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Phenotypic and molecular characterization of selected species of *Plantago* with emphasis on *Plantago ovata*

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Abstract

The extent of genetic diversity in crop plants is of prime concern to plant breeders and germplasm curators. Therefore, a study was employed to determine the genetic diversity and relationships among 38 genotypes of Plantago representing seven species using phenotypic and molecular markers. All 38 genotypes were obtained from various institutes of India. For phenotypic characterization, experiment was conducted in randomized block design with two replications. For RAPD and SSR analysis, DNA was isolated from bulk of 10 plantlets using CTAB method. At molecular level, a narrow range of genetic dissimilarity (0.01 - 0.29) was observed using morphological descriptors. However, a total 248 band positions with 100% polymorphism were amplified by 15 RAPD primers, whereas 33 allelic positions with 95% polymorphism were produced by 10 SSR primers with 100% reproducibility. The wide genetic distance was observed in Plantago species both at phenotypic as well as molecular level. The comparison of the clusters obtained based on the phenotypic parameters and marker data showed that the genotypes grouped differently in both cases. A poor correlation (r = -0.72) and lack of perfect concurrence between phenotype and marker-based analysis was revealed by the UPGMA dendrogram. The results of this study indicate that molecular markers are highly discriminatory, more precise, and more reproducible than some morphological traits used to estimate genetic relatedness among Plantago genotypes. Outcomes of diversity analysis revealed that though different species of genus Plantago included in present study significantly different from each other but variability with in *P. ovata* species was very limited. Therefore, induced mutation can be exploited to widen the range of variation.

Keywords: Isabgol; Mantel test; Phenotypic data; Plantago spp; RAPD; SSR.
Abbreviations: CV_ Coefficient of variance; GD_ Genetic distance; PCR_ polymerase chain reaction; RAPD_ Random Amplified Polymorphic DNA; SSR_ Simple Sequence Repeat; UPGMA_ Unweighted Pair-Group Method of Arithmetic Averages.

Introduction

Genus Plantago of family Plantaginaceae consists of nearly 200 species (Rahn 1996) most of which are predominantly cross-pollinated in nature. Although the center of diversity of plantago is deemed to be positioned somewhere in central Asia, some species have now become widespread far and wide with utmost concentration in temperate areas. Species of Plantago are small herbs, mostly growing as weeds, while some are exploited in varied modes in local medicines, two taxa namely *Plantago ovata* Forsk. and *P. psyllium* L. is economically imperative. These are cultivated for their mucilaginous seed husk which is pharmaceutically rated among the most effective laxatives. In India *P. ovata* is cultivated sporadically in parts of North Gujarat, South Rajasthan and some parts of Haryana. Globally, the contribution of India in the production and export of isabgol is 95 and 100%, respectively. India, therefore, ranks first in the production and trade of *P. ovata* in the world market. Consequently, India is earning more than 200 crores rupees forex annually. From the total production of husk in Gujarat province, 75% is exported. The largest buyer of isabgol from India is the United States, accounting for around 75% of the total husk exports from India. Isabgol, thus, is an industrially important cash crop for foreign trades of India. The seeds of *P. ovata* contain pale yellow oil (11.42%), large amount of mucilaginous matter, inorganic ash and reducing sugars (Pendse and Dutt, 1934). Besides being used as laxative, it is also having industrial importance especially in ice creams, chocolates, cosmetics and in printing and finishing industries. It lowers blood cholesterol levels considerably and has been used as an indigenous Ayurvedic and Unani medicine (Dhar et al., 2011). It is an out-crossing species and has a narrow genetic base on account of low chromosome number (2n=2x=8), small chromosome size (621 Mb), presence of lot of heterochromatin (constitutive) in all its chromosomes, low chiasmata frequency and low recombination index (Pandita, 2013). In spite of its immense medicinal and export value, the productivity of isabgol is under the constraints of biotic and abiotic stress causing heavy losses in seed/husk quality and yield. The wild Plantago species constitute a rich resource of important genes like biotic and abiotic stress tolerance, which if introgressed to *P. ovata*, can revolutionize the production of psyllium (Dhar et al., 2011). *P. ovata* has about 200 wild allies, some of which are medicinally important. These wild species are reservoir of 16 important genes, which if introgressed to cultivated species through marker-assisted breeding could revolutionize the production of isabgol.

However, for efficient interspecific crop improvement efforts, it is critical to assess the phylogenetic relationship and the amount of genetic diversity among species (Kotwal et al., 2013). Generally, morphological features of plants are being exploited by plant scientists to understand genetic diversity. But morphological markers are known to be significantly affected by edaphic and climatic conditions. Therefore, it is better to study phylogenetics at the DNA level, a true diversity indicator, using molecular markers. There are limited reports on exploitation of RAPD (Sanghmitra et al., 2011; Vala, et al,. 2011; Rohila et al., 2012) and combined RAPD and ISSR (Wolff and Morgan, 1998; Wolff et al., 2000; Kaswan et al., 2013) in assessing genetic diversity in this industrially important but resource poor crop. Equally, a very small number of microsatellite markers are available for many species of plantao viz. *P. major, P. lanceolata, P. coronopus, P. intermedia* (Wolff and Morgan, 1998; Koorevaar et al., 2002; Marie et al., 2003; Squirrell and Wolff, 2001) but not in *P. ovata*. Therefore, researchers could not efficiently exploit the variability available in secondary gene pool for this crop. The present paper highlights the efforts that have been made to study the molecular diversity in *P. ovata* and its selected wild species allies using RAPD and SSR markers followed by its comparison with phenotypic diversity.

Results and Discussion

The assessment of genetic diversity is not only important for crop improvement efforts but also for efficient management, characterization and protection of germplasm resources. Cultivated Plantago germplasm were low in genetic diversity and deficient for resistance to many biotic and abiotic stresses. Wild species of Plantago are potential sources of several important genes, which if transferred to *P. ovata*, can revolutionize the production of psyllium (Dhar et al., 2011). However, to develop successful interspecific hybrids it is essential to determine phylogenetic relationships among closely related species.

Morphological evaluation and cluster analysis

A total of nine morphological and yield related traits were recorded for assessment of relationship among Plantago genotypes. The results showed that the genotypes of Plantago were significantly different in nine traits (Table 1), suggesting that selection for relevant characters could be possible. All the phenotypic parameters with minimum, maximum, mean and CV values are presented in table 1. Thousand grain mass (TGM) which is very important yield component, showed wide variation among the species and ranged from 0.14 g (*P. serraria*) to 1.35 (*P. lanceolata*). Likewise, there was a significant variation in the seed yield per plant among the seven Plantago species studied. These finding are in congruous with previous reports (Patel and Saravanan, 2010). Highest variation (CV=2.65) was observed for number of spikes per plant followed by harvest index (CV=1.79), while it was minimum (CV=0.56) for plant height. The phenotypic variations among genotypes may be due to more than one factor and the degree of out-crossing is one such potent factor (Singh and Lal 2009). However, variation varies with genetic makeup of genotypes and environment. Isabgol is an introduced crop to India, the gene pool available in the country is very narrow and within the species of *P. ovata*, the variability for economically important traits is very narrow (Lal et al., 2000). Therefore, efforts should focus on generating genetic variability, through mutation breeding or wide-hybridization. Manhattan cluster analysis revealed that the highest dissimilarity index value of 0.29 was found between two mutant lines (RIM-18-15-64 & RIM-107-90-44) of *P.ovata* and *P. arenaria* of allied species and lowest dissimilarity index value of 0.01 was found among many of *P. ovata* genotypes. The dendrogram shows a clear-cut variation among the species as UPGMA-based dendogram had clearly put all the individual species into six major groups at an average cut of value of 0.07 (Fig. 1). According to the UPGMA-based clustering, group I had maximum (31) number of genotypes, while two each in each group II and VI. The accessions of *P. ovata* were found to be the least dissimilar and formed two sub-cluster within the major cluster 1 at a cut off value of 0.04. This substantiated the origin of most of the lines by mutagenesis which shared a common genome. The dendrogram constructed on the basis of phenotypic data clearly revealed DPO14, an early-flowering (30-35 days after sowing) and-maturing (80–85 DAS) mutant, as an out-group of *P. ovate*. Remaining released varieties were distributed randomly in the phenotypic-based dendrogram due to high dissimilarity coefficient. The result of low phenotypic diversity is in agreement with study of Sivaneson and Ranwah (2009). Moreover, the isabgol varieties released so far in India hardly show any phenotypic variation to discriminate them from each other and are similar in their yield potential due to narrow gene pool.

RAPD and SSR analysis

Using isozymes (Raychaudhari and Pramanik, 1997; Srimanta and Raychaudhuri, 1997; Kaswan et al., 2012), nuclear ribosomal DNA internal transcribed spacer (ITS) regions (Ronsted et al., 2002; Dhar et al., 2006) and amplified fragment length polymorphism (AFLP; Kotwal et al., 2013) very limited and scanty information has been generated on genetic diversity to determine phylogenetic relationships among Plantago accession.

RAPD Analysis

RAPD has been successfully used for differentiating species of a genus based on their similarities and geographical proximities (Sherry et al., 2011). Recently, RAPDs were employed for studying genetic relationships in several medicinal plants (Samantaray et al., 2010). In the present study for RAPD analysis, bulked DNA samples were used. Bulking of leaf samples prior to extraction of DNA for RAPD analysis will ensure that only the most frequent alleles are favoured during amplification compared to sparse ones (Michelmore et al., 1991). Thus the random arbitrary primers target the frequently occurring template regions in the bulked samples, preventing the amplification of rare sequences. All the chosen thirty RAPD primers amplified fragments during initial screening with five genotypes. Reproducibility of amplification profiles was tested for each primer and only 15 primers could be considered for the final analysis which could detect 248 amplicons from 38 genotypes and all were found polymorphic. The molecular size of the amplified RAPD products ranged from 150bp (OPA18, OPF02, OPF05

Table 1. Range, mean and coefficient of variance of different phenotypic traits in 38 Plantago genotypes.

S.No	Genotypes	Origin	DFF	PH (cm)	NSPP	SL (cm)	DtM	TGM (gm)	SYPP (gm)	NSPS	HI (%)
1	RIM-15-15-62		63.0	36.83	34.33	5.17	116.5	1.44	5.22	105.83	21.10
2	RIM-224-30-58		60.5	36.83	28.5	4.83	115.6	1.45	5.82	140.83	19.73
3	RIM-104-90-7		58.5	36.5	40.83	6.33	112.2	1.4	5.88	102.59	15.48
4	RIM-18-15-3		60.5	38.03	35.17	5.42	116.5	1.41	5.66	113.98	17.43
5	RIM-89-400-32		65.5	34.17	31.26	4.75	119.5	1.48	6.04	131.94	17.96
6	RIM-2-500-12		65.5	35.83	26.33	4.75	120.1	1.43	5.39	143.39	18.08
7	RIM-15-15-2		62.0	36.52	36.83	4.75	116.5	1.45	5.74	107.51	13.26
8	RIM-5-400-25		61.5	33.67	35.50	4.5	119.0	1.43	5.62	110.71	13.34
9	RIM-89-0-05-9		62.0	41.04	36.83	4.88	116.0	1.46	5.5	102.31	15.90
10	RIM-84-71-11		60.5	39.17	45.5	4.42	118.5	1.47	5.54	82.71	21.04
11	RIM-108-90-35	S.K.	61.5	40.51	38.50	5.08	120.2	1.47	5.75	101.8	17.95
12	RIM-89-800-35	Rajasthan	64.0	40.83	31.67	5.58	115.5	1.44	5.74	125.7	23.12
13	RIM-5-200-23	Agricultural	61.5	35.83	35.67	4.75	113.5	1.51	5.49	101.74	19.45
14	RIM-5-10-19	University,	60.0	42.17	40.33	4.42	113.3	1.49	5.87	97.65	19.74
15	RIM-108-90-10	Bikaner	62.0	38.83	40.52	4.83	114.5	1.51	5.37	88.31	17.24
16	RIM-5-500-26	(Rajasthan)	62.0	37.67	36.67	4.50	114.3	1.46	5.26	98.63	17.20
17	RIM-2-600-13		64.5	39.55	40.51	4.75	120.1	1.52	5.74	93.60	13.8
18	RIM-32-30-62		60.5	42.83	42.54	4.83	114.5	1.49	5.62	88.52	14.59
19	RIM-44-30-54		62.5	39.17	37.33	4.75	116.5	1.54	5.74	100.22	15.06
20	RIM-89-75-6		63.0	40.33	42.83	4.75	113.5	1.57	5.62	83.84	15.76
21	RIM-41-30-16		62.0	36.67	37.23	4.67	115.5	1.48	5.92	108.44	15.54
22	RIM-22-30-38		60.5	36.02	31.5	4.42	114.6	1.54	5.29	109.12	21.97
23	RIM-2-1400-19		58.0	40.02	35.12	4.17	114.5	1.59	5.31	95.63	16.69
24	RIM-18-15-64		64.0	36.51	31.33	4.92	118.7	1.55	5.64	115.87	19.34
25	RIM-107-90-44		65.5	34.67	33.13	4.67	119.5	1.49	5.73	116.53	15.28
26	RI-89		64.0	41.67	36.67	4.75	117.4	1.48	6.13	113.21	19.60
27	DPO 14	DMAPR,	47.3	36.83	46.83	6.6	89	1.73	5.7	70.6	34.09
28	EC-124345	Boriavi, Anand	62.5	35.33	33.51	4.88	115.5	1.58	7.73	146.57	30.54
29	Niharika	CIMAP,	62.0	36.67	41.33	4.67	115	1.24	6.75	132.23	17.09
30	Mayuri	U.P	64.1	36.67	40.67	4.92	116	1.24	5.89	116.54	22.55
31	HI-5	HAU, Haryana	66.2	40.67	42.67	4.75	119	1.48	7.2	114.16	22.67
32	GI-2	A.A.U., Anand	57.5	38.5	38.17	4.62	114	1.5	5.81	101.37	15.21
33	*P. arenaria*		71.0	40.33	256.67	1.43	128.5	1.01	1.41	5.44	4.1
34	*P. coronopus*	DMAPR, Boriavi, Anand	70.5	31.83	46.17	14.2	121.5	0.14	0.51	80.96	3.03
35	*P. indica*		82.3	73.67	70.51	1.82	134.5	0.9	1.81	28.69	4.2
36	*P. psyllium*		71.5	50.12	208.33	1.15	130.0	1.12	0.68	3.25	2.04
37	*P. serraria*		68.2	33.51	29.33	13.5	124.5	0.14	0.39	95.24	1.16
38	*P. lanceolata*		66.0	71.32	18.22	8.21	130.3	1.35	3.45	71.66	9.1
		Range	47-82	31.83-73.67	18-256.67	1.15-14.2	89-134.5	0.14-1.73	0.39-7.73	3.25-146.57	1.16-34.09
		Mean	63.22	39.91	47.73	5.16	117.38	1.36	5.10	98.61	16.35
		CV	0.95	0.56	2.65	0.63	0.90	1.0	1.48	1.38	1.79

DFF- Days to 50% flowering; PH- Plant height; NSPP- Number of spikes per plant; SL- Spike length; DtM- Days to maturity; TGM- Thousand grain mass; SYPP- Spike yield per plant; NSPS- Number of seeds per spike; HI- Harvest Index

and OPP18) to 1390bp (OPD-02). The number of amplicons produced per primer varied from 10 (OPF-02) to 28 (OPN-04) with a mean of 16.53 bands per primer which was higher than other studies (Vahabi et al., 2008; Vala et al., 2011; Rohilla et al., 2012, Kaswan et al., 2013). The RAPD marker OPA-09, OPD-02, OPF-02 and OPP-16 gave lowest of polymorphism i.e. 0% in *P. ovata* (data not shown). The PIC values, a reflection of allele diversity and frequency among the varieties, were uniformly higher for all the RAPD loci tested. The PIC for RAPD ranged from 0.72 (OPF02) to 0.91 (OPF05) with an average of 0.83 (Table 2). The highest PIC value obtained was 0.90 for OPF-05 marker and lowest PIC value was 0.67 for OPA-08, OPF-02 and OPF-03 in *P. ovata* (data not shown). A representative RAPD profile obtained by primer OPA-9 is shown in figure 2.

SSR analysis

SSRs are codominant in nature, multiallelic and uniformly dispersed throughout plant genomes. Development of SSRs is prohibitively expensive, but once the SSRs have been developed for a crop, they are cost- and time-effective in comparison to RAPD (Vinod and Sharma, 2011). Moreover, SSR primers show cross genus and cross species amplification (Whankaew et al., 2011).

Table 2. Characteristics of RAPD primers among Plantago species.

Sr. No.	Locus Name	Sequence (5´→ 3´)	Molecular size range (bp)	No. of Amplicons	No. of Polymorphic bands	PIC value
1	OPA-08	GTGACGTAGG	200-1290	17	17	0.83
2	OPA-09	GGGTAACGCC	250-1300	16	16	0.85
3	OPA-14	TCTGTGCTGG	280-1320	17	17	0.84
4	OPA-18	AGGTGACCGT	150-1320	16	16	0.83
5	OPC-11	AAAGCTGCGG	220-1350	16	16	0.88
6	OPD-02	GGACCCAACC	550-1390	18	18	0.82
7	OPD-11	AGCGCCATTG	200-1150	11	11	0.83
8	OPF-02	GAGGATCCCT	150-1300	10	10	0.72
9	OPF-03	CCTGATCACC	180-1200	12	12	0.78
10	OPF-05	CCGAATTCCC	150-1330	22	22	0.91
11	OPF-20	GGTCTAGAGG	300-1200	12	12	0.79
12	OPN-04	GACCGACCCA	320-1290	28	28	0.90
13	OPN-06	GAGACGCACA	500-1280	18	18	0.87
14	OPP-16	CCAAGCTGCC	250-1150	14	14	0.83
15	OPP-18	GGCTTGGCCT	150-1350	21	21	0.85
Total			-	248	248	-
Average			257-1283	16.53	16.53	0.83

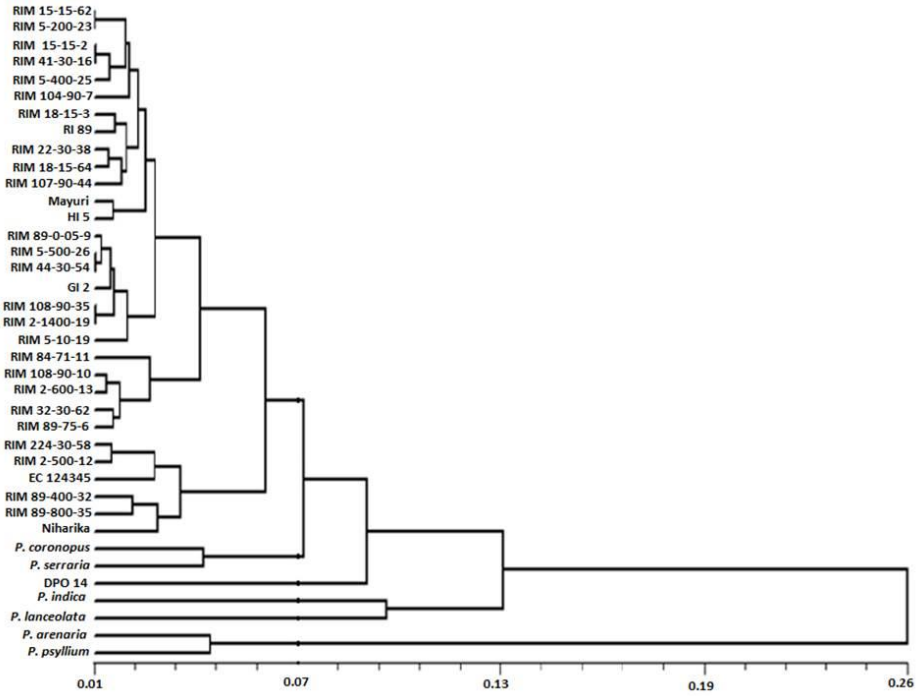

Fig 1. UPGMA dendrogram based on Manhattans coefficient from morphological analysis showing clustering of 38 Plantago genotypes into six major groups at an average cut of value of 0.07.

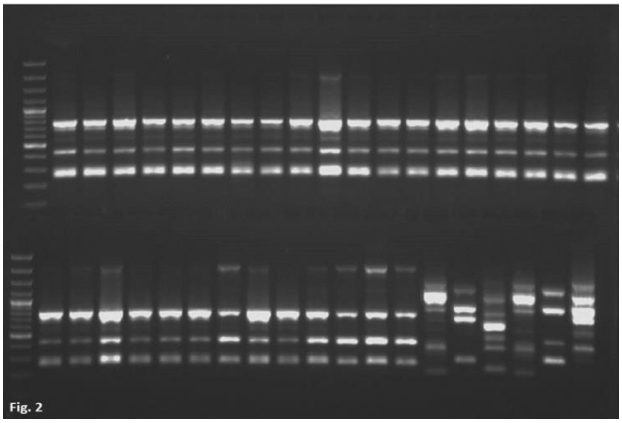

Fig 2. RAPD profile of Plantago genotypes generated by OPA-09; lane 1 - 100bp ladder; lane 2-20 - *P. ovata*; lane 21-100bp ladder; lane 22-34- *P. ovata*; lane 35 -*P. arenaria;* lane 36- *P. coronopus*; lane 37- *P. indica*; lane 38- *P. psyllium*; lane 39- P. *serraria*; lane 40- *P. lanceolata.*

SSRs, being a potential marker system to detect true hybrids and assessing genetic diversity, have not reported in *P. ovata*. Still, a limited work considering SSR from other plants crops and trees has recently carried out by Kotwal et al. (2013). In this study, simple sequence repeat (SSR) primer sets, developed using a CT/AG-enriched genomic library of *P. ovata* were also used to detect and estimate genetic polymorpism. Among the 10 SSR markers tested on 38 genotypes, 8 (80%) produced clear polymorphic bands (Table 3). A total of 33 polymorphic fragments were amplified with an average of 4.13 bands per primer. The PIC per marker ranged from 0.5% (APOM-15 and APOM-27) to 62% (APOM-4) with an average of 27.87%. A representative fingerprint pattern generated by primer APOM-25 is shown in (fig 3). Astonishingly all SSRs were monomorphic in *P. ovata*. It indicated very a low genetic diversity in cultivated plantago and results are in congruence with previous reports (Lal et al., 2000; Patel and Saravanan, 2010). Apparently, these markers were transferable among all species in the genus *Plantago*, even if the species were from different sections, indicates high level of sequence similarity in the DNA sequences flanking microsatellites. Approximately 80% were transferable in allied species. However, the failure of amplification could be due to lack of any conserved primer binding sites in the DNA for amplification to occur (Weising et al., 2005).Transferability of SSR markers among related species and among distant taxa have been observed previously (Yasodha et al., 2005; Sherry et al., 2011). This explains the potential of these primers to analyze polymorphism in crops like isabgol where no SSR, sequence data and genomic information is available. Among eight polymorphic SSRs, only three were amplified in all species. These results are in congruence with (Koorevaar et al., 2002; Wolff et al., 2009; Kotwal et al., 2013) where limited transferability of SSRs was observed. However, all eight SSRs showed amplification in *P. lanceolata* while only seven SSRs could produce amplicons in *P. coronopus*. The species-specific amplification of SSRs indicates the usefulness of these primers for fingerprinting purposes. In some species, an intense band can be seen with relatively less intense (stutter bands/multiple bands) ones above it. The nonspecific bands in some of the species are likely due to the primer mismatch positions from the different species DNA analysed here.

Combined RAPD and SSR based cluster analysis

Since phenotypic variation in natural or artificial populations is manifested as a result of genotype × environment interactions at all the genetic loci concerned, with qualitative and quantitative traits examined, the genomic differences between the accessions therefore may be small or large (Shasany et al., 2000). With the fact that RAPD and SSR target different portions of the genome; an UPGMA analysis was performed by combining these both marker systems to get better coverage. The resulting dendrogram (Fig. 4) defines the genomic relationships among analyzed genotypes. Genetic distance was very low due to the small number of polymorphic bands. The Jaccard's pair-wise similarity coefficient based on markers for the 38 pair-wise comparisons among accessions ranged from 0.04 (RIM-5-400-25/*P. psyllium*) to 0.96 (RIM-44-30-54/RIM-108-90-10 and RIM-5-500-26/RIM-108-90-10) with an average value of 0.62. Similar types of results have also been reported in other studies as well (Vahabi et al., 2008; Vala et al., 2011). The UPGMA based dendrogram grouped the genotypes in seven different clusters at a cut off value of 0.51.

The cluster one comprised of 32 genotypes from *P. ovata*. The genetic similarity was found very high (0.83) in cluster one and the similarity ranged from 0.63 (GI-2/ RIM-15-15-2) to 0.96 (RIM-44-30-54/RIM-108-90-10 and RIM-5-500-26/RIM-108-90-10). This low genetic distance can be explained because the plant is cultivated exclusively in Western India (Gujarat and Rajasthan) that ensures less impact of changing environmental conditions. The UPGMA based dendrogram generated exclusively for *P. ovata* could be further divided into six smaller subgroups at a very high cut off value of 0.82 which reflects the very low diversity on *P. ovata*. Low average Jaccard similarity coefficients indicated that marker profile of *P. ovata* especially mutant lines were similar, but not the same completely. The biggest sub-cluster in cluster one was harboring the maximum (24) number of genotypes. Marker based dendrogram revealed that there is no correlation between clustering pattern of genotypes of *P. ovata* and their geographical regions. The results are in agreement with the work done by Rohilla et al. (2012) and Kaswan et al. (2013). Most of the *P. ovata* genotypes showed high level of genetic similarity except GI2, EC12345, Mayuri, HI5 and Niharika, therefore, could be exploited in crop improvement program. A molecular relationship of the seven species showed that *P. arenaria* and *P. indica* formed a most closely related group followed by the second closely related group of *P. serraria* and *P. coronopus*. However, other two species such as *P. psyllium* and *P. lanceolata* distantly related. This pattern of clustering of other species corresponds with earlier studies of Sanghamitra et al. (2010). Results are in agreement with Wollf and Schaall (1992) chloroplast DNA markers study revealed that *P. coronopus* was distantly related to *P. lanceolata*.

Correlation between morphological and molecular markers

The results of this study indicate that molecular markers are highly discriminatory, more precise, and more reproducible than some morphological traits used to estimate genetic relatedness and/or identify germplasm. A close correspondence between the similarity matrices of RAPD and SSR was established by means of high value of matrix correlation (0.98), indicating that there is a pattern of association between the results obtained with these two analytical procedures (data not shown). Hence, the both marker systems either individually or combined can be effectively used in determination of genetic relationships among species. Moreover, a good diversity index was generated with low number of SSR over RAPD. The present study also comprehensively characterized the phenotypic and molecular genetic diversities of Plantago but the results were inconsistent. Furthermore, the phenotypic-based cluster did not correspond with the molecular-based cluster; the correlation coefficient (r = -0.72) for the two clustering matrices tested by a Mantel test showed a negative correlation between phenotype and molecular marker information. The negative correlation suggested that molecular measures of genetic diversity have a very limited ability to predict quantitative genetic variability and two methods were different and highly variable (Kumar et al., 2013). Moreover, negative correlation could be explained by the different properties of molecular and phenotypic expression (Navarro et al., 2005). Molecular markers are usually considered selectively neutral and thus do not necessarily reflect the diversity in functional characters (Li et

Table 3. Characteristics of SSR primers among Plantago species.

Sr. No.	Primers	Sequence (5′→ 3′)	Repeat motif	Total no. of loci	No. of polymorphic loci	PIC value	Transferability
1	APOM-3	F: GTTTACCTTGCTCAAGTGCTTGCT R: AACTCCTTCACCCTTCGCCTAACA	TG10	04	04	0.22	*P. coronopus, P. serraria,* and *P. lanceolata*
2	APOM-4	F: GTCACACACACACACACACACAC R: AGGGAAACTGCCATGACTCCTCTT	CA10	12	12	0.62	All species
3	APOM-13	F: CTATTTGTCCTTCTTTGCTA R: ATATATCTGACAAGTCGCAC	AGC7	03	02	0.51	All species
4	APOM-14	F: AAATTCTAAGGCTTGTACCT R: AAAATTTAGCACGCGA	TTC4	05	05	0.32	*P. coronopus, P. serraria,* and *P. lanceolata*
5	APOM-15	F: ATGGAGTAGGAAGTGAGAGT R: GAGATGCATATCTTAAGTGTAG	TTC4	02	02	0.05	*P. coronopus, P. serraria,* and *P. lanceolata*
6	APOM-25	F: TCCGGAGTATAATAAGTCAA R: ACAATAATGATGTGTTTGGT	TAA5	03	03	0.30	All species
7	APOM-27	F ACATTTTCCAACATTTAAGA R: CTAGACCAATAACAGCCTTA	TCA5	02	02	0.05	All species except *P. arenaria*
8	APOM-37	F: TGGTTTAGGCTTTCCTGCCTTTGC R: TTTCCTTCGGCCAAACACTCTTGC	TG7	02	02	0.16	*P. arenaria, P. psyllium,* and *P. lanceolata*
Total			-	33	32	-	-
Average			-	4.12	4	0.28	-

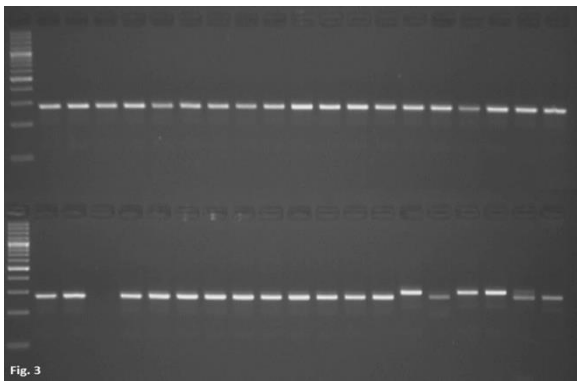

Fig 3. SSR profile of Plantago genotypes generated by APOM-25; lane 1 - 100bp ladder; lane 2-20 - *P. ovata*; lane 21-100bp ladder; lane 22-34- *P. ovata*; lane 35 -*P. arenaria;* lane 36- *P. coronopus*; lane 37- *P. indica*; lane 38- *P. psyllium*; lane 39- P. *serraria*; lane 40- *P. lanceolata.*

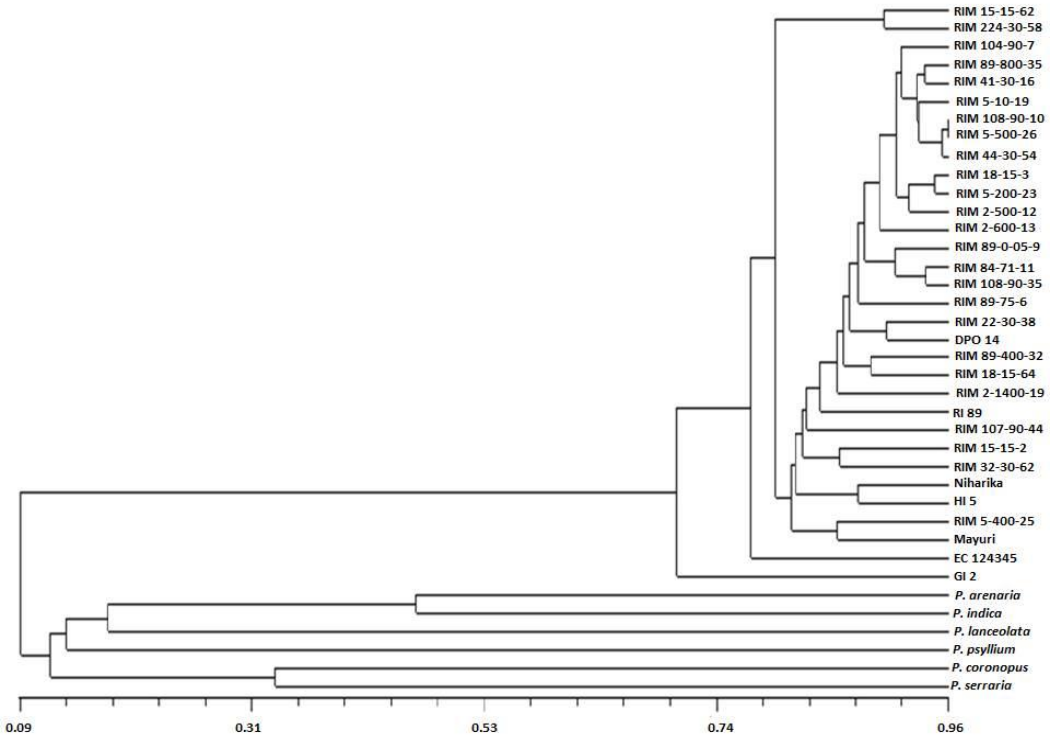

Fig 4. UPGMA dendrogram based on Jaccard coefficient from molecular maker analysis showing clustering of 38 Plantago genotypes into seven major groups at an average cut of value of 0.51.

al., 2008). Further, negative correlation suggests differences in the degree of genomic coverage between marker and phenotypic parameters. Moreover, the natural and human selective forces acting on molecular variation apparently differed from those acting on morphological traits (Alves et al. 2013). It would therefore, be justified to use both phenotypic-based and molecular-based techniques hand in hand rather than in isolation for genetic diversity assessments of the isabgol. However, with a negative correlation, few samples shared same group in both dendrograms e.g. RIM-5-500-26/RIM-44-30-54 and *P. serraria*/*P. coronopus.*

Materials and Methods

Plant material and DNA extraction

A total of 38 genotypes belonging to seven species were used in the present study which included commercially grown seven varieties of *P. ovata*, 25 mutant lines (M4) of RI-89 variety, one exotic line and six each of allied species of *P. ovata*. These genotypes were procured from different institutes (Table 1).

The genotypes were grown in field and young expanding leaves were harvested for DNA isolation. DNA from young leaves of a bulk of 10 plantlets was isolated by using CTAB technique (Doyle and Doyle, 1990), purified and quantified using Nanodrop (Thermo scientific, USA). DNA was diluted to 20 ng/µl with $T_{10}E_1$ buffer and stored at 4 ˚C.

RAPD and SSR amplification

A total of 40 primers (30 RAPD and 10 SSR) were used in PCR amplification. PCR amplification was carried out using 96 well micro titerplates in thermalcyclers (Biometra, Germany). RAPD amplification was performed according to Williams et al (1990) using decamer arbitrary primers (Operon technologies Inc, USA; SIGMA-D, USA) (Table 2). Amplifications were carried out in a 25ul reaction volume containing 2 µl DNA (50 ng), 12.5 µl Master Mix (Genei, Bangalore, India), and 1 µl of 10 pmol of primer. Amplification was performed in following steps: initial denaturation at 94 ˚C for 5 min, 42 cycles of denaturation at 94 ˚C for 1 min, primer annealing at 38 ˚C for 1 min, extension at 72 ˚C for 2 min, and final extension at 72 ˚C for 7 min.

For SSR analysis, primers were selected from our previous report (Fougat et al., 2014) and were originally tested at an annealing temperature of either 50 ˚C or 55 ˚C, and those that did not amplify well were then evaluated at 61 ˚C and/or 67 ˚C. With minor modifications, the PCR conditions used for detection of amplicons on agarose gels were performed in 20-µl reactions containing 25ng of template DNA, 0.2 µM of each primer, 200 µM of each dNTP, 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 1 unit of Taq polymerase. An MJ Research or PE9700 single or dual 96-well thermal cycler was used along with the following PCR programming: 94˚C for 5 min (denaturation), followed by 35 cycles of 94 ˚C for 1 min, 55 ˚C for 1 min, 72 ˚C for 2 min, and a final extension at 72 ˚C for 5 min. Amplified products were electrophoresed in 1.5% agarose in 1×TBE buffer. The gels were stained with ethidium bromide and documented using gel documentation system (Bio-Rad, Hercules, California). Each experiment was repeated two times with each primer and those primers which gave reproducible fingerprints (DNA bands) were only considered for further experimentation and data analysis.

Phenotypic analysis

Morpho-physiological data were collected during the entire growing season in a field trial carried out in 2012-13 (winter season) at Anand Agricultural University, Gujarat, India. Each accession was sown as single row in the field in two repetitions. Each row of 2m length which included 30 plants and plots were spaced 45 cm apart. All agronomic practices commonly adopted for isabgol cultivation were used. The observations were recorded on nine morphological traits viz., days to 50% flowering, plant height, number of spikes per plant, days to maturity, 1000 seed weight (gm), seed yield per plant (gm), number of seed per spike and harvest index(%) were collected. The data were recorded on five random but competitive plants except for days to 50% flowering and days to maturity, where it was taken on plot basis.

Data analysis

Genetic similarity based on genotypic data

All major DNA fragments were recorded as either 1 or 0 representing the presence or absence of the band, respectively. Faint or unclear bands were not considered. The binary data generated were used to estimate levels of polymorphism by dividing the polymorphic bands by the total number of scored bands. The pairwise genetic similarity coefficient (GS) was calculated using Jaccard coefficient (Jaccard, 1908) by the SIMQUAL program of NTSYS-pc software version 2.02 (Rohlf, 1998). The polymorphism information content (PIC) was calculated by the formula: PIC = 2Pi (1−Pi) (Bhat, 2002) where, Pi is the frequency of occurrence of polymorphic bands in different primers.

Genetic distances based on phenotypic data

A data matrix of 38 accessions from the means of nine yield and yield related traits was constructed. Phenotype data were standardized first by using the standardization (subtracting the average and dividing by the standard deviation) program of NTSYS-pc. Genetic distance (GD) was calculated using Manhattan coefficient and hierarchical clustering was done using the Unweighted Pair-Group Method of Arithmetic Averages (UPGMA) suggested by Sneath and Sokal (1973) using NTSYS-pc 2.1. In both dendrograms, average coefficient was used as cut-off value to define clusters.

Mantel's correlation analyses

Correlation between the matrices obtained with both marker types (RAPD and SSR primers) was estimated by means of Mantel test using MxComp module of NTSYS-pc (Mantel, 1967). Similarly, correlation between the two matrices derived from molecular markers data (based on the joint analysis) and phenotyping data was estimated using Mantel test.

Conclusion

Isabgol is commercially an important crop with different uses. However, scanty information is available concerning to morphology and genetic variability of isabgol and its wild relatives. Results of present study indicated that there was a

remarkable variation in the seed yield per plant and harvest index among the seven Plantago species studied. These characters may be considered in the wide crossing based breeding programs for the yield improvement of isabgol. Results of diversity analysis revealed that *P. ovata* showed high similarity and low genetic diversification. The limited existing genetic variability is major bottleneck for the improvement of the isabgol. Therefore, induced mutation can be exploited to widen the range of variation. However, as compare to phenotypic based clustering, marker study in these Plantago species proved helpful in clearly demarcating the species belonging to different sections. In conclusion, markers provide better information on genetic relatedness among the Plantago species and this knowledge of genetic variation would provide an important contribution in Plantago breeding strategies for its yield improvement and disease resistance.

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