Theor Appl Genet (2002) 105:829–833
DOI 10.1007/s00122-002-1055-5

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Genomic relationships between *Medicago murex* Willd. and *Medicago lesinsii* E. Small. investigated by in situ hybridization

Received: 2 July 2001 / Accepted: 4 February 2002 / Published online: 19 September 2002
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Abstract *Medicago murex* Willd. is an annual species (2n = 14) widespread in the wild and of remarkable interest for pastures in regions with a mediterranean climate. It is considered closely related to *Medicago lesinsii* E. Small (2n = 16) but, up to now, there is no evidence demonstrating their genetic affinity. This research was undertaken to investigate the genomic relationships between *M. murex* and *M. lesinsii* by using genomic in situ hybridization (GISH). In this study GISH experiments were performed using both species as sources of chromosomes and genomic probes. To better evaluate the results of the hybridization, the labelled DNA of each species was hybridized to chromosomes of the same species and to chromosomes of the diploid *Medicago littoralis* (2n = 16). Strong hybridization signals were found on chromosomes of *M. murex* and *M. lesinsii* after GISH. Differences in the hybridization strength were not observed when slides from interspecific hybridization were compared with the control preparations. These results suggest that consistent divergences of the DNA sequences did not occur after the separation of the two species. Instead very reduced cross hybridization was found on chromosome spreads of *M. littoralis* hybridized with the DNA of *M. lesinsii* or *M. murex*. The distribution of the ribosomal genes (rDNA) investigated by fluorescent in situ hybridization (FISH) appeared similar in both *M. murex* and *M. lesinsii*. The GISH technique may be a valuable approach to obtain information on evolution of the 2n = 14 species and on the origin of the polyploids *Medicago rugosa* (2n = 30) and *Medicago scutellata* (2n = 30). The first attempt to investigate the genomic composition of *M. scutellata* using a genomic probe is reported in this paper.

Keywords *Medicago murex* · *Medicago lesinsii* · Genomic in situ hybridization · Fluorescent in situ hybridization · rDNA

Introduction

Annual species constitute the most numerous component of the genus *Medicago*. They originated in the Mediterranean Basin from which they spread to the temperate regions of the world. The chromosome number 2n = 16 is the most frequently occurring in the group of diploid annual *Medicago* species, but there are a few species with 2n = 14 (Lesins and Lesins 1979). Contrary to the perennials which frequently exhibit polyploidy, the annuals are prevalently diploid with the exception of *Medicago scutellata* and *Medicago rugosa* that are polyploid with a chromosome number of 2n = 30.

Medicago murex Willd. is among the 2n = 14 species. It is spread extensively in the wild and is of considerable interest for pastures in some countries of the Mediterranean area and in Australia (Francis and Gillespie 1981; Gillespie 1988). For a long time the chromosome number of *M. murex* was given as 2n = 16 (Heyn 1956; Clement 1962) but, after the discovery of populations with 2n = 14 (Lesins et al. 1970), both chromosome numbers have been used to describe this species. Soon afterwards it was demonstrated that the 2n = 14 and 2n = 16 chromosome taxa can be identified morphologically. Hence, it appeared more appropriate to classify them as separate species (Small and Brookes 1985). The 2n = 14 form maintained the name *M. murex* Willd., while the 2n = 16 variant was described as a new species: *Medicago lesinsii* E. Small.

Although the two taxa are considered closely related, up to now, conclusive evidence has not been provided demonstrating their genetic relationships.

Communicated by F. Salamini

This research was funded by C.N.R. strategic project “Biodiversity”. The authors wish to thank Dr. Mary Traynor for checking the English form of the manuscript

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Molecular cytogenetic techniques, such as fluorescent in situ hybridization (FISH) have proved to be extremely valuable means for studying the genome organization of related species and for understanding their phylogenesis (Bennett 1995). The utilization of different types of DNA sequences as probes has offered a significant contribution to understanding the genome structure of several similar species (Mukai 1996). Moreover, the physical position of hybridization sites may provide useful markers for chromosome identification resulting in an improvement of karyotype analysis of species with small and uniform chromosomes (Maluszynska and Heslop-Harrison 1993; Schmidt et al. 1994). A more direct method for examining genome relationships is genomic in situ hybridization (GISH), i.e. the FISH technique using total genomic DNA as probes. It provides information on the similarity of the DNA of related species and, at the same time, reveals the physical location of the conserved sequences on chromosomes (Parakonny et al. 1992). It is currently applied to phylogenetic studies, above all for identification of parental genomes of hybrids and polyploid species (Bennett et al. 1992; Callimassia et al. 1994; Jiang and Gill 1994).

GISH technique used in this study represents a first approach to investigate the genomic homology between the diploid *M. murex* and *M. lesinsii*. The chromosome distribution of ribosomal genes 18S-5.8S-25S and 5S (rDNA) were also analyzed in both species by applying simultaneous fluorescent in situ hybridization.

Since this study is a part of a project dealing with evolutionary processes of *Medicago* species, GISH experiments were also conducted on *M. scutellata* (2n = 30) to investigate its genomic composition. *M. scutellata* as well as *M. rugosa* (2n = 30), is commonly considered an allopolyploid deriving from crosses between 2n = 16 × 2n = 14 species; this hypothesis, however, has never been confirmed since attempts to identify the parental forms have never been concluded (Brunner et al. 1995; Mariani et al. 1996).

Materials and methods

Plant material

The material used for this study consisted in the accessions PI 495351 and PI 495354 of *M. murex*, PI 534233 of *M. lesinsii*, and PI 292432 of *M. scutellata* obtained from the United States Department of Agriculture Plant Introduction Station, Pullman, Wash. (USA), and accession 985 of *Medicago littoralis* from the Department of Plant Biology and Agroenvironmetal Biotechnology of Perugia (Italy).

Chromosome preparations

Seeds were germinated in Petri dishes at room temperature. Root tips were excised when they were about 1 cm in length, pretreated in a saturated aqueous solution of α-bromonaphthalene for 4 h and then fixed in ethanol acetic acid (3:1) overnight.

For chromosome preparations, root tips were washed in enzyme buffer (10 mM citric acid/sodium citrate, pH 4.6) for 30 min and then placed on poly-L-lysine-coated slides with 1–2 drops of enzyme solution (4% cellulase Onozuka R10 and 1% pectolyase Sigma in distilled water) for 2 h at 37 °C. For each slide three root tips were used. After removing the enzyme with distilled water and eliminating excessive water, 1–2 drops of ethanol acetic acid (3:1) were added. Root tips were broken with a thin needle and spread on the slide. Preparations were air dried.

Preparation of probes

Total genomic DNA was extracted from young actively growing leaves of *M. murex* and *M. lesinsii* according the method of Dellaporta et al. (1983). For preparation of genomic probes DNA was sheared by vortexing for 20–30 s and then labelled with biotin-11-dUTP (Sigma) by nick translation. Heterologous probes were used for identification of ribosomal gene sites. Clone pTa71 contains a 9-kb *Eco*RI fragment of *Triticum aestivum* L. consisting of the 18S-5.8S-25S rRNA genes and nontranscribed spacer sequences (Gerlach and Bedbrook 1979). Clone pXVI contains the complete gene of 5S rRNA and the spacer region of *Beta vulgaris* L. (Schmidt et al. 1994). Clone pTa71 was labelled with digoxigenin-11-dUTP (Boehringer Mannheim) by nick translation, while pXVI was labelled with biotin-11-dUTP (Sigma) using the polymerase chain reaction.

In situ hybridization

Genomic in situ hybridization was accomplished by pretreating slides with 100 μg/ml of RNase A in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate) for 1 h at 37 °C and washed three times in 2 × SSC. After incubation with 80 units/ml of pepsin (Sigma) in 10 mM HCl for 15 min at 37 °C, the chromosome preparations were stabilized by immersion in freshly depolymerized 4% (w/v) paraformaldehyde in water for 10 min at room temperature, washed in 2 × SSC, dehydrated in a graded ethanol series and air dried. The hybridization solution consisting of 100 ng/μl of DNA probe, 50% (v/v) formamide, 10% (w/v) dextran sulphate, 0.1% (w/v) SDS (sodium dodecyl sulfate) and 300 ng/μl of sheared salmon sperm DNA, was incubated for 10 min at 70 °C and chilled on ice. Forty microliters of hybridization mixture were applied to each chromosome preparation and covered with a plastic coverslip. The hybridization mixture and the chromosomes were denatured together at 70 °C in a modified thermocycler for 5 min, then the temperature was gradually decreased to 37 °C. The hybridization was carried out overnight at 37 °C. One-day in situ hybridization was also performed; in this case the hybridization time was reduced to 3 h. Post-hybridization washes were carried out in 50% formamide (v/v) in 2 × SSC at 42 °C (high stringency) or 37 °C (low stringency). For detection of the biotinilated DNA, slides were transferred to the detection buffer (4 × SSC/0.1% Tween 20) for 5 min, treated with 5% (w/v) BSA (bovine serum albumin) in detection buffer for 5 min and incubated in 5 μg/ml of streptavidin conjugated with Cy3 in detection buffer containing 5% (w/v) BSA for 1 h at 37 °C. After incubation the slides were washed in detection buffer three times for 8 min each at 37 °C.

The same procedure as described for GISH was used for in situ hybridization of pTa71 and pXVI probes labelled with digoxigenin and biotin, respectively. Detection of the probes was carried out simultaneously with 20 μg/ml of sheep anti-digoxigenin antibody conjugated with FITC (Boehringer Mannheim) and 5 μg/ml of streptavidin conjugated with Cy3.

All the preparations were counterstained with 2 μg/ml of DAPI (4′,6-diamidino-2-phenylindole) and then mounted in antifade solution Vectashield (Vector Laboratories).

Slides were examined with a Microphot Nikon epifluorescence microscope. About 20 slides per accession were selected for the study. Photographs were taken using Fujichrome 400 color slide film.

Results and discussion

Genomic in situ hybridization

Total genomic DNAs from *M. murex* and *M. lesinsii* were used as probes. In situ hybridization experiments were performed using both species as sources of chromosomes and genomic probes. In order to better evaluate the results of the hybridization, the labelled DNA of each species was hybridized to its own chromosomes and to chromosomes of the diploid *M. littoralis* (2n = 16) which belongs to the same section of *M. murex* and *M. lesinsii*, but is not related to them (Small and Jompe 1989). The labelled DNA of *M. murex* was also used to investigate the genomic composition of *M. scutellata*, the polyploid species with 2n = 30.

Hybridization of *M. lesinsii* DNA to root tip preparations of *M. murex* produced strong hybridization signals on the metaphase chromosomes. The fluorescence was not distributed uniformly along the chromosomes but was concentrated at the centromeric regions (Fig. 1a, b). Preparations from reciprocal GISH using labelled genomic DNA of *M. murex* as a probe showed hybridization signals of high intensity in all 16 chromosomes of *M. lesinsii*. The pattern of the labelled chromatin was similar to that observed on *M. murex* chromosomes; centromeres and proximal regions fluoresced much stronger then distal regions (Fig. 1c, d). Modification of the temperature of stringency did not produce appreciable changes in the strength of the hybridization signals in any of the experiments. In order to confirm if this lack of labelling was due to genetic differences, the genomic DNA of each species was hybridized on chromosome preparations of the same species. The results clearly showed that also in this case the distal part of the chromosomes remained unlabelled.

Instead very reduced cross-hybridization was found on chromosome spreads of *M. littoralis* hybridized with the DNA of *M. lesinsii* or *M. murex*. The probe labelled only two sites corresponding to the secondary constrictions of the satellited chromosome pair; the centromeric regions as well as the chromosome arms did not reveal any signs of fluorescence (Fig. 1e, f). Similar results were obtained when the genomic DNA from *M. murex* was probed to preparations of *M. scutellata*; hybridization signals were detected on four chromosomes coinciding with the secondary constrictions, whereas the rest of the complement was totally unlabelled (Fig. 1g, h). In situ hybridization with pTa71 confirmed that these hybrid signals corresponded in number and position to the sites of the ribosomal genes.

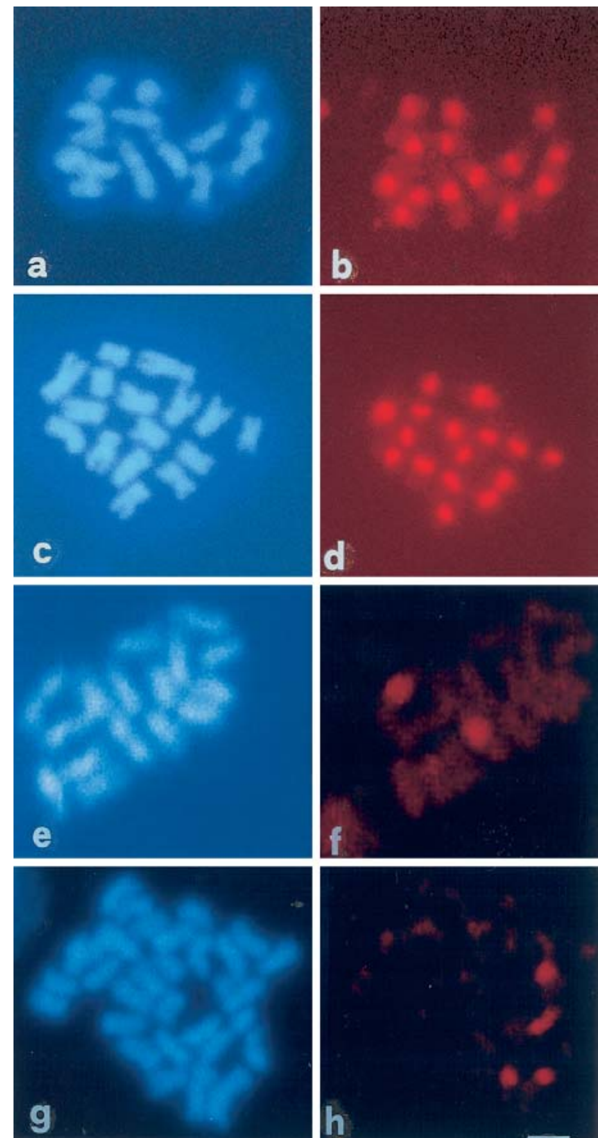

Fig. 1a–h GISH on metaphase chromosomes of *M. murex* (**a, b**), *M. lesinsii* (**c, d**), *M. littoralis* (**e, f**) and *M. scutellata* (**g, h**). Chromosomes of *M. murex* after DAPI staining (**a**) and after GISH using total DNA from *M. lesinsii* (**b**). Chromosomes of *M. lesinsii* after DAPI staining (**c**) and after GISH using total DNA from *M. murex* (**d**). Chromosomes of *M. littoralis* after DAPI staining (**e**) and after GISH using total DNA from *M. murex* (**f**). Chromosomes of *M. scutellata* after DAPI staining (**g**) and after GISH using total DNA from *M. murex* (**h**). In the two last species cross hybridization is restricted to the secondary constriction of the satellited chromosomes. The bar represents 2 μm

Fluorescent in situ hybridization. Localization of 18S-5.8S-25S and 5S rDNA

After in situ hybridization with probe pTa71 labelled with digoxigenin and probe pXVI labelled with biotin, the ribosomal genes 18S-5.8S-25S and 5S were mapped in the chromosome complements of *M. murex* and *M. lesinsii*. The simultaneous utilization of the two probes allowed the sites of the two clusters of genes to be identified in the same metaphase plates. Figure 2 shows metaphase chromosomes from the root tips of *M. murex* and *M. lesinsii* after fluorescent in situ hybridization with the two probes, and counterstaining with DAPI.

Two hybridization sites of probe pTa71 and probe pXVI were observed in both species. The green fluores-

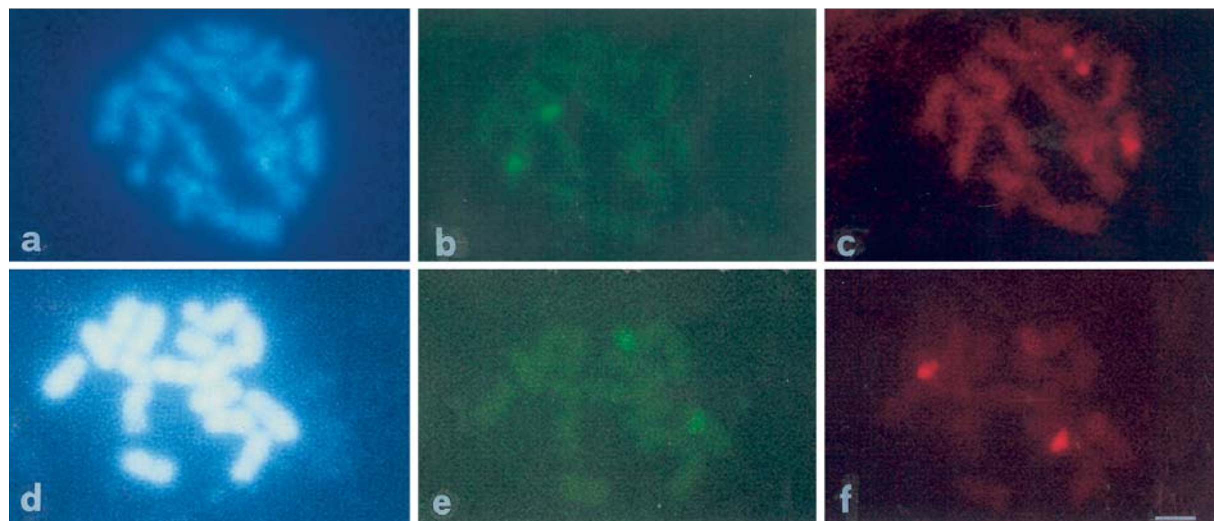

Fig. 2a–f Simultaneous in situ hybridization of two rDNA probes, pTa71 (*green*) and pXVI (*red*) to somatic chromosomes of *M. murex* (**a–c**) and *M. lesinsii* (**d–f**). Chromosomes of *M. murex* showing DAPI staining (**a**) sites of 18S-5.8S-25S rDNA (**b**) and sites of 5S rDNA (**c**). Chromosomes of *M. lesinsii* showing DAPI staining (**d**) sites of 18S-5.8S-25S rDNA (**e**) and sites of 5S rDNA (**f**). The bar represents 2 μm

cent signals indicated that loci of the 18S-5.8S-25S sequences are located in correspondence with the nucleolar organizer regions of the satellited chromosome pair identified with DAPI staining; signals outside these regions were never detected (Fig. 2a, b, 2d, e). Red fluorescent signals corresponding to 5S rDNA loci were localized in a couple of chromosomes near to the centromere (Fig. 2c, f). The loci were clearly detected because the signals were large and of high intensity. The size of the regions mapped with the two probes did not reveal appreciable variation between accessions or species. In both *M. murex* and *M. lesinsii* the clusters of 5S and 18S-5.8S-25S ribosomal genes were localized on different chromosomes.

Phylogenetic studies in the genus *Medicago* have been carried out primarily in the perennial taxa. Annual species have remained almost unexplored even though they exhibit characteristics of remarkable evolutionary significance. The presence of two basic numbers $x = 8$ and $x = 7$, for example, clearly indicates that mechanisms leading to modification of the basic set were involved in the evolution of this group. Besides *M. murex* the basic chromosome number $x = 7$ is found in *Medicago constricta*, *Medicago polymorpha*, *Medicago praecox* and *Medicago rigidula*.

Molecular cytogenetic investigations may contribute to understanding the evolution of the 2n = 14 species. The results of this study show that genomic in situ hybridization is an effective means to supply preliminary information on relationships between the 2n = 14 and 2n = 16 species. Strong hybridization signals were observed on metaphase chromosomes of *M. murex* and *M. lesinsii* after GISH. Differences in hybridization strength were not found when hybrid slides were compared with the control preparations. These results indicate that consistent genetic divergences did not occur after separation of the two species. Instead a total lack of labelling was observed on the chromosomes of *M. littoralis* after genomic probing.

The fact that *M. murex* and *M. lesinsii* are intersterile could depend on their different chromosome number or, more probably, on other mechanisms. It has been reported that evolution in annual species of *Medicago* favoured intersterility; the majority of closely related species, even with the same chromosome number, are absolutely intersterile (Small and Brookes 1985).

Genomic in situ hybridization may also be a powerful tool to investigate the chromosome organization of wild polyploid species of uncertain origin, such as *M. rugosa* and *M. scutellata*. An initial attempt to determine the genomic composition of *M. scutellata* was carried out by utilizing the genomic DNA of *M. murex* as a probe. The absence of labelling found after hybridization enabled *M. murex* and *M. lesinsii* to be excluded with certainty from the evolution of this polyploid species. Investigations to discover the diploid species which originated *M. scutellata* and *M. rugosa* are continuing. Identification of the parental genomes will also provide information to support their allopolyploid origin.

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