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Relationships of the Woody *Medicago* Species (Section *Dendrotelis*) Assessed by Molecular Cytogenetic Analyses

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• *Background and Aims* The organization of rDNA genes in the woody medic species from the agronomically important *Medicago* section *Dendrotelis* was analysed to gain insight into their taxonomic relationships, to assess the levels of infraspecific variation concerning ribosomal loci in a restricted and fragmented insular species (*M. citrina*) and to assess the nature of its polyploidy.
• *Methods* Fluorescence *in situ* hybridization (FISH) was used for physical mapping of 5S and 45S ribosomal DNA genes in the three species of section *Dendrotelis* (*M. arborea*, *M. citrina*, *M. strasseri*) and the related *M. marina* from section *Medicago*. Genomic *in situ* hybridization (GISH) was used to assess the genomic relationships of the polyploid *M. citrina* with the putatively related species from section *Dendrotelis.*
• *Key Results* The diploid ($2n = 16$) *M. marina* has a single 45S and two 5S rDNA loci, a pattern usually detected in previous studies of *Medicago* diploid species. However, polyploid species from section *Dendrotelis* depart from expectations. The tetraploid species ($2n = 32$) *M. arborea* and *M. strasseri* have one 45S rDNA locus and two 5S rDNA loci, whereas in the hexaploid ($2n = 48$) *M. citrina* four 45S rDNA and five 5S rDNA loci have been detected. No single chromosome of *M. citrina* was uniformly labelled after using genomic probes from *M. arborea* and *M. strasseri*. Instead, cross-hybridization signals in *M. citrina* were restricted to terminal chromosome arms and NOR regions.
• *Conclusions* FISH results support the close taxonomic interrelationship between *M. arborea* and *M. strasseri*. In these tetraploid species, NOR loci have experienced a diploidization event through physical loss of sequences, a cytogenetic feature so far not reported in other species of the genus. The high number of rDNA loci and GISH results support the specific status for the hexaploid *M. citrina*, and it is suggested that this species is not an autopolyploid derivative of *M. arborea* or *M. strasseri*. Further, molecular cytogenetic data do not suggest the hypothesis that *M. arborea* and *M. strasseri* were involved in the origin of *M. citrina*. FISH mapping can be used as an efficient tool to determine the genomic contribution of *M. citrina* in somatic hybrids with other medic species.

Key words: *Medicago arborea*, *M. citrina*, *M. strasseri*, rRNA genes, 18S-5·8S-25-S, 5S, FISH mapping, GISH, polyploidy.

INTRODUCTION

Medicago is a genus of the legume family (Fabaceae) that includes agriculturally and economically important species (e.g. *M. sativa*, alfalfa) and model organisms for legume biology (*M. truncatula*). About 85 species (and 18 infraspecific taxa) included in 12 sections have been recognized in world-wide systematic revisions (Small and Jomphe, 1989). In contrast with the remaining medics, which are annual or perennial herbs, section *Dendrotelis* comprises woody shrubs up to 4 m tall, with perennial stems characterized by annual rings of wood and bark produced by cambia (Small and Jomphe, 1989).

Up to three allopatric species, restricted to rocky and cliff faces in coastal places of the Mediterranean basin, have been included within section *Dendrotelis*, viz. *M. arborea*, *M. citrina* and *M. strasseri*. However, the taxonomic status of the latter two species has been controversial and some authors have included them as infraspecific variants of *M. arborea* (Bolòs and Vigo, 1974; Lesins and Lesins, 1979; Small and Jomphe, 1989; Sobrino *et al.*, 2000). These three woody medics are polyploids, *M. arborea* and *M. strasseri* being tetraploid [$2n = 32$; Falistocco (1987) and González-Andrés (1999), respectively] and *M. citrina* hexaploid ($2n = 48$; Boscaiu *et al.*, 1997).

Medicago arborea has been widely cultivated as a forage plant in the Mediterranean region (Olives, 1969), and it has been introduced as an ornamental in other areas of Europe, North Africa and Asia, blurring the boundaries of its natural distribution. In fact, Greuter (1986) suggested that *M. arborea* was endemic to small islets of the Aegean Sea being later introduced throughout most places of its current Mediterranean range. *Medicago strasseri* is endemic of Crete, being known from only two limestone gorges in the central part of the island (Greuter *et al.*, 1982), whereas *M. citrina* is restricted to a few small islets surrounding the Balearic Islands (Alomar *et al.*, 1997) and the volcanic Columbretes archipelago (Bolòs and Vigo, 1984).

Legume shrubs growing in Mediterranean environments arouse an increasing interest as forage plants adapted to drought and salinity. Woody medics from section *Dendrotelis* constituted suitable organisms for these purposes due to their perennial habit, polyploidy, as well as their adaptation to water- and salt-stressed environments

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(Chebbi *et al.*, 1994; Koning *et al.*, 2000; Sibole *et al.*, 2003, 2005). Thus, the transfer of these agriculturally important traits to other unrelated medic species for crop improvement through somatic hybridization (protoplast fusion) is a challenge for biotechnology. Only a single woody medic, *M. arborea*, has been used to produce somatic hybrids with other species (Cluster *et al.*, 1996; Calderini *et al.*, 1997; Busti *et al.*, 2001). However, *M. citrina* is more drought and salt tolerant than *M. arborea* (Correal, 1997; Sibole *et al.*, 2003; Lefi *et al.*, 2004) and could be more efficiently used for alfalfa improvement.

The present study aims to assess (*a*) the molecular cytogenetic organization of ribosomal loci (45S and 5S) and (*b*) the genomic relationships between medics from section *Dendrotelis* by *in situ* hybridization techniques (FISH and GISH). These approaches have the potential to contribute to (*a*) assessment of the taxonomic relationships of these woody species, (*b*) studies to gain insights into the auto- or allopolyploid origin of the hexaploid *M. citrina*, and (*c*) assessment of the levels of infraspecific variation concerning ribosomal loci in a restricted and fragmented insular species (*M. citrina*). The variation in number and localization of these multigene families has been used as genomic landmarks to study infraspecific genome evolution (Cerbah *et al.*, 1999; Falistocco and Falcinelli, 2003), identify the genomic contribution in somatic hybrids (Calderini *et al.*, 1997), construct molecular cytogenetic maps (Kulikova *et al.*, 2001), and to assess genomic relationships between closely related species (Falistocco *et al.*, 2002) in several *Medicago* species. Further, the genomic *in situ* hybridization (GISH) technique has been reported to be a valuable approach to assess the origins of annual medic species (Falistocco *et al.*, 2002).

MATERIALS AND METHODS

Plant material

Accessions from *M. arborea*, *M. citrina* and *M. strasseri* were obtained from field populations or from botanical gardens (Table 1). Another species from section *Medicago*, *M. marina*, that is phylogenetically close to members of section *Dendrotelis* (Downie *et al.*, 1998; Juan, 2002), was used for comparative purposes.

Chromosome preparations

Seeds were germinated on agar (0·6 %) in Petri dishes at 20 °C. The actively growing root tips were removed from seedlings and pre-treated with 0·002 M 8-hydroxyquinoline for 2 h at 20–25 °C and then 2 h at 4 °C. The root tips were fixed in 3:1 ethanol:acetic acid and stored at −20 °C until used. The root tips were washed in 10 mM citrate buffer, pH 4·6, and then macerated in mixture of 2 % (v/v) cellulase (Calbiochem) in citrate buffer, pH 4·6, and 20 % pectinase (from *Aspergillus niger*) in 40 % glycerol in 10 mM citrate buffer, pH 4·6, for 1 h at 37 °C. The spreading procedure was made according to Zhong *et al.* (1996) to prepare nuclei and chromosomes for *in situ* hybridization. The slides were stained with 4 % Giemsa solution diluted with 0·2 M Sörensen phosphate buffer, pH 6·9.

TABLE 1. *Species and accessions used in this study and the chromosome number of the accessions analysed*

	Provenance	Chromosome number
Medicago arborea	Botanical Garden of Valencia University	$2n = 32$
Medicago strasseri	Botanical Garden of Valencia University	$2n = 32$
Medicago citrina	Cabrera, Ses Bledes islet (Balearic Islands)	$2n = 48$
	Ibiza, Es Malvins islet (Balearic Islands)	$2n = 48$
	Ibiza, S'Espartar islet (Balearic Islands)	$2n = 48$
	Grossa islet (Columbretes Islands)	$2n = 48$
	La Mona islet (Iberian Peninsula)	$2n = 48$
Medicago marina L.	Mareny de Vilches (France)	$2n = 16$

DNA probes and labelling for fluorescent in situ *hybridization (FISH)*

The two multigene families of rDNA were localized with two different DNA probes. Clone pTa71 is a 9-kb *Eco*RI fragment containing the 18S–5·8S–26S rDNA genes and the intergenic spacer regions from *Triticum aestivum* (Gerlach and Bedbrook, 1979). The 5S rDNA was localized using clone pTa794, containing a 410-bp *Bam*HI fragment of the 5S rDNA gene and intergenic spacer from *T. aestivum* (Gerlach and Dyer, 1980). The 5S rDNA fragment was amplified with the universal primer M13 forward and reverse. PCR products were purified using Montage PCR Centrifugal filter devices (Millipore). The pTa71 and pTa794 probes were labelled with either digoxigenin-11dUTP or biotin-11-dUTP by nick translation based on the protocols of the manufacturer (Roche, Germany).

FISH

The protocols were carried out according to Chiavarino *et al.* (2000) with minor modifications. The chromosome preparations stored at 37 °C overnight were incubated in RNase A (1 μg mL^{-1}) in 2× SSC for 1 h at 37 °C. Subsequently, the slides were rinsed three times for 5 min in 2× SSC, fixed in 4 % paraformaldehyde in 1× SSC for 10 min at room temperature, washed three times for 5 min in 2× SSC, and then dehydrated in an ethanol series and air-dried. Prior to hybridization, the chromosome preparations were denatured in 70 % (v/v) formamide in 2× SSC at 68 °C for 2 min, dehydrated trough an ice-cold ethanol series and air-dried.

The hybridization mixture, containing 2 μg mL^{-1} of each labelled probe (45S rDNA and 5S rDNA) was denatured by boiling for 10 min, quenched on ice for 7 min, and added to the denatured chromosome

preparations. Hybridization was carried out overnight at 37 °C in a humid chamber. Post-hybridization washes of the slides were done two times for 2 min at 42 °C in 2× SSC, two times for 5 min at 42 °C in 0·1× SSC, and three times for 3 min at 42 °C in 2× SSC.

Digoxigenin-labelled probe detection was performed with anti-digoxigenin antibodies conjugated to fluorescein isothiocyanate (Roche). Biotin-labelled probe detection was performed with streptavidin conjugated with Texas Red (Vector Laboratories).

After detection, the slides were rinsed two times for 5 min at 37 °C and one time at room temperature in detection buffer [4× SSC, 0·2 % (v/v) Tween 20]. Finally, the slides were counterstained with DAPI and mounted in Vectashield (Vector Laboratories). Hybridization signals were analysed using an epifluorescence Olympus microscope, with appropriate filter set, equipped with an Olympus Camedia C-2000-Z digital camera. The images were optimized for best contrast and brightness by image-processing software (Adobe Photoshop v. 7·0).

GISH: genomic DNA probes

Total genomic DNA from young leaves of *M. arborea* and *M. strasseri* was isolated using a modified CTAB protocol of Doyle and Doyle (1987). For experiments using genomic blocking, DNA fragments, 100–300 bp in length, were obtained by autoclaving the total genomic DNA from each species. Probe DNA from *M. arborea* and *M. strasseri* was labelled with digoxigenin-dUTP and biotin-dUTP, respectively, by nick translation following the manufacturer instructions (Roche). Labelled DNA of *M. arborea* and *M. strasseri*, used to analyse the genomic relationship with chromosomes of *M. citrina*, was mixed in different combinations with unlabelled blocking DNA from the other species at a concentration of 50-fold excess and 70-fold excess, or *Escherichia coli* DNA. When both species were used in the same experiment genomic probes were used in the same concentration.

GISH

The method of *in situ* hybridization followed Leitch *et al.* (1994) and Schwarzacher and Heslop Harrison (2000). The hybridization mixture containing 2 μg mL^{-1} of each digoxigenin and biotin-labelled genomic DNA, 50 % (v/v) formamide, 10 % (v/v) dextran sulfate and 2× SSC and unlabelled blocking DNA (50- and 70-fold excess) was denatured by boiling for 10 min and placed on ice for 7 min. When both the probe and the chromosomes had been denatured the probe was applied to the slide and DNA–DNA *in situ* hybridization was carried out at 37 °C in a humid chamber for up to 20 h. Post-hybridization washes of the slides were carried out two times for 2 min each at 42 °C in 2× SSC, two times for 5 min each at 42 °C in 0·1× SSC, and three times for 3 min each at 42 °C in 2× SSC. The other stringency wash solution used was: two times for 2 min each at 42 °C in 2× SSC, in 20 % formamide and 0·1× SSC for 10 min at 42 °C, two times for 5 min each at 42 °C in 2× SSC.

RESULTS

The chromosome number counts of species from section *Dendrotelis* agreed with previous determinations and showed the tetraploid level ($2n = 32$) for *M. arborea* and *M. strasseri*, and the hexaploid level for all *M. citrina* accessions ($2n = 48$). *Medicago marina* was diploid and showed $2n = 16$ chromosomes. In all species, chromosome complements were constituted mainly by metacentric and submetacentric chromosomes of similar size and shape, making chromosome identification difficult.

FISH: 45S and 5S rDNA loci

The diploid *M. marina* showed a single 45S ribosomal locus (two large signals), located at secondary constrictions (Fig. 1A, B). *Medicago arborea* (Fig. 1C–F) and *M. strasseri* (Fig. 1G–I) showed two hybridization sites of probe pTa71 in both interphase and metaphase cells. In these two species the hybridization signals at interphase nucleus were usually observed as de-condensed chromatin strings associated with the nucleolar domain. These gene puffs showed a condensed and bright portion connected to de-condensate labelled chromatin extensions that were also present in metaphase cells. At metaphase, the FISH signals were terminal, and were located at secondary constrictions. All accessions of *M. citrina* showed the same pattern of 45S hybridization, involving eight signals (five large and three small) located in metacentric (one locus), submetacentric (two loci), and subtelocentric (one locus) chromosomes (Fig. 1J, L). All loci were located at terminal sites, and four of the strongest signals were associated with the secondary constrictions. Apparently, a different number of rDNA copies is located in the subtelocentric chromosome pair since the intensity of hybridization signals differs in each of the homologous chromosomes analysed in 50 cells. In all samples of *M. citrina* analysed, contrary to what has been observed in the other two species from section *Dendrotelis*, FISH signals appeared consistently as compact blocks at interphase and metaphase cells.

In all the species analysed, 45S rDNA and 5S rDNA loci were not collinear. In *M. marina* four hybridization sites of probe pTa794, corresponding to two 5S rDNA loci, were observed in both interphase and metaphase cells (Fig. 1A, B). These were located in the proximal regions of two different metacentric chromosome pairs. The strongest and brightest signals were located proximally in the longest of these chromosomes, whereas the minor locus was observed proximally in the median metacentric pair. Two loci were also observed in *M. arborea* (Fig. 1E, F) and *M. strasseri* (Fig. 1H, I). In *M. arborea*, the 5S loci were proximal, located on the long arms of metacentric chromosomes, and one locus showed a stronger FISH signal than the other. However, in *M. strasseri* the 5S loci were proximal, one of them located on a metacentric and the other on a submetacentric chromosome. In this species all loci showed similar FISH signals concerning intensity and size. *Medicago citrina* showed five 5S rDNA loci that mapped to five metacentric chromosome pairs (Fig. 1K, L). FISH signals, observed in interphase

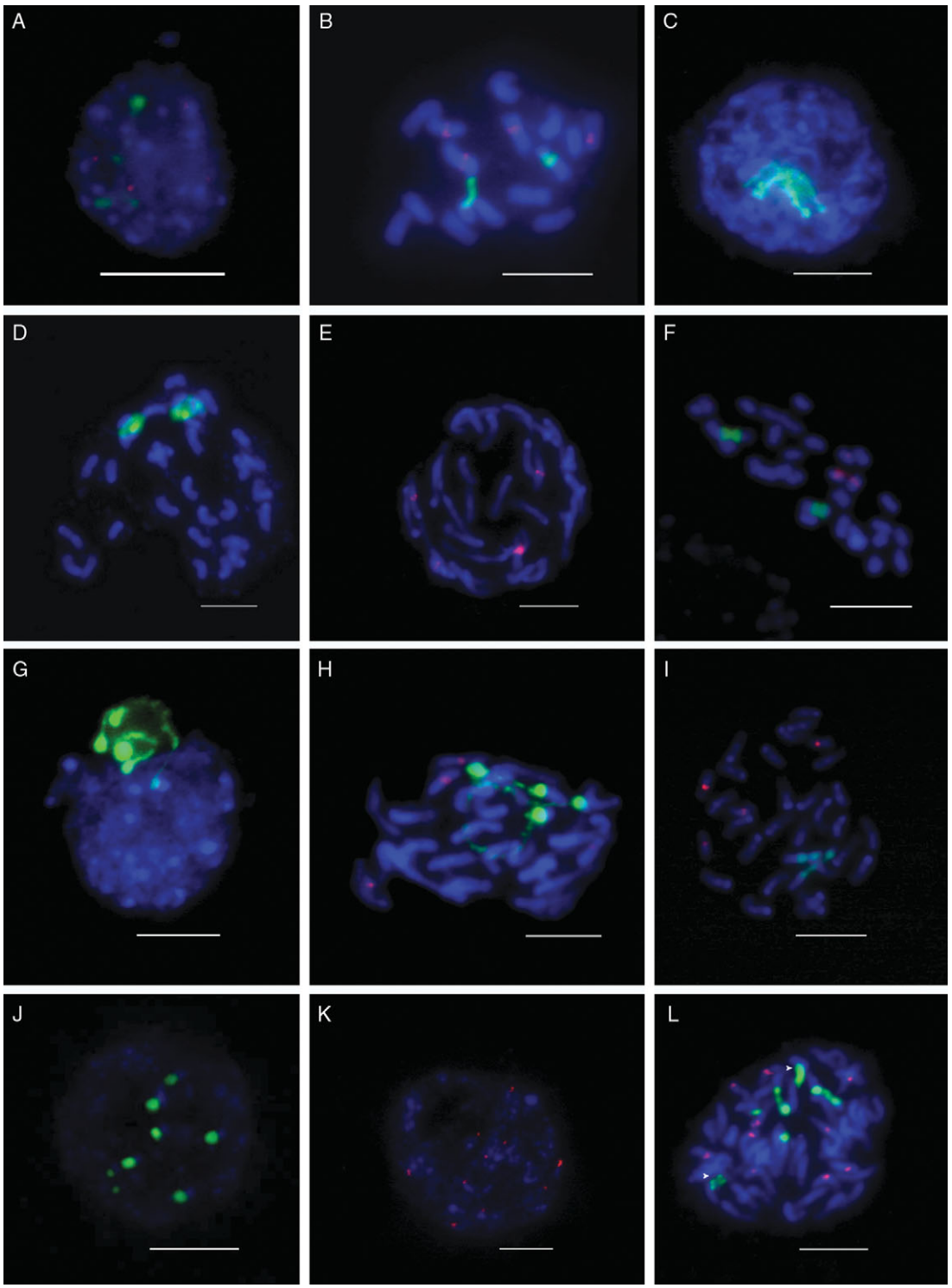

FIG. 1. Fluorescent *in situ* hybridization with 45S (green) and 5S (red) rDNA probes on root tip cells of *Medicago marina* (A, B), *M. arborea* (C–F), *M. strasseri* (G–I) and *M. citrina* (J–L). All cells were counterstained with DAPI (blue). (A) *Medicago marina* interphase nucleus showing two 45S rDNA sites (green) and four 5S rDNA sites (red); (B) *M. marina*, double FISH in metaphase cells; (C) *M. arborea*, interphase nucleus showing the decondensate 45S rDNA locus; (D) *M. arborea*, metaphase cell showing the sub-terminal 45S rDNA locus; (E) *M. arborea*, metaphase cell showing four proximal 5S rDNA sites; (F) *M. arborea*, double FISH at metaphase, showing two 45S rDNA and four proximal 5S rDNA sites; (G) *M. strasseri*, interphase nuclei showing the decondensed 45S locus; (H) *M. strasseri*, interphase nucleus showing four 5S rDNA hybridization signals; (I) *M. strasseri*, double FISH in a metaphase cell showing four proximal 5S rDNA sites (red) and two sub-terminal 45S rDNA sites (green); (J) *M. citrina*, interphase nucleus showing FISH signals of 45S rDNA; (K) *M. citrina*, interphase nucleus showing ten compact 5S rDNA sites; (L) *M. citrina*, metaphase cells with FISH signals of 5S and 45S rDNA, two pairs of chromosome exhibited very small hybridization 5S signals. Arrowheads indicate a heteromorphic chromosome pair showing different intensities in 45S hybridization signals. Scale bars = 10 μm.

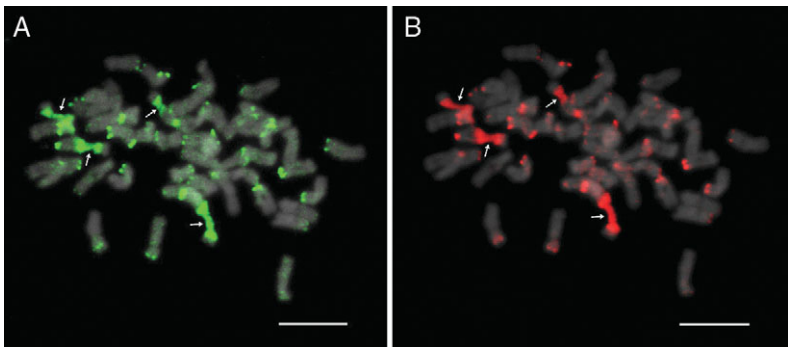

FIG. 2. GISH on metaphase chromosomes of *M. citrina* simultaneous hybridized with *M. arborea* (A) and *M. strasseri* (B) genomic probes. All cells were counterstained with DAPI (grey). In *M. citrina* cross-hybridization was restricted to terminal segments and secondary constriction of the satellited chromosomes (indicated by the arrows). Scale bars = 10 μm.

and metaphase cells, differ in size and intensity. The six strongest signals were localized in proximal regions whereas the remaining four FISH sites were observed in interstitial regions. Variation concerning the number, intensity, and size of 5S loci was not detected among all accessions of *M. citrina*.

GISH

GISH experiments on mitotic chromosomes of *M. citrina* using genomic DNA probes from the other species of section *Dendrotelis* yielded similar results. GISH results were reproducible, despite modifications in experimental conditions concerning the source of unlabelled blocking DNA, time of hybridization (up to 20 h), stringency parameters, and concentration of labelled genomic DNA. No single chromosome of *M. citrina* was uniformly labelled after using genomic probes from *M. arborea* and *M. strasseri*. Instead, cross-hybridization signals in *M. citrina* were restricted to terminal chromosome arms (usually one arm per chromosome) and secondary constriction of the satellited chromosomes (Fig. 2). Nine chromosomes of *M. citrina* did not reveal any signs of fluorescence when *M. arborea* and *M. strasseri* were used as probes.

DISCUSSION

In plants, FISH mapping of repeated DNA sequences has become the most accurate technique for localizing specific nucleic acid sequences on chromosomes, allowing the identification of individual chromosomes and providing molecular understanding of the evolution of plant genomes and organisms (Heslop-Harrison, 1991). The ribosomal RNA genes (5S and 45S rDNA loci), which occur as tandem repeats, are good markers for *in situ* hybridization, as they have conserved DNA sequences that are present in high copy numbers, allowing the use of cloned heterologous probes. Further, they usually show considerable variation in number, size and position among closely related species (Appels *et al.*, 1980), providing useful molecular landmarks to track chromosomal changes accompanying plant evolution and speciation (Weiss-Schneeweiss *et al.*, 2007), even in groups showing uniform karyotype structure (Adams *et al.*, 2000).

However, concerns regarding the appropriateness of mapping rDNA loci by FISH for plant evolutionary and taxonomic studies have been voiced. These caveats are mainly based on the extensive variation detected in the location and number of rDNA sites and NORs within individual plants and within *Allium* species (Bougourd and Parker, 1976; Schubert, 1984; Schubert and Wobus, 1985). It has been suggested that in this genus rDNA sequences are mobile predominantly at the chromosome ends (Schubert, 1984), allowing the presence of intragenomic polymorphisms and hence precluding the use of rDNA loci as stable evolutionary markers. The direct involvement of transposons in the transfer of rDNA within genomes has been suggested by several authors (Raskina *et al.*, 2004; Datson and Murray, 2006). However, these findings do not dismiss the value of rDNA as evolutionary markers, rather, they provide molecular evidence on the evolution of rDNA loci and chromosome repatterning in plant lineages.

FISH data concerning the number of nuclear ribosomal loci present in *Medicago* are only available for eight species including three out of 12 sections of the genus (Table 2). In fact, most of the molecular cytogenetic research conducted in *Medicago* has focused on two lucernes, *M. sativa* and *M. truncatula*, using mainly non-wild accessions. The data available for the whole genus are not sufficient to elucidate the evolutionary dynamics of rDNA site number in *Medicago*, as has been done in other genera (e.g. Mishima *et al.*, 2002). Furthermore, all species for which the number of ribosomal loci is known appear to be derived in a phylogenetic context (Bena *et al.*, 1998; Downie *et al.*, 1998; Bena, 2001). The lack of FISH data concerning ribosomal loci for basal species of medics makes reconstruction of the evolutionary history of rDNA site number based on the maximum parsimony method difficult.

Infraspecific variation concerning the number and location of rDNA loci has rarely been reported in *Medicago*, and each taxon is characterized by a single molecular cytogenetic pattern. However, substantial infraspecific variation in 45S and 5S rDNA loci has been reported in the model species *M. truncatula* (Cerbah

TABLE 2. *Cytogenetic features (chromosome number and ploidy level) and number of reported 45S and 5S rDNA loci in* Medicago

	Chromosome number	45S rDNA	5S rDNA	Reference
Sect. *Dendrotelis*				
M. arborea	$2n = 32$ (4*x*)	1	nr	Cluster *et al.* (1996)
		1	2	This work
M. strasseri	$2n = 32$ (4*x*)	1	2	This work
M. citrina	$2n = 48$ (6*x*)	4	5	This work
Sect. *Medicago*				
M. sativa subsp. *coerulea*	$2n = 16$ (2*x*)	1	nr	Cluster *et al.* (1996)
		1	2	Falistocco (2000)
		1	2	Abirached-Darmency *et al.* (2005)
M. sativa subsp. *glomerata*	$2n = 16$ (2*x*)	1	2	Falistocco (2000)
M. sativa subsp. *falcata*	$2n = 16$ (2*x*)	1	2	Falistocco (2000)
		1	2	Abirached-Darmency *et al.* (2005)
	$2n = 32$ (4*x*)	2	4	Falistocco (2000)
M. sativa subsp. *sativa*	$2n = 32$ (4*x*)	2	nr	Cluster *et al.* (1996)
		2	4	Falistocco (2000)
M. marina	$2n = 16$ (2*x*)	1	2	This work
Sect. *Spirocarpos*				
M. truncatula	$2n = 16$ (2*x*)	1	1–3	Falistoco and Falcinelli (2003)
		1	2–3*	Cerbah *et al.* (1999)
		1	3*	Kulikova *et al.* (2001)
		2	3*	Abirached-Darmency *et al.* (2005)
M. lesinsii	$2n = 16$ (2*x*)	1	1	Falistocco *et al.* (2002)
M. murex	$2n = 14$ (2*x*)	1	1	Falistocco *et al.* (2002)

nr = not reported.
* One of the 5S rDNA loci is collinear with a 45S rDNA locus.

et al., 1999; Kulikova *et al.*, 2001; Falistocco and Falcinelli, 2003; Abirached-Darmency *et al.*, 2005). This variation could be partially explained by the artificial origin of the accessions used (e.g. Jemalong J5 and R-1081 lines; Cerbah *et al.*, 1999). Also, taxonomic misidentifications of wild-collected accessions by plant breeders and agricultural researchers are possible, since the identification of annual taxa of medics is not an easy task (e.g. Small and Jomphe, 1989). In this work, no infraspecific variation concerning ribosomal loci has been detected in the hexaploid *M. citrina* despite the fact that (*a*) geographically structured variation, as inferred by AFLP markers, is present in this narrowly endemic species (Juan *et al.*, 2004), and (*b*) gene-flow is virtually absent among populations (Pérez-Bañón *et al.*, 2003), promoting the fixation of rare genetic variants.

With the single exception of the reported *M. truncatula*, from section *Spirocarpos*, diploid taxa of *Medicago* show a single 45S locus and two 5S loci (Table 2). Tetraploid taxa from section *Medicago*, in turn, showed the expected number of rDNA signals that should be present after a polyploidization event from diploid ancestors (two of 45S rDNA and four of 5S rDNA).

However, taxa from section *Dendrotelis* depart from expectations. On the one hand, the presence of a single 45S rDNA locus in the tetraploid taxa of section *Dendrotelis* (*M. arborea* and *M. strasseri*) suggests that ribosomal loci have experienced a diploidization event through physical loss of the sequences and not just loss of function. In addition, the chromosomal diploidization of rDNA loci has not been consistently operating in this section, since the hexaploid *M. citrina* showed the highest number of rDNA loci reported in the genus so far.

Extensive genome rearrangements and modifications of gene expression occurring during polyploid formation have been documented in several newly synthesized polyploids, including the gain or loss of parental DNA sequences, gene silencing, histone modifications, chromatin remodelling, DNA methylation, and activation of transposable elements (Song *et al.*, 1995; Liu *et al.*, 1998; Kashkush *et al.*, 2003; Lukens *et al.*, 2006). Elimination, silencing and rearrangement of rDNA genes occur in early generations after polyploidization events (Pontes *et al.*, 2004; Skalicka *et al.*, 2005), and nonadditive contribution of rDNA loci during the evolution of natural polyploid complexes has been reported in several plant genera (e.g. Kim *et al.*, 1993; Badaeva *et al.*, 1996; Hanson *et al.* 1996; Krishnan *et al.*, 2001).

Medics from section *Dendrotelis* are morphologically isolated within *Medicago*, and no single species outside the section has been postulated to be related to them (Small and Jomphe, 1989). However, phylogenetic analyses using nuclear ribosomal ITS (internal transcribed spacer region) and ETS (external transcribed spacer) of 45S rDNA have consistently shown that (*a*) this section is unlikely to be monophyletic, and (*b*) species from section *Dendrotelis* are not basal and are not phylogenetically isolated, being included within a largely unresolved section *Medicago* (Bena *et al.*, 1998; Downie *et al.*, 1998; Bena, 2001). These results were corroborated by plastid DNA sequences on a smaller sample set (Juan, 2002). A lack of diploids in section *Dendrotelis* suggests that some of the ancestors of *M. arborea*, *M. strasseri* and *M. citrina* may be extinct. Alternatively, species from other sections could have been involved in the origin of these polyploid

taxa. FISH data are not conclusive about the auto- or allopolyploid status of the tetraploids *M. arborea* and *M. strasseri*.

Both species are closely related on morphological grounds, and some authors have even reported that *M. strasseri* should be included within *M. arborea* at the infraspecific level (Sobrino *et al.*, 2000). The fact that both species share two unique cytogenetic features (the loss of an rDNA locus at the polyploid level, and the presence of highly decondensed NOR sequences at interphase and metaphase stages) agree with their close evolutionary relationships. It has been found that, in *Medicago*, the presence of decondensed NORs is not technique-dependent (as reported for *Trifolium* by Ansari *et al.*, 1999), but rather related to species boundaries since they have not been observed in any accession of *M. citrina*.

The alleged close relationships of *M. citrina* with the other species of section *Dendrotelis* are not supported in this study by GISH data. The facts that (*a*) no mitotic chromosome from *M. citrina* was evenly labelled, and (*b*) less than half of their chromosomes showed cross-hybridization when probed with *M. arborea* and *M. strasseri*, suggests that consistent genetic divergence has occurred between *M. citrina* and the pair *M. arborea*–*M. strasseri*. The high number of 45S rDNA loci and the organization of NORs detected in the hexaploid *M. citrina*, together with the GISH results, hardly supports the hypotheses that this species has originated (*a*) through autopolyploidy from any other species of section *Dendrotelis*, (*b*) through interspecific hybridization between *M. arborea* and *M. strasseri*, and (*c*) from a cross, and posterior polyploidization event, between either *M. arborea* or *M. strasseri* and an unrelated diploid species from section *Medicago*. A broader sampling from section *Medicago* (constituted by 14 species; Small and Jomphe, 1989) is necessary to assess further the origin of *M. citrina*.

Despite uncertainties concerning its origin, FISH and GISH results obtained from woody medics strongly suggest the taxonomic independence of *M. citrina* from *M. arborea* and *M. strasseri*. Although few features discriminate *M. citrina* from the other two species (leaflet shape, corolla colour, number of pollen colpi, number of ovules and pod shape; Juan *et al.*, 2003), they are footprints of a distinct evolutionary history, as evidenced by molecular cytogenetic techniques.

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