83), followed by absorbance ratio of 1.65 and 1.60 for SDS-pot acetate and SDS miniprep methods, respectively (Table 1).

Very high amount of polysaccharides present in the DNA expected, as reflected by the dark brown color, were removed by phenol-chloroform extraction. The amount of RNA present in the DNA extracted by further methods was very high, except CTAB method in which it was comparatively low. The ratio of absorbance at A260/A280 showed that DNA was free from contaminants like polyphenols, polysaccharides, protein, RNA etc. A single discrete band of high molecular weight showed that genomic DNA extracted with CTAB method was free from any mechanical or enzymatic degradation, and was intact and of good quality (Figure 2). DNA was also free from RNA contamination. The DNA isolation protocol was standardized with different concentration of CTAB and incubation period. Optimum DNA yield and quality (Table 2) were obtained when incubation time was one and half hour and CTAB concentration was 1.5% for Isabgol genotypes. Similar types of results were obtained in other medicinal plants such as Tansy (Keskitalo et al., 1998) and Almond (Bartolazzi et al., 1998). However, Wolff et al. (1998) extracted total genomic DNA from young leaves of *Plantago major* by using miniprep CTAB method.

PCR amplification conditions

For standardization of PCR amplification conditions, optimization was carried out to determine the optimal concentration of template DNA, $MgCl_2$, Taq DNA polymerase and annealing temperature. The influence of

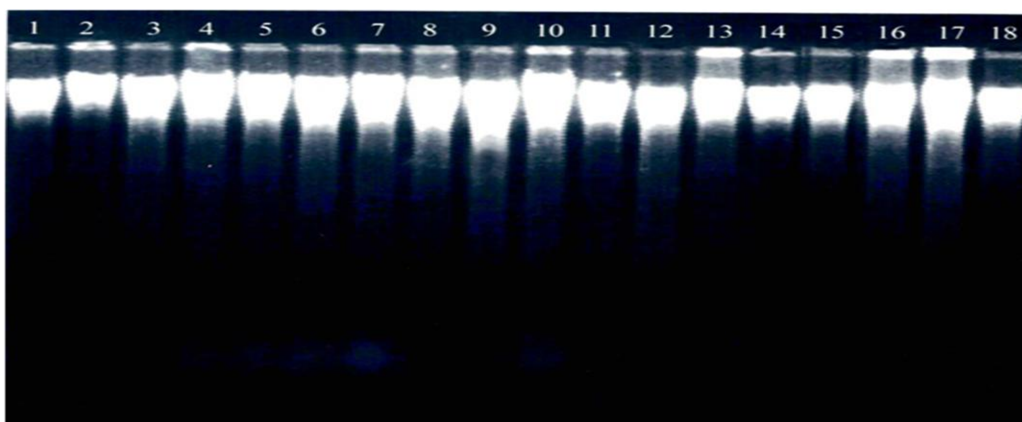

Figure 2. Electrophoretic pattern of purified high molecular weight genomic DNA extracted from Isabgol genotypes using CTAB method.

Table 2. Quantity and quality of genomic DNA of Isabgol genotypes extracted following CTAB methods.

Genotype	Quantity (µg/ml)	Quality (A_{260}/A_{280})
Ahmedabad	2905	1.82
HI-34	2100	1.80
GI-1	2370	1.79
HI-4	2445	1.76
EC-42706	2680	1.81
Palampur-3	3180	1.80
RI-49	2030	1.79
MIB-125	2550	1.83
P-7-12	1340	1.77
HI-5	2550	1.80
DM-10	1925	1.75
GI-2	1165	1.79
PB-10-4	1100	1.80
HI-1	1865	1.82
HI-96	820	1.77
EC 7739	1565	1.82
RI-9709	965	1.80
EC-41181	2650	1.80

various combinations of genomic DNA (50, 100 and 200 ng), $MgCl_2$ (1.0, 1.5, 2.0 mM), Taq DNA polymerase (1.0, 1.5, 2.0 U) and annealing temperature (35, 40, 45°C) on amplified products were worked out. In general, either no product or products with inconsistent bands were obtained when the concentration of the template DNA was 50 ng or $MgCl_2$ was 1.0 mM, with high annealing temperature of 45°C. When higher concentration of template DNA (200 ng) and $MgCl_2$ (2 mM) were used, smeared DNA banding patterns were produced. The optimal concentration of template DNA and $MgCl_2$ were found to be 100 ng and 2 mM. The optimal concentration of Taq DNA polymerase was observed to be one 1.5 unit/reaction. An annealing temperature of 40°C was found to be optimum for generation of clear and reproducible bands. However, with lower annealing temperature (35°C), false bands were observed.

Random amplified polymorphic DNA (RAPD) analysis

Thirty eight random decamer primers obtained from Operon Technologies USA, were used for RAPD analysis of different cultivars to detect polymorphism. Out of 38

Table 3. Random primers showing polymorphism among Isabgol genotypes.

Primer Code	Sequence	No. of genotypes amplified	Total band	Polymorphic band	Monophorphic band	Percent polymorphism
OPA-1	CAGGCCCTTC	18	9	7	2	77.77
OPA-2	TGCCGAGCTG	16	4	4	0	100
OPA-4	AATCGGGCTG	17	8	8	0	100
OPA-5	AGGGGTCTTG	16	5	5	0	100
OPA-6	GGTCCCTGAC	18	10	10	0	100
OPA-7	GAAACGGGTG	16	8	8	0	100
OPA-8	GTGACGTAGG	18	11	10	1	90.9
OPA-9	GGGTAACGCC	18	12	8	4	66.6
OPA-10	GTGATCGCAG	18	13	12	1	92.3
OPA-11	GAATCGCCGT	18	9	5	4	55.5
OPA-12	TCGGCGATAG	18	10	5	5	50%
OPA-13	CAGCACCCAC	18	7	3	4	42.8
OPA-14	TCTGTGCTGG	18	10	4	6	40
OPA-15	TTCCGAACCC	18	11	9	2	81.8
OPA-16	AGCCAGCGAA	18	12	4	8	33.3
OPA-17	GACCGCTTGT	18	9	6	3	66.6
OPA-18	AGGTGACCGT	18	10	3	7	30
OPA-19	CAAACGTCGG	18	7	6	1	85.7
OPA-20	GTTGCGATCC	17	10	10	0	100
OPB-1	GTTTCGCTCC	18	10	4	6	40
OPB-2	TGATCCCTGG	18	12	6	6	50
OPB-3	CATCCCCCTG	18	12	8	4	66.6
OPB-4	GGACTGGAGT	18	9	5	4	55.5
OPB-5	TGAGCCCTTC	18	9	6	3	66.6
OPB-6	TGATCTGCCC	18	11	4	7	36.6
OPB-7	GGTGACGCAG	18	10	8	2	80
OPC-1	TTCGAGCCAG	18	6	5	1	83.3
OPC-2	GTGAGGCGTC	17	6	6	0	100
OPC-1 I	AAAGCTGCGG	18	6	0	6	0
OPC-12	TGTCTCCCC	18	5	2	3	40
OPC-13	AAGCCTCGTC	17	5	5	0	100
OPC-14	TGCGTGCTTG	17	5	5	0	100
OPC-15	GACGGATCAG	18	6	3	3	50
OPC-16	CACACTCCAG	18	5	0	5	0
OPD-3	GTCGCCGTCA	18	10	4	6	40
Total			**302**	**198**	**104**	**65.5**

random primers screened, 35 primers showed amplification in all genotypes while 3 primers (OPA-3, OPC-8 and OPC-9) did not show any amplification in any of the genotypes. These primers perhaps did not find any complementary binding sequence on genomic DNA of these Isabgol genotypes. Moreover, these primers might have some special requirements for amplification as suggested by Weeden et al. (1992) and Ahmad (1999). Out of these 35 primers, 33 primers show polymorphism and 2 primers produced all monomorphic bands only. Twenty seven primers amplified all the 18 genotypes whereas 5 primers; OPA-4, OPA-20, OPC-2, OPC-13 and OPC-14 amplified at least 17 genotypes. The genotypes which were not amplified by these five primers were MIB-125 (OPA-4), DM-10 (OPA-20) and HI-34 (OPC-2, OPC-13, and OPC-14). Three primers; OPA-2, OPA-5 and OPA-7 amplified 16 genotypes only (Table 3). Vahabi et al. (2008) observed 142 polymorphic PCR product (average 4.05 bands for each primer) by using 35 RAPD primers in *P. ovata*. Similarly, a total of 102 bands were scored against 36 genotypes of *P. ovata* by using 20 random primers. Of 102 bands, 89 (87.25%) were

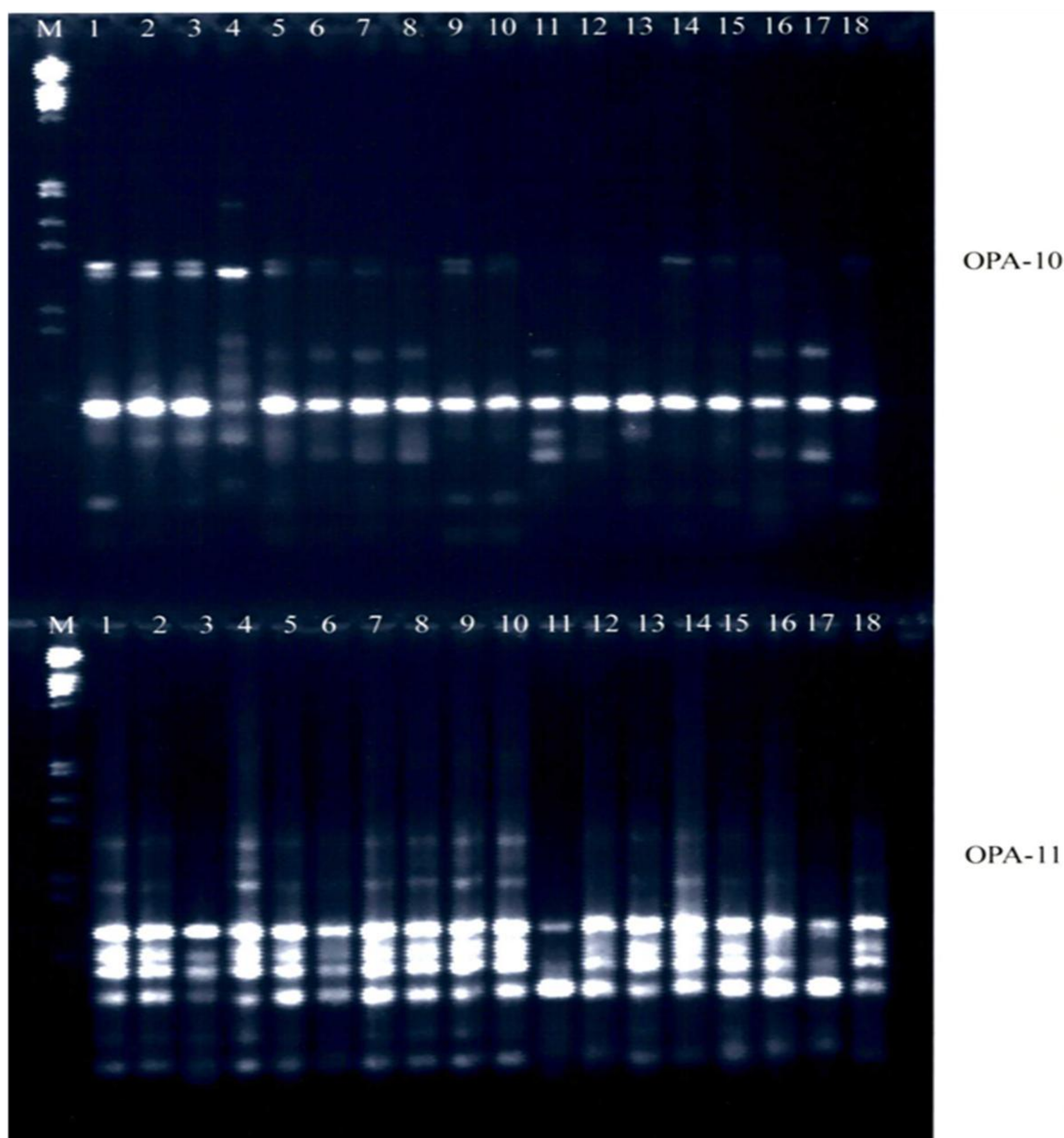

Figure 3. Electrophoretic pattern of Isabgol genotypes with primers OPA-10 and OPA-11 [(Lane M) represents λ DNA/Eco RI + HindIII Marker. (Lane 1 to 18): eighteen Isabgol genotypes amplified with random primers].

found to be polymorphic, while 5 (4.9%) were monomorphic and 8 (7.8%) were unique as studied by Singh et al. (2009) (Figure 3).

Genetic dissimilarity and cluster analysis

The dissimilarity indices based on Nei and Li equation for different genotypes ranged from 0.07 to 0.29, indicating moderate level of genetic variability among the genotypes that might be due to the morphological similarity among the various genotypes of Isabgol (Table 4). Two genotypes GI-2 and P-7-12 had maximum genetic distance that is, 0.29, whereas two genotypes P-7-12 and HI-5 had minimum genetic distance that is, 0.07. Maximum genetic similarity between two genotypes was 93% whereas the minimum similarity was 71%.

The association amongst different genotypes was presented in the form of dendrogram prepared using rescaled distances. With SPSS hierarchical cluster analysis based on RAPD markers, the 18 Isabgol genotypes studied were grouped into three major clusters

Table 4. Pairwise dissimilarity indices of Isabgol genotypes.

Genotypes	Ahmadabad	HI-34	GI-1	HI-4	EC-42706	Palam-pur-3	RI-49	MIB-125	P-7-12	HI-5	DM-10	GI-2	PB-10-4	HI-1	HI-96	EC-7739	RI-9709	EC-41181
Ahmadabad	0	0.16	0.22	0.12	0.19	0.17	0.16	0.19	0.12	0.17	0.23	0.15	0.19	0.24	0.15	0.14	0.22	0.18
HI-34		0	0.22	0.25	0.16	0.17	0.17	0.19	0.23	0.19	0.22	0.16	0.19	0.16	0.18	0.21	0.19	0.19
GI-1			0	0.21	0.14	0.23	0.23	0.20	0.29	0.28	0.24	0.19	0.20	0.24	0.21	0.28	0.20	0.23
HI-4				0	0.16	0.09	0.10	0.13	0.14	0.17	0.23	0.13	0.14	0.12	0.11	0.15	0.17	0.16
EC-42706					0	0.13	0.14	0.10	0.18	0.18	0.16	0.13	0.16	0.18	0.16	0.19	0.17	0.18
Palampur-3						0	0.08	0.15	0.10	0.11	0.19	0.11	0.15	0.11	0.12	0.12	0.16	0.17
RI-49							0	0.12	0.14	0.16	0.17	0.10	0.12	0.14	0.09	0.15	0.13	0.16
MIB-125								0	0.18	0.18	0.16	0.12	0.11	0.17	0.14	0.20	0.16	0.17
P-7-12									0	0.07	0.21	0.11	0.18	0.11	0.14	0.14	0.21	0.20
HI-5										0	0.21	0.14	0.19	0.15	0.17	0.15	0.23	0.21
DM-10											0	0.15	0.16	0.23	0.20	0.21	0.14	0.19
GI-2												0	0.13	0.12	0.11	0.16	0.15	0.17
PB-10-4													0	0.15	0.15	0.2	0.15	0.12
HI-1														0	0.11	0.15	0.16	0.14
HI-96															0	0.15	0.12	0.14
EC-7739																0	0.17	0.16
RI-9709																	0	0.12
EC-41181																		0

(Figure 4). First cluster comprised of ten genotypes; P-7-12, HI-5, Palampur-3, RI-49, HI-4, HI-96, GI-1, HI-1, Ahmedabad and EC-7739. Seven genotypes fell in second cluster whereas the remaining genotype that is,

HI-34 out grouped as a separate third cluster at a genetic distance of 25% on hierarchical scale. The first cluster had further two subgroups with two genotypes in one subgroup and eight genotypes in another subgroup. In the first cluster, four genotypes (P-7-12 and HI-5 and Palampur-3 and RI-49) were very close to each other and have lowest genetic distance value that is, 1% on hierarchical scale. On the other hand, two genotypes Ahmedabad and EC-7739 were far from others with a genetic distance of about 16% on the hierarchical scale. Similarly in cluster II, two genotypes MIB-125 and PB-10-4 are close to each other and have lower genetic distance value that is, 6% while the genotypes GI-2 and EC-42706 have genetic distance value of 11%. In the cluster III, the only genotype HI-34 exists at maximum genetic distance (25%) on hierarchical scale.

Using RAPD markers, Vahabi et al. (2008) studied genetic relationships among 22 populations of *P. ovata*. In the RAPD based clustering, all populations that belonged to near area formed close groups. The phylogenetic tree revealed a clear differentiation of two main groups. Zubair et al. (2012) detected genetic and phytochemical differences between and within a total of five populations of *Plantago major* by performing RAPD analysis. Similarly, Singh et al. (2009) assessed germplasm diversity among 80 accessions of *Plantago* spp. through RAPD profiling. Based on the degree of divergence, 80 accessions were grouped into seven clusters.

Conclusion

The present study has proved to be successful in standardizing the DNA extraction protocol to isolate high quantity and good quality DNA from Isabgol and to optimize the PCR reaction conditions for the same and the results revealed that RAPD is an efficient technique to characterize and classify different Isabgol genotypes. It was also indicated that RAPD analysis has determined the genetic relationships and estimated the

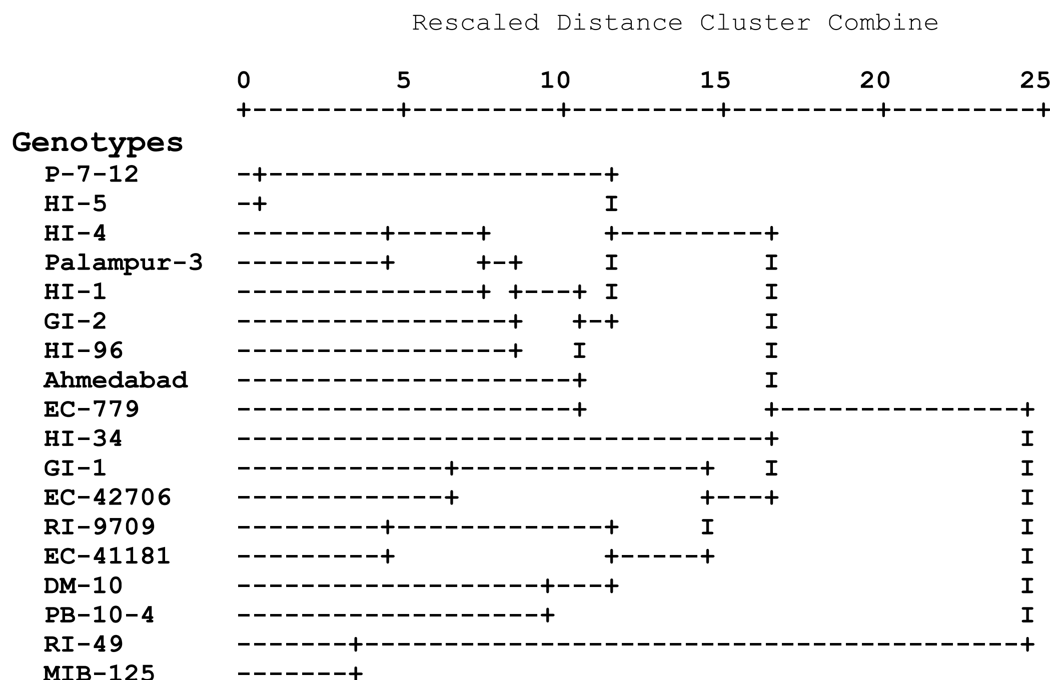

Figure 4. Hierarchical cluster analysis dendrogram using average linkage (between groups).

genetic diversity among the genotypes of Isabgol. This can be further used for the improvement of Isabgol for various traits through different breeding methods.

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