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Cytological, morphological and molecular analyses of controlled progenies from meiotic mutants of alfalfa producing unreduced gametes

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Abstract A program of sexual polyploidization was carried out in alfalfa using plants from wild diploid species that produced male or female unreduced gametes. Sixteen progenies from 2x – 4x and 2x – 2x crosses were examined with a combination of morphological, cytological and molecular analyses. The chromosome counts revealed diploid, tetraploid and aneuploid plants. Plants with B chromosomes were also detected. The leaf area of the plants was a useful characteristic for distinguishing tetraploid from diploid plants obtained by unilateral or bilateral sexual polyploidization. Leaf shape and leaf margin were not correlated with the ploidy levels. Plants with supernumerary chromosomes displayed obovate or elliptic leaves which differed markedly from the range of forms typical of diploid and tetraploid alfalfa plants. RAPD markers were investigated in all progeny plants to determine maternal and paternal amplification products. Three alfalfa-specific primers proved to be effective in revealing the hybrid origin of the plants. A combination of cytological, morphological and molecular analyses is essential for a detailed genetic characterization of progenies in programs of sexual polyploidization.

Key words Alfalfa breeding · Aneuploids · B chromosomes · Morphological traits · RAPD markers · Sexual polyploidization · Unreduced gametes · Wild germplasm

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Introduction

Cultivated alfalfa (*Medicago sativa* L., 2n = 4x = 32) is an open-pollinated species characterized by tetrasomic inheritance and pronounced inbreeding depression (Busbice and Wilsie 1966). Heterosis in tetraploid alfalfa is dependent on heterozygosity involving allelic and non-allelic gene interactions (Bingham 1980; Bingham et al. 1994). Quantitative genetic theory and experimental data have demonstrated the importance of maximizing tri- and tetra-allelic loci for increasing forage yield and fertility (Demarly 1963; Dumbier and Bingham 1975) and have also shown that inbreeding depression is a function of the loss of both higher-order interactions from tri- and tetra-allelic loci and complementary gene interactions among loci associated in linkats (Busbice and Wilsie 1966; Demarly 1979; Bingham et al. 1994).

The use of diploid meiotic mutants that produce unreduced (2n) gametes is one of the methods available for maximizing heterozygosity in cultivated alfalfa (Bingham 1980; Veronesi et al. 1986). Studies on diploid alfalfa species have revealed that the formation of 2n pollen and 2n eggs is due to cytological alterations that result in first-division restitution (FDR) and second-division restitution (SDR) (Vorsa and Bingham 1979; Pfeiffer and Bingham 1983; Tavoletti et al. 1991a). Mechanisms that give rise to FDR are generally considered more advantageous than those which lead to SDR for transferring parental heterozygosity and retaining epistatic interactions in progeny by means of unilateral or bilateral sexual polyploidization (Bingham 1980). The high levels of heterosis in both forage- and seed-yield in alfalfa hybrids produced by sexual polyploidization, reported in numerous studies (McCoy and Rowe 1986; Groose et al. 1988), have prompted attempts to develop acceptable 2n-pollen and 2n-egg producers for use in breeding (Tavoletti et al. 1991 b). Because the selection of 2n-pollen producers is quite simple, most data on the performance of tetraploids generated by unilateral sexual polyploidization have

been collected in populations from 4x – 2x crosses involving male FDR-type gametes (McCoy and Rowe 1986; Motzo et al. 1994). Owing to the greater difficulties encountered in selecting 2n-egg producers, and because to-date only the mechanism leading to SDR-type gametes has been identified (Pfeiffer and Bingham 1983; Tavoletti 1994), there are few reports on alfalfa progenies from 2x – 4x crosses (Bingham 1990) and none on bilateral sexual polyploidization (2x – 2x crosses).

The objective of the present study was to set up a program of sexual polyploidization in alfalfa using plants from the diploid species *Medicago falcata* (L.) Arcang. (2n = 2x = 16) and *Medicago coerulea* (Less.) Schm. (2n = 2x = 16), which produce male or female 2n gametes. The performance of progenies from 2x – 4x crosses and the potential of 2x – 2x crosses to furnish tetraploid plants were investigated by combined morphological, cytological and molecular analyses. Several morphological traits related to leaves as well as the chromosome number were examined. RAPD markers (Williams et al. 1990), whose development, comparability and potential applications in the genus *Medicago* were recently reported by Barcaccia (1994), were adopted for genomic analysis.

Materials and methods

Plant materials

The plant materials used for controlled matings consisted of diploid meiotic mutants as well as normal diploid and tetraploid plants of alfalfa (Table 1).

The plant of *M. falcata* that produced 2n eggs, termed PGF-9, was selected by Veronesi et al. (1988) and later characterized for the occurrence of two mechanisms of 2n-egg formation: second-division restitution and apomeiosis (Tavoletti 1994). Five 2n-egg and jumbo-pollen producers (plants H-21, H-23, H-25, H-27 and H-29) were selected from an experimental population by Mariani et al. (1993). Since these types of mutants are functionally male sterile, they were used as seed parents. The 2n-pollen producers (plants 3-P, 4-P, 11-P, 12-P, 13-P) were obtained after two cycles of phenotypic recurrent selection for 2n-pollen formation (Tavoletti et al. 1991 b). The pollen donors used also included normal diploids (control plants 2-DI, 6-DI and 7-DI) of *M. coerulea* and normal tetraploids of *M. sativa*, cv 'Adriana' (plants 10-TE and 16-TE), as well as plants Rm-1 and Rm-2 of the Italian ecotype 'Romagnola'.

Controlled 2x – 2x and 2x – 4x matings were carried out in a growth chamber crossing every seed parent with all pollen donors. Thirty seeds from each of the 72 cross combinations were germinated in Petri dishes and the resulting plantlets grown in a greenhouse during the spring of 1994. A total of 160 plants, obtained by random collection of 16 progenies, and ten plants from each progeny, were used for cytological, morphological and molecular investigations.

Cytological analysis

Cytological specimens were prepared from root tips excised from plants kept in the greenhouse. For chromosome counts, the root tips were pre-treated in a saturated solution of α-bromonaphthalene at room temperature for 3 h and then fixed overnight in ethanol-acetic acid (3:1). After staining in Feulgen, the meristematic region was squashed in 1.5% acetic orcein and attached to a cover slip with glycerin-albumin.

Morphological analysis

Ten fully expanded central leaves were collected from each plant and the mean leaf area (cm^2) determined with a leaf-area meter (Hithaci). Leaves were collected at blooming time from shoots 2–3 nodes under the apex. Leaf shape and type of margin were also recorded. The following numbers designate the leaf shape: 1 = oblong; 2 = oblong-cuneate; 3 = oblong-obovate; 4 = elliptic; 5 = obovate; while the leaf margin was defined as continuous (c) or with serrations (s).

RAPD analysis

For DNA isolation, three leaflets from each plant were harvested and frozen in liquid nitrogen. Total genomic DNA was isolated according to the procedure described by Edwards et al. (1991). Spectrophotometric estimation was used to quantify the amount of genomic DNA, evaluate its purity, and adjust samples to a concentration of 2.5 ng/μl.

The sequences of oligonucleotide primers (5'–3') used in this study were: N. 2-GGTCGCAGGC; N. 5-CTCACCGTCC; and N. 6-GTGGTGCTAT. These 10-base-long nucleotide primers (M-Medical/Genenco) were the best from a set of primers selected in previous investigations on the basis of their ability to find homologous binding sites among alfalfa genomic templates (Barcaccia et al. 1994). The polymerase chain reaction (PCR) occurred in a 25-μl vol, which included 10 mM Tris-HCl (pH 8.8), 50 mM KCl and 1.5 mM $MgCl_2$, with 100 μM each of dCTP, dGTP, dATP and dTTP, 400 nM of a

Table 1 Specifications of the alfalfa genotypes used in the controlled crosses

Plant material	Germplasm source	Genotype code	Chromosome number
[a] Apomeiotic and SDR 2n-egg producer	*Medicago falcata* (L.) Arcang. acc. # 258754	PGF-9	2n = 2x = 16
[a] Jumbo pollen and 2n-egg mutants	*M. falcata* × *M. sativa* clone HY6 × CADL	H-21, H-23, H-25, H-27, H-29	2n = 2x = 16
[b] 2n-pollen mutants	*Medicago falcata* acc. # 258754 and acc. # 262532	3-P, 4-P 11-P, 12-P, 13-P	2n = 2x = 16
[b] Normal diploids	*Medicago coerulea* (Less.) Schm. acc. # 2956	2-DI, 6-DI, 7-DI	2n = 2x = 16
[b] Normal tetraploids	*Medicago sativa* L. cv "Adriana" ecotype "Romagnola"	10-TE, 16-TE Rm-1, Rm-2	2n = 4x = 32

[a] Seed parents
[b] Pollen donors

single primer, and 1 U of Taq DNA polymerase (Stratagene). Amplification was performed in an OmniGene thermal cycler (HyBaid) under cycling conditions similar to those described by Barcaccia (1994).

Products generated by PCR amplification were separated by electrophoresis in 1.4% agarose gels run with TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Photographs (Polaroid type 667) were taken of the ethidium bromide-stained gels visualized by UV light illumination.

Results

Cytological analysis

The determination of the chromosome number revealed diploid, tetraploid and aneuploid plants.

The chromosome numbers of plants obtained from crosses in which PGF-9 was used as seed parent are reported in Table 2. When the pollen donor was the tetraploid 10-TE, eight plants with 2n = 32, one plant with 2n = 16, and one plant with 33 chromosomes, were produced (Fig. 1a). When the pollen donor was the tetraploid Rm-1, all plants were 2n = 32, with the exception of plant no. 14, which had 30 chromosomes. Crosses involving the diploid producers of 2n pollen gave six tetraploid plants from the combination PGF-9 × 3-P, one tetraploid plant from the combination PGF-9 × 4-P, and three tetraploid plants from the combination PGF-9 × 13-P (Table 2). One plant with 19 chromosomes was also generated within the PGF-9 × 3-P progeny (Fig. 1b). The cross of PGF-9 with the normal

Table 2 Morphological, cytological and molecular data of six progenies from crosses involving the mutant PGF-9

Progeny	Leaf morphology			Chromosome number (2n)	Polymorphic bands		Shared bands
	Area (cm^2)	Shape	Margin		Maternal	Paternal	
PGF9 × 10TE							
2	0.517	3	s	32	6	8	8
5	0.767	2	s	32	8	5	9
6	0.275	1	s	16	12	0	11
7	0.717	3	s	32	9	3	11
8	0.575	1	s	32	4	2	7
11	0.634	3	s	32	3	1	6
13	0.467	3	s	32	6	5	5
14	1.008	5	c	33	4	3	4
15	0.717	3	s	32	3	3	9
16	0.959	3	s	32	7	4	10
PGF9 × Rm 1							
1	0.675	3	s	32	6	5	7
2	0.450	2	s	32	5	4	4
3	0.758	2	s	32	6	7	6
7	0.825	3	s	32	4	5	6
9	0.750	3	s	32	4	5	5
10	1.383	3	s	32	8	4	8
13	1.100	3	s	32	6	5	7
14	0.458	2	s	30	5	7	5
18	0.992	2	s	32	7	5	7
22	0.542	2	s	32	5	6	6
PGF9 × 3P							
1	0.300	3	c	16	5	3	8
3	0.158	3	c	16	5	4	8
6	0.383	3	s	32	5	4	8
8	0.367	3	s	32	4	3	8
11	0.367	3	c	32	6	2	7
14	0.258	3	c	16	5	2	8
17	0.433	2	s	32	4	5	7
19	0.375	3	s	32	6	4	9
21	0.390	3	s	32	6	5	8
23	0.217	4	c	19	3	4	6
PGF9 × 4P							
1	0.158	1	c	16	7	5	10
3	0.292	3	c	16	4	3	11
7	0.233	3	c	16	4	3	10
8	0.483	2	s	16	3	3	12
10	0.550	3	c	16	5	4	9
12	0.617	2	s	32	4	3	8
14	0.233	1	c	16	3	2	9
16	0.342	1	c	16	4	3	7
20	0.450	3	s	16	4	1	10
23	0.217	1	c	16	3	2	9

Table 2 (Continued)

Progeny	Leaf morphology			Chromosome number (2n)	Polymorphic bands		Shared bands
	Area (cm^2)	Shape	Margin		Maternal	Paternal	
PGF9 × 13P							
4	0.375	3	s	16	7	6	5
5	0.292	1	c	16	7	4	4
6	0.333	1	s	16	8	6	6
8	0.300	1	s	16	6	5	5
12	0.492	1	s	32	5	5	6
15	0.333	2	s	16	3	8	7
16	0.592	3	s	32	4	6	5
19	0.392	2	s	16	8	7	5
20	0.483	3	s	32	6	4	7
22	0.333	1	s	16	5	7	5
PGF9 × 7DI							
2	0.358	2	s	16	4	5	10
5	0.308	2	s	16	4	6	9
7	0.692	3	s	16	6	4	8
10	0.450	2	s	16	5	8	8
12	0.358	2	s	16	2	4	11
14	0.392	1	s	16	5	5	8
17	0.408	1	s	16	5	3	11
18	0.392	5	s	25	4	4	9
19	0.317	3	s	16	4	3	9
21	0.400	2	s	16	5	4	10

diploid parent 7-DI produced nine diploid plants and one plant with 25 chromosomes. On the basis of karyotype analysis the latter plant was classified as a tetrasomic triploid (Fig. 1c). The chromosome numbers of plants derived from controlled crosses in which the male-sterile plants were used as seed parent are reported in Table 3. All plants obtained by crossing H-25 with 16-TE and with Rm-2 turned out to be $2n = 32$. The H-21 × 2-DI cross yielded six plants with B chromosomes, four with $2n = 16 + 1B$ (Fig. 1d) and two with $2n = 16 + 2B$ (Fig. 1e), while the remaining four plants were $2n = 16$. All plants produced by the remaining crosses (H-21 × 11-P, H-23 × 12-P, H-25 × 11-P, H-25 × 12-P, H-27 × 12-P, H-29 × 11-P, H-29 × 6-DI) were diploid.

Morphological analysis

Measurement showed that the leaf area of tetraploid plants derived by crossing 2n-egg producers with tetraploid pollen donors was markedly greater than that of the diploid parent and similar to that of the tetraploid parent (Table 2). The tetraploid plants obtained by crossing PGF-9 with 2n-pollen producers had a considerably greater leaf area than the diploid plants of the same progeny or either of the parents (Fig. 2a).

Leaf shape and margin did not appear to be correlated with the ploidy level of plants. In all the progenies the shape of leaves varied from oblong to oblong-obovate, while the margin was continuous or with serrations without any distinction between diploid and tetraploid plants. The only plants that showed leaves of different shape were those with aneuploid chromosome numbers: plants with $2n = 33$ and $2n = 25$ had obovate leaves (Fig. 2b), and the plant with $2n = 19$ had elliptical leaves (Table 2).

The flower colour of the analyzed plants ranged from purple to yellow. In particular, plants from crosses between *M. falcata* and *M. coerulea* or *M. sativa* had variegated flowers which, due to the co-dominant expression of the yellow (*M. falcata*) and purple (*M. coerulea* and *M. sativa*) genes, are typical of this cross combination (Barnes 1966).

RAPD analysis

The selected primers were used to generate genome-specific fingerprints by means of RAPD markers. The detection of both maternal and paternal traits allowed the hybrid origin of the plants to be determined. On the whole, the plants of the 16 progenies exhibited a number of amplification products of paternal origin ranging from a minimum of one to a maximum of eight (Tables 2 and 3). Figure 3 shows the genomic fingerprints of parents PGF-9 and 13-P (Fig. 3a), and H-25 and 16-TE (Fig. 3b) and their progenies following RAPD analysis with primer N. 5. The only exception was plant no. 6 of the PGF-9 × 10-TE combination, where no amplification products of paternal origin were found. Since three alfalfa-specific primers revealed all RAPD bands to be of

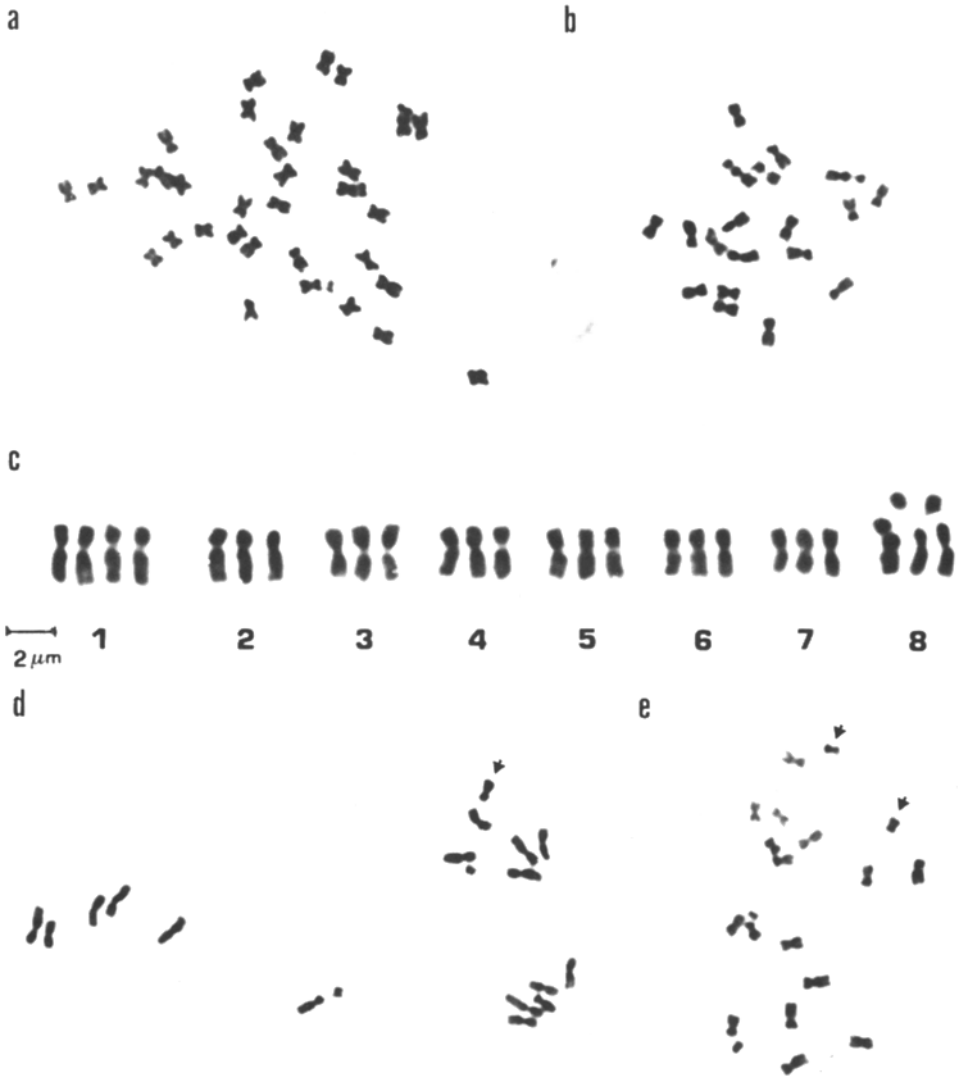

Fig. 1 a–e Mitotic metaphases (2200 x) of a plant with 33 chromosomes **(a)** and a plant with 19 chromosomes **(b)**; karyotype (3660 x) of the tetrasomic triploid, plant no. 18 of the progeny from PGF-9 × 7-DI **(c)**; **(d, e)** mitotic metaphases (2200 x) displaying one and two B chromosomes (*arrows*)

maternal origin, it can be assumed that this plant originated from selfing, which agrees with the results of cytological and morphological analyses (Table 2).

A number of amplification products which were conserved among parents and plants of the relevant progeny were detected with all the primers used. The average number of shared amplification products varied among progenies from 5.5 to 9.5.

Discussion

The results of this study demonstrate that 2n-pollen and 2n-egg producers can be effectively used for carrying out bilateral sexual polyploidization and that the production of tetraploids from 2x – 2x crosses depends on parental genotypes, 2n-gamete rates being similar. They also offer further evidence of the possibility of using plants producing 2n eggs for direct gene exchange from wild diploid relatives into cultivated alfalfa by means of 2x – 4x crosses.

The identification of tetraploid plants is a fundamental step in this type of alfalfa breeding. It is known that the ploidy levels can be identified on the basis of phenotypic traits, such as plant vigour and pollen size (Veronesi et al. 1986). Although these characteristics give an indication of the ploidy level of progenies they do not permit the effective chromosome number to be ascertained. Cytological analysis allowed the effective tetraploid status of plants to be verified and the aneuploids, which are expected to form among progenies of meiotic mutants, to be identified. Since aneuploids are undesirable in programs of sexual polyploidization, their identification is essential. Nevertheless, aneuploid series could be useful for locating genes on chromosomes and for research on genic dosages (McCoy and Echt 1992). Aneuploids were found at the diploid and

Table 3 Morphological, cytological and molecular data of three progenies from crosses of jumbo-pollen and 2n-egg producers with normal tetraploid and diploid pollen donors

Progeny	Leaf morphology			Chromosome	Polymorphic bands		Shared bands
	Area (cm^2)	Shape	Margin	number (2n)	Maternal	Paternal	
H25 × 16TE							
1	0.833	3	s	32	4	6	9
2	1.067	3	s	32	5	5	8
3	0.717	2	c	32	5	4	9
4	0.658	2	s	32	4	5	10
5	0.900	3	s	32	3	3	10
6	0.692	3	s	32	4	3	8
7	1.525	4	s	32	6	2	9
9	1.225	4	s	32	3	4	9
11	0.825	3	s	32	4	4	8
12	0.867	3	s	32	5	5	9
H25 × Rm2							
1	0.792	3	s	32	4	5	5
2	0.867	3	s	32	5	6	5
3	0.800	3	s	32	3	4	8
4	0.667	3	s	32	6	5	4
5	1.017	3	s	32	4	7	7
6	0.692	3	s	32	4	5	6
9	0.775	3	s	32	3	4	7
10	0.725	3	s	32	5	6	5
11	0.717	3	s	32	2	5	6
12	0.608	3	s	32	3	4	8
H21 × 2DI							
1	0.417	1	s	16	5	4	6
2	0.433	2	s	16	3	4	7
3	0.450	2	c	16 + 2B	3	4	7
4	0.275	1	c	16 + 2B	4	8	8
6	0.583	3	s	16 + 1B	1	5	5
7	0.297	1	c	16 + 1B	3	6	7
8	0.308	3	c	16 + 1B	3	4	8
9	0.475	2	s	16 + 1B	2	3	6
10	0.367	3	c	16	3	4	8
11	0.483	2	s	16	2	5	8

tetraploid levels. The plant with 25 chromosomes was derived from the fertilization of a 2n egg cell with a male n gamete and was the only triploid among the 160 plants examined. The occurrence of triploid plants in interploid crosses of alfalfa is a rare event that has been explained by the presence of an effective triploid block which eliminates almost all the triploid embryos (Veronesi et al. 1986), probably as a consequence of endosperm imbalance (Johnston et al. 1980).

Plants with 17 and 18 chromosomes were not included in the category of aneuploids because the supernumerary chromosomes were not homologous to any chromosome of the basic set and were, therefore, considered as B chromosomes. Chromosome analysis of both parents revealed that the B chromosomes were transmitted by the pollen donor 2-DI, which was $2n = 16 + 2B$. The fact that B chromosomes often do not undergo disjunction at the first pollen mitosis (Jones and Rees 1967) may account for the finding of two B chromosomes in two progeny plants. Although B chromosomes have been reported in annual species of the genus *Medicago* (Heyn 1956, 1963; Agarwal and Gupta 1983), to the best of our knowledge this is the first time that they have been discovered in species of the *M. sativa-coerulea-falcata* complex.

The leaf area was the only morphological characteristic correlated with the ploidy level of plants; it was consistently greater in tetraploids than in either diploids of the same progeny or diploid parents. This relationship can be applied to preliminary screening of large progenies to select plants with a large leaf area that can then be tested for chromosome number. Leaf shape and margin were not correlated to the ploidy level, since they varied within the range typical of diploid and tetraploid alfalfa. Different leaf shapes were observed only in the aneuploid plants with additional chromosomes; plants with 25 and 33 chromosomes had obovate leaves while the plant with 19 chomosomes had elliptical leaves. The

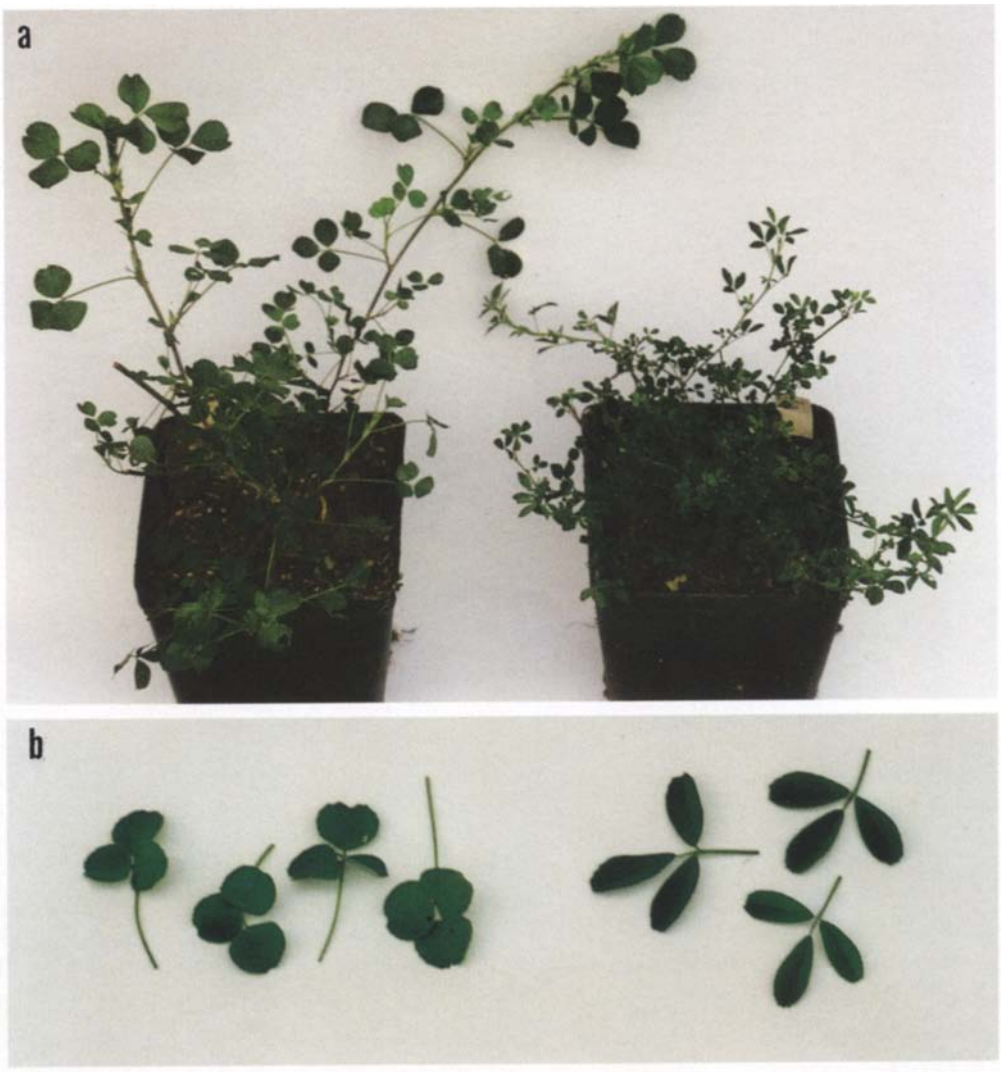

Fig. 2 a Growth habit of plants no. 12 (2n = 4x = 32) (left) and no. 7 (2n = 2x = 16) (right) from the PGF-9 × 4-P progeny, the different leaf and stem sizes are clearly evident; **b** obovate leaves of the plant with 25 chromosomes (left) and oblong-cuneate leaves of a normal diploid plant (right) from the same PGF-9 × 7-DI progeny

leaves of plants that lacked two chromosomes and those of plants with one or two B chromosomes were normal. These results indicate that, in some cases, aneuploids can be used to relate morphological features to specific chromosomes.

Molecular analysis with RAPD markers has been used for various purposes in alfalfa (Barcaccia 1994). The parental plants employed in the present study were characterized by RAPD markers in a preliminary study (Barcaccia et al. 1994) and the information on genomic polymorphisms utilized for analyzing progenies in the present investigation. In particular, RAPD markers that were polymorphic between the parents were used to detect maternal and paternal amplification products and thus verify the hybrid origin of each plant. The evaluation of RAPD markers also allowed the introgression of wild germplasm into cultivated alfalfa to be ascertained. Their capacity to reveal DNA polymorphisms at an early stage of plant development makes RAPD markers a valuable tool for the precocious identification of hybrids and plants from selfing. The fact that several polymorphic and conserved amplification products were detected within all progenies is a further demontration that RAPD markers can be employed for investigating between-species phylogenetic relationships and within-population genetic variability.

In conclusion, a combination of cytological, morphological and molecular analyses appears to be essential for obtaining a detailed genetic characterization of progenies in programs of sexual polyploidization. The results obtained in our experiments will be exploited to isolate tetraploid plants from the other 56 available progenies. All tetraploid plants generated from both unilateral and bilateral polyploidization will finally be used in field trials to assess their forage and seed production in order to compare them with those of tetraploid plants of alfalfa varieties.

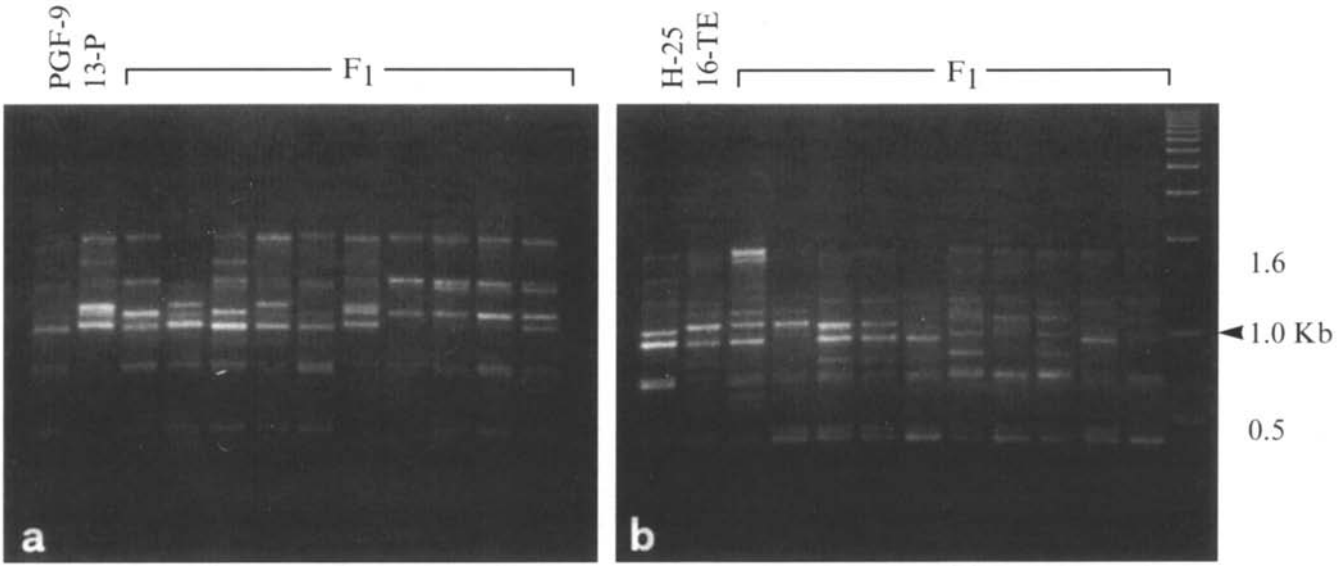

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Fig. 3 a,b RAPD patterns generated from PGF-9 and 13-P and their progeny **(a)**, and from H-25 and 16-TE and and their progeny **(b)**, using primer N. 5. Amplification products of maternal and paternal origin are shown for both progenies

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