Microsporogenesis of Alfalfa Cultivars and Selected Genotypes II

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Abbreviations: PMCs, pollen mother cells.

ABSTRACT

The degree of regularity at each stage of microsporogenesis in alfalfa (2n=4x=32) was studied to understand what is typical in alfalfa cultivars. Materials included 11 'Vernal', four 'Arrow', and three 'WISFAL' plants, an elite clone P from 'Blazer XL', and a male sterile clone 6-4ms developed from 'Saranac'. Regular meiotic stages predominated in all plants, and they had similar meiotic profiles; however, every plant also had some degree of irregularity. Univalents were observed in about 15% of the PMCs in all plants. The various ways univalents behaved throughout meiosis caused the main irregularities. The basic mechanism producing univalents was disassociation of some multivalents between pachytene and metaphase I. When present at diakinesis there was mainly one ring or chain quadrivalent, and when still present at metaphase I the most common type was a chain quadrivalent or thrivalent plus the obligatory univalent. Univalents caused irregularities by lying on or off the division plate in metaphase I, dividing precociously, moving to one division pole, or simply remaining at the cell equator at anaphase I. Bivalents occasionally lagged in anaphase I generating subsequent irregularities as well. Rare bridges at anaphase I and anaphase II were interpreted as due to delayed chromatid separation from chromosomes that were either bivalents or univalents in earlier stages. All types of irregularities resulted in tetrads with micronuclei containing microspores, and microspores and pollen grains of varying sizes. Thus, the tetrad stage was a defining stage in terms of degree of meiotic irregularity. Although pollen was a good indicator of male fertility, it was considered less defining because some plants that had lost chromatin in micronuclei had pollen that appeared normal.

INTRODUCTION

Cultivated alfalfa *Medicago sativa* behaves as an autotetraploid (2n=4x=32) (Stanford et al., 1972). The four homologous chromosomes pair mainly as bivalents, with fewer than one quadrivalent per cell on the average in eight independent studies cited by McCoy and Bingham (1988). Tetrasomic genetic ratios (Stanford, 1951; Bingham, 1973; Quiros, 1982) indicates that the bivalent pairing is random and not preferential. Most studies of microsporogenesis in cultivated alfalfa usually described either the early stages of meiosis, or focused on the direct outcomes of tetrads and pollen (Reeves, 1930; Gillies, 1970; Armstrong, 1971; Gillies and Lesins, 1971; Smith and Murphy, 1986). Patterns of irregularity exhibited by the meiotic chromosomes through the duration of the

microsporogenesis have been reported (Atwood and Grun, 1951; Grun, 1951), but have not received major attention until now.

'Vernal' alfalfa was featured in the present study for a number of reasons. Vernal is a benchmark cultivar released in 1953 and still on the market. Moreover it is a parent or grandparent in at least two-thirds of 802 certified varieties released between 1962 and 1998 (NAAIC, http://naaic.org). *Medicago falcata* from a 2x-4x cross and from 'Cossack' was used in the development of Vernal (Barnes et al., 1977). The other cultivars used in this study all have Vernal derivatives in their pedigree and thus have at least a trace of *M. falcata*. A derived tetraploid *M. falcata*, WISFAL, was also examined.

The objective of the present study was to establish a base-line for the degree of meiotic regularity in northern-adapted alfalfa cultivars that have a natural level of fertility. This basic knowledge will be useful in the evaluation of materials after cyclic selection for fertility, and after hybridization with other *Medicago* species.

MATERIALS AND METHODS

Vernal plants used in the study were grown from certified seed and sampled in the year of establishment (Graber, 1956). Thus, natural selection was minimal. The complex pedigree of Vernal is discussed in the introduction. 'Arrow' is a synthetic cultivar the parents of which contained at least 6% Vernal. Methods were the same as for Vernal. The following selected clones and plants were also examined. Clone 'P' of synthetic variety 'Blazer XL' was selected several years ago as outstanding in vigor, self fertility, and self progeny performance. In addition, clone P is heterozygous (duplex) for the cauliflower head-simple leaf trait. The male sterile clone '6-4ms' was obtained from crosses within 'Saranac' alfalfa (Murphy and Lowe, 1966) and has been maintained by cuttings for more than 40 years. It is known that it has uniparental transmission of the mitochondrial genome, and biparental transmission of the chloroplast genome (Forsthoefel et al., 1992). 'WISFAL' is tetraploid *M. falcata* developed from several diploid *M. falcata* plant introductions (Bingham, 1990, 1993).

Flower buds of 11 Vernal alfalfa plants were collected from spaced plants in the field in August 1995. They were fixed in three parts 95% ethanol to one part glacial acetic acid solution for 24 hours and then were stored in 70% ethanol at room temperature. Cytological smear preparations were made according to standard acetocarmine technique. A minimum of 100 pollen mother cells (PMCs) at each stage of meiosis was examined in all 11 Vernal plants. In the other materials fewer than 100 PMCs per stage were sometimes examined, but the total PMCs was more than 100 in all cases. Each type of irregularity was recorded on the basis of PMCs exhibiting it compared to the total number of PMCs examined. Microsporogenesis in Arrow plants, WISFAL plants, and in the selected clones was studied using buds collected in the greenhouse between January and April 1996. Statistical analysis included computation of means, standard deviations (SD), coefficient of variation (CV), Fisher test for equality of variances (F variances), T-test and multiple t-test for equality of means (T multiple). In addition to that, abnormalities observed through the duration of meiosis in the set of Arrow and WISFAL plants were subjected to correlation analysis based upon Bonferrony procedure.

RESULTS

      The characteristics of regular stages of microsporogenesis in tetraploid alfalfa from diakinesis to microspores are shown in Figure 1. A complete set of regular stages in tetraploid alfalfa has not been published previously. Most PMCs in all plants studied had 16 bivalents at diakinesis (Fig. 1a) and metaphase I (Fig. 1b). Similarly, most PMCs were free of lagging chromosomes and chromosome bridges at anaphase I (Fig. 1c), the division spindles were aligned properly at metaphase II (Fig. 1d), and there were no lagging chromosomes at anaphase II (Fig. 1e). Simultaneous cytokinesis following telophase II formed four microspores free of micronuclei (Fig. 1f).

      Although regular stages predominated in all materials, essentially every plant also had some degree of irregularity at all stages. Departures from regular meiosis typical for all materials are featured in Fig. 2 and 3. Photomicrographs are based on Vernal plants and are considered representative of the irregularities in other alfalfa tetraploids. At pachytene, it was difficult to trace the behavioral patterns of the chromosomes due to tight chromosome grouping and overlapping, and no quantitative data were collected. Nonetheless, some abnormalities were photographed including less than perfect pairing (Fig. 2a), an apparent translocation (Fig. 2b), and occasional switching of pairing partners (Fig. 2c). In addition, small loops sometimes were observed which may have resulted from a segment deficiency in one of the pairing partners.

      Diakinesis (Fig. 2d) is also difficult to analyze in alfalfa, but bivalents, quadrivalents, and univalents could be identified. At metaphase I (Fig. 2e), a chain multivalent of three chromosomes accompanied by univalent was common for Vernal plants. A PMC typical of those containing only bivalents and univalents is illustrated in Fig. 2f. Lagging chromosomes at anaphase I (Fig. 2g) were associated with the separation of lagging univalents and bivalents. Univalents that divided precociously at anaphase I (Fig. 2h) did not move toward the cell pole before the second meiotic division started. In that regard, Fig. 3a illustrates chromatin entities positioned off the plate at metaphase II that likely originated from univalents which remained at the cell equator at anaphase I. Bridges at anaphase II (Fig. 3b) were interpreted as due to delayed chromatid separation. Cells with numerous laggards at anaphase II (Fig. 3c) occurred consistently across genotypes. Those laggards accounted for the variation in size of extranuclear chromatin entities at telophase II (Fig. 3d) which proceeded to form micronuclei occurring with tetrads of microspores (Fig. 3e) or contributed to the formation of pollen grains of various sizes (Fig. 3f).

      A large number of PMCs per plant per stage were analyzed to establish the typical chromosome pairing behavior in alfalfa during first meiotic division from diakinesis to anaphase I (Table 1). The multivalent frequency in all plants tended to decrease from diakinesis to metaphase I. Some quadrivalents became two bivalents, a bivalent and two univalents, or occasionally a trivalent and a univalent, apparently due to insufficient crossing over to maintain the original quadrivalent. At metaphase I, chain and ring quadrivalents were observed at about equal frequencies across plants, but no quantitative data were collected. Within Vernal population, the multivalent frequency ranged from 0.11 to 0.28, and each plant had to be considered individually due to highly significant differences between the variances. The average frequency of multivalents at metaphase I

for Arrow and WISFAL populations was 0.14 and 0.16, respectively, that for clone Saranac 6-4ms was 0.27, while in clone Blazer XL-P it was only 0.01. Univalents were recorded in every plant (Table 1) with a range of 0.03 - 0.16 in Vernal, an average of 0.14 in Arrow, 0.17 in WISFAL, 0.07 in clone Blazer XL-P, and a low of 0.02 in clone Saranac 6-4ms. In WISFAL a noticeable correlation r = 0.893, yet not significant at $P = 0.05$ (r = 0.970), between frequencies of multivalents at diakinesis and univalents at metaphase I confirmed the relationship between these irregularities.

Other irregularities associated with chromosome pairing were lagging bivalents and univalents in anaphase I (Table 1). Lagging bivalents were found in 10% or less of the PMCs of any given plant, while the frequency of lagging univalents was consistently higher with means of 0.17 in Vernal, 0.16 in Arrow, 0.30 in WISFAL, 0.22 in Saranac 6-4ms, and as rare as 0.02 in clone Blazer XL-P.

Analysis of second meiotic division was in many cases based on an even larger number of PMCs (Table 2) because the second division is easier to analyze. At metaphase II, every PMC could be easily classified for the presence of mispositioned chromosomes. In Vernal their frequency ranged 0.06 - 0.31, in Arrow and WISFAL it was 0.10 and 0.34, respectively, clone Blaser XL-P had an average of 0.06, and clone Saranac 6-4ms had 0.29. At anaphase II, lagging chromosomes again ranged 0.06 - 0.45, predominantly due to the presence of univalents and their erratic behavior at first meiotic division. This interpretation is supported by strong correlation, r = 0.992 at $P = 0.05$, between the frequency of lagging univalents at anaphase I and mispositioned chromosomes at metaphase II in WISFAL, for example.

The frequencies of lagging chromosomes at anaphase II and micronuclei at tetrad stage are similar in each respective plant with the exception of male sterile clone Saranac 6-4ms where the frequency of metaphase irregularities rose sharply, probably due to the onset of male sterility. The frequency of tetrads with micronuclei was as low as 0.06 in Vernal-36 and as high as 0.44 in Vernal-11 (Table 2). In Arrow plants the mean frequency was 0.17, while in WISFAL it was 0.29. That difference between the means of the two populations was highly significant, T = 11.43. Clone Blazer XL-P had a frequency of 0.10, while the male sterile clone Saranac 6-4ms had a high frequency of 0.69. Interestingly, in Arrow and WISFAL populations the correlations between tetrads with micronuclei containing microspores and lagging chromosomes at anaphase II was highly significant at a $P = 0.05$, r = 0.997 and r = 0.978, respectively.

A graph of the degrees of regularity of microsporogenesis of the various tetraploid alfalfa materials, Fig. 4, provides profiles of the materials but does not establish cultivar differences because of the different sample sizes. Arrow started at 0.75 level of regularity and slightly improved toward the end of meiosis, 0.83, with some frequency fluctuations on the way, between 0.72 and 0.90. WISFAL is tetraploid recently developed from diploid *Medicago falcata* and had the highest frequencies of irregularities at each stage among the materials. Clone BlazerXL-P selected for its excellent reproductive characteristics was the most regular one with recorded frequencies always around the 0.90 mark. Saranac 6-4ms was selected for its stable male sterility, and was as regular as other materials until anaphase II, at which time irregularities increased probably related to the onset of male sterility, and the eventual failure of pollen formation. The overall meiotic behavior of Vernal plants represented by the dotted line, was intermediate to the other plants in the study.

DISCUSSION

Alfalfa chromosomes are described as short and subtelocentric with 2.26 average arm ratio (Gilles, 1970; Gilles and Lesins, 1971). According to Stanford et al. (1972), these features could limit chiasma formation, especially in the shorter chromosome arm. The disassociation of some quadrivalents between pachytene and metaphase I as reported by Armstrong (1971) and in this study, is evidence of insufficient chiasma formation to maintain quadrivalents. The disassociation of quadrivalents resulted in mostly bivalents as well as univalents supporting the conclusion that alfalfa is a bivalent-forming autotetraploid (Stanford et al., 1972; McCoy and Bingham, 1988). Importantly, univalents also occurred in some meiocytes of every plant.

Atwood and Grun (1951), and Armstrong (1954, 1971) reported multivalent frequencies ranging from 0.21 to 0.89 per cell in plants from cultivars. The same general range was found in this study with an average of 0.29 at diakinesis and 0.16 at metaphase I. Interestingly, this was also the frequency of multivalents observed in colchicine-doubled cultivated diploids (Obajimi and Bingham, 1973). That of the derived tetraploid WISFAL was slightly higher, 0.37, but still in the same range.

Univalents appeared to be the principal cause of irregularities throughout the stages of microsporogenesis in alfalfa in this and previous studies by Julen (1944), Atwood and Grun (1951), Grun (1951), Armstrong (1971), and Smith and Murphy (1986). One univalent is always produced when there is insufficient crossover to hold a bivalent together. Our investigations on populations of Vernal and Arrow revealed univalent frequency of about 0.11 at metaphase I, and 0.17 at anaphase I. Considering all materials in this study, univalents were observed in about 15% of all the PMCs. Results from four Arrow and three WISFAL genotypes also indicated strong correlations between the frequency of PMCs with lagging univalents at anaphase I, mispositioned chromosomes at metaphase II, and lagging chromosomes at anaphase II. Furthermore, for both populations there was a highly significant correlation between the frequency of PMCs with lagging chromosomes at anaphase II and the tetrads containing microspores with micronuclei. A similar pattern was detected in the population of 11 Vernal plants. The tetrad stage was considered more useful than pollen in evaluating regularity of microsporogenesis because some plants that had micronuclei in the tetrad stage went on to produce pollen that appeared normal in size and stainability.

A comparison of degree of regularity exhibited throughout microsporogenesis in Vernal, Arrow, WISFAL, and the individual clones Blazer XL-P and Saranac male sterile 6-4ms indicates that they have similar meiotic profiles. However, clone Blazer XL-P which was selected as a very fertile clone was consistently the most regular of all entries in the study. The derived tetraploid nature of WISFAL probably accounted for the high frequency of lagging univalents, and, consequently, the mispositioned chromosomes at metaphase II. Toward the end of microsporogenesis, the PMCs of Saranac male sterile clone 6-4ms were the most irregular, likely reflecting the expression of cytoplasmic male-sterility.

Regularity of microsporogenesis in alfalfa appears to be a matter of degree, with even the most regular plant containing occasional univalents and microspores with micronuclei. As discussed by Smith and Murphy (1986), a threshold level of irregularity

apparently must be reached before fertility is significantly affected. In the Vernal and Arrow populations, plants with a similar background of meiotic irregularities ranged from nearly self-sterile to quite self-fertile (data not reported). This indicated that genetic factors affecting other fertility parameters had a greater impact on fertility than the range of meiotic regularity observed in this study. Smith and Murphy (1986) suggested that when selection is practiced for improved fertility, meiotic regularity is improved to a much smaller degree. In fact this is what has been observed in an ongoing analysis of the materials in the present study after cycles of selection for improved fertility (Dilkova and Bingham, 2003, unpublished).

What is the impact of univalents and associated irregularities? Aneuploidy is one consequence, and 4 - 5% aneuploidy has been reported in alfalfa over the years (Stanford, 1959; Bingham, 1968, and unpublished; and Bauchan et al., 2002). Aneuploidy in turn can mimic double reduction (Bingham, 1968), and could exaggerate inbreeding depression.

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Table 1. Chromosome pairing irregularities and their average frequency during first division of meiosis in PMCs from 11 Vernal plants, four Arrow plants, clone Blazer XL-P, male sterile Saranac clone 6-4ms, and three WISFAL plants.

Plant ID	PMCs †	Diakinesis	Metaphase I		Anaphase I	
	in Meiosis I	Multivalents	Multivalents	Univalents	Lagging bivalents	Lagging univalents
Vernal- 4	336	0.29	0.19	0.07	0.08	0.09
Vernal- 7	324	0.36	0.14	0.11	0.09	0.16
Vernal-11	411	0.30	0.13	0.09	0.06	0.21
Vernal-18	332	0.37	0.25	0.07	0.06	0.24
Vernal-20	348	0.34	0.11	0.16	0.08	0.20
Vernal-27	467	0.35	0.24	0.03	0.06	0.15
Vernal-36	343	0.31	0.16	0.10	0.03	0.12
Vernal-41	397	0.33	0.12	0.05	0.04	0.19
Vernal-43	484	0.36	0.20	0.07	0.01	0.25
Vernal-45	326	0.30	0.10	0.05	0.005	0.11
Vernal-46	342	0.49	0.28	0.07	0.07	0.18
Mean		0.34	0.17	0.08	0.04	0.17
F variances ‡		2.86**	4.05***	2.22*	1.46	1.09
T multiple §		-	-	-	0.38	0.42
Arrow- 1	298	0.25	0.19	0.09	0.00	0.12
Arrow- 6	230	0.19	0.13	0.12	0.02	0.19
Arrow-18	135	0.26	0.10	0.20	0.04	0.17
Arrow-24	150	0.30	0.14	0.13	0.00	0.15
Mean		0.25	0.14	0.14	0.01	0.16
F variances		1.00	1.93	2.88	0.81	0.33
T multiple		0.57	0.51	0.70	1.08	0.49
Blazer XL-P	507	0.16	0.01	0.07	0.00	0.02
SD ¶		0.01	0.01	0.04	0.00	0.02
CV #		0.06	1.00	0.57	0.00	1.00
Saranac 6-4ms	123	0.38	0.27	0.02	0.00	0.22
SD		0.13	0.09	0.02	0.00	0.12
CV		0.34	0.33	1.00	0.00	0.55
WISFAL- 1	237	0.43	0.16	0.20	0.14	0.37
WISFAL- 2	228	0.32	0.21	0.12	0.10	0.23
WISFAL- 6	174	0.36	0.10	0.19	0.05	0.29
Mean		0.37	0.16	0.17	0.10	0.30
F variances		1.08	0.55	0.68	2.10	1.01
T multiple		0.80	0.23	0.33	1.07	0.50

* Evidence of significant differences at probability level $P = 0.05$.
** Evidence of significant differences at probability level $P = 0.01$.
*** Evidence of significant differences at probability level $P = 0.001$.
† PMCs, number of examined pollen mother cells.
‡ F variances, Fisher test for equality of variances.

§ T multiple, multiple t-test for equality of means.
¶ SD, standard deviation.
# CV, coefficient of variation.

Table 2. Chromosome irregularities and their frequencies during second division of meiosis in PMCs from 11 Vernal plants, four Arrow plants, clone Blazer XL-P, male sterile Saranac clone 6-4ms, and three WISFAL plants.

Plant ID	PMCs †	Metaphase II	Anaphase II	Tetrads
	in Meiosis II	Mispositioned chromosomes	Lagging chromosomes	Micronuclei
Vernal- 4	477	0.12	0.22	0.16
Vernal- 7	436	0.25	0.25	0.19
Vernal-11	487	0.31	0.45	0.44
Vernal-18	363	0.29	0.25	0.20
Vernal-20	416	0.22	0.37	0.31
Vernal-27	414	0.23	0.15	0.17
Vernal-36	390	0.09	0.15	0.06
Vernal-41	437	0.21	0.20	0.16
Vernal-43	416	0.16	0.16	0.19
Vernal-45	433	0.06	0.09	0.10
Vernal-46	406	0.20	0.14	0.08
Mean		0.20	0.22	0.19
F variances ‡		5.32***	14.30***	26.83***
Arrow- 1	334	0.05	0.15	0.23
Arrow- 6	348	0.21	0.17	0.17
Arrow-18	254	0.08	0.18	0.16
Arrow-24	150	0.10	0.22	0.12
Mean		0.10	0.18	0.17
F variances		1.96	0.18	0.60
T multiple §		1.13	0.10	0.27
Blazer XL-P	772	0.06	0.10	0.10
SD ¶		0.06	0.04	0.02
CV #		1.00	0.40	0.20
Saranac 6-4ms	284	0.29	0.54	0.69
SD		0.01	0.11	0.04
CV		0.03	0.20	0.06
WISFAL-1	258	0.42	0.32	0.29
WISFAL-2	282	0.28	0.45	0.32
WISFAL-6	390	0.33	0.25	0.25
Mean		0.34	0.34	0.29
F variances		4.49	4.24	3.23
T multiple		1.65	0.98	0.99

*** Evidence of significant differences at probability level P = 0.001.

† PMCs, number of examined pollen mother cells.

‡ F variances, Fisher test for equality of variances.

§ T multiple, multiple t-test for equality of means.

¶ SD, standard deviation.

CV, coefficient of variation.

Fig. 1. Regular meiosis in PMCs from cultivar Vernal: a. Late prophase showing 16 bivalents (Vernal-18); b. Metaphase I (Vernal-41); c. Late anaphase I (Vernal-46); d. Metaphase II (Vernal-43); e. Anaphase II (Vernal-27); f. Association of four microspores (Vernal-43); (100/1.32).
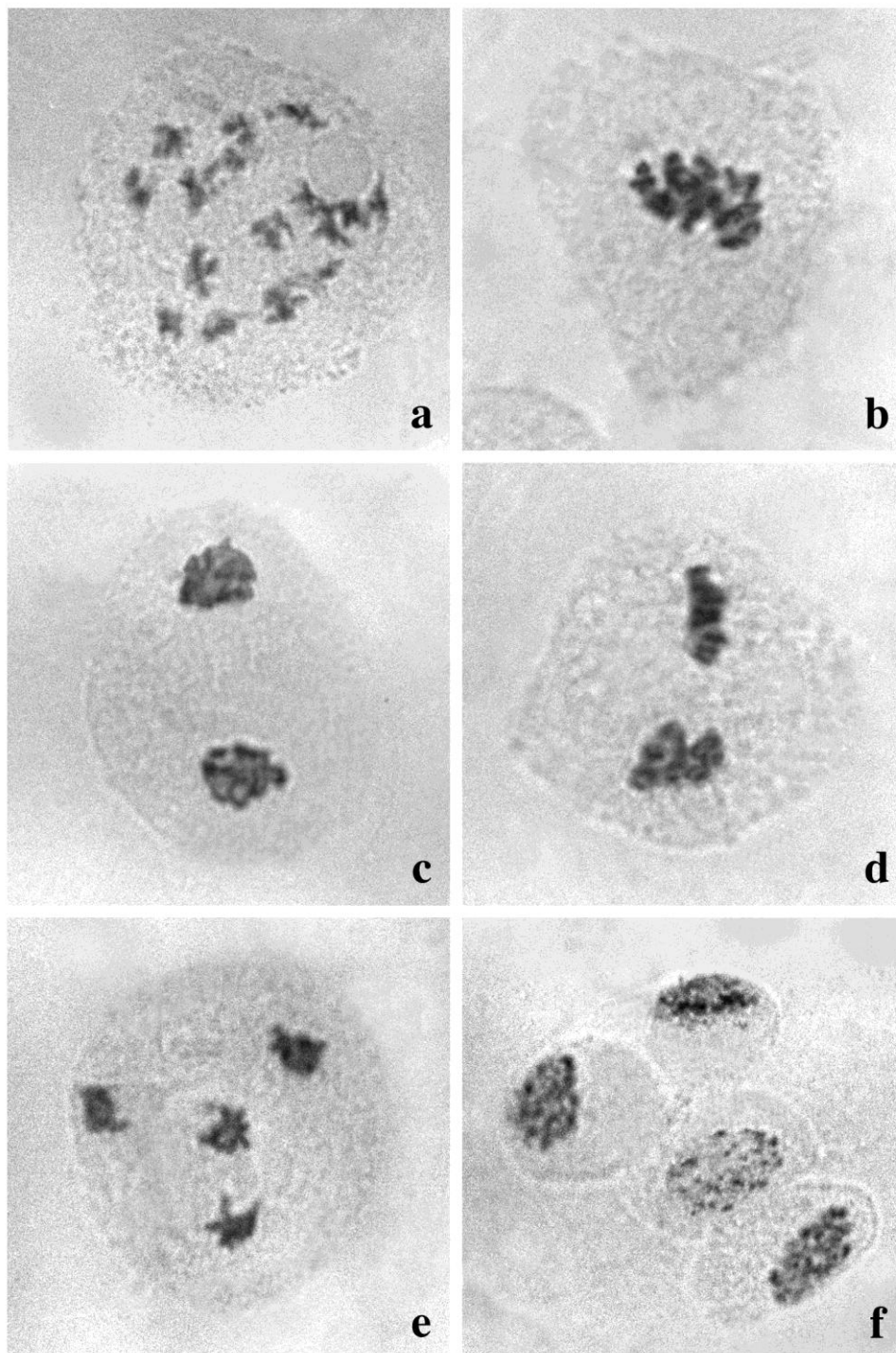
Fig. 2. First meiotic division in PMCs from cultivar Vernal featuring various patterns of chromosome pairing: a. Pachytene chromosomes with a less than perfect pairing, arrow (Vernal-20); b. Pachytene chromosomes involved in an apparent translocation, arrow (Vernal-20); c. Pachytene chromosomes engaged in a double nonhomologous arm exchange, arrow (Vernal-7); d. Late prophase chromosomes, bivalent and quadrivalent associations, arrow (Vernal-11); e. Metaphase I with multivalent and univalent (Vernal-7); f. Early metaphase I with two univalents (Vernal-43); g. Late anaphase I with multiple laggards undergoing delayed division (Vernal-20); h. Telophase I with univalents remaining at the cell equator (Vernal-4); (100/1.32).
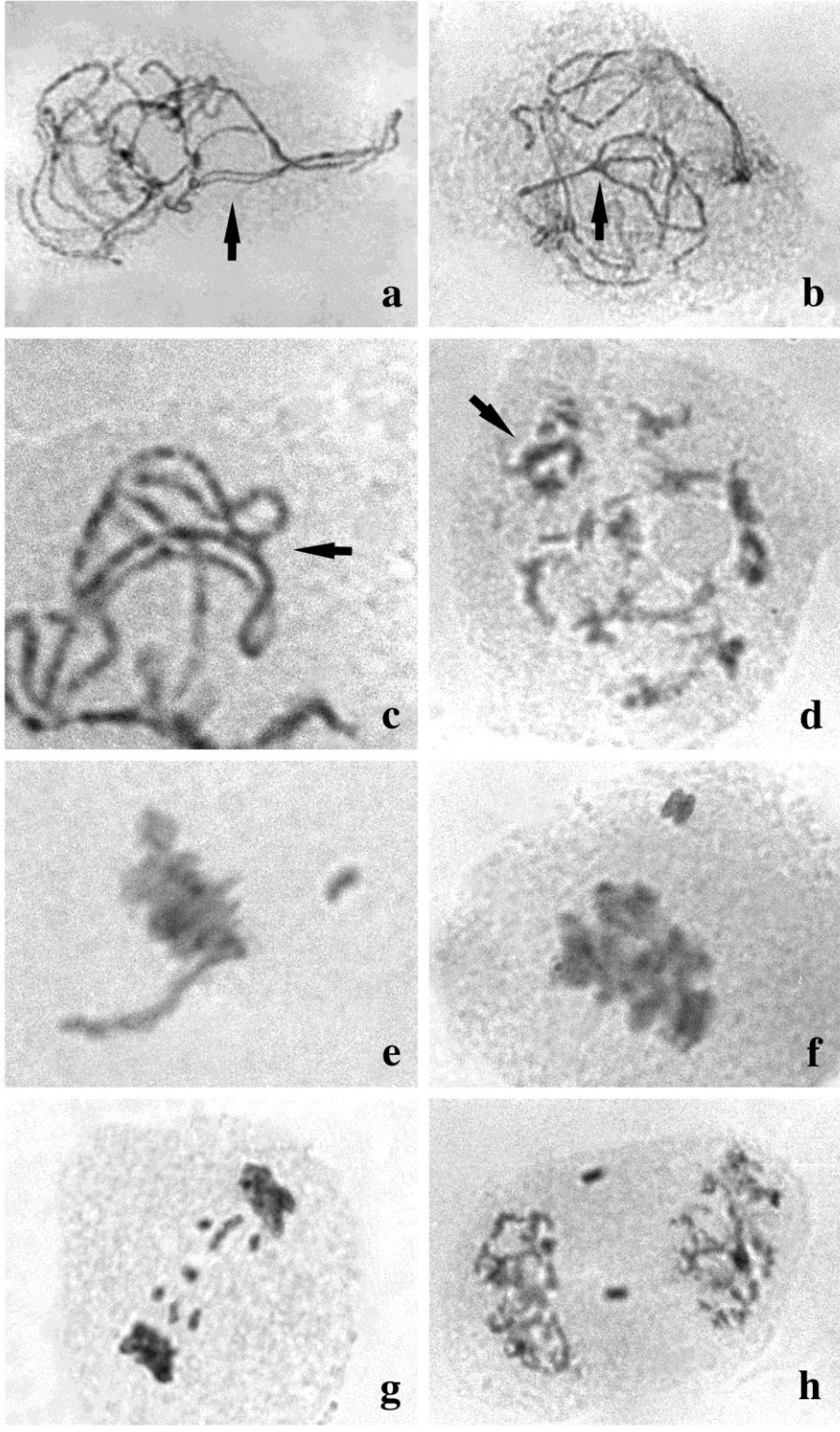
Fig. 3. Second meiotic division irregularities in PMCs from cultivar Vernal: a. Metaphase II with chromosomes positioned distantly of the metaphase plate, arrows (Vernal-43); b. Anaphase II with a chromosomal bridge (Vernal-20); c. Late anaphase II with numerous laggards (Vernal-11); d. Late telophase II with laggards associated in a separate chromatin body, arrow (Vernal-43); e. Microspores of various sizes and a micronucleus, arrow (Vernal-7); f. Pollen grains of heterogeneous size and stainability (Vernal-18); (100/1.32).
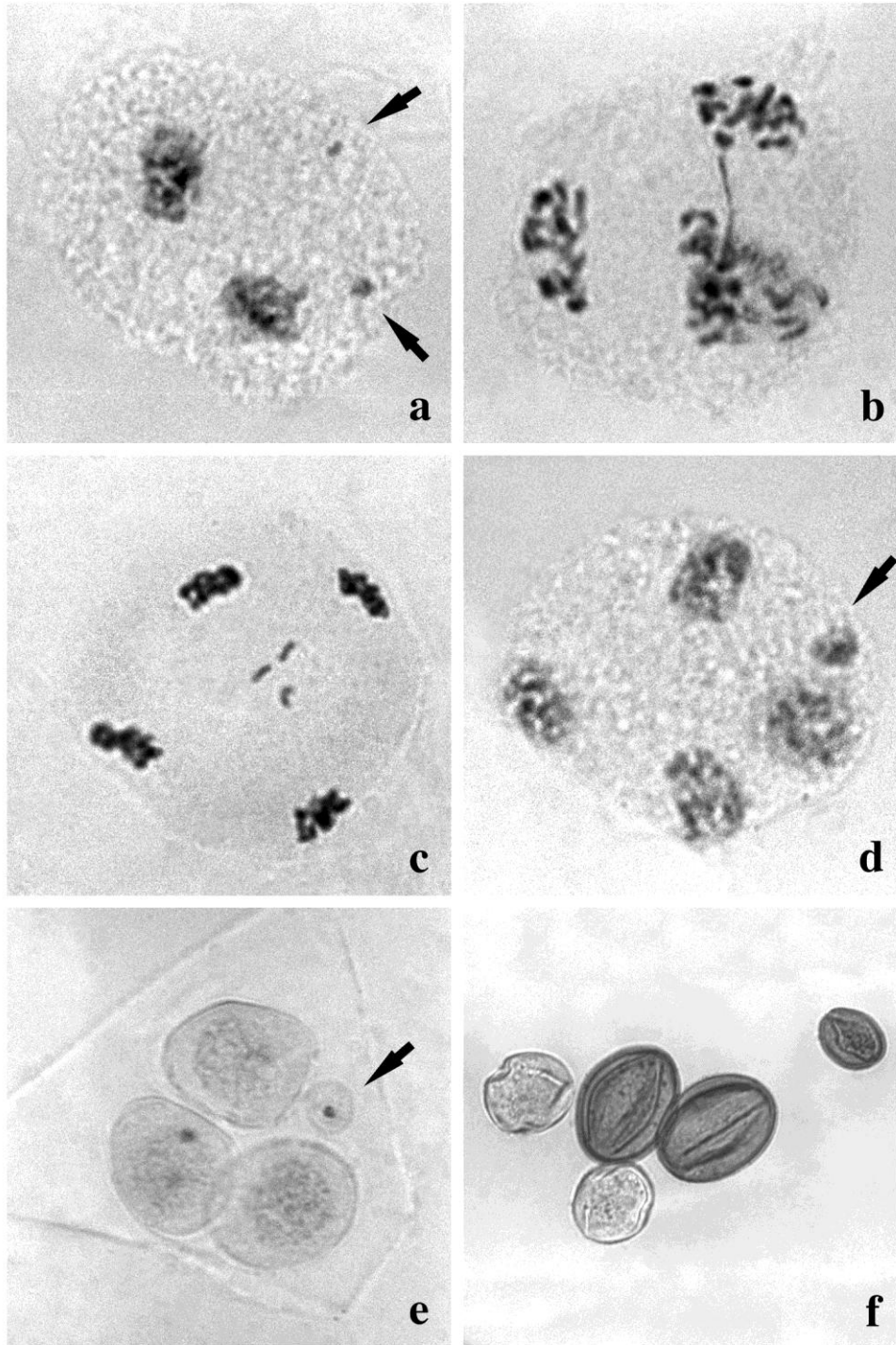
Fig. 4. Meiotic profiles of populations of Arrow and WISFAL plants, and clones Blazer XL-P and male sterile Saranac 6-4ms based on data from PMCs with regularly progressing division. The dotted line features a generalized profile of cultivar Vernal, where the variation among individual plants was significant.