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RFLP marker analysis supports tetrasonic inheritance in *Lotus corniculatus L.*

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Abstract *Lotus corniculatus* is a tetraploid (2*n*=4*x*=24) perennial forage legume and has been reported to have tetrasomic inheritance for several traits, although it has also been reported to show disomic inheritance. Molecular markers were used to clarify whether tetrasomic inheritance, disomic inheritance, or a combination of both, was found within an F_2 population arising from a cross between two diverse *L. corniculatus* accessions. The inheritance of "tetra-allelic" RFLP markers (markers with four segregating bands) indicated that disomic inheritance could not account for the phenotypic F_2 classes observed, and that only tetrasomic inheritance would explain the observed results. Goodness of fit tests for "tetra-allelic" and "tri-allelic" (three segregating bands) RFLP marker data suggested support for chromosomal-type tetrasomic inheritance. RFLP genotypes interpreted from autoradiographic signal intensity provided additional support for tetrasomic inheritance and the occurrence of preferential pairing between parental chromosomes. Bivalent pairing was predominant in the two parental lines and their F_1 hybrid in cytological analyses. *L. corniculatus* has been classified as both an autotetraploid and an allotetraploid species. RFLP evidence of tetrasomic inheritance gives support for *L. corniculatus* being classified as an autotetraploid species. Even though bivalent pairing occurs, as seen in other autotetraploid species, pairing between any of the four homologous chromosomes is possible. Preferential pairing in the F_1 hybrid suggests that genome differentiation appears to be minimal between homologs within an accession, while genome differentiation is greater between homologs from different accessions of this genetically diverse species.

Keywords Tetrasomic inheritance · RFLP markers · *Lotus corniculatus* · Birdsfoot trefoil · Autotetraploid

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Introduction

Lotus corniculatus L. (birdsfoot trefoil) is a tetraploid (2n=4*x*=24) (2*n*=4*x*=24) forage legume that is grown throughout temperate regions of the world. The mode of inheritance in *L. corniculatus* is reported as being either disomic or tetrasomic. Tetrasomic inheritance was first documented by Dawson (1941) in his study of cyanogenesis. Although other morphological traits such as leaf size (Donovan 1959) and leaf color (Pootsci and MacDonald 1961) have subsequently been interpreted to be inherited tetrasomically, the categorization of such quantitative traits into discreet classes may not irrefutably support tetrasomic inheritance. Brown keel tip color was concluded to show single-gene tetrasomic inheritance (Hart and Wilsie 1959; Buzzell and Wilsie 1963; Ramnani and Jones 1984), but other modifier genes were postulated to exist to and may account for incompatible fits to tetrasomic inheritance with this trait (Hart and Wilsie 1959, Buzzell and Wilsie 1963). Alternatively, inconsistent inheritance of brown tip keel color of brown tip keel color could be explained by disomic inheritance (Donovan 1957). Disomic inheritance also was reported for seed color mottling (Donovan 1957) and tannin production (Dalrymple et al. 1984). Tetrasomic inheritance was indicated for pubescence, chlorophyll deficiency, flower color, and corolla striping by (Bubar and Miri (1965), but the supporting data were not published. Molecular markers could provide more definitive evidence on the nature of inheritance in *L. corniculatus* since genotypes and phenotypes can be assigned to distinct classes that do not involve divisions of phenotypic variation. The only previous molecular marker inheritance study in *L. corniculatus* indicated that *Pgi*2 isozyme inheritance

was best explained by a disomic inheritance model (Raelson et al. 1989). Understanding the nature of inheritance is fundamental for making crop improvements through breeding efforts. The purpose of the present study was to use molecular markers to clarify the mode of inheritance in *L. corniculatus*.

Materials and methods

Plant materials

Molecular marker inheritance was based on the analysis of segregation from 82 F_2 progeny derived from a cross between two diverse tetraploid (2n=24) *L. corniculatus* accessions. The male parent was a vegetatively propagated rhizomatous Moroccan accession, 'G31276' (Beuselinck et al. 1996). The female parent was from an autogamous accession, 'AG-S4' (Steiner 1993), selected out of 'MU-81', an open-pollinated heterogeneous population developed from a broad base of *L. corniculatus* germplasm (Beuselinck and McGraw 1986). Parental, F_1, and F_2 progeny DNA was isolated from lyophilized leaf and stem tissue using a CTAB extraction technique (Doyle and Doyle 1990). Isolated nucleic acids were RNase-treated and 30% ethanol was used to selectively precipitate polysaccharides (Michaels et al. 1994) before addition of isopropanol to precipitate DNA.

RFLP marker detection

Genomic clones used for RFLP inheritance analysis were produced from 0.5 to 1.5 kbp-length fragments of *Pst*I-digested G31276 DNA ligated into pSPORT1 (Life Technologies). *Lotus corniculatus* inserts were PCR-amplified from plasmid mini-prep DNA (Sambrook et al. 1993) using pUC forward- and reverse-sequencing primers. The genomic copy number prevalence of cloned inserts was assessed by electrophoresing amplified inserts on 1×TAE 1.2% agarose gels, Southern transferring them to nylon membranes (Hybond N, Amersham), and hybridizing them to radiolabeled total genomic *L. corniculatus* DNA (Landry and Michelmore 1985). Inserts showing low radioactive signal strength (approximately 95% of inserts) were used for screening parental DNA for RFLPs.

Approximately 6 µg of high-molecular-weight *L. corniculatus* DNA was digested with 40 U of *Eco*RI or *Eco*RV for 6 h, electrophoresed in 1×TAE 0.9% agarose gels run overnight at 0.6V cm^{-1}, and transferred onto nylon membranes (Hybond N+, Amersham) according to the supplier's specifications. Radioactive RNA probes were made using a T7 RNA polymerase probe kit (Sigma) according to the manufacturer's specifications with 300 ng of a PCR-amplified insert. Blot hybridization was performed overnight according to Church and Gilbert (1984) or Murray et al. (1992), with both methods yielding equivalent results. Blots used the method of Murray et al. (1992). Washed blots were exposed to film (BioMax MS, Kodak) for 8 to 16 h at –80°C using one intensifying screen.

RFLP phenotypes were scored manually from autoradiographs. RFLP genotypes were scored based on band intensity interpretation manually, and with a desktop computer (PowerMacintosh, Apple) using public domain image analysis software (NIH Image, U.S. National Institutes of Health, http://rsb.info.nih.gov/nih-image/).

Segregation analyses

Segregation analyses of tetrasomic inheritance models used the expectations for chromatid (Haldane 1930)- and chromosomal

Table 1 Expected disomic and tetrasomic genotypic and phenotypic progeny class frequencies of F_2 segregation for a "tetra-allelic" RFLP (ABCD) F_1 genotype. Disomic inheritance is based on expectations fitting disomic segregation at two loci with no common allelic bands as shown in Fig. 1 (i.e., A_1B_1 and C_2D_2). For this table, alleles A and B are assigned to be at the same locus and C and D are at the other (homoeologous) locus. Tetrasomic inheritance is based on expectations fitting chromatid (Ct-Tet)- or chromosomal (Cs-Tet)-type tetra-allelic segregation at a single locus (i.e., ABCD). Expectations for homozygous progeny (e.g., AAAA) were not included because of their rare (1/784) occurrence rate

Genotype	Expectation			Phenotype	Expectation		
	Disomic	Ct–Tet	Cs–Tet		Disomic	Ct–Tet	Cs–Tet
AABB	0	9/392	1/36	AB	0	17/392	1/36
AAAB	0	1/98	0	–[a]	–	–	–
ABBB	0	1/98	0	–	–	–	–
AACC	1/16	9/392	1/36	AC	1/16	17/392	1/36
AAAC	0	1/98	0	–	–	–	–
ACCC	0	1/98	0	–	–	–	–
AADD	1/16	9/392	1/36	AD	1/16	17/392	1/36
AAAD	0	1/98	0	–	–	–	–
ADDD	0	1/98	0	–	–	–	–
BBCC	0	9/392	1/36	BC	1/16	17/392	1/36
BBBC	0	1/98	0	–	–	–	–
BCCC	0	1/98	0	–	–	–	–
BBDD	1/16	9/392	1/36	BD	1/16	17/392	1/36
BBBD	0	1/98	0	–	–	–	–
BDDD	0	1/98	0	–	–	–	–
CCDD	0	9/392	1/36	CD	0	17/392	1/36
CCCD	0	1/98	0	–	–	–	–
CDDD	0	1/98	0	–	–	–	–
ABCC	1/8	5/98	1/18	ABC	1/8	15/98	1/6
AABC	0	5/98	1/18	–	–	–	–
ABBC	0	5/98	1/18	–	–	–	–
ABDD	1/8	5/98	1/18	ABD	1/8	15/98	1/6
AABD	0	5/98	1/18	–	–	–	–
ABBD	0	5/98	1/18	–	–	–	–
ACDD	0	5/98	1/18	ACD	1/8	15/98	1/6
AACD	1/8	5/98	1/18	–	–	–	–
ACCD	0	5/98	1/18	–	–	–	–
BCCD	0	5/98	1/18	BCD	1/8	15/98	1/6
BBCD	1/8	5/98	1/18	–	–	–	–
BCDD	0	5/98	1/18	–	–	–	–
ABCD	1/4	6/49	1/6	ABCD	1/4	6/49	1/6

[a] Phenotypic expectations are grouped into the above class

(Muller 1914)-type segregation for "tetra-allelic" (ABCD) and "tri-allelic" (AABC) genotypes (Tables 1 and 2), as well as the expectation for "complete" (Mather 1935) equational tetrasomic segregation (also known as "maximal" equational segregation, Burnham 1962). The phrases "tetra-allelic" and "tri-allelic" are in quotes because neither tetrasomic nor disomic inheritance was assumed beforehand. Observed F_2 progeny numbers in phenotypic classes were tested for goodness of fit to expected ratios of chromatid- and chromosomal-type segregation by computing χ^2 values with *df*=10 for "tetra-allelic" markers and *df*=4 for "tri-allelic" markers.

Expectations of RFLP segregation using disomic models at "tetra-allelic" markers (Table 1) were based on disomic inheritance at two independent homoeologous loci with no common allelic bands (e.g., A_1B_1 and C_2D_2). The conditions for these disomic models were: (1) G31276 was assigned the phenotype AC; (2) AG-S4 was assigned the phenotype BD; and (3) their F_1 had the phenotype ABCD. The assumption was made that no progeny could have phenotypes AC or BD, if A and C were at the same locus (or conversely, phenotypes AD or BC, if A and D were at the same locus). In other words, for disomic inheritance, plants could not have four allelic copies at one locus and no allelic copies at its homoeologous locus (Fig. 1). Allele assignment to loci was performed as follows: (1) because alleles A and C both came from the parent G31276, for disomic inheritance they would have to be at different (homoeologous) loci; (2) because alleles B and D both came from AG-S4, they would have to be at different loci; (3) since it was not known whether A and B or A and D were at the same locus, alleles were assigned so that A and B were at the same locus which gave the best agreement with disomic expectations.

Expectations of RFLP segregation using disomic models at "tri-allelic" markers were based on disomic inheritance at two independent homoeologous loci, with each locus sharing one common allelic band (Table 2). The conditions for these disomic models were: (1) one parent had identical alleles at homoeologous loci (e.g., A_1A_1 and A_2A_2) and had phenotype A; (2) the other parent was homozygous for unique alleles at both homoeologous loci (e.g., B_1B_1 and C_2C_2) and had phenotype BC; and (3) their F_1 was heterozygous at two homoeologous loci (e.g., A_1B_1 and A_2C_2) and had phenotype ABC. Observed F_2 progeny numbers in phenotypic classes were tested for goodness of fit to expected disomic segregation ratios by computing χ^2 values with *df*=8 for "tetra-allelic" markers and *df*=4 for "triallelic" markers.

Repulsion linkage analysis was performed after assigning interpreted genotypes to the F_2 progeny, and using public domain software (MapManager, http://mcbio.med.buffalo.edu/mmXP.html) to determine if the presence of codominantly scored alleles were linked to the absence of any codominantly scored alternative alleles using the same RFLP marker. For this analysis, the presence of two or more copies of an allele was scored as a homozygote, one copy as a heterozygote and no copies as a non-parental homozygote for parental alleles; while conversely scored alternative alleles were scored as a parental homozygote if no copies were present, a heterozygote if one copy was present, and as a non-parental homozygote if two or more copies were present. Repulsion linkage distances were measured as the Kosambi cM map distance between the parental allele and two (for "tri-allelic" RFLP markers) or three (for "tetra-allelic" RFLP markers) conversely scored alternative alleles.

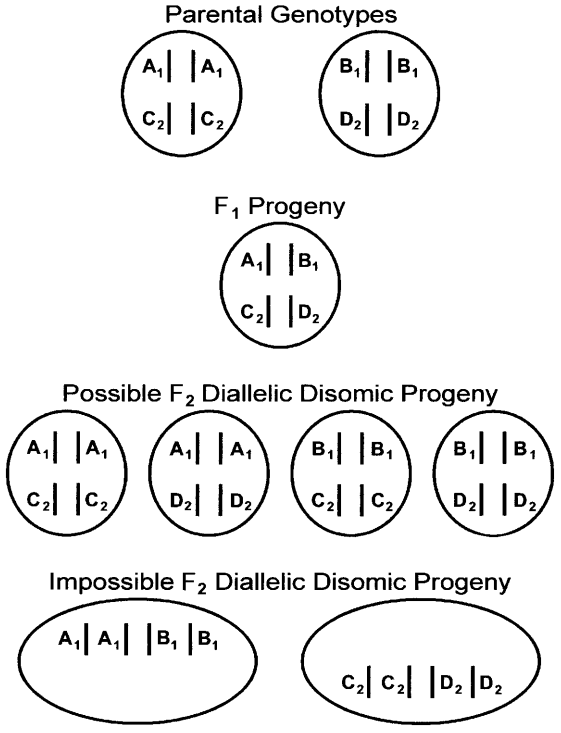

Fig. 1 Graphical representation on possible and impossible diallelic progeny produced from a "tetra-allelic" disomic F_1 plant segregating at two homoeologous loci. Chromosomes carrying alleles A and B are on one set of homologous chromosomes with subscript 1, and alleles C and D are on a separate (homoeologous to A and B) set of homologous chromosomes with subscript 2

Table 2 Expected genotypic and phenotypic progeny class frequencies for F_2 segregation of a "tri-allelic" RFLP (AABC) F_1 genotype. Disomic inheritance is based on expectations fitting disomic segregation at two homoeologous loci having identical molecular weight bands for one allele at each of the loci (A_1B_1 and A_2C_2) and tetrasomic inheritance is based on expectations fitting chromatid (Ct–Tet)- or chromosomal (Cs–Tet)-type tetrasomic segregation at a single tri-allelic (AABC) locus

Genotype	Expectation			Phenotype	Expectation		
	Disomic	Ct–Tet	Cs–Tet		Disomic	Ct–Tet	Cs–Tet
AAAA	1/16	9/196	1/36	A	1/16	9/196	1/36
AAAB	1/8	6/49	1/9	AB	3/16	47/196	2/9
AABB	1/16	19/196	1/9	–[a]	–	–	–
ABBB	0	1/49	0	–	–	–	–
AAAC	1/8	6/49	1/36	AC	3/16	47/196	2/9
AACC	1/16	19/196	1/36	–	–	–	–
ACCC	0	1/49	0	–	–	–	–
BBCC	1/16	9/392	1/36	BC	1/16	17/392	1/36
BBBC	0	1/98	0	–	–	–	–
BCCC	0	1/98	0	–	–	–	–
AABC	1/4	11/49	5/18	ABC	1/2	3/7	1/2
ABBC	1/8	5/49	1/9	–	–	–	–
ABCC	1/8	5/49	1/9	–	–	–	–

[a] Phenotypic expectations are grouped into the above class

Meiotic pairing analysis

Meiotic staining of chromosomes was performed by the method of Nualsri et al. (1998). Configurations in meiotic microspore cells were scored for the parental lines, G31276 (31 cells) and AG-S4 (46 cells), and their F_1 hybrid (191 cells) that gave rise to the F_2 progeny population.

Results

RFLP segregation

Over 120 probes from a *Pst*I genomic library were screened for the presence of suitable "tetra-allelic" or "tri-allelic" RFLP banding patterns in *Eco*RI- and *Eco*RV-digested DNA from G31276, AG-S4, and their F_1 progeny. Only ten suitable probes were found having four unique allelic bands in the F_1 and two from each parent which that could provide readily interpretable segregation analysis of "tetra-allelic" RFLPs. Several other probes gave four or more bands in the F_1 progeny, but multiple banding patterns and probable gene duplication events prevented adequate phenotype and allele assignment. Fourteen potential "tri-allelic" banding patterns were observed, but only five were studied in detail. Emphasis was placed on detecting "tetra-allelic" loci since these could provide greater comparative evidence of tetrasomic versus disomic inheritance.

All ten "tetra-allelic" RFLP markers had progeny in classes that could not be expected from disomic segregation (Table 3). For "tetra-allelic" RFLP markers, if pairing in *L. corniculatus* was tetrasomic, where a chromosome could pair with any of three possible homologous chromosomes, then six diallelic classes could result. As displayed in Fig. 1, if pairing was disomic, where a particular chromosome could only pair with a single homologous chromosome during meiosis, then only four diallelic classes would be possible. All six classes would be impossible, since two of the classes would result from having no member chromosomes present from one of the homoeologous sets. Three of the RFLP markers (L2075, L3026, and L3058) had progeny in all six diallelic phenotype classes and six RFLP markers had progeny in five diallelic classes. Only one RFLP marker had four diallelic classes, although one of the missing classes was a parental type. A total of four markers were missing progeny in parental classes (no AC or BD progeny).

Goodness of fit tests indicate that chromosomal-type tetrasomic inheritance ratios typically gave better χ^2 values than chromatid-type tetrasomic inheritance ratios. Chromosomal-type tetrasomic inheritance was significantly rejected at $P \geq 0.05$ for three of the ten RFLP markers, while chromatid-type tetrasomic inheritance was rejected for nine of the RFLP markers. Eight of the markers had χ^2 values that were lowest for chromosomal-type tetrasomic ratios and two markers had χ^2 values that were lowest for chromatid-type tetrasomic ratios. The goodness of fit to "complete" or "maximal" equational tetrasomic segregation was calculated for each

Table 3 F_2 phenotypic segregation of "tetra-allelic" RFLP loci with chi-square values from expectations fitting disomic segregation at two loci with no common allelic bands (e.g., A_1B_1 and C_2D_2) and chromatid (Ct–Tet)- or chromosomal (Cs–Tet)-type tetrasomic segregation at a single tetra-allelic locus (i.e., ABCD). Parental phenotypes were assigned as G31276: AC, AG–S4: BD, F_1: ABCD

Locus	Number of F_2 progeny with allelic phenotype											χ^{2a} Disomic	χ^{2b} Ct–Tet	χ^{2c} Cs–Tet
	AB[d]	AC	AD	BC	BD	CD[d]	ABC	ABD	ACD	BCD	ABCD	n			
L2002	4	2	1	4	2	0	12	15	5	11	25	81	>[e]13.55***	34.80***	22.12**
L2037	2	3	3	5	0	2	17	8	13	12	16	81	>13.81***	12.62	9.97
L2041	1	0	4	1	1	1	9	10	12	19	23	81	>20.32***	32.90***	17.88*
L2075	1	1	3	1	2	1	8	13	8	19	23	80	>20.06***	31.15***	15.56
L3003	2	0	3	4	1	2	6	18	8	17	21	82	>22.15***	27.82***	17.35*
L3006	3	0	3	2	1	0	16	11	14	9	23	82	>16.34***	28.77***	14.64
L3009	2	3	2	2	1	0	11	9	13	16	23	82	>14.40***	26.34***	12.24
L3026	7	2	1	6	1	1	24	10	3	12	15	82	>34.81***	31.93***	35.61***
L3037	3	1	3	4	0	4	9	8	16	15	19	82	>17.52***	17.72*	14.33
L3058	7	2	1	6	1	1	24	10	4	11	14	81	>33.27***	30.11***	34.63***

*, **, *** Significance at the 0.10, 0.05, and 0.01 probability levels, respectively

[a] χ^2 for expected values fitting a 0:1:1:1:1:0:2:2:2:2:4 segregation ratio (df=8). See Materials and methods for details on the assignment of classes with expectations of zero progeny

[b] χ^2 for expected values fitting a 17:17:17:17:17:17:60:60:60:60:48 chromatid-type segregation ratio (*df*=10)

[c] χ^2 for expected values fitting a 1:1:1:1:1:1:6:6:6:6:6 chromosomal-type segregation ratio (*df*=10)

[d] Represents an impossible disomic class

[e] >Signifies that the actual χ^2 would exceed this value since one class has an expectation of 0, but has 1 or more observed progeny in this class giving a χ^2 value of infinity. The summed χ^2 values shown do not include the infinite values obtained for disomic expectation

marker and all had greater χ^2 values than those calculated for chromosomal- or chromatid-type segregation (data not shown).

For the "tri-allelic" RFLP markers, chromosomal-type tetrasomic inheritance gave somewhat better fits to the observed progeny values than chromatid-type tetrasomic and disomic inheritance. Two of the markers were rejected for both chromosomal-type tetrasomic and disomic inheritance ratios and four of the markers were rejected for chromatid-type tetrasomic inheritance at the P=0.05 level. Four of the markers had their lowest χ^2 values for chromosomal-type tetrasomic inheritance, one marker had its lowest χ^2 value for chromatid-type tetrasomic inheritance, while no markers had their lowest χ^2 values for disomic inheritance.

Even though RFLP banding patterns can provide more reliable phenotypic class information, it can also provide genotypic class information, albeit subjectively based on band intensity. RFLP genotypic progeny classes exist that could only arise from tetrasomic segregation (Figs. 2 and 3). Table 1 shows that for disomic "tetra-allelic" F_2 segregation, each progeny class having a tri-allelic phenotype can only have one possible genotype with two identical alleles. For example, with progeny having the ABC phenotype, disomic segregation could not give rise to all three genotypic classes of AABC, ABBC and ABCC offspring, since two of these classes would result from having three alleles present at one locus and only one allele present at the other (homoeologous) locus. For the selected progeny segregation at marker L2041 (Fig. 2), lanes 9, 13 and 16 show ABC phenotype progeny in each of these three genotypic classes. Additionally, lanes 12 and 17 show two types of genotypic classes (ACCD and AACD, respectively) for the ACD phenotype, and lanes 4 and 14 show two types of genotypic (AABD and ABBD, respectively) classes for the ABD phenotype. Nine "tetra-allelic" RFLP markers could be reliably scored for band signal intensities and each displayed two or more genotypic progeny classes in all of the four tri-allelic phenotype classes scored.

Disomic segregation also could not account for the apparent occurrences of double reduction identified by one "tri-allelic" (L3117) and two "tetra-allelic" (L3009 and L3037) RFLP markers. Double reduction is observed in chromatid-type segregation after a crossover between an allele and the centromere, non-disjunction of sister alleles during the first meiotic division, followed by passage of both sister alleles to the same gamete in the second meiotic division. The presence of three copies of one allele in F_2 progeny when only one allelic copy was present in the F_1 (e.g., two progeny having the genotype ABBB scored with L3117, Fig. 3, lanes 9 and 11) could only result from the occurrence of chromatid-type tetrasomic segregation.

For the RFLP markers in general, there is was a noticeable lack of progeny with parental phenotypes. This lack of parental type progeny accounted for 54.7%, 40.7%, and 39.2% of the "tri-allelic" RFLP marker χ^2 values for disomic, chromatid-type tetrasomic, and chromosomal-type tetrasomic segregation, respectively. For the "tetra-allelic" RFLP markers, high numbers of tetra-allelic (ABCD) class progeny often predominated and made up the largest class with high χ^2 values in both chromatid- and chromosomal-type segregation, accounting for 43.8% and 26.4% of the χ^2 values, respectively.

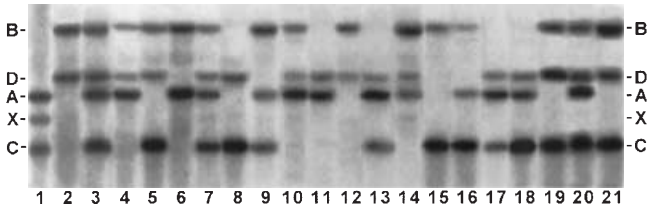

Fig. 2 Composite image of RFLP autoradiograph for "tetra-allelic" marker L2041. Plant identity and interpreted genotypes are provided as follows: *lane 1*-G31276, AACX; *lane 2* AG-S4, BBDD; *lane 3* AG-S4×G31276 F_1 hybrid, ABCD; *lanes 4 to 21* F_2 progeny, AABD, BCCD, AABB, ABCD, CCDD, ABBC, AABD, AADD, BBDD, AACD, ABBD, BBCC, ABCC, AACD, ACCD, BCDD, ABCD, BBCD. G31276 carries three alleles (**A, C, and X**) and the F_1 hybrid inherited two of them (**A and C**). Note that each band does not give equal autoradiographic intensity (C≅A>B≅D) and that genotypes must be interpreted from intensity ratios relative to that found in tetra-allelic plants

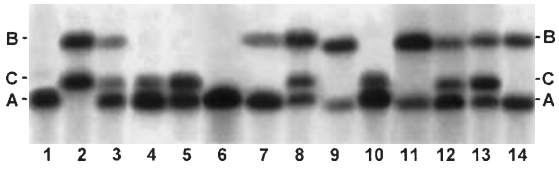

Fig. 3 Composite image of RFLP autoradiograph for "tri-allelic" marker L3117. Plant identity and interpreted genotypes are provided as follows: *lane 1* G31276, AAAA; *lane 2* AG-S4, BBCC; *lane 3* AG-S4×G31276 F_1 hybrid, AABC; *lanes 4 to 13* F_2 progeny, AAAC, AACC, AAAA, AAAB, ABBC, ABBB, AAAC, ABBB, AABC, ABCC, AABB. Each band was estimated to have equal autoradiographic signal intensity

Repulsion-linkage estimation is a method to investigate chromosomal assortment during meiosis. Marker alleles scored "in repulsion" (as explained in Materials and methods) should appear linked with alternative alleles when preferential assortment occurs between chromosome pairs (Al-Janabi et al 1993). Repulsion-linkage analysis showed that seven out of the ten "tetra-allelic" RFLP markers and four of the five "tri-allelic" RFLP markers exhibited some degree of repulsion linkage of parental (maternal with maternal and paternal with paternal) alleles. It was typically seen that one of the AG-S4 alleles was in loose repulsion linkage with the other AG-S4 allele, and likewise with the two G31276 alleles (average repulsion linkage value of 22.0 cM), indicating that parental alleles were preferentially pairing during meiosis.

An additional point of interest is that the F_1 hybrid between G31276 and AG-S4 inherited most of the AG-S4 bands from over 120 scored RFLP markers, but not

Table 4 F_2 phenotypic segregation of "tri-allelic" RFLP loci with chi–square (χ^2, *df*=4) values from expectations fitting disomic inheritance at two homoeologous loci with one common allelic band (i.e., A_1B_1 and A_2C_2) and chromatid (Ct–Tet)- or chromosomal (Cs–Tet)-type tetrasomic inheritance at a single tri-allelic locus (i.e., AABC)

Locus	No. of F_2 progeny with allelic phenotype						χ^{2a} Disomic	χ^{2b} Ct–Tet	χ^{2c} Cs–Tet
	A	AB	AC	BC	ABC	n			
L2004	1	18	21	6	35	81	6.86	3.99	8.09*
L2019	0	8	23	0	50	81	19.77***	21.35***	13.68***
L2027	1	12	15	2	51	81	8.50*	14.14***	5.94
L2043	0	6	21	2	53	82	18.32***	23.11***	14.44**
L3117	3	13	20	0	46	82	8.38*	9.50**	4.79

*, **, *** Significance at the 0.10, 0.05, and 0.01 probability levels, respectively

[a] χ^2 for expected values fitting a 1:3:3:1:8 segregation ratio (Table 2)

[b] χ^2 for expected values fitting a 18:94:94:17:168 segregation ratio (Table 2)

[c] χ^2 for expected values fitting a 1:8:8:1:18 segregation ratio (Table 2)

all of the bands from G31276 (data not shown). This implies that AG-S4 displays noticeable "fixed heterozygosity" (Soltis and Riesberg 1986), with its gametes commonly containing all the alleles present in the parental plant giving rise to them, whereas G31276 was most likely heterozygous.

Meiotic pairing analysis

Bivalents (mean value=11.6) made up the majority of the chromosomal configurations observed in diakinesis and metaphase-I stage microspores (data not shown). Multivalents were not common (0.1 per cell) in the parental lines or their hybrid. Univalents were more frequent in the F_1 hybrid (0.9 per cell) than in either parent (0.3 per cell), perhaps indicating incomplete chromosome homology between these diverse *L. corniculatus* accessions.

Discussion

RFLP marker analyses provided evidence that tetrasomic inheritance is the most likely mode of segregation that generated the phenotypes observed in the F_2 progeny. Because of the codominant nature of RFLP markers, phenotypic and genotypic classes were observed that could clearly distinguish disomic from tetrasomic inheritance. Even though "tetra-allelic" RFLP markers were relatively difficult to identify because of their low frequency in the mapping population, their phenotypic data gave strong support for tetrasomic segregation in *L. corniculatus*. Disomic inheritance was rejected for the "tetra-allelic" RFLP markers because of the presence of progeny in classes that are unexpected from disomic segregation. For "tri-allelic" RFLP markers, it was more difficult to discriminate disomic from tetrasomic inheritance because of the low frequency of parental-type offspring (which would be in classes that give rise to the largest proportion of χ^2 differences between disomic and chromosomal-type tetrasomic inheritance patterns) and the limited progeny population size. Genotypic data for both "tetra-allelic" and "tri-allelic" RFLP markers, although being more subjective than phenotypic data due to the judgement required to assign plant genotype based on RFLP band intensity, gave further proof against disomic inheritance in *L. corniculatus*. Tetrasomic inheritance appeared to occur throughout the genome of *L. corniculatus* as the 15 RFLP markers used have been mapped to five different linkage groups in this *x*=6 species (data not shown).

Chromosomal-type tetrasomic inheritance frequently gave a better fit to the observed RFLP data than chromatid-type tetrasomic inheritance. Despite the limited population size of 82 progeny (with over half of the loci having one or two missing progeny), seven out of the 15 markers studied were accepted as having chromosomal-type inheritance while being rejected for having chromatid-type segregation at *P*=0.05. Alternatively, none of the markers were rejected for chromosomal-type segregation and accepted for chromatid-type segregation. For markers displaying perfect chromosomal-type ratios, 336 and 349 progeny would be needed to reject (at *P*=0.05) chromatid-type segregation for "tetra-allelic" and "tri-allelic" loci, respectively. Less progeny, 36 and 186, displaying perfect chromosomal-type tetrasomic inheritance would be required to reject disomic inheritance for "tetra-allelic" and "tri-allelic" loci, respectively. It should be noted that the 82 progeny we studied did not display perfect chromosomal-type tetrasomic inheritance. Our F_2 population had a large number of tetra-allelic and triallelic progeny for the "tetra-allelic" and "tri-allelic" RFLP markers, respectively, which frequently caused rejection of chromatid-type, and acceptance of chromosomal-type, tetrasomic segregation.

Chromosomal- type tetrasomic segregation was reported in the inheritance studies of Dawson (1941), Donovan (1959), Hart and Wilsie (1959), Pootsci and MacDonald (1961), Buzzell and Wilsie (1963), and Bubar and Miri (1965). However, our genotypic RFLP data indicated that several progeny arose from double-reduction gametes which could only arise from chromatid-type tetrasomic segregation. It can be noted that markers detecting progeny produced from double reduction (L3009, L3037, and L3117) have been mapped as distal to the centromere on two different *L. corniculatus*

linkage groups (data not shown), as would be expected for loci displaying chromatid-type segregation. The genotypic data in this study provides the first evidence of double reduction in *L. corniculatus*. Even though chromosomal-type segregation predominates, chromatid-type segregation may occur in *L. corniculatus*.

It has been debated whether *L. corniculatus* is an "autotetraploid" or an "allotetraploid" species. As in previous cytological studies of *L. corniculatus* (Wernsman et al. 1964), we have seen bivalent pairing to be predominant in the *L. corniculatus* accessions studied. The combination of tetrasomic inheritance and bivalent pairing present in *L. corniculatus* has led *L. corniculatus* to be categorized as a "segmental allopolyploid" (Stebbins 1950). In segmental allopolyploids, the polyploid contains two genomes which that possess a considerable number of homologous segments. In segmental allopolyploid multivalent formation, homogenetic and heterogenetic pairing between homologous and homoeologous chromosomes, respectively, would result. The frequency of homogenetic to heterogenetic associations would depend on the homology of the chromosomes involved. Stebbins (1950) suggesteds that *L. corniculatus* has a high differentiation between genomes, leading to preferential pairing which that results in a predominance of bivalents at meiosis.

Our results for "tetra-allelic" RFLP markers suggest that there is preferential pairing in *L. corniculatus*, but not of the type previously proposed by Stebbins (1950). For the classical definition of a segmental allotetraploid, if each of our parental lines contains contained two different genomes, we would expect that the heterogenetic parental chromosomes (carrying the "heterogenetic" alleles A and C from G31276 or alleles B and D from AG-S4) would typically not pair with each other in their F_1 hybrid. We would therefore expect to see independence of their segregation and no lack of parental phenotypes in the F_2 progeny population. The results we obtained, however, imply that parental chromosomes preferentially paired with each other (and not with the chromosomes from the other parent) to give us a lack of F_2 progeny in the parental phenotype classes. This preferential pairing of parental-type chromosomes would also account for an increased number of tetra-allelic $F_2$2 progeny and repulsion linkage between parental alleles.

The fixed heterozygosity seen in AG-S4 implies this accession has a higher differentiation of chromosomes, with greater homogenetic pairing versus heterogenetic pairing than in the G31276 parental line. This differentiation is, however, not absolute and pairing in the F_1 hybrid readily occurs between the formerly heterogenetic chromosomes. The assignment of chromosomes as being homogenetic or heterogenetic in the parental lines seems to fall apart in their F_1 hybrid, making these classifications somewhat arbitrary. It can be argued that AG-S4, displaying fixed heterozygosity and autogamy, could be one of the most "diploidized" of *L. corniculatus* accessions. Even with these diploid-like characteristics that are commonly seen in many traditional allopolyploid species (e.g., wheat and cotton), differentiation is not strong enough to prevent pairing in F_1 hybrids between the parental genomes of AG-S4. Like G31276, we have not detected the fixed heterozygosity displayed by AG-S4 in other *L. corniculatus* accessions in which we studied molecular-marker inheritance (Fjellstrom and Steiner, unpublished).

The formation of bivalents in *L. corniculatus* could be due to causes other than genome homogeneity or heterogeneity. A lack of multivalents in *L. corniculatus* could be due to the relatively small size of chromosomes in this species (Wernsman et al. 1964). Furthermore, true autotetraploids often display a predominance of bivalent pairing, with few multivalents (e.g., *Tolmiea*, *Coreopsis*, and *Woodsia*, reviewed in Soltis and Riesberg 1986). Soltis and Soltis (1993) make the convincing argument that bivalent pairing in tetraploids is not an indication of allotetraploidy, but is a method by which regular chromosome division at meiosis can be enforced in autotetraploids in autotetraploids. Bivalent pairing could be such an adaptive trait in *L. corniculatus*.

From the evidence presented in this research, it appears that *L. corniculatus* could well be an autotetraploid species. Alternatively, if it is a segmental allotetraploid species , the small degree of genome differentiation present is not strong enough to prevent pairing of chromosomes between the genomes. It is likely that there have been several independent origins of tetraploid *L. corniculatus*, similar to what has been observed in numerous polyploid species (Soltis and Soltis 1993). The F_1 hybrid between G31276 and AG-S4 could then carry two to four haploid genomes, with the G31276 haploid genomes being more related to each other than the AG-S4 haploid genomes, and vice-versa. Chromosomes from any one of these genomes are able to pair with those from any other genome, but preferentially pair with chromosomes from its own parental line. It is interesting to note that *Medicago sativa*, another forage legume, is well accepted as an autotetraploid species (McCoy and Bingham 1988), although it once had a similar history of disputed tetrasomic and disomic inheritance and was also categorized as a segmental allotetraploid species (Little 1958).

Since few diploid *L. corniculatus* populations have been identified (Small et al. 1983), it has been difficult to accept *L. corniculatus* as an autotetraploid species. However, it has been equally difficult to classify *L. corniculatus* as an allotetraploid species because of a similar lack of obvious diploid progenitors (reviewed in Grant and Small 1996). This research can not address the topic of what species may have led to the evolution of *L. corniculatus* or how many independent origins of tetraploid *L. corniculatus* may have occurred in the lineage of this species. However, it can support a scenario of at least two origins of *L. corniculatus* arising from chromosome doubling of a diploid form or hybrids between two highly related diploid progenitor species that have given rise to the tetraploid forms found today.

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