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Inheritance of parental genomes in progenies of *Poa pratensis* L. from sexual and apomictic genotypes as assessed by RAPD markers and flow cytometry

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Abstract Moving gene(s) responsible for the apomictic trait into crop plants that naturally reproduce through a sexual process would open up new areas in plant breeding and agricultural systems. Kentucky bluegrass (*Poa pratensis* L.) is one of the most important forage and turf grasses in temperate climates. It reproduces through facultative aposporous parthenogenesis, but the reproductive behaviour ranges naturally from nearly obligate apomixis to complete sexuality. In addition to apomictic reproduction, sexual hybridization may take place. Selfing may also occur, and occasionally reduced egg cells may develop through parthenogenesis generating (poly)haploids. The inheritance of parental genomes was assessed in Kentucky bluegrass progenies by employing RAPD markers in combination with flow cytometry (FCM). Nine progenies from different crosses carried out between completely sexual and highly apomictic genotypes were evaluated in order to probe the reproductive behaviour of the mother plants and to distinguish the different classes of aberrant plants. Not only were maternals and balanced B_{II} hybrids recorded, but so were (poly)triploid B_{III} hybrids, selfs, and (poly)haploids. The application of these techniques demonstrated that FCM analysis accurately distinguishes the n, 2n, and 3n ploidy levels of progenies, and that RAPD markers unequivocally recognize progenies of apomictic and hybrid origin. The occurrence of aneusomaty was documented in one of the selected sexual genotypes, whose crossed progeny plants manifested two distinct classes of ploidy. The nomenclature B_I was adopted to refer to hybrids with a hypodiploid nuclear condition. On the whole, the FCM analysis confirmed most of the RAPD data. The combined evaluation of DNA markers and DNA contents proved to be an efficient screening tool for scoring maternal plants, assessing the genetic origin of aberrant plants, and quantifying the inheritance of parental genomes in Kentucky bluegrass. Hybrid populations from sexual × apomictic matings that segregate for the mode of reproduction represent a valuable basis for attempting to identify molecular markers linked to the apomixis gene(s).

Key words Kentucky bluegrass · Apospory · Sexuality · Genomic DNA markers · Nuclear DNA contents · Ploidy · Aneusomaty

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Introduction

Kentucky bluegrass (*Poa pratensis* L.) is one of the most important forage and turf grasses in temperate climates (Bashaw and Funk 1987). Although it has a mode of reproduction ascribable to facultative aposporous parthenogenesis, its reproductive behaviour ranges naturally from nearly obligate apomixis (frequent) to complete sexuality (rare). Not only apomictic reproduction, which requires the fertilization of polar nuclei (i.e., pseudogamy) to initiate endosperm development and to produce a viable embryo of maternal origin, but also sexual hybridization may occur. A convenient nomenclature used to define progenies of aberrant origin is B_{II} for n + n (poly)diploid hybrids and B_{III} for 2n + n (poly)triploid hybrids (i.e., fertilization of reduced and unreduced egg cells, respectively) (Rutishauser 1948). Selfing may take place, and

occasionally reduced egg cells may develop through parthenogenesis generating (poly)haploids.

Although the mode of reproduction is known to be under genetic control, detailed genetic analysis is extremely difficult due to the association of facultative apomixis with a high and irregular ploidy and a high and variable level of heterozygosity. An analysis of progenies from controlled crosses between completely sexual genotypes and highly apomictic cultivars by the auxin test (Matzk 1991) revealed (1) a dominant expression of parthenogenesis (apomixis) over obligatory zygotic embryogenesis (amphimixis); (2) the presence of one or more dominant alleles in apomictic plants; (3) the lack of genes/alleles for parthenogenesis in pure sexual plants; and (4) a segregation ratio of sexual and apomictic equal to 1 : 1.

Establishing the ploidy level and ascertaining the hybrid origin are essential for the determination of the mode of reproduction of single plants and the assessment of the inheritance of parental genomes in progenies of *P. pratensis*. In this species it is quite difficult to screen among progeny plants of apomictic origin, selfs, and hybrid constitutions when the parents have similar ploidy levels and/or do not display discriminant genetic polymorphisms.

A nearly complete polyploid series can be obtained in *P. pratensis*, with the 2n chromosome number ranging from 28 to 140 (Love and Love 1975). The counting and karyotyping of somatic chromosomes is a laborious task owing to the metaphasic chromosomes being very small with barely distinguishable morphology. The discrimination among aberrants of different genetic origin, such as between selfs and (poly)haploids, and between B_{II} and B_{III} hybrid constitutions can be addressed by flow cytometry (FCM) using effective methods for the rapid mechanical extraction of intact nuclei (Galbraith et al. 1983). Analysis of the fluorescence intensity emitted from isolated nuclei in suspension stained with DNA-specific fluorochromes and comparison of the values with an internal standard (Tiersch et al. 1989; Lucretti et al. 1992) enables precise estimations of the nuclear DNA content and ploidy level of *P. pratensis* plants (Huff and Bara 1993; Naumova et al. 1993; Mazzucato et al. 1994).

Random amplified polymorphic DNA (RAPD) markers (Williams et al. 1990) provide a valuable means for genomic analysis in facultative apomicts. The frequency of maternal plants and the effective occurrence of aberrant plants of hybrid and non-hybrid origin in *P. pratensis* progenies can be determined by genomic DNA fingerprinting (Huff and Bara 1993; Mazzucato et al. 1995). The use of markers randomly distributed in the genome, such as RAPD sequences, coupled with an approach which does not need saturated linkage maps and near-isogenic lines, such as the bulked segregant analysis (Michelmore et al. 1991; Ozias-Akins et al. 1993; Leblanc et al. 1995), appears very promising for identifying DNA markers linked with the apomixis gene(s) even in remarkably complex species like *P. pratensis*.

We attempted to quantify the inheritance of parental genomes in *P. pratensis* by combining RAPD markers with FCM analysis to test progenies from crosses between selected sexual and apomictic genotypes in order to assess the reproductive behaviour of mother plants, to screen maternal plants, and to distinguish the different classes of aberrant plants, such as B_{II} and B_{III} hybrids, selfs and (poly)haploids.

Materials and methods

Plant sources

Controlled matings were carried out without emasculation between completely non-parthenogenetic (Matzk 1991) and highly apomictic (Mazzucato 1995) genotypes of *P. pratensis* L. Three sexual (S1, S2, and S4) and five apomictic (RS7, RS9, RS18, RS20, and MM12) seed parents were crossed in different combinations within pollination cells.

Stain-clearing

To quantify aposporic tendencies, panicles of two sexual (S1 and S2) and three apomictic (RS9, RS18, and MM12) seed parents, spanning the stages from archesporium formation to embryo sac maturation, were collected, fixed in FAA, and stored at $+4°C$. Cytoembryological investigations of mega-sporogenesis and gametogenesis were carried out using the stain-clearing technique described by Stelly et al. (1984) with some changes. Ovaries were dissected under a stereo-microscope, submitted to a 2-h staining phase, mounted on slides with a drop of methyl salicylate and examined under a light-microscope. Overall, 54–82 ovaries from each selected parent were investigated. The degree of apospory was calculated as the frequency of ovules with clear evidence of aposporic initials or embryo sacs at stages from the megaspore mother cell (MMC) to the four-nucleated embryo sac.

RAPD markers

Nine progenies, for a total of 202 plants, were evaluated by screening for RAPD markers. Total genomic DNA was extracted from etiolated leaf-blade samples according to the protocol described by Edwards et al. (1991). Spectrophotometric estimation was used to quantify the amount of genomic DNA and to evaluate its purity. The amplification reactions were performed in a OmniGene thermal cycler (HyBaid) using the polymerase chain reaction (PCR) parameters defined by Barcaccia (1994) with some modifications to optimize the analysis in *P. pratensis* (Mazzucato et al. 1995). Six 10-mer primers (C/09, F/20, P/01, P/09, R/04, and R/05, Operon Technologies) that gave a reasonable number of strong amplification products and genomic polymorphisms between parents were selected during preliminary experiments. Amplification products were separated by electrophoresis in 1.4% agarose gels run with TBE buffer. Photographs (Polaroid type 667) were taken of the ethidium bromide-stained gels, visualized by UV-light illumination. RAPD fingerprint evaluation considered only bands ranging between 200 bp and 2.2 kb in length that were reproducible and could be scored unambiguously. Progenies were classified as maternal when their DNA fingerprints were identical with those of the seed parent and as aberrant when bands of paternal origin or new bands were present or when maternal bands were lacking in at least one

fingerprint. Within the aberrant progenies, three putative classes were scored: B_{II} hybrids, when the fingerprint showed maternal and paternal markers, in which at least one maternal marker segregated; B_{III} hybrids, when all maternal markers were conserved and one or more paternal markers were present; non-hybrids, when paternal markers were absent and one or more maternal markers were lacking (which could include plants from selfing or from haploid parthenogenesis). Genetic similarity estimates (Nei and Li 1979) between the parental plants and between each pair-wise comparison of aberrant plants of the progenies and both parents were calculated after excluding bands shared between parents and non-segregating in the progenies. The similarity estimates of the B_{II} and B_{III} hybrid and non-hybrid plants from all progenies were subjected to analysis of variance (Statistical Analysis System, SAS Institute, Cary, N.C.) to evaluate differences among types of aberrants. Statistical differences among means were tested by least significant difference at the 5% level.

Flow cytometry

All seed and pollen parents and 59 individuals selected from all progenies, which included a large number of the aberrant (hybrid and non-hybrid) plants and a sample of maternal plants, as assessed by RAPD analysis, were used to ascertain the nuclear DNA content by FCM analysis. Nuclei were isolated from approximately 30–50 mg of etiolated basal stems by chopping with a sharp scalpel in a glass petri dish containing 1 ml of LB01 lysis buffer (Doležel et al. 1989). Unstained chicken red blood cell (CRBC) nuclei were added to the isolation buffer as internal standard. Cellular debris was removed by filtering suspensions of released nuclei through a 21-μm nylon mesh; fluorochrome propidium iodide (PI) and RNase were added according to Lucretti et al. (1992); samples were then incubated 30 min at room temperature, before analysis. Nuclei suspensions were processed with a FACStar Plus flow cytometer and sorter (Becton Dickinson) equipped with an argon ion laser. PI-stained nuclei were excited at 514 nm with a laser adjusted to 200 mW output power. The fluorescence emitted from nuclei was collected through a 620/30-nm band-pass filter, and fluorescence pulses were acquired according to their height, width, and area. The instrument amplification was adjusted so that the peak corresponding to single CRBC nuclei was positioned approximately at channel 50 on a 256-channel histogram. For each sample, the plant nuclear 2C DNA content, measured in picograms, was determined by taking the ratio of the G_0/G_1 peak mean and the red blood cell peak mean, and multiplying by the known nuclear DNA content of chicken red blood cells (Huff and Bara 1993). The mean and standard errors were calculated for the nuclear DNA contents related to maternal plants and all types of aberrants scored in each progeny. The nuclear DNA content of each plant, as estimated by FCM analysis, was compared with that expected on the basis of the genetic origin assessed by RAPD markers. A sample of 11 maternal plants was used to estimate confidence intervals between observed and expected nuclear DNA contents. Plants that departed significantly from the expected value as defined by RAPD fingerprint evaluation were reclassified in the most appropriate aberrant class according to their nuclear DNA content. The mean deviation between expected and observed values was calculated for all classes of aberrant plants within each progeny using a number of estimations ranging from 2 to 6.

Results

Stain-clearing analysis

The occurrence of meiotic or aposporic cells in ovules of the five selected seed parents was determined by cytological analysis. Plants S1 and S2, selected as completely non-parthenogenetic according to the auxin test and the morphological progeny test (Matzk 1991), had to be also considered non-aposporic since aposporic initials were never detected (Fig. 1a–f). Moreover, plants RS9, RS18, and MM12, which were selected as apomictic, and thus parthenogenetic, on the basis of the isozyme progeny test (Mazzucato 1995), displayed consistent aposporic tendencies: the degree of apospory observed was 66%, 60% and 74%, respectively. Aposporic initials were found during both meiosis and gametogenesis (Fig. 1e).

Analysis of RAPD fingerprints

The reproductive characteristics of the seven seed parents as determined by the RAPD progeny test are summarized in Table 1. Plants S1, S2, and S4, selected as non-parthenogenetic (Matzk 1991), proved to be exactly sexual after the evaluation of RAPD fingerprints (Table 1). Plants RS7, RS9, RS18, and MM12, selected as apomictic (Mazzucato 1995), exhibited consistent levels of apomixis even after the RAPD analysis of progenies: 90%, 90%, 93%, and 80%, respectively (Table 1).

Figure 2 shows RAPD fingerprints of hybrid (Fig. 2 a–b) and maternal (Fig. 2c) origin generated by progenies S2 × RS20, S1 × S2, and MM12 × RS7, respectively.

The combination of six banding patterns showed that all of the aberrant plants detected in the progenies of S1, S2, S4, RS9, and RS18 were of a hybrid origin, while 3 aberrants produced by MM12 and 1 by RS7, which did not show any specific paternal amplification product, were scored as non-hybrids and probably originated from selfing or haploid parthenogenesis (Table 1). Moreover, 1 aberrant plant of the MM12 progeny, which presented no departure from the maternal profiles but had amplification products of the pollen parent, was considered to be a putative B_{III} hybrid; 2 other putative B_{III} hybrids were revealed, 1 each in the progeny of RS18 and RS9 (Table 1).

The number of RAPD markers scored for each cross-combination, either polymorphic between parents or shared between parents but segregating in the progeny, differed in sexual and apomictic parental plants. Within the progeny of sexual plants, the number of bands ranged from 33 to 47 (average 40.2), whereas in the progeny of apomictic plants the number of bands scored ranged from 12 to 24 (average 17.3). Polymorphic bands between parental plants ranged from 66% to 100% in combinations where seed parents were apomictic genotypes and from 58% to 81% when they were sexual genotypes. New bands were always detected in the progenies of sexual genotypes, up to seven in the progeny of S2, but, with one exception in the progeny of RS18, never observed in the progenies of apomictic genotypes.

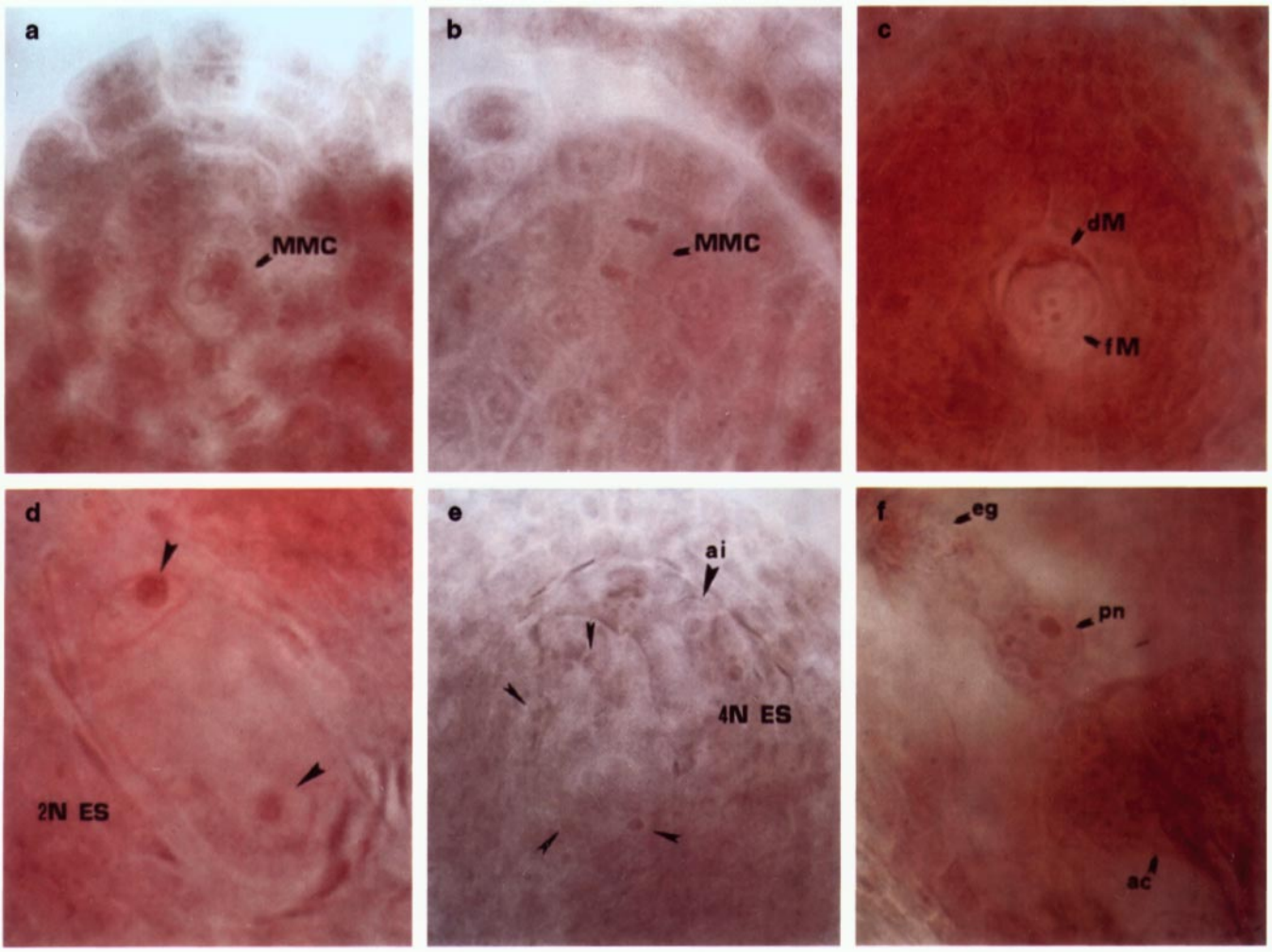

Fig. 1a–f Sexual development in *Poa pratensis*. **a,b** Megaspore mother cell (*MMC*) at prophase I **(a)** and anaphase I (**b**); **c** functional megaspore (*fM*) and degenerating megaspores (*dM*) **d**; binucleated (2N ES), **e** tetranucleated (4N ES), **f** mature embryo sac. Aposporic initials or embryo sacs (*ai*) were detected only in apomictic plants (**e**). *eg* egg cell, *pn* polar nuclei, *ac* antipodals

Similarity estimates between parents ranged from 0.58 to 0.75, with an average value of 0.67. The similarity estimate of putative B_{II} hybrids varied between 0.35 and 0.88 with the seed parent, and between 0.35 and 0.89 with the pollen parent; in putative B_{III} hybrids it ranged from 0.92 to 0.95 with the seed parent and from 0.15 to 0.59 with the pollen parent. Non-hybrids had a lower similarity with the pollen parent (0.00–0.28), but some of the highest estimates with the seed parent (0.90–0.94). The average similarity estimates of B_{II} hybrids with seed and pollen parents were comparable, while in B_{III} hybrids and non-hybrids they were higher with the seed parent than with the pollen parent (Table 1). The differences among mean similarity estimates of aberrants with the seed parent were not significant between B_{III} hybrids and non-hybrids; B_{II} hybrids differed significantly from both. With the pollen parent, B_{II} hybrids showed the highest value and differed significantly from B_{III} hybrids and non-hybrids (Table 1).

Flow cytometric analysis

The yield of nuclei in suspension from the selected progeny plants was largely controlled by the age of the stems used. The best isolations were achieved by chopping the basal part of young stems; using 40 mg of vegetative tissue a typical yield of 2×10^5 nuclei per milliliter was obtained. The coefficient of variation (CV) of plant samples and internal reference standard spanned from 2.5% to 7.1%; lower coefficients were obtained when DAPI was used as fluorochrome due to its high specificity for bases A and T (Lucretti and Doležel 1995). However, to avoid any potential misinterpretation of fluorescence intensities related to unknown changes in the base composition of *Poa* genome, we used PI as DNA fluorochrome because of its non-specificity for DNA base pairs (Doležel et al. 1992).

FCM analysis enabled the discrimination of nuclear DNA contents between parental plants and among

Table 1 Results from the analysis of DNA fingerprints generated by RAPD markers: genetic similarity estimates between seed and pollen parents, frequency of maternal plants, genetic origin and frequency of aberrant plants, and mean similarity estimates between aberrant progeny plants and both parents

Cross-combination	Progeny size	Similarity estimate between parents	Number of maternals	Genetic origin and no. of aberrants		
				B_{II} hybrid	B_{III} hybrid	Non-hybrid
S1 × S2	9	0.70	0	9	0	0
S2 × S1	20	0.70	0	20	0	0
S1 × RS9	23	0.66	0	23	0	0
RS9 × S1	30	0.66	27	2	1	0
S2 × RS20	18	0.58	0	18	0	0
S4 × RS18	13	0.64	0	13	0	0
RS18 × S4	29	0.64	27	1	1	0
MM12 × RS7	30	0.75	24	2	1	3
RS7 × MM12	30	0.75	27	2	0	1
Mean similarity estimate with the seed parent *				0.67b	0.93a	0.92a
Mean similarity estimate with the pollen parent*				0.68a	0.37 b	0.21b

* Means followed by different letters in the same row are significantly different at $P \leqslant 0.05$

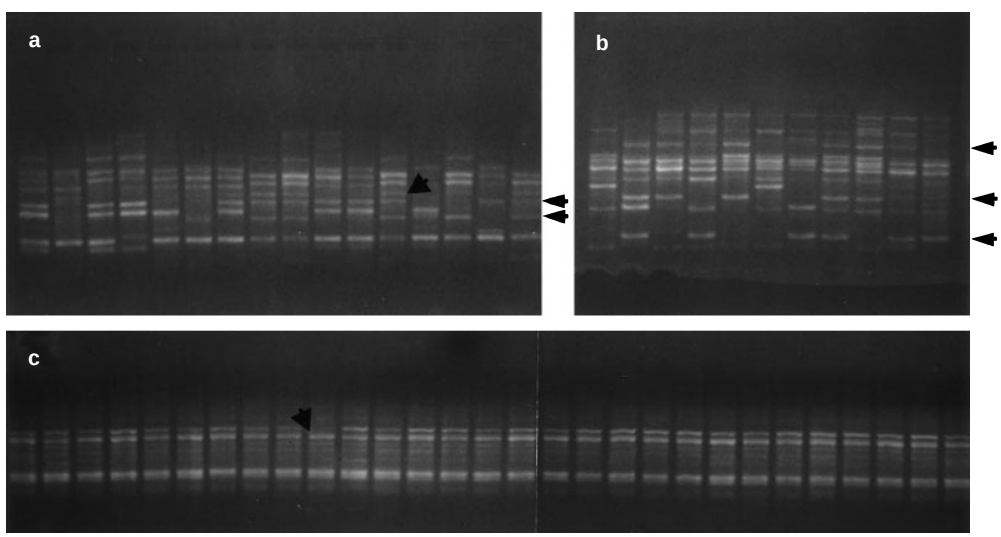

Fig. 2a, b Aberrant (hybrid) RAPD fingerprints of selected individuals of *P. pratensis* progenies S2 × RS20 (**a**) and S1 × S2 (**b**). **c** Maternal RAPD fingerprints of selected individuals of *P. pratensis* progeny RS7 × MM12. *Arrows* indicate maternal and/or paternal bands that discriminate aberrant profiles

progeny plants. The regression line between DNA content and chromosome number was estimated by counting metaphase plates in the root apical meristems of plants S2 and RS9, which had 44 and 56 chromosomes and scored 5.04 and 6.54 pg of nuclear DNA, respectively (Fig. 3). The lowest nuclear 2C DNA content was registered in plant MM12 (4.76 pg), while the highest was documented in plant S4 (6.69 pg), values which correspond to about 40 and 58 chromosomes, respectively (Fig. 3). Previous FCM analysis conducted on plant S2 permitted two distinct peaks corresponding to different ploidy levels to be revealed in somatic tissues: in addition to the diploid peak, a hypodiploid peak was observed with a nuclear DNA content of 2.74 pg (Fig. 4). This variable nuclear condition was reconcilable to the phenomenon of aneusomaty already documented in *P. pratensis* (Speckmann and van Dijk 1972).

Maternal plants of apomictic origin, aberrant plants such as hybrids, and (poly)haploids were unambiguously identified by FCM analysis (Table 2). The vast majority of plants classified as maternal and B_{II} hybrids on the basis of RAPD fingerprints displayed, respectively, nuclear DNA contents comparable to the seed parent or intermediate between the parents (Table 2). Some anomalous ploidy levels were also found in the progenies where parent S2 was involved. In the cross combinations S1 × S2, S2 × S1, and S2 × RS20, 12 out of 24 progeny plants analysed had a ploidy level significantly lower than that expected for B_{II} hybrids. Such ploidy was explainable by the involvement of gametes produced by a hypodiploid branch of plant S2. We have adopted and expanded the nomenclature system by including B_I hybrids to refer to plants from cross-fertilization with a hypodiploid nuclear condition

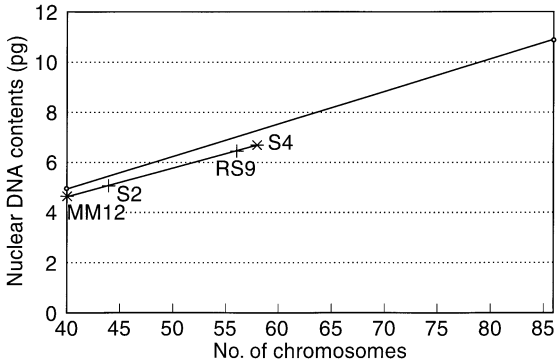

Fig. 3 Regression line between nuclear DNA content (pg) and number of chromosomes, as reported by Huff and Bara (1993) (○), as estimated in the present study (+) and derived (∗) using the regression equation

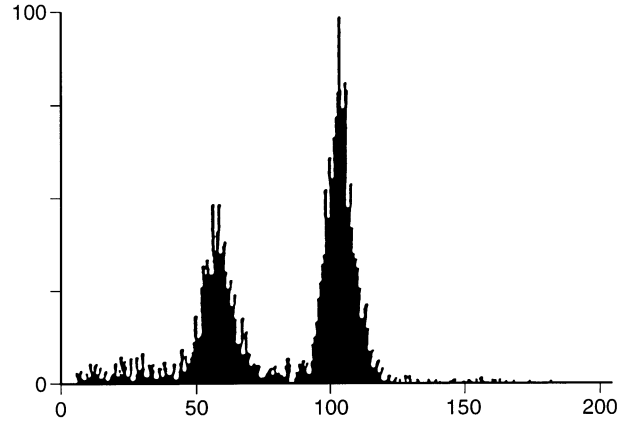

Fig. 4 FCM histograms produced by the aneusomatic plant S2: the higher peak corresponds to normal diploid nuclear DNA content (5.04 pg); the lower one to a hypodiploid nuclear DNA content (2.74 pg). The analysis of metaphases of diploid root apical meristems revealed 44 chromosomes; the number of chromosomes of hypodiploid vegetative apical meristems was proportionally determined to be 26

(Table 2; Fig. 5d). The nuclear DNA content of the remaining progeny plants was typical of B_{II} hybrids (Table 2; Fig. 5c). Of the 3 plants classified as putative B_{III} hybrids according to RAPD analysis, 1 proved to be a B_{II} hybrid after FCM analysis. Moreover, FCM analysis disclosed 5 other plants with the B_{III} hybrid complement (Fig. 6d–f); 4 had been scored as B_{II} hybrids and 1 as maternal by RAPD analysis. Among the non-hybrids, 3 plants displayed maternal ploidy level at FCM analysis and, in consequence, were classified as selfs; another exhibited the (poly)haploid complement (Fig. 6a–c). The other (poly)haploid plant identified by FCM analysis had been documented as maternal by RAPD analysis.

Mean deviation of observed from expected values in terms of nuclear DNA content was inside the confidence limits estimated as the reference. A little discrepancy between observed and expected values was documented in progenies RS9 × S1, its reciprocal S1 × RS9, and S4 × RS18 where all crossed plants had a nuclear DNA content lower than, respectively, B_{III} and B_{II} hybrid constitutions (Table 2).

Table 2 Results from the evaluation of nuclear 2C DNA contents of seed and pollen parents and progenies by FCM analysis: mean observed (pg ± SE), expected values, and calculated mean deviation

Cross-combination	Number of plants	Nuclear DNA content (pg)		Genetic origin and no. of progeny plants		Nuclear DNA content (pg)		
		Seed parent	Pollen parent			Observed (± SE)	Expected	Deviation[a]
S1 × S2	9	5.61	2.74–5.04	B_{II} hybrid	3	5.07 ± 0.42	5.33	0.48
				B_I hybrid	6	3.77 ± 0.08	4.16	0.39
S2 × S1	9	2.74–5.04	5.61	B_{II} hybrid	5	5.06 ± 0.12	5.33	0.35
				B_I hybrid	4	3.58 ± 0.12	4.16	0.58
S1 × RS9	5	5.61	6.54	B_{II} hybrid	3	6.06 ± 0.25	6.08	0.47
				B_{III} hybrid	2	7.63 ± 0.02	8.88	1.26
RS9 × S1	6	6.54	5.61	Maternal	3	6.52 ± 0.23	6.54	0.30
				B_{III} hybrid	3	7.83 ± 0.42	8.88	1.81
S2 × RS20	6	2.74–5.04	5.18	B_{II} hybrid	4	5.21 ± 0.15	5.11	0.27
				B_I hybrid	2	4.25 ± 0.22	3.96	0.32
S4 × RS18	4	6.69	6.26	B_{II} hybrid	4	5.49 ± 0.13	6.48	1.19
RS18 × S4	4	6.26	6.69	Maternal	2	6.34 ± 0.13	6.26	0.13
				B_{III} hybrid	2	9.57 ± 0.17	9.61	0.17
MM12 × RS7	10	4.76	5.89	Maternal	4	4.92 ± 0.15	4.76	0.23
				Selfed	3	4.65 ± 0.14	4.76	0.16
				B_{II} hybrid	3	5.18 ± 0.09	5.33	0.15
RS7 × MM12	6	5.89	4.76	Maternal	2	5.60 ± 0.19	5.89	0.30
				Haploid	2	2.99 ± 0.15	2.95	0.14
				B_{II} hybrid	2	5.81 ± 0.24	5.33	0.49

[a] Confidence interval estimated on a reference sample is ± 0.54 pg

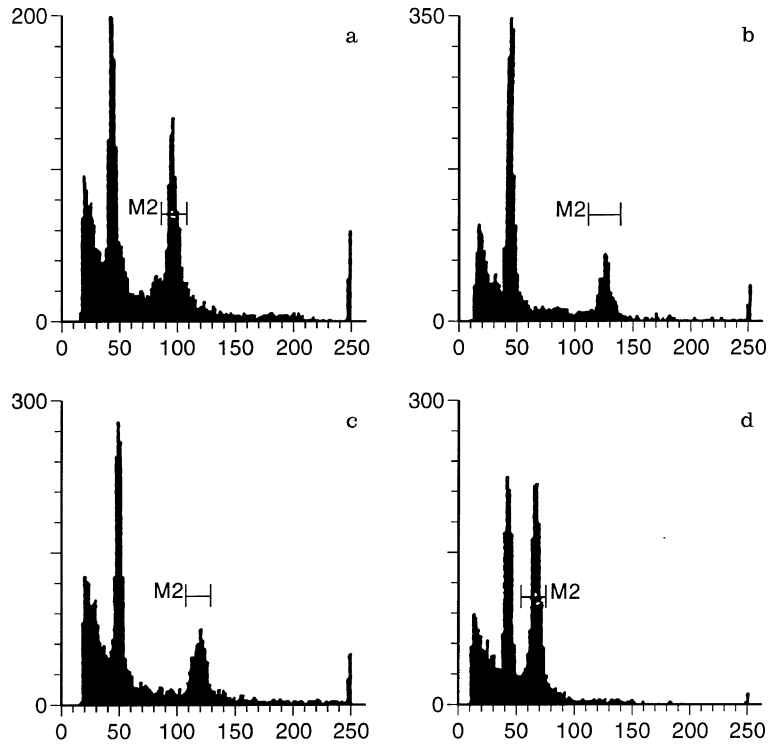

Fig. 5a–d FCM histograms of sexual parents S2 (**a**) and S1 (**b**) and two progeny plants, one B_{II} hybrid (**c**) and one B_I hybrid (**d**). DNA integral fluorescence intensities are shown on 256 channel histograms; *Poa* sample nuclei are reported as the *M2* peak. The peak corresponding to CRBC nuclei (internal standard) is positioned approximatively at channel 50

Discussion

Sexual, non-parthenogenetic plants completely lacked aposporic activity in ovules. As expected, apomictic plants were highly aposporic and parthenogenetic. Therefore, the cytoembryological steps leading to apomixis appeared strictly associated in *P. pratensis*.

The RAPD analysis was an efficient screening tool for discriminating maternal plants and recognizing the hybrid constitution of aberrant plants. The use of this type of molecular marker required preliminary characterization of both seed and pollen parents in order to select primers that give DNA polymorphisms and thus originate maternal- and paternal-specific amplification products detectable in the RAPD fingerprints of progenies. All progeny plants of sexual female parents arose from cross-fertilization (B_I and B_{II} hybrids). In contrast, not only maternals, but also plants from selfing, haploid parthenogenesis, and cross-fertilization (B_{II} and B_{III} hybrids) were found in the progenies of apomictic female parents. The FCM analysis confirmed most of the RAPD data, although discrepancies were found between hybrids of the B_{II} and B_{III} types. However, all plants scored as from sexual hybridization at genomic DNA fingerprinting were revealed to have hybrid nuclear DNA contents.

An internal reference standard has to be used to avoid bias due to staining and instrumental changes when estimating nuclear DNA ploidy by flow cytometric analysis (Vindelov et al. 1983). CRBC nuclei were selected as this reference because their DNA content permits a fair discrimination of different ploidy levels in *Poa*. Since the nuclear DNA content of G0/G1 cells models the ploidy status of a plant tissue, the chopping technique and FCM analysis with reference standards may be used for rapid estimation of the ploidy level in plants (Arumuganathan and Earle 1991). FCM analysis proved to be useful for rapid and convenient determination of nuclear DNA contents in *P. pratensis*. The discrepancies between observed and expected values in some hybrid constitutions could indicate the occurrence of aneuploids by chromosome elimination. For aneuploidy assessment, a high quality nuclei isolation procedure should be coupled with high-resolution FCM to permit the differentiation of DNA content changes lower than 2%, as already shown in wheat (Pfosser et al. 1995). This holds true especially for plants where classical karyological techniques are difficult to perform, as is the case in sugar beet or banana (De Laat et al. 1987; Doležel 1991) and Kentucky bluegrass. The non-destructive FCM method should find applications in various areas of basic and applied research in *Poa*, ranging from taxonomy to biotechnology, and also support molecular marker-aided breeding programmes.

It is known that ploidy levels may change in *P. pratensis* during plant development and over generations, since the chromosome number of vegetative meristems often varies due to cytological aberrations of

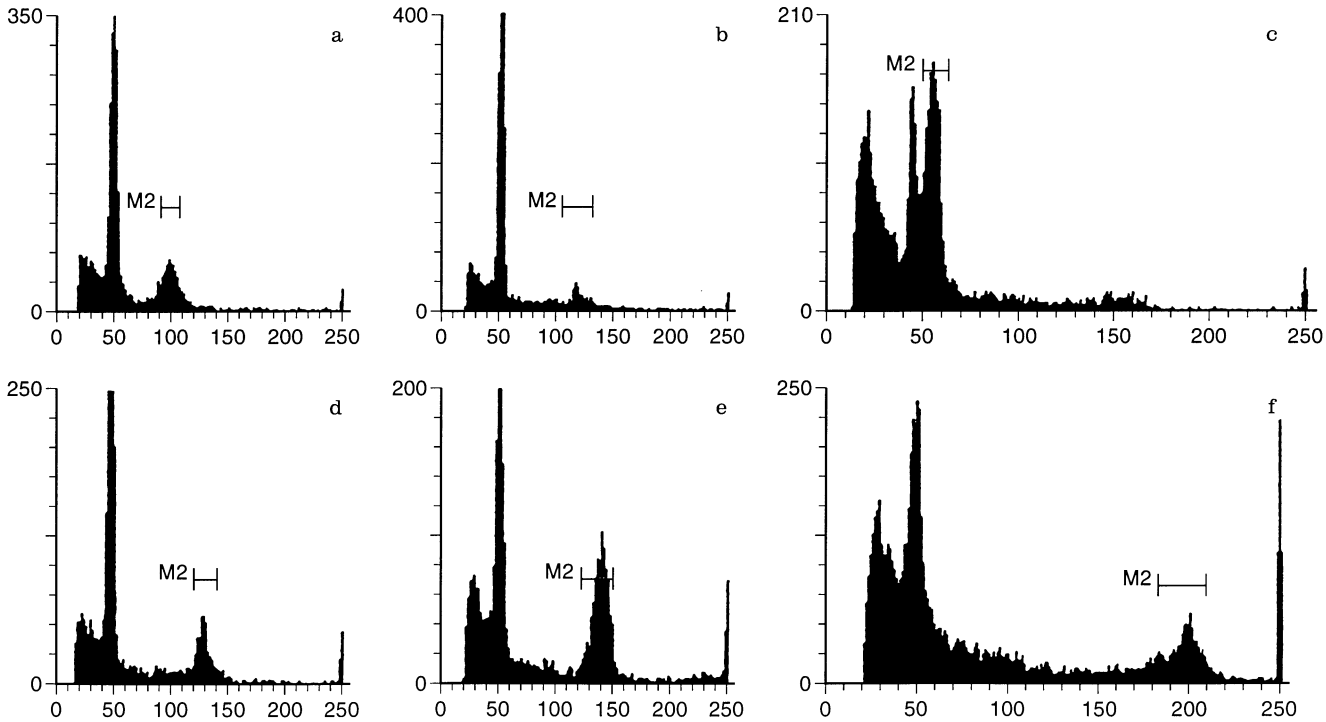

Fig. 6a–f FCM histograms of apomictic parents MM12 (**a**) and RS7 (**b**) and one (poly)haploid (**c**) of the progeny MM12 × RS7; FCM histograms of apomictic and sexual parents RS18 (**d**) and S4 (**e**) and one B_{III} hybrid (**f**) of the progeny RS18 × S4

mitosis (D'Amato 1995). Thus, the chromosome number of gametes produced by a given genotype may be variable. Speckman and van Dijk (1972) studied *P. pratensis* collected in various places and found that the vast majority of plants within a given progeny were aneusomatic, with an average variation of 4.7 chromosomes and a maximum of 9. Aneusomaty, with an estimated deviation of 18–20 chromosomes, was documented in plant S2, characterized by sexual reproduction, where the normal nuclear condition included 44 chromosomes. Moreover, its progeny manifested two distinct classes of ploidy, thereby demonstrating that the two types of gametes were functional and so effective in producing viable seed through allogamous fertilization. Since plant S2 never displayed the double peak of nuclear DNA content when the basal part of stems was used for FCM analysis, aneusomaty could have originated directly in the apical meristems through such mitotic mechanisms as nondisjunction of chromosomes, precocious separation of chromatids, or lagging chromosomes (reviewed by D'Amato 1995).

On the whole, the application of these techniques demonstrates that FCM accurately distinguishes the ploidy levels of haploid, selfed, and B_{II} and B_{III} hybrid progenies, and that RAPD markers unequivocally recognize apomictic and crossed progenies. The combination of RAPD and FCM techniques should, therefore, provide an efficient screening tool of progenies for scoring maternal plants, assessing the genetic origin of aberrant plants, and quantifying the contribution of parental genomes. Since segregation of the mode of reproduction was proven in hybrid populations of *P. pratensis* (Matzk 1991), hybrid populations from 'sexual × apomictic' matings which segregate for the mode of reproduction represent a valuable basis for attempting to identify DNA markers linked with the apomixis gene(s) by bulked segregant analysis (Michelmore et al. 1991).

The targeting of the gene(s) encoding for apomictic reproduction would increase the chances of cloning and transferring this valuable genetic trait and, at the same time, offer new perspectives to plant breeding.

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