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Characterization and Physical Mapping of Ribosomal RNA Gene Families in *Plantago*

MANOJ K. DHAR[1,*], BERND FRIEBE[2], SANJANA KAUL[1] and BIKRAM S. GILL[2]

[1]*Department of Biotechnology, University of Jammu, Jammu — 180 006, J & K, India and* [2]*Department of Plant Pathology, Wheat Genetics Resource Center, 4024 Throckmorton Plant Sciences Center, Kansas State University, Manhattan, KS 66506-5502, USA*

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• *Background and Aims* The organization of rRNA genes in cultivated *Plantago ovata* Forsk. and several of its wild allies was analysed to gain insight into the phylogenetic relationships of these species in the genus which includes some 200 species.
• *Methods* Specific primers were designed to amplify the internal transcribed spacer (ITS1 and ITS2) regions from seven *Plantago* species and the resulting fragments were cloned and sequenced. Similarly, using specific primers, the 5S rRNA genes from these species were amplified and subsequently cloned. Fluorescence *in-situ* hybridization (FISH) was used for physical mapping of 5S and 45S ribosomal RNA genes.
• *Results* The ITS1 region is 19–29 bp longer than the ITS2 in different *Plantago* species. The 5S rRNA gene-repeating unit varies in length from 289 to 581 bp. Coding regions are highly conserved across species, but the non-transcribed spacers (NTS) do not match any database sequences. The clone from the cultivated species *P. ovata* was used for physical mapping of these genes by FISH. Four species have one FISH site while three have two FISH sites. In *P. lanceolata* and *P. rhodosperma*, the 5S and 45S (18S-5·8S-25S) sites are coupled.
• *Conclusions* Characterization of 5S and 45S rRNA genes has indicated a possible origin of *P. ovata*, the only cultivated species of the genus and also the only species with $x = 4$, from a species belonging to subgenus *Psyllium*. Based on the studies reported here, *P. ovata* is closest to *P. arenaria*, although on the basis of other data the two species have been placed in different subgenera. FISH mapping can be used as an efficient tool to help determine phylogenetic relationships in the genus *Plantago* and show the interrelationship between *P. lanceolata* and *P. lagopus*.

Key words: *Plantago*, *Psyllium*, rRNA genes, internal transcribed spacers, FISH mapping, phylogeny.

INTRODUCTION

The genus *Plantago*, of family Plantaginaceae, includes some 200 species (Rahn, 1996). Although its centre of diversity is believed to lie in central Asia, some species have now become dispersed widely, with maximum concentration in the temperate regions. Species of *Plantago* are small herbs, mostly growing as weeds, while some are of medicinal value. *Plantago ovata* is the only cultivated species. The seed husk, called isabgol in Hindi and psyllium in English, is not only a highly effective laxative but is also used in lowering blood cholesterol levels, ice cream making and cosmetics (Dhar *et al*., 2005).

Ribosomal DNA variations have been described in many plant species (Rogers and Bendich, 1987). The 45S rDNA associated with the NOR consists of tandem repeat units, comprising coding regions and the intergenic spacer (ITS) regions (Appels and Honeycutt, 1986). Individual genes within a unit are separated by two internal transcribed spacer regions, ITS1 and ITS2. The high copy number, rapid concerted evolution, and small size of the ITS regions makes them informative in determining evolutionary relationships between closely related species (see Baldwin *et al*., 1995, for a review; Susanna *et al*., 1999; Anamthawat-Jonsson and Bodvarsdottier, 2001).

5S ribosomal RNA (rRNA) genes are arranged in tandem arrays of hundreds to thousands of copies. Each 5S rRNA gene unit consists of an approx. 120-bp coding region and a non-transcribed spacer (NTS) (Long and Dawid, 1980). In plants, NTS of 95–730 bp have been reported (Hembleden and Werts, 1988). The ribosomal genes have been studied in several plant species (Appels *et al*., 1992; Lapitan, 1992; Singh *et al*., 1994; Kellogg and Appels, 1995). While the coding regions are highly conserved among taxa, the NTS show a large amount of intra- as well as interspecific variability.

Development of FISH techniques have provided a valuable tool for physical mapping of single-copy and repetitive DNA sequences including rRNA genes (Mukai *et al*., 1990; Leitch and Heslop-Harrison, 1992; Jiang and Gill, 1994*a*; Castilho and Heslop-Harrison, 1995; Linares *et al*., 1996; Raina and Mukai, 1999). These studies have provided valuable information regarding chromosome identification, genome organization and chromosome evolution in several plant species (for a review, see Jiang and Gill, 1994*b*).

Plantago is a unique group of plants whose phylogenetic relationships are still under investigation. In an exhaustive phylogenetic study of the family Plantaginaceae based on morphological, embryological and chemical data, Rahn (1996) was not able to find a sister-family for this monophyletic group. More recently, Ronsted *et al*. (2002) have determined phylogenetic relationships in *Plantago* based

* For correspondence. E-mail manojkdhar@rediffmail.com

TABLE 1. Plantago *species studied*

Name	Chromosome number	Habit	Place of collection
Plantago ovata	$2n = 8$	Cultivated	Jammu, India
P. lanceolata	$2n = 12$	Wild	Manhattan, USA
P. lagopus	$2n = 12$	Wild	Jammu, India*
P. major	$2n = 12$	Wild	Jammu, India
P. rugelii	$2n = 24$	Wild	Manhattan, USA
P. rhodosperma	$2n = 24$	Wild	Manhattan, USA
P. arenaria	$2n = 12$	Wild	USDA

* The seed was originally obtained from Royal Botanic Gardens, Kew during 1980.

on the sequence data of ITS regions and plastid *trn*L-F. In the present investigation, the organization of rRNA genes in the cultivated species *P. ovata* and six of its wild allies was analysed. Fluorescence *in-situ* hybridization (FISH) was used to determine the chromosomal location of rRNA genes in these species.

MATERIALS AND METHODS

Plant material and DNA isolation

Seven species of *Plantago* were used for the present investigation (Table 1). Genomic DNA was isolated from young leaves of several plants of each species as described by Saghai-Maroof *et al*. (1984) with minor modifications.

Polymerase chain reaction

The primers used to amplify the 5S rDNA were designed based on sequences of different taxa retrieved from the GenBank. These were P1: 5′-GGGTGCGATCATACCAGCGT-3′ and P2: 5′-GGGTGCCAACACTAGGACTTC-3′. The ITS regions in each species were amplified by using specific primers S1: 5′-TCCTCCGCTTATTGATATATATGC-3′ and S2: 5′-GGAAGTAAAAGTCGTAACAAGG-3′. PCR reactions contained 10 mM TRIS–HCl pH 8·3, 50 mM KCl, 1·5 mM $MgCl_2$, 200 μM of each dNTP, 1 μM of each primer, 2 U of *Taq* polymerase (Gibco-BRL, USA) and approx. 20 ng genomic DNA in a volume of 50 μL. Thermal cycler conditions were 4 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 40–60 s at 50 °C, 1 min at 72 °C and a final extension step of 7 min at 72 °C on a Perkin Elmer 9700. PCR products were either cloned directly or first gel purified using the GENECLEAN (BIO 101, USA) kit. PCR products of three independent reactions in each species were cloned using the AdvantAge PCR cloning kit (CLONTECH Laboratories Inc., USA) as per the manufacturer's instructions. The clones were sequenced using M13 forward and reverse primers. The 5S rRNA sequences of seven species have been deposited in GenBank under accession numbers, AF464933, AF464934, AY684360, AY684361, AY684362, AY684363 and AY684364, while ITS sequences have been deposited under accession numbers, AY692076, AY692077, AY692078, AY692079, AY692080, AY692081 and AY692082.

Southern blotting

RFLP of 5S rDNA was carried out with two restriction enzymes, *Sca*I and *Msp*I, while that of 45S with three enzymes, *Eco*RI, *Xba*I and *Dra*I. For Southern blotting the procedure outlined in Gill *et al*. (1991) was followed. The restriction endonuclease digested genomic DNA was transferred to Hybond N+ membrane (Amersham Biosciences, UK). For hybridization, 25 ng of probe was labelled with ^{32}P by random primer labelling. Hybridization was performed at 65 °C for 20 h. The washing stringency was 1 × SSC, 1 % SDS twice at room temperature for 10 min each and once at 65 °C for 15 min. Autoradiography was done by exposing the blot to X-ray film.

DNA sequence and phylogenetic analyses

The DNA sequences of three clones from each species were aligned to generate a consensus sequence for that species. The similarity searches were made using the BLAST program and sequences were aligned using the CLUSTAL W program. These alignments were slightly modified manually. The aligned sequence data matrix was further analysed using the PAUP version 3·1.1 program (Swofford, 1993) or Jalview provided with CLUSTAL W. To estimate the amount of phylogenetic information in the parsimony analysis, consistency index, retention index, and rescaled consistency index (Farris, 1989) were computed. The distance matrices were calculated and the g1 statistic was also computed by using the Random trees option of PAUP.

FISH analysis

Root tips were pre-treated with ice-water for 24 h and fixed in ethanol and glacial acetic acid (3 : 1). Root tips were squashed in 45 % acetic acid and cover slips removed after quick freezing. FISH was performed following the protocol of Dhar *et al*. (2002). For mapping 45S rRNA genes, the wheat probe pTa71 (Gerlach and Bedbrook, 1979) was directly labelled by nick translation with tetramethyl-rhodamine-6-dUTP (Roche Molecular Biochemicals, USA) following the manufacturer's instructions. For mapping 5S rDNA, the probe pPov1 (Dhar *et al*., 2002) was labelled with digoxigenin −11-dUTP (Roche Molecular Biochemicals, USA) as per the manufacturer's instructions. The slides were counterstained with DAPI or propidium iodide in Vectashield (Vector Laboratories, USA). However, in order to improve the presentation of images, DAPI-stained blue chromosomes were pseudocoloured as red and signals as yellow green. Slides were examined with a Zeiss axioplan epifluorescence microscope. Images were captured with a SPOT CCD camera using the appropriate SPOT 2·1 software (Diagnostic Instruments Inc., USA).

RESULTS

Cloning and sequencing

PCR amplification of 5S rDNA resulted in several bands, the major band being the basic repeating unit in each

TABLE 2. *Characteristic features and parsimony analysis of ITS1-5·8S-ITS2 regions of* Plantago *species*

	Length range	G/C content range (%)	No. of indels	No. of variable sites	No. of informative sites	Transitions	Transversions	Ratio
(A) Analysis of sequence data								
ITS1	223–224	52·4–56·5	4	62	43	48	24	2 : 1
5. 8S	165	55·1–56·9	0	5	1	2	1	2 : 1
ITS2	193–204	51·5–56·2	12	47	30	25	27	1 : 1·1
Complete	581–593	53·3–56·4	16	113	74	75	52	1·4 : 1

			Consistency index				
	No. of parsimonious trees	Shortest tree length	Including uninformative	Excluding uninformative	Retention index	Rescaled index	Skewness
(B) Parsimony analysis of data							
ITS1	64	88	0·864	0·810	0·797	0·688	–0·965812
5. 8S	144	06	1·000	1·000	0	0	
ITS2	136	61	0·885	0·833	0·857	0·759	–0·755038
Entire sequence	156	0·872	0·813	0·815	0·710	–0·835274	

species while other faint bands represent dimers and trimers of the monomer. The major fragment, in each species, was cloned and sequenced. The clones were designated as pPov1 (derived from *P. ovata*), pPln1 (*P. lanceolata*), pPlg1 (*P. lagopus*), pPmj1 (*P. major*), pPrg1 (*P. rugelii*), pPrd1 (*P. rhodosperma*) and pPan1 (*P. arenaria*). The repeat unit length varies from 289 bp in *P. rhodosperma* to 581 bp in *P. arenaria*. The repeat units of *P. lanceolata, P. lagopus* and *P. major* are invariably 365 bp.

While the coding region is highly conserved among the seven species, the NTS shows much heterogeneity. BLAST searches of the coding region resulted in hits with 5S sequences of many plant species; however, the spacer regions were unique. Sequence comparison among *Plantago* species reveals eight variable sites even in the coding region while there are numerous such sites in the spacer region. Besides, several indels (insertion–deletion) of 1 to few bp, upstream of the 5′ end of the gene are two very large insertions of 121 bp in the repeat unit of *P. ovata* and 180 bp in the repeat unit of *P. arenaria*, as compared with other species.

Similarly, the ITS1-5·8S-ITS2 regions cloned from the seven species were designated as pPov2, pPln2, pPlg2, pPmj2, pPrg2, pPrd2 and pPan2, derived from *P. ovata, P. lanceolata, P. lagopus, P. major, P. rugelii, P. rhodosperma* and *P. arenaria*, respectively. The sequences obtained from three clones of each species were compared with published sequences of other plant species and the boundaries of the spacer regions were confirmed. The length and other characteristics of spacer regions and 5·8S rRNA gene are given in Table 2.

The consensus sequences of all the species were aligned using Clustal W. The differences among species ranged from a minimum of 11 bases between *P. lagopus* and *P. lanceolata* to a maximum of 69 bases between *P. ovata* and *P. rugelii.* The results of parsimony analysis are given in Table 2. In addition to the entire sequence, the ITS1, 5·8S and ITS2 sequences were used separately to develop phylogenetic trees. Similarly, the tree was also derived from 5S sequences. The strict consensus tree derived from all these regions was in total agreement. Among *Plantago* species, three groups emerge: group 1, *P. ovata* and *P. arenaria*; group 2, *P. lanceolata* and *P. lagopus*; group 3, *P. major*, *P. rugelii* and *P. rhodosperma* (Fig. 1).

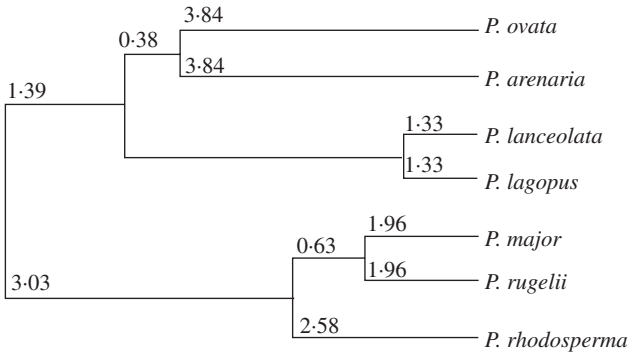

FIG. 1. Phenetic tree summarizing relationships among *Plantago* species based on 5S, ITS1 and ITS2 sequences.

FISH analysis

The chromosomes in all species have been numbered on the basis of descending order of length. *Plantago ovata* ($2n = 2x = 8$) has two pairs each of submetacentric and subtelocentric chromosomes numbered as chromosomes 1 to 4. FISH analysis reveals four 45S rDNA signals at the ends of the short arms of subtelocentric chromosomes, while the 5S FISH sites were located near the middle of the long arm of both chromosomes 1 (Fig. 2A, B). The chromosome complement of *P. lanceolata* and *P. lagopus* consists of four pairs of submetacentric and two pairs of subtelocentric chromosomes; 45S rDNA FISH sites were observed at the ends of the short arms of the subtelocentric chromosomes (Fig. 2C and E). In *P. lanceolata* two 5S loci

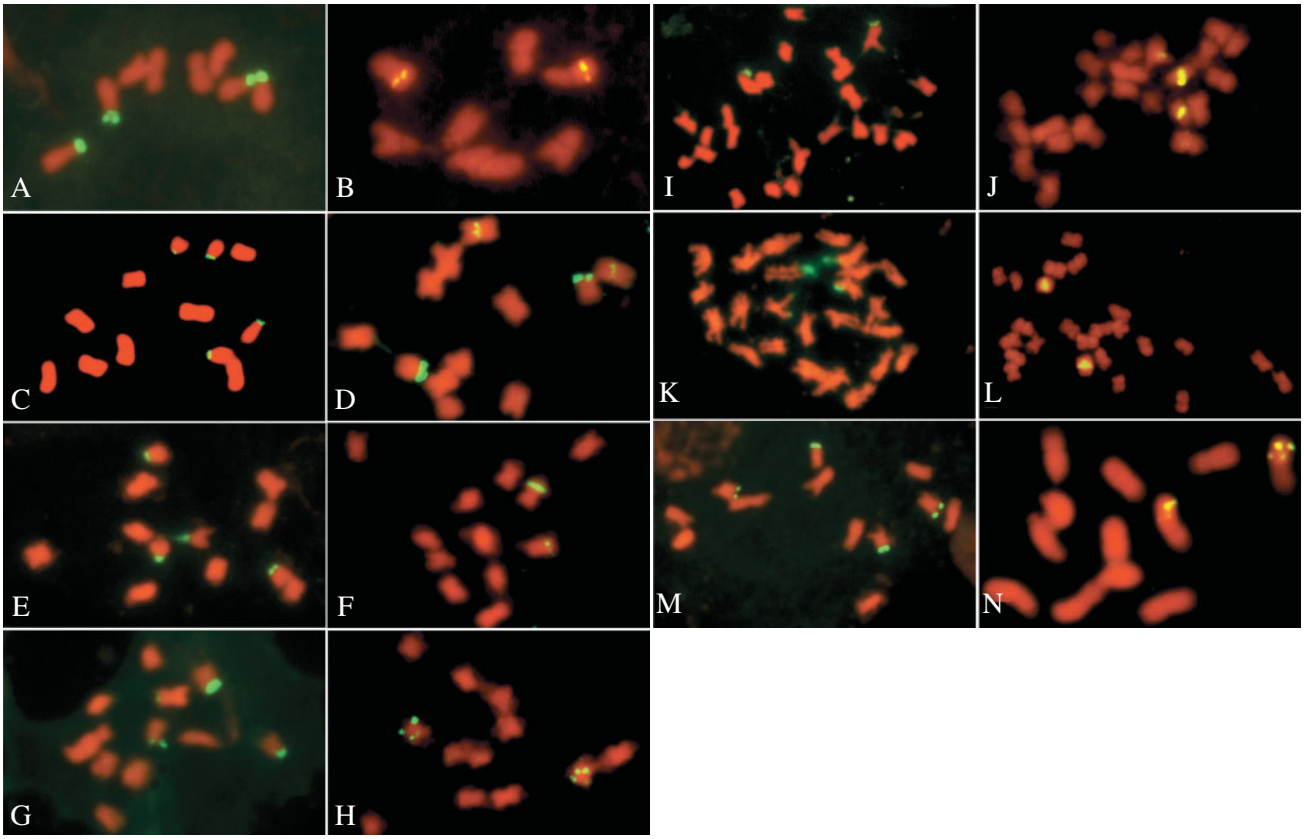

FIG. 2. Physical mapping of 45S (A, C, E, G, I, K and M) and 5S (B, D, F, H, J, L and N) rRNA, sequences, respectively, in seven *Plantago* species: *P. ovata* (A and B), *P. lanceolata* (C and D), *P. lagopus* (E and F), *P. major* (G and H), *P. rugelii* (I and J), *P. rhodosperma* (K and L) and *P. arenaria* (M and N).

were detected; one close to the centromere in the long arm of both chromosomes 1, and another in the short arm of both chromosomes 5 (Fig. 2D). In *P. lagopus* the 5S site was located in the long arm of both chromosomes 2 (Fig. 2F). The chromosome complement of *P. major* consists of six pairs of submetacentric chromosomes. Chromosome pairs each of 4 and 6 bear 45S rDNA hybridization signals at the ends of the short arms (Fig. 2G). Two prominent 5S loci were detected in the long arm of both chromosomes 2 (Fig. 2H). *Plantago arenaria* has 12meta- to submetacentric chromosomes and is unique because 45S rDNA FISH signals were observed at the ends of the long arms of four chromosomes. Two 5S loci were detected in the long arm of both chromosomes 1 (Fig. 2 M and N). The remaining two species, *P. rugelii* and *P. rhodosperma*, with $2n=2x=24$ chromosomes differ in the number of hybridization sites. While *P. rugelii* has one 45S rDNA FISH site in the short arm of both chromosomes 1 and one 5S hybridization site in the long arm of both chromosomes 8 (Fig. 2I, J), *Plantago rhodosperma* has two 45S rDNA sites one in the short arm of both chromosomes 2 and another at the end of the long arm of both chromosomes 10 (Fig. 2K). The 5S hybridization site was also located in the long arm of both chromosomes 10 (Fig. 2L). The final picture with respect to 45S and 5S sites is presented in Fig. 3. The FISH sites in all the species correspond with the NOR regions observed in Feulgen-stained preparations (results not shown).

Southern blot analysis

To understand the organization of 5S rRNA genes, genomic DNA of seven species was completely digested with the restriction enzymes *Sca*I and *Msp*I and hybridization with pPov1 revealed the ladder pattern. The results are shown for *Msp*I in Fig. 4. The sizes of basic repeat units varied considerably among these species. Similarly, to determine variability in the organization of 18S-5·8S-25S rRNA genes, genomic DNA of seven species was completely digested with three restriction enzymes and probed with pTa71. A high level of interspecific variation was observed among these species. Restriction with *Eco*RI generates a 3·8-kb fragment in all the species (Fig. 5). Another prominent fragment of 6·5 kb was observed in only two species, *P. ovata* and *P. lanceolata*. *Plantago lagopus* has a unique fragment of 2·0 kb, while *P. rugelii* shows a minor 0·9-kb fragment in addition to a 3·8-kb common fragment. *Dra*I generated a 9-kb fragment in all the species except *P. rhodosperma*, which shows two fragments of 7- and 4-kb (Fig. 5). *Xba*I generated an interesting pattern. Fragments of 11 and 9 kb were observed in *P. lanceolata*, *P. lagopus* and *P. major*, while *P. ovata* and *P. rhodosperma* had fragments of 9 kb and 8·5 kb, *P. rugelii* also had 9-kb and 7-kb fragments, while *P. arenaria* stands out from the rest by showing a single 9-kb fragment (Fig. 5).

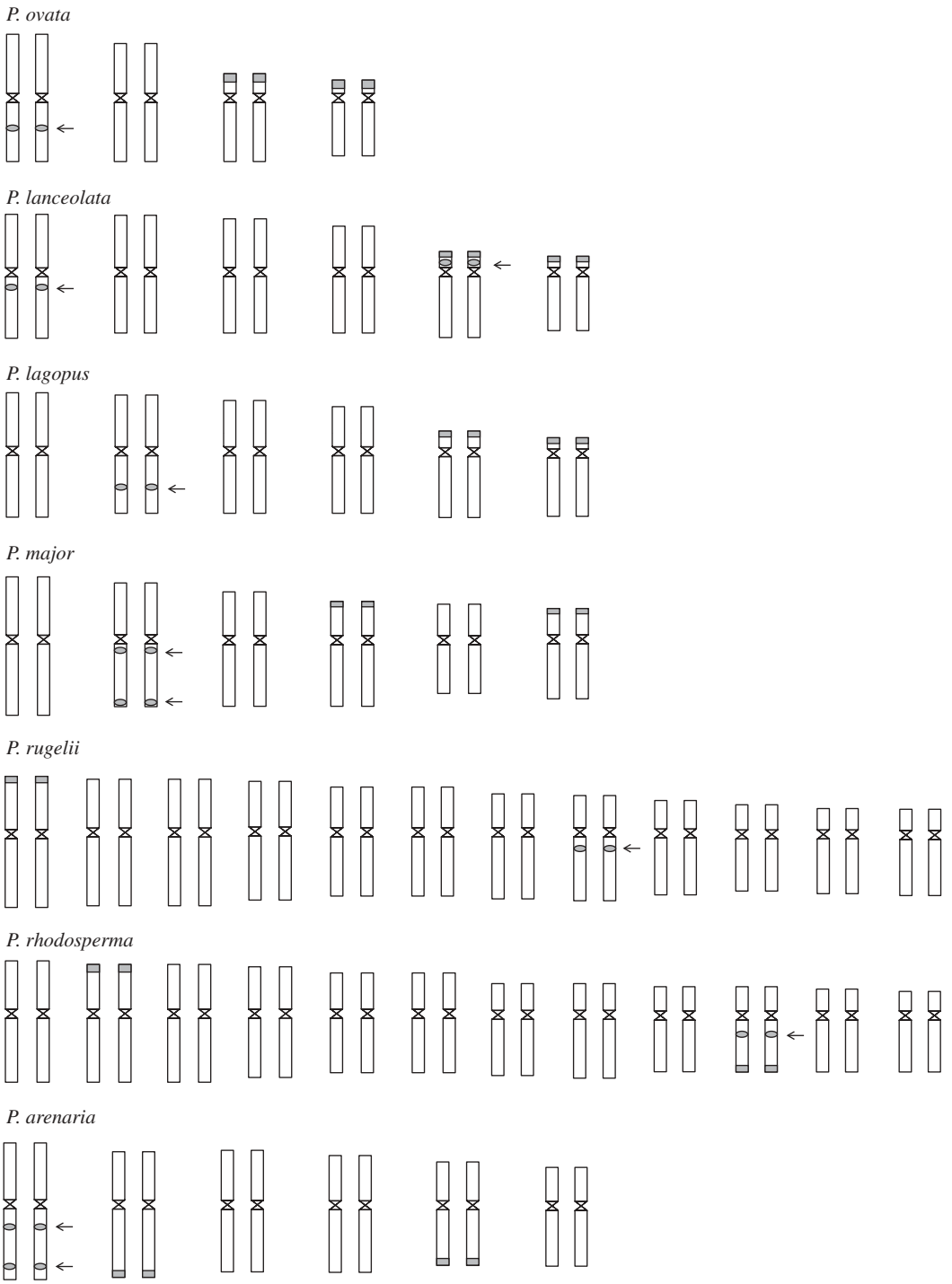

FIG. 3. Idiograms of *Plantago* species showing the position of 18S-5.8S-25S (indicated by a rectangle) and 5S rRNA genes (indicated by a circle and arrow).

DISCUSSION

rRNA genes

The study shows that FISH mapping of rRNA genes can be an excellent addition to the armory of tools for determining phylogenetic relationships in *Plantago*. In plants, 5S rRNA genes are arranged in tandem arrays. The number of arrays generally vary from one to two (Kellogg and Appels, 1995) with a copy number ranging from several hundred to several thousand. In the tribe Triticeae, the 5S rRNA gene arrays are present at two distinct chromosomal locations which can be unambiguously distinguished in terms of length and sequence of spacers (Kellogg and Appels, 1995). In the present sample, three species have two 5S rRNA gene arrays each. Interestingly, the two arrays are present in the same chromosome in *P. major* and *P. arenaria*, whereas

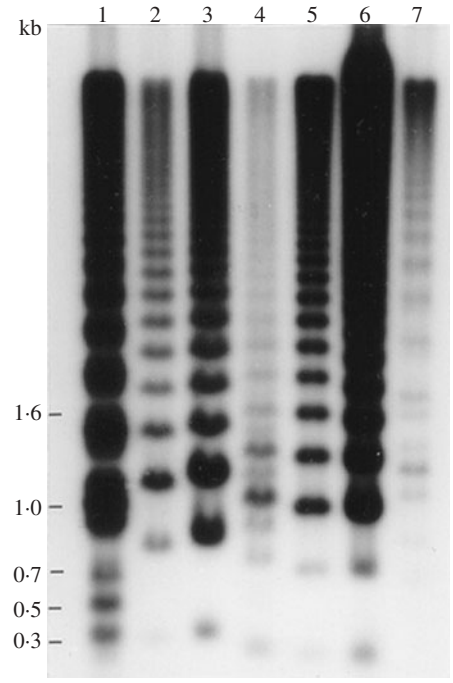

FIG. 4. Southern hybridization pattern of *Msp*I-digested DNA probed with pPov1. The molecular weight marker is indicated in the left margin. Lane 1, *P. ovata*; lane 2, *P. lanceolata*; lane 3, *P. lagopus*; lane 4, *P. major*; lane 5, *P. rugelii*; lane 6, *P. rhodosperma*; lane 7, *P. arenaria.*

they are present on two different chromosomes in *P. lanceolata.* In *P. lanceolata* and *P. rhodosperma*, the 5S and 45S rDNA sites are coupled. This result is surprising because based on the ITS data (authors' observations) and other characteristics (Rahn, 1996) *P. lanceolata* and *P. lagopus* are considered to be closely related phylogenetically. However, FISH data do not agree as the latter species shows only one 5S site in contrast to two in *P. lanceolata*.

Hybridization with pPov1 reveals a characteristic ladder pattern true for 5S rRNA genes. Plant DNA is known to be highly methylated at C residues, so the ladder pattern may be the result of partial digestion due to inactivation of some of the restriction sites by methylation, since *Msp*I is methylation sensitive. In *P. ovata* and *P. arenaria*, each rung on the ladder can be resolved into three bands which can be explained by the fact that their spacer regions have an additional site for *Msp*I.

The size range of ITS regions of *Plantago* species is in agreement with reports on other plant species (Baldwin *et al*., 1995). Table 2 shows that in *Plantago* species, ITS1 is 19–29 bp longer than ITS2, less than *Sinapsis* and *Arabidopsis* where the difference is 77–81 bp (Rathgeber and Capesius, 1989; Unfried and Gruendler, 1990).

The average GC content of spacer regions in *Plantago* species ranges between 51·5 % and 56·5 %, which is in conformity with other angiosperms. Interestingly, the species that has the highest GC content in ITS1 has the highest percentage in ITS2 as well. Like other angiosperms, in *Plantago* species, the 5·8S rRNA gene is 165 bp long. The interspacer similarity has been considered to reflect some degree of co-evolution of ITS1 and ITS2 sequences (Baldwin *et al*., 1995).

Liu and Schardl (1994) reported a highly conserved sequence GGCRY-GYGYCAAGGAA in ITS1 of 88 species belonging to ten angiosperm families. The conserved motif has been speculated to play a key role in processing of rRNA transcripts. In *Plantago* species the conserved motif is located in the central region; however, it does not match completely with that of other species. On the basis of similarity of motifs, *P. ovata* and *P. arenaria* constitute one group while the rest of the five species form a second group.

RFLP analysis

RFLP analysis revealed interspecific variation (Fig. 5), as found in other species (Shiran and Raina, 2001). While *Eco*RI and *Dra*I generate unique patterns in *P. rugelli*, *Xba*I digestion distinguishes *P. arenaria* from the rest. *Eco*RI generates 6·5 kb fragment in *P. ovata* and *P. lanceolata*. These two species show similarity in the location of 45S and 5S genes on chromosomes, although the latter species has an additional site of 5S genes. *Plantago lagopus* shows a unique 2-kb fragment, thereby supporting the FISH data that shows it to be less related to *P. lanceolata*, a conclusion drawn by earlier workers (Rahn, 1996; Ronsted *et al*., 2002) and the present ITS sequence data.

Phylogeny

Rahn (1996) recognized only one genus, *Plantago*, with approx. 200 species, in family Plantaginaceae, and raised six subgenera. The subgenera are subgen. *Plantago* (131 species), subgen. *Coronopus* (11 species), subgen. *Littorella* (three species), subgen. *Psyllium* (16 species), subgen. *Bougueria* (one species) and subgen. *Albicans* (51 species). Figure 1 reveals a close relationship between *P. lanceolata* and *P. lagopus*, and *P. major* and *P. rugelii*. This is in agreement with the morphological and embryological data (Rahn, 1996; Ronsted *et al*., 2002). Similarly, *P. ovata* seems to be closest to *P. arenaria*, although on the basis of other data the two species have been placed in different subgenera (Ronsted *et al*. 2002).

The genus *Plantago* is tribasic; 4, 5 and 6 are the three base numbers (Dhar and Sharma, 1999). *Plantago ovata* is the only species based on $x = 4$ and its evolution has to be traced from a species based on $x = 6$. So far, it was suggested that *P. ovata* originated from species belonging to subgenus *Plantago.* However, the present data indicate that it may have originated from a species belonging to subgenus *Psyllium* (*P. arenaria* belongs to *Psyllium*). This is substantiated by the conservation of the location of 5S rRNA sites on chromosome 1 in both the species. However, after the origin of *P. ovata*, the spacer region seems to have diversified in terms of the base composition as well as the length. Also, during the course of evolution one of the 5S sites seems to have been lost. According to Dhar and Sharma (1999) *P. ovata* has originated from a species with $2n = 10$ chromosomes, suffering a loss of one chromosome pair.

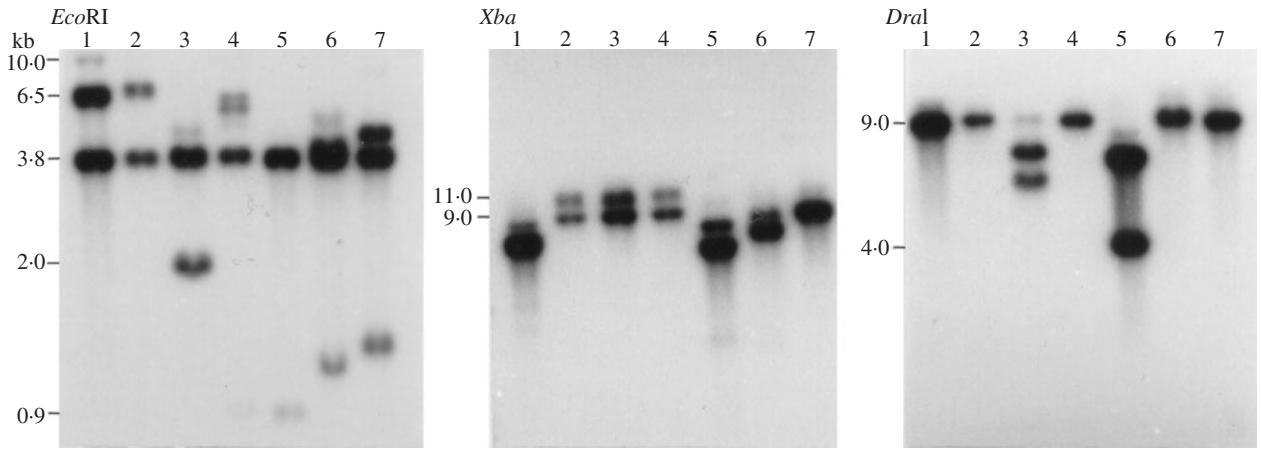

FIG. 5. Southern hybridization using pTa71 as the probe. Lane 1, *P. ovata*; lane 2, *P. lanceolata*; lane 3, *P. lagopus*; lane 4, *P. major*; lane 5, *P. rugelii*; lane 6, *P. rhodosperma*; lane 7, *P. arenaria*.

BLAST sequence similarity searches revealed that ITS1 and ITS2 regions of *Plantago* species have maximum similarity with corresponding regions of species belonging to family Scrophulariaceae. Interestingly, the 5·8S rRNA gene sequence is 98% similar to *Heliohebe hulkeana*, also a member of Scrophulariaceae. As pointed out earlier, Rahn (1996) conducted an exhaustive phylogenetic study based on 91 morphological and embryological characters, and concluded that the family Plantaginaceae belongs to the core of monophyletic Lamianae. Recently, Ronsted *et al*. (2002) used ITS and trnL-F sequences from 57 *Plantago* species, for molecular phylogenetic studies. Their data identified *Aragoa* as a sister to genus *Plantago* diversified 7·1 million years ago. The present data, based on seven representative species, adds to other studies and suggests that *Plantago* falls in a clade that includes *Veronica* and *Hebe* of family Scrophulariaceae.

The results of parsimony analysis have revealed that the tree length and other parameters have inverse linear correlation (Farris, 1989; Kollipara *et al*., 1997 for other species). The g1 statistic value is negative, indicating significant skewness of tree length distribution to the left. Therefore, it can be concluded that there is a greater probability of parsimony analysis determining the true phylogeny (Hillis and Huelsenbeck, 1992).

Molecular phylogenetic analysis will require data from more species in order to arrive at sound phylogenetic conclusions.The present study shows that molecular cytogenetics analysis adds to the tools for determining phylogenetic relationships in *Plantago* and supports some models of their evolution.

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