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Tetraploid alfalfa mapping using AFLP markers and research of markers of pollen fertility

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SUMMARY – Molecular markers and mapping methods were applied in tetraploid alfalfa. The mapping population included 180 F1 individuals, obtained by the cross of two plants (one coming from the Provence variety Magali and the other from the Flemish variety Mercedes). With seven primer pairs, 186 dominants AFLP markers were obtained. For mapping, the markers used were those which segregated in F1, present in one parent (as simplex Aaaa or as duplex AAaa) and absent in the other, but also those present as simplex in both parents (biparental simplex markers). The calculation for this case was described. These biparental simplex markers make it possible to combine the maps of the two parents. For each parent, 8 linkage groups among the 32 expected were obtained. More markers are needed to complete the map. Pollen fertility of the F1 individuals and of the parents was measured. Seven markers explained between 9 and 22% of the phenotypic variation each. Feasibility of mapping and research of markers linked to agronomic traits was proved in alfalfa. The program of the next months is to find more AFLP markers and codominant markers to increase the saturation of the map.

Key words: *Medicago sativa*, molecular markers, polyploid.

RESUME – *"Cartographie de la luzerne tétraploïde à l'aide de marqueurs AFLP et recherche de marqueurs liés à la fertilité pollinique". Les outils de marquage moléculaire et de cartographie ont été appliqués à la luzerne autotétraploïde. La population de cartographie est constituée de 180 individus F1 obtenus par le croisement de deux plantes (l'une issue de la variété de type Provence Magali et l'autre issue de la variété flamande Mercedes). Avec 7 couples d'amorces, 186 marqueurs AFLP, dominants, ont été obtenus. Pour la cartographie, les marqueurs retenus sont ceux qui ségrègent en F1, présents chez un des parents (en simplex Aaaa ou en duplex AAaa) et absents chez l'autre, mais aussi ceux présents en simplex chez les deux parents (marqueurs simplex biparentaux). Les calculs de ségrégation attendue ont été formulés pour ce dernier cas. Ces marqueurs simplex biparentaux permettent de regrouper les cartes des deux parents. Pour chacun des parents, 8 groupes de liaison parmi les 32 attendus ont été identifiés. D'autres marqueurs sont donc nécessaires pour compléter la carte. La fertilité pollinique des individus F1 a été mesurée. Sept marqueurs expliquent chacun entre 9 et 22% de la variation phénotypique pour ce caractère. La faisabilité de la cartographie et de la recherche de marqueurs de caractères d'intérêt agronomique est donc prouvée chez la luzerne. Nous avons prévu de continuer la recherche de nouveaux marqueurs AFLP et de marqueurs codominants pour augmenter le degré de saturation de la carte.*

Mots-clés : Medicago sativa, *marqueur moléculaire, polyploïde.*

Introduction

Mapping of autopolyploid species is far more complicated than on diploids. However, theories were developed to analyse dominant markers in autotetraploids (Wu *et al*., 1992; Hackett *et al*., 1998; Ripol *et al*., 1999). In alfalfa, molecular mapping would help in understanding of the agronomic traits inheritance and to assist breeding by the use of markers (MAS: Molecular Assisted Selection). In alfalfa the varieties are synthetic populations, and the heritability of the agronomic traits is usually low, even if the additivity effect is higher than the dominance effect. The range of variation, among or within varieties is large, due to both the allogamy and tetraploidy of the species. Brouwer and Osborn (1999) developed a linkage map on tetraploid alfalfa, and identified markers associated to winter hardiness, autumn growth and freezing injury (Brouwer *et al*., 2000).

In this paper, we report the results obtained on molecular mapping using dominant markers (AFLP) in alfalfa, and the possibility to identify markers linked with agronomic trait that is related to seed production and to the pollen fertility.

Materials and methods

The mapping population (F1) was produced by the cross of two heterozygous plants. One parent is a plant from Magali variety, a French Provence type variety, and this plant was known to be lodging susceptible, nematode susceptible, and to have a high forage quality. The other parent originated from Mercedes, a Flemish type variety, was selected for lodging and nematode resistance. 230 F1 individuals were obtained, but only 180 were used in this experiment.

Seven AFLP primer pairs were selected among 18 tested, for the quality of the electrophoretic patterns and the diversity among the two parents and the 10 F1 individuals. These are identified as the following: ACG/CAT, AAC/CTA, AAC/CTG, ACT/CTT, ACC/CTC, ACA/CAG and AAC/CAG. The bands were scored if there was polymorphism among the F1s and among the parents (present in one parent, absent in the other), and also if there was polymorphism among the F1s and presence in both parents. In the first case, the marker is present either as simplex (Aaaa) or as duplex (AAaa). In the second case, we focused on the markers present in simplex in both parents, namely biparental simplex. The parents produced half of the gametes with the marker, and half of the gametes without the marker. The expected frequency of F1 individuals with the marker is 3/4, and without the marker of 1/4.

Table 1. Phenotypic frequencies for a biparental simplex marker (Sbi) and a monoparental simplex marker (S), linked in coupling and repulsion, and the variance of the estimate of the rate of recombination, r (n: number of individuals)

Linkage type			Phenotypic marker classes in F1 progeny				Variance
Marker A	Marker B	Phase	AB	AO	OB	OO	
Sbi	S	Coupling	$\frac{(2-r)}{4}$	$\frac{(1+r)}{4}$	$\frac{r}{4}$	$\frac{(1-r)}{4}$	$\frac{r(2-r)(1+r)(1-r)}{n(1/2+r-r^2)}$
Sbi	S	Repulsion	$\frac{(4+r)}{12}$	$\frac{(5-r)}{12}$	$\frac{(2-r)}{12}$	$\frac{(1+r)}{12}$	$\frac{(4+r)(5-r)(2-r)(1+r)}{n(13/2+r-r^2)}$

Linkage between markers was estimated as described by Hackett *et al*. (1998), considering coupling and repulsion configurations. For biparental simplex markers, similar calculations of expected frequencies of the phenotypic classes in F1 were made (Table 1), and the variance of the estimate of recombination rate (r) was calculated. The estimate of *r* and the standard error of *r* estimate for biparental simplex and monoparental simplex markers in coupling and in repulsion were calculated. The log-likelihood for the linkage between two markers A and B was:

$$L(r) = constant + a\log(p_{AB}) + b\log(p_{AO}) + c\log(p_{OB}) + d\log(p_{OO})$$

being p_{AB}, p_{AO}, p_{OB}, p_{OO} the expected frequencies of the phenotypic classes AB, AO, OB and OO respectively and *a*, *b*, *c* and *d* the number of individuals in each class. The *r* estimate was obtained by maximising the log-likelihood with respect to *r*. Solving the equation $\frac{dL}{dr} = 0$, we obtained the following formulae and solved them:

Coupling: $n\hat{r}_c^3 \pm (3b + 2c + d)n\hat{r}_c^2 - (a - 2b + c + 2d)\hat{r}_c + 2c = 0$

Repulsion: $n\hat{r}_r^3 - (6a - 3b + 3d)\hat{r}_r^2 + (3a - 6b - 21c - 18d)\hat{r}_r + 10a - 8b - 20c + 40d = 0$

with $n = a + b + c + d$.

The asymptotic variance of the estimator of *r* (Table 1) was given by:

$$^2 = 1\Big/ E\,\frac{d^2L}{dr^2}$$

The standard error of *r* estimate for biparental simplex and monoparental simplex markers in coupling was slightly higher than for two monoparental simplex markers in coupling but lower than for duplex-

duplex markers in coupling (Fig. 1). The standard error of *r* estimate was high for biparental simplex and monoparental simplex markers in repulsion.

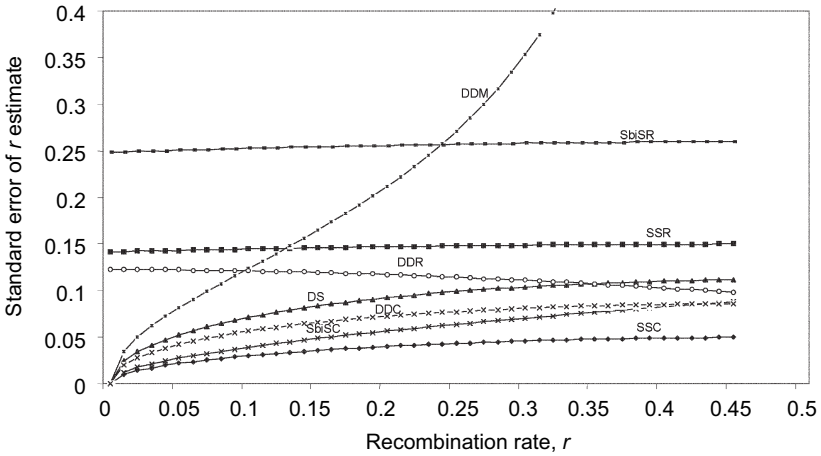

Fig. 1. Standard errors of *r* estimates for biparental simplex markers and simplex markers in coupling (SbiSC) and in repulsion (SbiSR) compared by other linkages (SSC: simplex-simplex in coupling, SSR: simplex-simplex in repulsion, DDC: duplex-duplex in coupling, DDS: duplex duplex in repulsion, DS: duplex-simplex, DDM: duplex-duplex mixed).

A linkage map was made for each parent with JoinMap software.

The F1 individuals were planted in an isolated plant design in the field during 2000. In summer, flowers were collected from each plant, and pollen fertility was tested on 500 pollen grains, with two sampling dates, using the Alexander test. By analysis of variance, the effect of each marker on the variation for pollen fertility was tested. The r^2 of the model gave the part of the variation explained by each marker.

Results and discussion

The number of obtained bands for each pair of primers varied from 60 to 120, but the number of polymorphic ones varied from 16 to 31. The segregation of the markers in the progeny was tested by a ^{2} test at the level of 5%. The expected frequencies were 1:1 for simplex, 5:6 for duplex and 3:4 for biparental simplex. A total of 186 markers was obtained (Table 2), with a large proportion of simplex markers. For 54 markers, the frequencies in the F1 indicated a segregation distortion. This distortion could be due either to technical problems such as gel reading or to abnormal meiosis and zygotic selection. The distortion was not too large, but surprisingly nearly a quarter of the markers were distorted.

We obtained almost the same number of markers from each parent, and the proportion of the markers types were similar too (Table 2). The level of heterozygozity is probably similar for both parents.

Table 2. Proportion of each type of marker, in the F1 population, and for each parent

Marker	F1 population	Magali	Mercedes
Simplex	92	49	43
Duplex	28	14	14
Biparental simplex	13	13	13
Distorted[†]	53	28	30
Total	186	104	100

[†]Five distorted markers were biparental markers.

Using a LOD score of 2, we obtained 8 linkage groups for each parent, among the 32 linkage groups expected (Figs 2 and 3). The duplex markers gathered some linkage groups in homology groups. One biparental marker was present in a linkage group in each parent, indicating that these two linkage groups belonged to the same group of homology. The number of markers was far too low to obtain a saturated map.

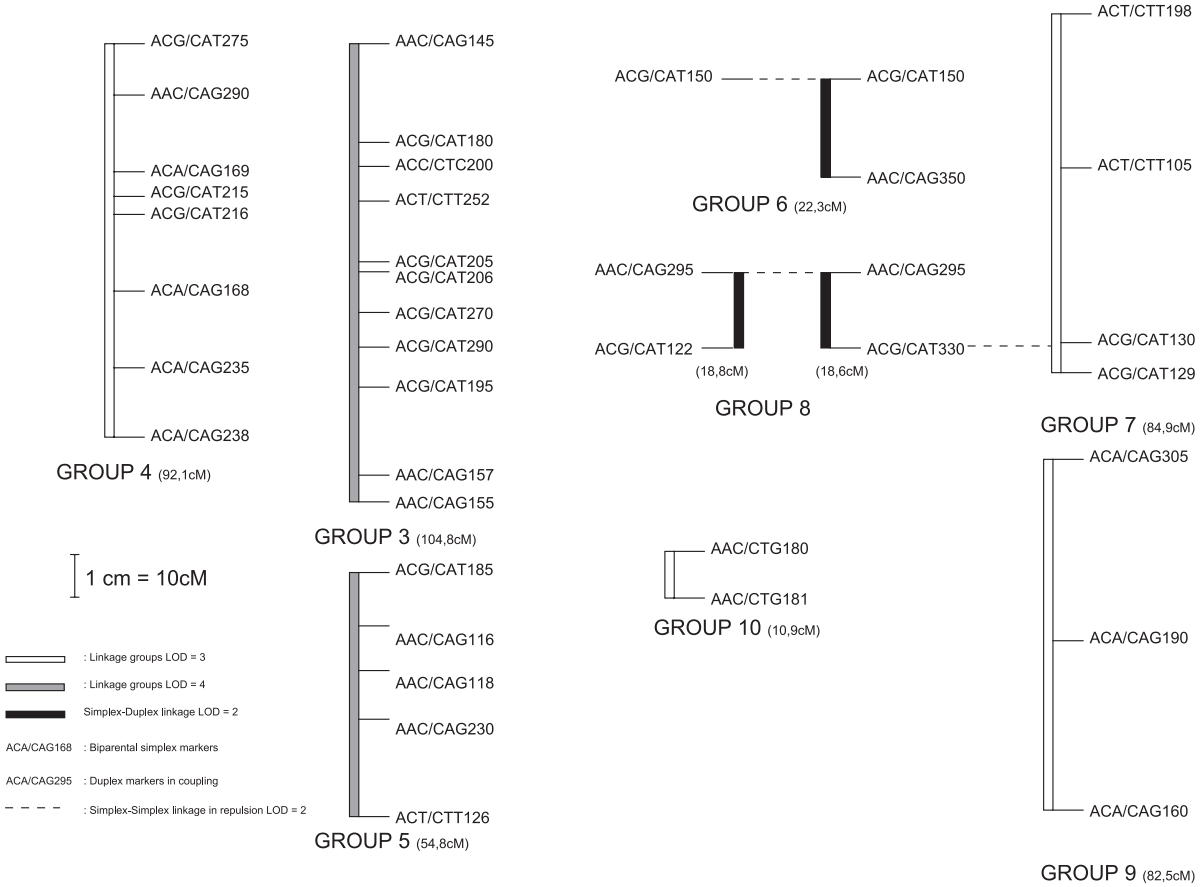

Fig. 2. Linkage map of the markers present in Magali.

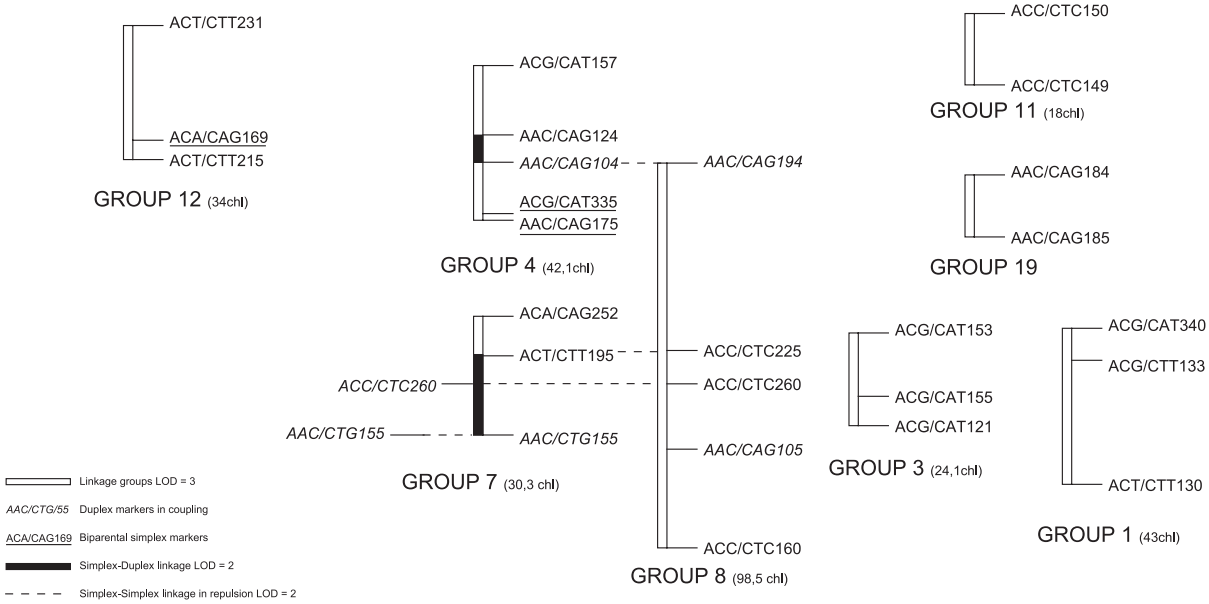

Fig. 3. Linkage map of the markers present in Mercedes.

Both parents had the same level of pollen fertility (94.0%). In the F1 progeny, the variation was from 45.4 to 98.6%. For quantitatively inherited traits, in a cross between heterozygous and tetraploid parents, this situation illustrates the recombinations that arisen during meiosis. In the analysis of variance for each marker, we selected 22 markers for which the probability of the model was lower than 1%. They explained from 4.5 to 22% of the variation for pollen fertility (Table 3). Eight markers were duplex, 11 were simplex and three were distorted. Twenty markers originated from Magali parent and two from Mercedes. For all the markers originating from Magali, the allele "presence" had a positive effect for pollen fertility, while for the 2 markers from Mercedes, the allele "presence" had a negative effect. This discrepancy between the two parents could not be related to their own pollen fertility or to difference in number of markers from each parent.

Table 3. List of the markers that contributed significantly to variation for pollen fertility

Marker	Code	R^2	Origin	Dose†	Mean "absence"	Mean "presence"
AAC/CTA141	50	0.220	Magali	D	75.6	88.5
ACA/CAG113	102	0.204	"	–	79.3	89.2
ACA/CAG112	103	0.196	"	–	79.5	89.2
ACG/CAT115	131	0.120	"	D	79.1	88.0
AAC/CTG460	58	0.113	"	S	81.8	89.3
ACT/CTT500	149	0.107	"	D	79.5	87.9
ACA/CAG202	92	0.095	"	–	82.7	88.8
ACT/CTT151	165	0.091	"	S	83.3	89.2
AAC/CAG295	6	0.080	"	D	78.4	87.3
ACA/CAG230	89	0.076	"	D	79.6	87.7
ACG/CAT216	111	0.066	"	S	84.2	89.3
ACA/CAG160	96	0.063	"	S	84.5	89.4
ACG/CAT215	112	0.062	"	S	84.2	89.2
ACC/CTC165	141	0.059	"	D	81.5	87.5
ACC/CTC142	146	0.054	"	S	83.4	88.2
ACT/CTT180	162	0.052	"	D	81.2	87.5
ACG/CAT129	127	0.052	"	S	84.3	88.9
ACG/CAT185	117	0.051	"	S	84.2	88.7
ACA/CAG235	87	0.051	"	S	84.8	89.2
AAC/CAG104	27	0.049	Mercedes	D	91.0	85.2
ACA/CAG238	86	0.049	Magali	S	84.6	88.8
ACC/CTC195	140	0.045	Mercedes	S	88.3	84.0

†S = simplex, D = duplex, – = distorded.

Conclusions

In this preliminary research of molecular markers in tetraploid alfalfa, we found a large number of AFLP markers. Their number was insufficient to build a saturated map or even to gather the linkage groups in groups of homology. We develop calculations to take into account a new type of simplex marker, present in both parents, and named biparental simplex markers. Using these markers it is possible to combine the maps obtained for each parent with dominant markers.

We found 22 markers linked to variation in pollen fertility. Although both parents had the same pollen fertility, the 20 markers present in Magali had a positive effect and the 2 markers present in Mercedes had a negative effect. In alfalfa, other markers related to a disease resistance and to somatic embryogenesis have been reported (Yu and Pauls, 1993; Obert *et al*., 2000).

More markers are needed to improve the map. AFLP markers will be added, and some codominant markers, originating from diploid species *Medicago* species (Diwan *et al*., 1997; Thoquet *et al*., in prep.), will be too mapped. Codominant markers are more informative for mapping in autopolyploid species and statistical analyses were adapted by Xie and Xu (2000), Luo *et al*. (2000) and Skinner *et al*. (2000). The

mapping population will be studied for other agronomic traits, as stem elongation, stem histology and digestibility.

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