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Half tetrad analysis in alfalfa using multiple restriction fragment length polymorphism markers

(centromere mapping/2*n* gametes/maximum likelihood)

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ABSTRACT **A maximum likelihood approach of half tetrad analysis (HTA) based on multiple restriction fragment length polymorphism (RFLP) markers was developed. This procedure estimates the relative frequencies of 2*n* gametes produced by mechanisms genetically equivalent to first division restitution (FDR) or second division restitution and simultaneously locates the centromere within a linkage group of RFLP marker loci. The method was applied to the diploid alfalfa clone PG-F9 (2*n* = 2x = 16) previously selected because of its high frequency of 2*n* egg production. HTA was based on four RFLP loci for which PG-F9 was heterozygous with codominant alleles that were absent in the tetraploid tester. Models including three linked and one unlinked RFLP loci were developed and tested. Results of the HTA showed that PG-F9 produced 6% FDR and 94% second division restitution 2*n* eggs. Information from a marker locus belonging to one linkage group was used to more precisely locate the centromere on a different linkage group. HTA, together with previous cytological analysis, indicated that in PG-F9, FDR 2*n* eggs are likely produced by diplospory, a mechanism common among apomictic species. The occurrence of FDR 2*n* eggs in plant species and their importance for crop evolution and breeding is discussed together with the potential applicability of multilocus HTA in the study of reproductive mutants.**

In diploid species, half tetrad analysis (HTA) can be carried out when two of the four chromatids of a meiotic tetrad can be recovered and scored for genetic markers heterozygous in the diploid parent. Half tetrads have been used to analyze recombination for attached X chromosomes of *Drosophila melanogaster* (1) and autosomal trisomies of humans (2). First polar bodies and secondary oocytes have been used to analyze first meiotic division products in mammals (3, 4), and mutants producing gametes with somatic chromosome number (2*n* gametes) have been used in plant species such as *Zea mays* L. (5, 6), *Solanum tuberosum* L. (7), and *Medicago sativa* L. (8). These plant mutants have been extensively studied because of their importance in the evolution of polyploid series (9, 10) and their potential use in advanced breeding programs of polysomic polyploids, such as potato (11) and alfalfa.

There are two major modes for 2*n* gamete formation, based on the genetic constitution of the 2*n* gametes: first division restitution (FDR) and second division restitution (SDR) (12). The different cytological mechanisms that can be included in each mode were summarized by Veilleux (10). Nonrecombinant 2*n* gametes due to the absence of crossing-over followed by FDR type 2*n* gamete formation (FDR-NCO 2*n* gametes) would maintain the parental genotype and represent another interesting class of 2*n* gametes, especially for breeding purposes (12).

The theory of HTA using FDR or SDR 2*n* gamete mutants was developed by Mendiburu and Peloquin (7). It involves analyzing tetraploid progeny obtained by crossing a diploid parent that produces 2*n* gametes to a tetraploid parent with normal meiosis. Both codominant and dominant markers can be used, but codominant markers are advantageous because the heterozygous class of 2*n* gametes can be identified (13). HTA can be used for gene-centromere mapping (14), and results from mapping single loci with respect to the centromere have been described for mutants producing 2*n* gametes by one mode, e.g., FDR or SDR, but not both modes (7, 15, 16). Multilocus HTA, based on the analysis of first meiotic division products, has been proposed for use in mammals to locate the centromere within a group of linked marker loci (4), and this method could be applied to plants in which SDR-only mutants have been identified. If a centromeric marker is already available, HTA also can be easily used to determine if FDR and/or SDR 2*n* gametes are produced by one mutant and to estimate the relative frequency of FDR and SDR when both modes are present (6, 17). Thus, HTA is a powerful method for mapping centromeres, or for determining the mode(s) of 2*n* gamete formation. However, a method for determining these factors simultaneously has not been described.

In this paper, a multilocus maximum likelihood method of HTA is presented; this method permits the estimation of both the relative frequencies of FDR and SDR 2*n* gametes and the centromere location within a linkage group without relying on previously identified centromeric markers. This method was applied to the diploid alfalfa mutant PG-F9, which produces mainly SDR 2*n* eggs together with a low frequency of cells developing directly into the female gametophyte without undergoing meiotic divisions (18).

MATERIALS AND METHODS

Plant Materials and Restriction Fragment Length Polymorphism (RFLP) Loci Used for HTA. An RFLP linkage map of PG-F9 was produced at the diploid level using a pseudotestcross strategy to find codominant RFLP markers heterozygous in the mutant and to determine their linkage relationships (19). Subsequently, PG-F9 was crossed as the seed parent with two tetraploid plants of the inbred line MAG7 (20). MAG7 was chosen as the source of pollen because it derives from somatic doubling of a single diploid genotype and, therefore, should have fewer alleles per locus than noninbred tetraploid alfalfa. Use of MAG7 should minimize the possibility of having RFLPs from the pollen parent that comigrate with RFLPs from PG-F9, which could interfere in assigning marker genotypes to 2*n* eggs. A

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Abbreviations: HTA, half tetrad analysis; RFLP, restriction fragment length polymorphism; FDR, first division restitution; SDR, second division restitution; FDR-NCO, nonrecombinant FDR.

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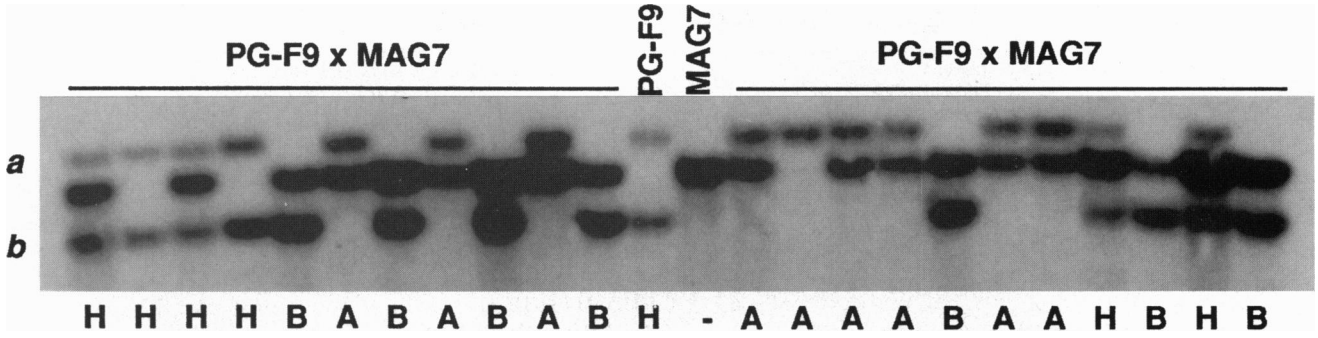

FIG. 1. RFLP patterns for PG-F9, MAG7, and PG-F9 × MAG7 tetraploid progeny probed with *MTSc9*. PG-F9 has two RFLP alleles (*a* and *b*) which are not present in MAG7. A and B designate 2*n* eggs homozygous for RFLP alleles *a* and *b*, respectively; H designates heterozygous 2*n* eggs. Progeny without MAG7 restriction fragments apparently received a null allele from MAG7 and are not parthenogenic because they had restriction fragments from MAG7 for other probes.

population of 164 tetraploid progeny obtained from PG-F9 × MAG7 crosses was analyzed using previously described RFLP procedures (21). The HTA was applied to four RFLP loci at which PG-F9 was heterozygous for codominant RFLP alleles that MAG7 plants lacked. Three of these marker loci (*UWg119*, *MTSc9*, and *UWg65*) were closely linked in linkage group 1, whereas the RFLP locus *UWg69* mapped on linkage group 6 (19, 21).

Scoring Genotypes of 2*n* Egg Cells at the RFLP Marker Loci. For the three linked marker loci, the two alleles from PG-F9 at each locus were coded as *a* and *b*, respectively, and alleles of different loci that were linked in coupling were labeled with the same letter. The linkage phase of RFLP alleles was known from the PG-F9 linkage map developed previously. For each marker locus, PG-F9 2*n* eggs were scored as A or B when they were homozygous *aa* or *bb*, respectively, or as H when they were heterozygous *ab* (Fig. 1). The linkage order *UWg119–MTSc9–UWg65* was based on the PG-F9 map (19). Genotypes of 2*n* egg cells were also scored as A, B, or H for *UWg69* even though the *a* and *b* codes were arbitrarily assigned to the two RFLP alleles, given the independence of *UWg69* from the other three loci.

Likelihood Analysis. Genetic models including SDR and FDR as possible modes of 2*n* egg formation in PG-F9 were developed. Knowledge of the map order for the three marker loci linked on group 1 reduced the number of models to be compared. These models corresponded to the four possible locations of the centromere within this linkage group (Fig. 2). Due to the short map distance covered by these RFLP loci (19), it was assumed that multiple crossovers do not occur (complete chiasma interference) in the region of the marker loci. The frequency of meioses with one crossover in a chromosome region delimited by the centromere and one marker locus or by two marker loci was defined as p_k (k = 1, 2, or 3 for the three linked markers, respectively, and k = 4 for *UWg69*). Genotypic classes of 2*n* eggs showing the same expected frequency were grouped together.

The log likelihood for each possible centromere location can be expressed as follows (the multinomial coefficient has been omitted):

$$\mathrm{Log}(L) = \sum_i n_i \times \mathrm{Log}(G_i),$$

in which n_i is the number of observations for the ith group of genotypic classes and G_i is the probability an individual is in the ith genotypic class group.

The probability G_i can be expressed as follows:

$$G_i = \sum_{r=1} \sum_{m=1} P(g_i \mid m, r) \times P(m) \times P(r),$$

with the sum being over the possible recombination events (r) and modes of 2*n* gamete formation (m = FDR or SDR). $P(g_i \mid m, r)$ is the probability of each genotypic class (g_i) belonging to the ith group given the mth mode of 2*n* gamete formation and the rth recombination event. The probability of the rth recombination event $P(r)$ depends in an obvious way on the crossover probabilities p_k (k = 1, 2, 3, or 4) under the assumption of complete chiasma interference.

The likelihood analysis was carried out in two steps. Initially, only the three marker loci belonging to linkage group 1 were used, and subsequently, *UWg69* was also added to the models. Genotypic class probabilities [$P(g_i \mid m, r)$] for each recombination event were obtained considering that homologous chromosomes with sister and nonsister centromeres will remain together in 2*n* gametes produced by SDR and FDR, respectively. Maximization of each likelihood function was carried out by a grid search (22). Models were compared using the $\log_{10}$ of the odds ratio (LOD score). The maximum log likelihood corresponding to the most probable order suggested the location of the centromere within linkage group 1.

RESULTS

Models with Three Linked Marker Loci (*UWg119–MTSc9–UWg65*). Genotypes at all four marker loci could be assigned unambiguously to 152 PG-F9 × MAG7 tetraploid plants. Genotypic classes that could derive only by multiple crossover events were not observed, indicating complete chiasma interference. Assuming complete chiasma interference, six groups of genotypic classes (i = 1, . . . , 6) of 2*n* gametes are possible,

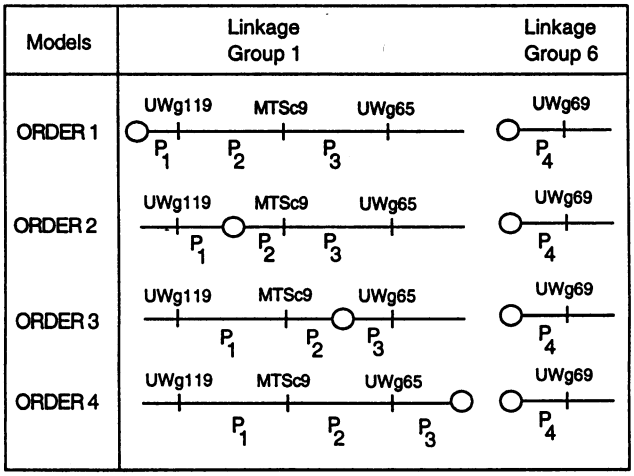

FIG. 2. Models tested for centromere location in linkage group 1.

Table 1. Number of progenies derived from different 2*n* egg groups according to models with three and four marker loci

UWg119–MTSc9–UWg65			*UWg119–MTSc9–UWg65/UWg69*		
Group	Genotypic classes	No. of progeny observed	Group	Genotypic classes	No. of progeny observed
1	AAA+BBB	94	1	AAA/A+AAA/B+BBB/A+BBB/B	91
			2	AAA/H+BBB/H	3
2	AAH+BBH	30	3	AAH/A+AAH/B+BBH/A+BBH/B	30
			4	AAH/H+BBH/H	0
3	HHA+HHB	1	5	HHA/A+HHA/B+HHB/A+HHB/B	0
			6	HHA/H+HHB/H	1
4	AHH+BHH	12	7	AHH/A+AHH/B+BHH/A+BHH/B	12
			8	AHH/H+BHH/H	0
5	HAA+HBB	1	9	HAA/A+HAA/B+HBB/A+HBB/B	0
			10	HAA/H+HBB/H	1
6	HHH	14	11	HHH/A+HHH/B	7
			12	HHH/H	7

Shown are the genotypic classes of 2*n* eggs expected for models with three linked marker loci (*UWg119–MTSc9–UWg65*) and four marker loci, three linked and one unlinked (*UWg119–MTSc9–UWg65/UWg69*), assuming complete chiasma interference in the region covered by the linked markers, and the number of progeny plants observed for each genotypic class of 2*n* eggs.

and at least one progeny plant was observed for each group (Table 1). The likelihood analysis confirmed previous cytological observations that SDR is the prevalent mode of 2*n* egg formation in PG-F9 (Table 2). However, orders 1 and 2 showed the same log likelihood values, which in turn were significantly higher than order 3 (LOD > 3). Therefore, the likelihood analysis carried out using only the three markers linked on group 1 indicated that the centromere is near locus *UWg119*, but we could not determine on which side of *UWg119* it is located.

Models with Three Linked (*UWg119–MTSc9–UWg65*) and One Unlinked (*UWg69*) Marker Loci. If a mutant produces a mixture of FDR and SDR 2*n* gametes, the relative frequency of heterozygous 2*n* gametes depends on both the distance of the marker from the centromere and the relative frequency of FDR and SDR. In particular, when the relative frequency of FDR tends to 0 (and therefore the relative frequency of SDR tends to 1), the closer the marker locus is to the centromere, the lower the frequency of heterozygous 2*n* gametes for this locus (7). *UWg69* showed a very low percentage of heterozygous 2*n* eggs (7.4%; Table 3), and therefore, it should be very close to its centromere on linkage group 6. The frequency of heterozygous 2*n* eggs also confirmed that *UWg119* (11.6%) was the closest to the centromere among the three markers of linkage group 1 (Table 3). Moreover, the percentage of heterozygous 2*n* eggs was 20.1% and 33.7% for *MTSc9* and *UWg65*, respectively, which supported the assumption that the map order previously reported was the correct order for developing the models.

Markers close to the centromere are very useful for discriminating between FDR and SDR (6, 11, 17). Therefore, the four models developed with three linked marker loci were extended to include the unlinked locus *UWg69*. Assuming complete chiasma interference, 12 groups of genotypic classes of 2*n* eggs are expected for the four marker loci, and at least one progeny plant was observed for eight of the groups (Table 1). The analysis with four marker loci allowed a clear location of the centromere on linkage group 1, as indicated by an LOD of 7.50, calculated as the difference between the log likelihoods of the two most likely orders (Table 4). Because under complete chiasma interference the map distance is $1/2(p_k)$, a map of linkage group 1 that included the centromere was obtained as follows: centromere–2.5–*UWg119*–4.4–*MTSc9*–10.4–*UWg65*, and for *UWg69* (linkage group 6), centromere–0.9–*UWg69*, where map distances are expressed in recombination units. Moreover, the estimated frequency of FDR 2*n* eggs (f_{FDR}) was 6% and the frequency of SDR 2*n* eggs ($f_{SDR} = 1 - f_{FDR}$) was 94%.

DISCUSSION

Mutants producing 2*n* pollen and/or 2*n* eggs have been identified in many plant species, and such mutants probably have played an important role in the origin of many polyploids (9). Several mechanisms of 2*n* gamete formation have been observed (10) and can be grouped according to their genetic consequences into the following modes: FDR, FDR-NCO, and SDR. Based on the parental heterozygosity transmitted to the tetraploid progeny, FDR and FDR-NCO 2*n* gametes are generally considered superior to SDR 2*n* gametes for plant breeding applications (12).

In many plant species characterized by simultaneous cytokinesis at the end of male meiosis, FDR 2*n* pollen is produced because of parallel or fused spindles at metaphase and anaphase II (23–27). In these species, SDR mutants having abnormal cytokinesis also have been described and individual sporophytes producing both FDR and SDR 2*n* pollen have been found (10). FDR-NCO 2*n* pollen is produced when synaptic mutants are coupled with the parallel spindles mutation (12). Formation of 2*n* eggs has been observed to occur mainly by SDR mechanisms in *Datura* (28), maize (5), *Hordeum vulgare* L. (29), potato (30), and

Table 2. Parameter estimates and log likelihood values for models with three linked marker loci

Model	Centromere location*	p_1	p_2	p_3	f_{FDR}	Log(L)
1	C–(1)–*UWg119*–(2)–*MTSc9*–(3)–*UWg65*	0.022	0.089	0.213	0.086	−114.48
2	*UWg119*–(1)–C–(2)–*MTSc9*–(3)–*UWg65*	0.002	0.088	0.216	0.103	−114.48
3	*UWg119*–(1)–*MTSc9*–(2)–C–(3)–*UWg65*	0.090	0.000	0.216	0.177	−126.48
4	*UWg119*–(1)–*MTSc9*–(2)–*UWg65*–(3)–C	0.090	0.215	0.000	0.368	−147.78

Shown are the maximum likelihood estimates of the frequencies of meioses with one crossover in the kth chromosome region (p_k, k = 1, 2, 3) and of the frequency of FDR 2*n* eggs (f_{FDR}), and maximum log likelihood values.
*C, centromere; chromosome regions 1, 2, and 3 indicated in parentheses.

Table 3. Observed numbers of homozygous and heterozygous 2*n* eggs produced by PG-F9 for each of four RFLP loci

RFLP locus	LG*	2*n* egg genotypes†		Total‡
		A+B	H (%)	
UWg119	1	137	18 (11.6)	155
MTSc9	1	131	33 (20.1)	164
UWg65	1	106	54 (33.7)	160
UWg69	6	150	12 (7.4)	162

*Linkage group number.
†(A+B), homozygous 2*n* eggs; H, heterozygous 2*n* eggs; %, percentage of heterozygous 2*n* eggs.
‡Total number of 2*n* eggs classified.

alfalfa (8). Extensive cytological investigations carried out in diploid potato showed that FDR or FDR-NCO 2*n* eggs can be produced only by synaptic mutants that undergo nuclear restitution after the first meiotic division (31) or pseudohomotypic division (32). Mutants producing mixtures of both FDR and SDR 2*n* eggs also have been found (5, 30, 33).

These previous studies showed that cytological analyses of micro- and macrosporogenesis were extremely informative when mutants could be classified based on the mechanisms of 2*n* gamete formation. However, Ramanna (34) and Tavoletti *et al.* (35) pointed out that when abnormal cytokinesis is involved, it is difficult to establish cytologically if FDR and/or SDR dyads of 2*n* microspores are produced. Multilocus HTA can effectively complement cytological analysis of mutants having mixtures of modes for 2*n* gamete formation. Previous applications of HTA have used centromeric markers to estimate the frequency of different modes (17). Using the procedures described in this paper, the relative frequencies of different modes resulting in functional 2*n* gametes can be determined without the need for centromeric markers, and simultaneously, the centromere can be mapped within a group of linked marker loci.

Results of the HTA reported here indicate that the alfalfa mutant PG-F9 produces mainly SDR 2*n* eggs (94%) and a low frequency of FDR 2*n* eggs (6%). These results agree with previous cytological observations showing that PG-F9 produces a high frequency of SDR functional macrospores due to the absence of the second meiotic division (18). The cytological analysis of macrosporogenesis also showed some ovules with cells that developed directly in the female gametophyte without undergoing meiotic divisions. This mechanism closely resembles diplospory of several apomictic species (36). However, because it is very difficult to analyze the very early stages of prophase I during alfalfa macrosporogenesis, it was not possible to determine if these eggs developed by FDR from megaspore mother cells that underwent recombination before developing into a diplosporic female gametophyte. Our observation of 2*n* eggs belonging to the HAA/HBB and HHA/HHB genotypic classes (Table 1, three markers, groups 3 and 5) demonstrates, for the first time (to our knowledge), that FDR 2*n* eggs are produced in diploid alfalfa.

Some of the 2*n* eggs analyzed in this study may have arisen by FDR-NCO. These eggs would have the same genotype as PG-F9 and would be heterozygous at all marker loci heterozygous in PG-F9. However, recombinational FDR and SDR also would result in 2*n* eggs heterozygous for the four marker loci considered in this study. Since FDR-NCO 2*n* eggs could not be identified using such a small number of marker loci, this mode of 2*n* egg production was excluded from the models tested. Use of additional RFLP loci that are distributed throughout the genome and are heterozygous in PG-F9 would allow confident detection of FDR-NCO 2*n* eggs derived from this mutant.

In addition to determining the relative frequencies of FDR and SDR, the method described here also allows for mapping of centromeres. Using data from PG-F9 2*n* eggs for three RFLP loci on linkage group 1, we positioned the centromere near *UWg119*. Inclusion of RFLP data for a marker near the centromere of another linkage group provided additional information to more precisely map the centromere on linkage group 1. Data from centromere markers has been used previously in yeast (37) to help position the centromere on other chromosomes; however, our method does not require markers that are at the centromere.

The order and the overall distance covered by the marker loci on linkage group 1 in this study are in agreement with the previous map of PG-F9 (19). However, the linkage distance estimates for adjacent markers based on 2*n* egg data were different than reported previously for pseudotestcross data: *UWg119*–9.4–*MTSc9*–1.9–*UWg65* for PG-F9 (19); *UWg119*–9.2–*MTSc9*–1.8–*UWg65* for W2x-1 (19); *UWg119*–20–*MTSc9* for the recurrent parent; and *MTSc9*–2.5–*UWg65* for the F_1 parent (21). These discrepancies may be due to the use of different population samples and/or different parental genotypes.

Unlike previously described methods, the maximum likelihood procedure reported in this paper does not rely on knowledge of the genetic mode for 2*n* gamete formation or centromere-marker map distances. Both of these parameters are estimated simultaneously, and therefore, this procedure can be applied to mutants producing 2*n* gametes by different modes at the same time. Our models assumed complete chiasma interference; however, they could be extended to include multiple crossover events if the marker loci cover a large region on one or both chromosome arms. Models also can be extended to include FDR-NCO 2*n* gametes if genetic information for marker loci uniformly distributed throughout the genome is available. Thus, this procedure will be useful for characterizing plant chromosomes and for defining the modes of reproduction in meiotic mutants. In plant breeding, this information could help in exploiting gametes that maximize hybrid vigor and for assembling a diplosporic apomictic system.

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Table 4. Parameter estimates and log likelihood values for models with three linked and one unlinked marker loci

Model	Centromere location*	p_1	p_2	p_3	p_4	f_{FDR}	Log(L)
1	C–(1)–*UWg119*–(2)–*MTSc9*–(3)–*UWg65* + C–(4)–*UWg69*	0.050	0.088	0.209	0.019	0.061	−167.13
2	*UWg119*–(1)–C–(2)–*MTSc9*–(3)–*UWg65* + C–(4)–*UWg69*	0.000	0.090	0.216	0.068	0.105	−174.63
3	*UWg119*–(1)–*MTSc9*–(2)–C–(3)–*UWg65* + C–(4)–*UWg69*	0.090	0.000	0.215	0.156	0.178	−202.10
4	*UWg119*–(1)–*MTSc9*–(2)–*UWg65*–(3)–C + C–(4)–*UWg69*	0.090	0.215	0.000	0.365	0.368	−246.87

Maximum likelihood estimates of the frequencies of meioses with one crossover in the kth chromosome region (p_k, k = 1, 2, 3, 4) and of the frequency of FDR 2*n* eggs (f_{FDR}), and maximum log likelihood values.
*C, centromere; chromosome regions 1, 2, and 3 indicated in parentheses.

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